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DOCTORAL DISSERTATION

Improving the Efficacy of Daratumumab in Multiple Myeloma: Assessment of a New Therapeutic Combination and Characterization of Resistance Mechanisms

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LIST OF ABBREVIATIONS

Ab	Antibody
ADC	Antibody-drug conjugate
ADCC	Antibody dependent cellular cytotoxicity
ADCP	Antibody dependent cellular phagocytosis
ADO	Adenosine
AP-1	Activator protein 1
APC	Antigen presenting cell
Arg-1	Arginase 1
ASCT	Autologous stem cell transplantation
BCMA	B-cell maturation antigen
BM	Bone marrow
BMSC	Bone marrow stromal cell
Breg	Regulatory B lymphocyte
CALR	Calreticulin
CAR-T	Chimeric antigen receptor T-cell
CCND1	Cyclin D1
CCND3	Cyclin D3
CDC	Complement dependent cytotoxicity
CIP	Complement inhibitory protein
COX-2	Cyclooxygenase 2
CRBN	Cereblon
CTLA-4	Cytotoxic T lymphocyte antigen 4
CXCR4	C-X-C Chemokine Receptor Type 4
DC	Dendritic cell
DNMT	DNA methyltransferase
DR	Death receptor
EC	Endothelial cell
ERK	Extracellular signal-regulated kinase
Fab	Fragment antigen binding domain
Fc	Fragment crystallizable
FcγRIIIA	Immunoglobulin Gamma Fc Region Receptor III-A
FDA	United States Food and Drug Administration
FGFR3	Fibroblast growth factor receptor 3
FLC	Free light chain
G-MDSC	Granulocytic myeloid-derived suppressor cell
GLOBOCAN	Global Cancer Observatory
Has1	Hyaluronan synthase 1
HAT	Histone acetyltransferase

HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitor
HHLA2	HERV-H LTR-Associating 2
HIF-1	Hypoxia inducible factor 1
HMGB1	High mobility group box 1
HRD	Hyperdiploid
HSP	Heat shock protein
ICD	Immunogenic cell death
IDO	Indoleamine-2,3-dioxygenase
Ig	Immunoglobulin
IGF-1	Insulin like growth factor 1
IGH	Immunoglobulin heavy chain
IKZF1	Ikaros
IKZF3	Aiolos
IMiD	Immunomodulatory drug
IMWG	International Myeloma Working Group
IRF4	Interferon regulatory factor 4
ISS	International staging system
ITAM	Immunoreceptor tyrosine-based activation motif
KIR	Killing inhibitory receptor
LAG3	Lymphocyte-activation gene 3
M-MDSC	Monocytic myeloid-derived suppressor cell
mAb	Monoclonal antibody
MAC	Membrane attack complex
MAF	Avian musculoaponeurotic fibrosarcoma
MAFB	Avian musculoaponeurotic fibrosarcoma homolog B
MAPK	Mitogen-activated protein kinase
mDC	Myeloid dendritic cell
MDE	Myeloma defining events
MDSC	Myeloid-derived suppressor cell
MEK	Mitogen-activated protein kinase
MGUS	Monoclonal gammopathy of undetermined significance
MHC	Major histocompatibility complex
miRNA	microRNA
MM	Multiple myeloma
MMAF	Monomethyl auristatin F
MMP	Metalloproteinase
MMSET	Multiple myeloma SET domain protein
MoA	Mechanism of action
MRI	Magnetic resonance imaging
Myc	V-myc avian myelocytomatosis viral oncogene homolog
NCR	Natural cytotoxicity receptor

NDMM	Newly diagnosed MM
NFκB	Nuclear factor kappa B
NGS	Next generation sequencing
NK cell	Natural killer cell
NO	Nitric oxide
NSD2	Nuclear receptor binding SET domain protein 2
OB	Osteoblast
OC	Osteoclast
OPN	Osteopontin
ORR	Overall response rate
OS	Overall survival
PC	Plasma cell
PD	Programmed death
pDC	Plasmacytoid dendritic cell
PFS	Progression free survival
PI	Proteasome inhibitor
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PLCγ	Phospholipase C-gamma pathway
PMN-MDSC	Polymorphonuclear myeloid-derived suppressor cell
PTPN11	Protein tyrosine phosphatase, nonreceptor type 11
RB1	Retinoblastoma 1
ROS	Reactive oxygen species
RRMM	Relapse and/or refractory multiple myeloma
RTK	Receptor tyrosine kinase
scFv	Single-chain variable fragment
SDF-1	Stromal cell-derived factor 1
SLAMF7	SLAM Family Member 7
SMM	Smoldering multiple myeloma
Syk	Spleen tyrosine kinase
TAM	Tumor associated macrophage
TCR	T cell receptor
TIGIT	T cell immunoreceptor with Ig an ITIM domains
TMIGD2	Transmembrane and immunoglobulin domain-containing 2
TNF-α	Tumor necrosis factor alpha
TRADD	TNFR1 associated death domain
Treg	Regulatory T lymphocyte
VEGF	Vascular endothelial growth factor
VPA	Valproic acid
XPO1	Exportin-1

INTRODUCTION

1.1. MULTIPLE MYELOMA

Multiple myeloma (MM) is a hematological malignancy characterized by the clonal expansion of plasma cells (PCs) in the bone marrow (BM). MM is the second most common hematological neoplasm accounting for the 0.9% of all cancer diagnoses in 2018 according to Global Cancer Observatory (GLOBOCAN) statistics[1]. The median age of MM patients at diagnosis is around 65 years being slightly more common in men than in women and twice as common in African-Americans in comparison to Caucasians[1,2] (Figure 1).

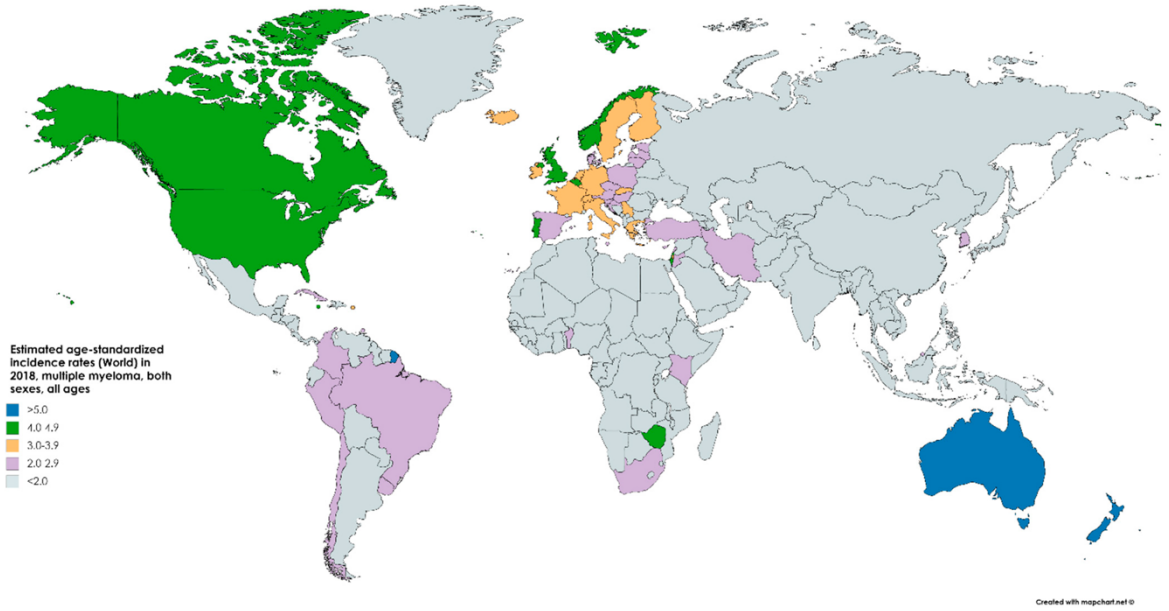

Figure 1. Global estimated age-standardized incidence rates per 100,000 inhabitants (worldwide) of MM for both sexes and all ages[1].

According to the International Myeloma Working Group (IMWG), the diagnosis of the disease is based on both infiltration of PCs in the BM (≥10% cellularity in BM morphological examination or a biopsy proven extramedullary plasmacytoma) and the presence of one or more myeloma defining events (MDEs). MDEs consist of: (i) end organ damage defined by CRAB features (acronym derived from the clinical manifestations of the disease: hypercalcemia, renal insufficiency, anemia and lytic bone disease); (ii) clonal BM plasma cell percentage ≥60%; (iii) involved:uninvolved

serum free light chain (FLC) ratio ≥100; and (iv) the presence of more than one focal lesion on magnetic resonance imaging (MRI)[3,4].

Almost all patients with MM evolve from an asymptomatic premalignant stage named monoclonal gammopathy of undetermined significance (MGUS)[5,6], which progresses to MM at a rate of 1% per year[7]. Since MGUS is asymptomatic, over 50% of individuals who are diagnosed with MGUS have had the condition for over 10 years prior to the clinical diagnosis[8]. According to the IMWG, MGUS is defined by serum monoclonal protein (IgM or non-IgM type) <3 g/dL, <10% of plasma cells in the BM and the absence of CRAB features[3]. The main factors associated with the progression to MM are: the presence of more than 5% of malignant PCs in the BM, the increase in serum M protein concentration, the type of M protein (patients with IgA and IgM isotypes show a risk of progression greater than those with IgG isotype), and the abnormal ratio of Kappa-Lambda FLC in serum[7].

In some patients, an intermediate asymptomatic but more advanced premalignant stage referred to as smoldering multiple myeloma (SMM) can be diagnosed. SMM is defined by the presence of a serum monoclonal protein of ≥3 g/dL or urinary monoclonal protein ≥500 mg per 24 h and/or 10% to 60% clonal bone marrow PCs with no evidence of any other MDE[9]. SMM progresses to MM at a rate of approximately 10% per year over the first 5 years following diagnosis and decreases thereafter[10]. Progression risk factors include, but are not limited to: high serum M protein concentration, IgA isotype, high PC infiltration in the BM, a high proportion of FLC in serum, immunoparesis and the presence of more than 95% of malignant plasma cells in the BM[11].

1.2. MULTIPLE MYELOMA PATHOPHYSIOLOGY

Multiple myeloma is a heterogeneous disease resulting from multiple genomic events that lead to tumor development and progression. Together with genomic abnormalities, epigenetic alterations and the interaction of plasma cells with the BM

microenvironment play pivotal roles in disease progression and drug resistance appearance.

1.2.1. Genetic alterations

Molecular cytogenetic studies have shown that almost every MM patient harbors cytogenetic alterations leading to the genomic instability that characterizes this disease. These genetic events of MM can be mainly divided into two different groups: primary and secondary (Figure 2).

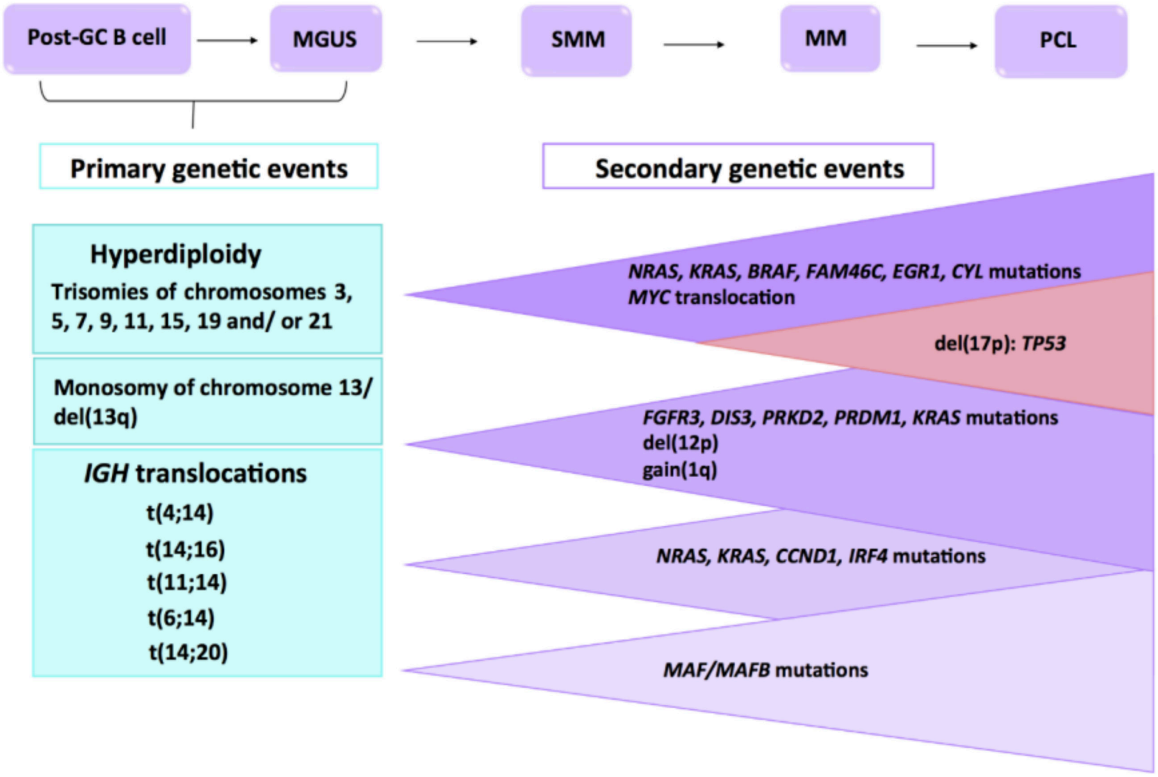

Figure 2. Primary and secondary genetic events involved in the MM transformation and progression from the precursor entities to MM[12].

1.2.1.1. Primary genetic alterations

With respect to the primary genetic events, myelomas are usually divided into hyperdiploid (HRD) and non-HRD subtypes. Almost half of MM tumors are in the HRD subtype preferentially with trisomies of some or all of chromosomes 3, 5, 7, 9, 11, 15, 19 and 21[13].

Among the non-HRD tumors, deletion of the short arm of chromosome 13 [del(13q)] can be found in 45-50% of MM patients and is also present at MGUS stage[14]. Patients with del(13q) have lower levels of the tumor suppressor gene Retinoblastoma (*RB1*) allowing cell cycle progression[15].

Finally, one of the central characteristics of non-HRD tumors are chromosomal translocations being present in up to 50% of patients. Indeed, the large majority (>90%) of translocations affect chromosome 14, specifically the immunoglobulin heavy chain (*IGH*) locus with other partners[14]. Due to these translocations the resulting fusion product ends under the control of the *IGH* enhancer, which is one of the most active ones in plasma cells[16]. The most frequent of these translocations are:

- Translocations t(11;14) and t(6;14) juxtapose the *IGH* enhancer with cyclin D genes (*CCND1* and *CCND3*, respectively), subsequently inactivating the *RB1* gene[12]. Patients harboring these translocations are categorized as standard risk[17].

- Translocation t(4;14) is observed in 15% of patients and leads to simultaneous overexpression of two genes: Fibroblast Growth Factor Receptor 3 (*FGFR3*) and Nuclear Receptor Binding SET Domain Protein 2 (*NSD2*), commonly known as MMSET (Multiple Myeloma SET Domain Protein)[14]. The overexpression of these two genes contributes to an increased tumor proliferation and high tumorigenicity. This translocation is associated with bad prognosis and patients are classified in the high-risk category[12].

- Translocations t(14;16) and t(14;20) deregulate Avian Musculoaponeurotic Fibrosarcoma (*MAF*) and Avian Musculoaponeurotic Fibrosarcoma Homolog B (*MAFB*) genes, respectively. The resulting overexpression induces proliferation and protects cells from drug-induced apoptosis. Patients with these translocations are also considered high-risk patients[12].

1.2.1.2. Secondary genetic alterations

Next generation sequencing (NGS) development has allowed the detection and identification of many different genetic mutations that could play a role in MM progression. Despite the mutational heterogeneity that characterizes MM, the most recurrent mutated genes can be grouped into specific signaling pathways. Mutations affecting the mitogen-activated protein kinase (MAPK) pathway (i.e. mutations in *KRAS*, *NRAS*, *BRAF, EGR1* and *FGFR3* genes) are the most frequent ones[14]. Moreover, the DNA repair pathway is mutated in 15% of patients observing mutations in genes such as, *TP53*, *ATR*, *ATM* and *ZFHX4*[18]. Also, mutations in genes belonging to the nuclear factor kappa B (NFκB) pathway have been reported, such as, *TRAF3*, *LTB* or *CYLD*[19].

In addition to mutations, translocations affecting *MYC* (V-Myc Avian Myelocytomatosis Viral Oncogene Homolog) are also considered secondary genetic events since they are found at low frequencies in MGUS or SMM patients (3-4%), whereas in newly diagnosed MM (NDMM) the frequency rises to 15-20%[20] and to a 50% in relapse and/or refractory multiple myeloma (RRMM)[21]. *MYC* has been described to juxtapose to different loci, but in all situations, the translocation leads to the overexpression of *MYC* which increases DNA damage and reactive oxygen species (ROS)[22]. Importantly, *MYC* rearrangement has become a central event in the transition from premalignant conditions to symptomatic MM[12].

Finally, other secondary genetic alterations are:

- Deletion of the short arm of chromosome 17 [del(17p)]. This has been described as a secondary event since although only 10% of NDMM patients harbor this deletion, it reaches up to 80% in later stages of the disease[23]. This cytogenetic alteration entails the loss of *TP53* gene which is a tumor suppressor gene responsible for cell cycle control and DNA damage response[12]. Therefore, del(17p) is a poor prognostic factor for overall survival (OS) in MM[14].

- Deletion of the short arm of chromosome 12 [del(12p)] could also be a secondary event in disease progression. Indeed, according to the IMWG this deletion could be a high-intermediate risk prognostic marker[24].

- The long arm of chromosome 1 is gained [gain(1q)] in almost 50% of NDMM patients and in around 68% in RRMM[12]. This alteration is associated with poor prognosis. In fact, gain(1q) alteration is part of the concept of "double-hit" myeloma together with international staging system (ISS) stage 3. Patients with "double-hit" are considered to have an ultra-high risk disease[25]. The gene or genes responsible for the poor prognosis of gain(1q), however, remain unknown[13].

1.2.2. Epigenetic alterations

Apart from the well-known genetic alterations already described, recent data suggest that epigenetic aberrations could also play an important role in MM pathogenesis. Main epigenetic alterations described in cancer cells are aberrant DNA methylation, histone modifications and abnormal micro-RNA (miRNA) expression.

1.2.2.1. DNA methylation

DNA methylation involves the covalent addition of a methyl group to the aromatic ring of a cytosine residue of CpG dinucleotide. This process is regulated by a family of DNA methyltransferases (DNMTs) that includes DNMT1, DNMT3a and DNMT3b, among others. This DNA modification has generally been related to transcriptional silencing.

In the context of MM, as in other cancer types, a global hypomethylation across the genome has been described, due to the rapid cell proliferation in the germinal center[26]. Despite the general and extensive hypomethylation, myeloma cells frequently present regional DNA hypermethylation. This is the case of the promoter of the cyclin-dependent kinase inhibitor genes *CDKN2B* and *CDKN2A* leading to a transcriptional silencing of these genes and therefore to cell cycle progression[27].

Moreover, promoter hypermethylation has also been found in some members of WNT signaling pathway, as well as in components of the JAK/STAT signaling[26]. In both pathways, the transcriptional silencing of the genes may play an important role in the progression of MM by unleashing the two signaling pathways.

1.2.2.2. Histone post-translational modifications

Histones are proteins that together with DNA constitute the chromatin. Chromatin is organized in nucleosomes that are formed of a histone octamer around which 147bp of DNA are wrapped. The N-terminal domain of histones may be subjected to different post-translational modifications such as methylation or acetylation[28].

As already mentioned, one of the most commonly translocated genes in MM is *MMSET* [t(4;14)]. This gene encodes for a H3K36 methyltransferase, so the translocation of this gene to the *IGH* enhancer induces an H3K36me2 mark and consequently an increase in chromatin accessibility[29]. In addition to t(4;14), several mutations have been identified in genes with a relevant role in epigenetic regulation. Indeed, mutations in *IDH1* and *IDH2* genes have recently been described[30]. These genes encode isocitrate dehydrogenases that normally produce α-ketoglutarate, but when mutated they lead to the accumulation of 2-hydroxyglutarate, a substrate that inhibits histone demethylase enzymes. These mutations lead, ultimately, to an altered function of transcription factors such as c-Myc and MAX, which are sensitive to DNA methylation status[31].

Together with being methylated, histones may also be acetylated. Histone acetylation is a general mark of transcriptional activation that is regulated by histone acetyltransferases (HATs) and deacetylases (HDACs). In MM, an overexpression of HDACs contribute to an aberrant acetylome playing a role in the malignant MM phenotype[28]. For instance, HDAC1 overexpression could result in proliferation through repression of tumor suppressor cyclin-dependent kinase inhibitors, and angiogenesis by repression of p53 and induction of Hypoxia Inducible Factor (HIF-

1) and hypoxia responsive genes such as Vascular Endothelial Growth Factor (VEGF)[32].

1.2.2.3. Micro-RNAs (miRNAs)

Micro-RNAs (miRNAs) are regulatory, short, single-stranded, non-coding RNAs that control gene expression through post-transcriptional silencing of target genes[26]. It is estimated that the expression of one third of genes may be regulated by miRNAs.

The development of RNA-sequencing in the last years has allowed the identification of different important groups of miRNAs in the context of MM. Indeed, as reviewed by Amodio et al. distinctive miRNA signatures, such as, the increase of the "oncogenic miRNAs" miR-18, miR-21, miR-125a-5p, miR-32, miR-92a, among others and the down-regulation of miR-29b, miR-125b, miR-199a-5p, miR-15a, miR-16 and miR-370 have been described[26]. Furthermore, *MMSET* overexpression that occurs in t(4;14) reduces levels of miR-126, ultimately favoring c-Myc upregulation[33]. In addition, it has been shown that the expression of circulating miRNAs may have prognostic value in MM[34].

Regarding disease progression, miRNA expression profile studies performed using normal, MGUS, and MM PCs revealed different miRNA signatures for each of these stages. In fact, Pichiorri et al. identified miR-21, miR-106b/-25 cluster and miR-181a/b to be overexpressed in PCs from MM and MGUS patients in comparison to healthy donors. Moreover, the upregulation of miR-32 and miR-17/92 cluster was only detected in the context of MM[35].

1.2.3. Interaction of PCs with BM microenvironment

Together with the (epi)genetic alterations already mentioned, the interaction between myeloma PCs and their microenvironment also plays a role in the pathogenesis of MM. The BM microenvironment is constituted by different components such as, immune cells [i.e., T cells, natural killer (NK) cells, dendritic cells, etc.], non-immune cells [i.e., bone marrow stromal cells (BMSCs), osteoblasts, osteoclasts, etc.], matrix proteins (i.e., fibronectin, laminin, etc.) and secreted soluble

factors (i.e., cytokines, growth factors, etc.). The majority of the studies describing MM microenvironment are focused on the interplay between tumor cells and BMSCs, which may occur through direct cell-to-cell contact, soluble factors and, as it has been recently described, through extracellular vesicles that contribute to the crosstalk through the transfer of proteins, non-coding RNAs and even lipids. These interactions play a role in different biological processes in the context of MM, including, among others, tumor growth and survival, inhibition of osteoblast differentiation, drug resistance, dissemination and homing and immunosuppressive and proinflammatory environment (Figure 3).

The crosstalk between MM cells and BM microenvironment actively contributes to the pathogenesis of the disease as reviewed by Maiso et al.[36]. The interaction of myeloma cells with the BM niche occurs through a set of adhesion molecules, including α and β1 integrins (VLA-4, VLA-5 and VLA-6) on myeloma cells, with the intercellular adhesion molecule ICAM-1, as well as the vascular cell adhesion molecule VCAM-1 on BMSCs[37]. In addition, BMSCs produce factors such as SDF-1 (Stromal Cell-Derived Factor 1), which binds to the CXCR4 receptor (C-X-C Chemokine Receptor Type 4) on MM cells[37]. Also, molecules expressed on myelomatous cells such as CD138, CD38, CD44 and CD106 facilitate this interaction with BMSCs[36].

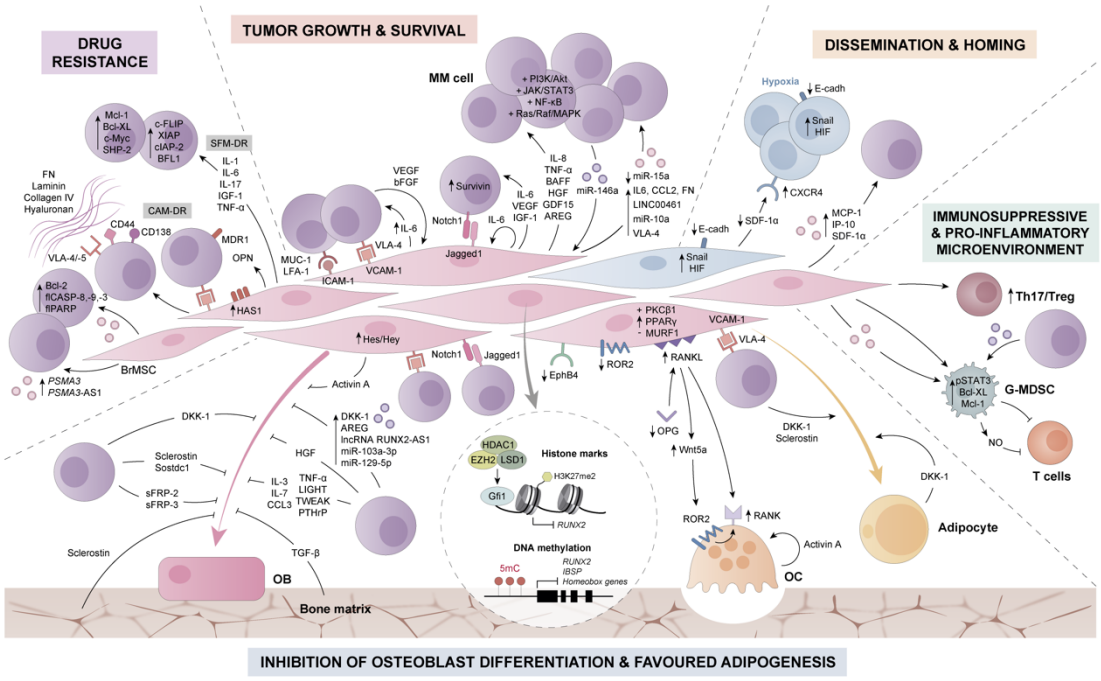

Figure 3. BMSC mediated biological activity in the BM microenvironment of MM[36].

The production and secretion of cytokines by the cells of the microenvironment and by myeloma cells themselves, such as, TNF-α (Tumor Necrosis Factor Alpha), IL6, IL21, IGF1 (Insulin Like Growth Factor 1), SDF-1α and VEGF among others, activate multiple signaling cascades on myeloma cells, (MAPKs, PI3K/AKT, NFκB and JAK/STAT), which, in turn, induce apoptosis inhibition and tumor cell survival and proliferation[38].

In addition, MM cells also interact in the BM with osteoclasts (OCs) and osteoblasts (OBs), among others. Briefly, the interaction of MM cells with BMSCs changes the transcriptomic profile and induces epigenetic changes, eventually reducing the bone-forming capacity of OBs and favoring both the differentiation and the resorptive activity of OCs, leading to the characteristic osteolytic lesions associated to MM[39,40].

Regarding the involvement of the microenvironment in drug resistance, it is known that both soluble factors such as IL-6, IGF-1, IL-1, IL-17 and TNF-α; and adhesion molecules including fibronectin, laminin, hyaluronan and collagen IV are responsible for it[41]. On the one hand, IL-6 is one of the mediators of dexamethasone

resistance in myeloma cells via protein tyrosine phosphatase, nonreceptor type 11 (PTPN11)[42]. Similar to IL-6, IGF-1 is another important mediator of drug resistance in MM context. Indeed, IGF-1 induces the activation of multiple signaling pathways in myeloma cells (PI3K/AKT, MAPK, and NFκB) that results in increased telomerase activity and upregulation of antiapoptotic molecules[43]. On the other hand, binding of myeloma cells to extracellular components through adhesion molecules has been shown to further contribute to drug resistance. Specifically, fibronectin binding through $\alpha4\beta1$ or $\alpha5\beta1$ upregulates p27, induces NFκB activation, and has been shown to alter the expression of 469 genes in MM cells. In addition, it has also been described increased production of osteopontin (OPN) and hyaluronan synthase 1 (Has1) by BMSCs conferring drug resistance to MM cells[36].

In addition, MM progression involves a continuous mobilization of MM cells into the PB and homing back to the BM. Malignant PCs depend on the BM microenvironment to survive and expand; nevertheless, as myeloma progresses, both myeloma cells and the microenvironment become hypoxic, leading to the mobilization of tumor cells to PB. As shown in Figure 3, later homing of MM cells to the BM depends on chemokines, such as SDF-1α, that regulate the adhesion of MM to BMSCs. It has been shown that reduction of SDF-1α or up-regulation of CXCR4 by hypoxia induces the mobilization of PCs out of the BM[36].

The contribution of the tumor microenvironment in the induction of an immunosuppressive tumor niche will be reviewed in the following section.

1.3. IMMUNE SYSTEM ALTERATIONS IN MULTIPLE MYELOMA

Patients with monoclonal gammopathies, including MM, have shown a general immunosuppression condition characterized by the alteration of different immune populations and cytokine profile. This alteration is the result of both cell-to-cell contacts and the presence of several extracellular components which ultimately inhibit cytotoxic cells and recruit and activate immunosuppressive populations[44].

Hereunder, we will discuss the main alterations that have been described in the number and function of the major immune populations (Figure 4).

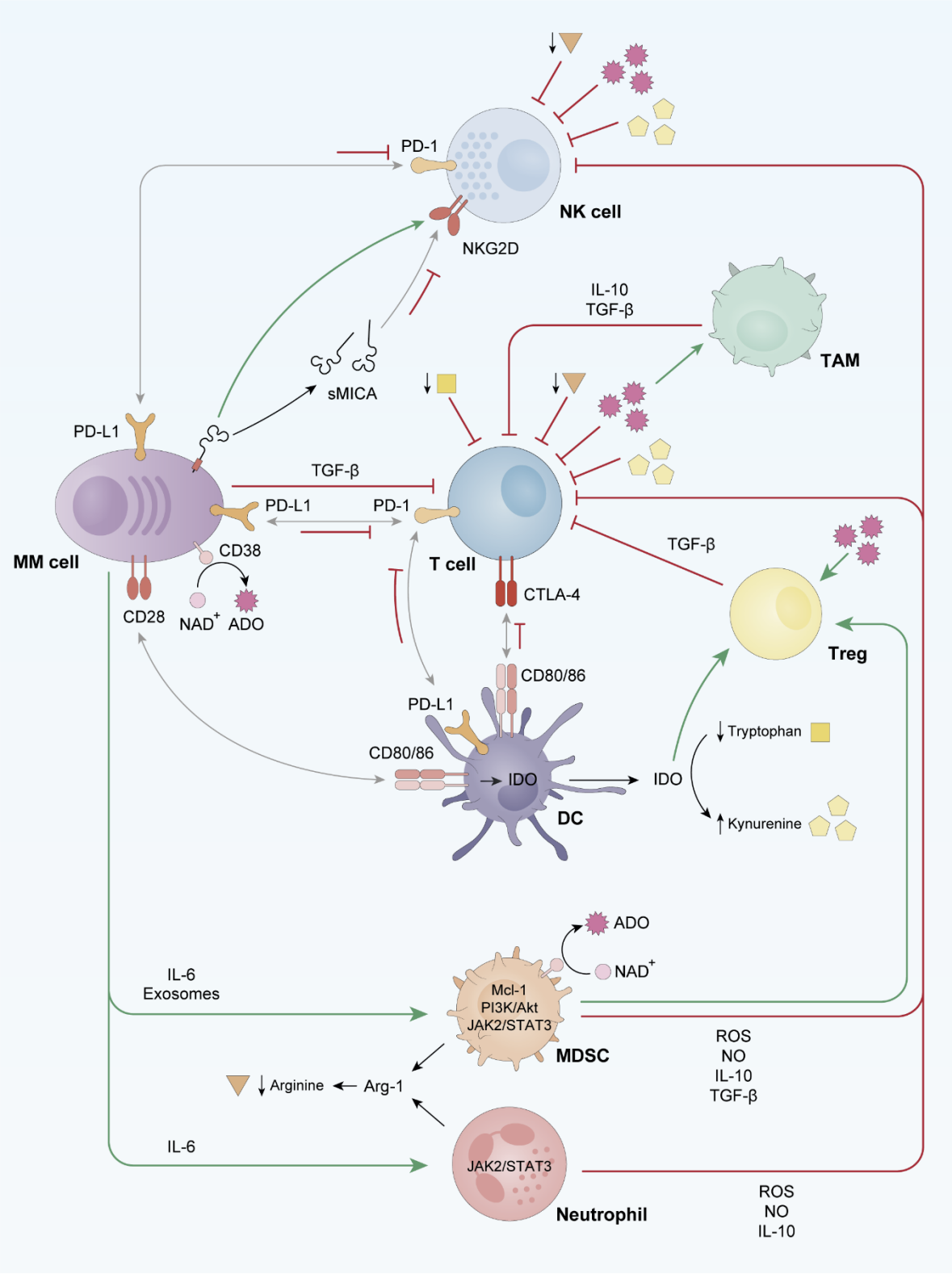

Figure 4. Schematic representation of the main immune system alterations described in MM patients[44].

1.3.1. T lymphocytes

T lymphocytes, both T helper $CD4^+$ and T cytotoxic $CD8^+$, are major components of the adaptive immune system that act as coordinators and effectors of immunity[45]. Both subsets play a crucial role in the anti-tumoral immunity. Regarding the alterations found in these populations in MM patients, the most frequently described one is the decrease in $CD4^+$ cell counts, which is associated with lower progression free survival (PFS) and OS and a higher probability of relapse[46,47]. Additionally, the production of TGF-β and IL-6 from MM cells and BMSCs induces the differentiation of naïve T cells to Th17 cells[48], an immunosuppressive $CD4^+$ T cell subset which, as a positive feedback loop, secretes different cytokines promoting MM cell expansion[49].

With respect to the population of cytotoxic T cells, an increase in the total number of these cells in both MGUS and symptomatic MM in comparison with healthy donors[50] has been reported. More recently Zavidij et al. have shown that during disease progression a depletion of memory $CD8^+$ T cell subset is observed, thus, being more abundant in MGUS individuals compared to SMM and active MM[51]. Soluble factors have been observed to modulate the activity of cytotoxic T cells. In this sense, TGF-β inhibits IL-2-dependent proliferation and maturation of T cells and prevents naïve T cells from acquiring effector functions[52,53]. The activity of effector T lymphocytes is also inhibited by the immunosuppressive nucleoside adenosine (ADO), derived from ATP or NAD^+ after sequential catalytic reactions initiated by, among others, the surface molecule CD38 in MM cells[54–56].

The activation of T cells is initiated through antigen recognition by the T cell receptor (TCR), and then regulated by a balance between co-stimulatory and co-inhibitory signals denominated immune checkpoints[57]. Although immune checkpoints are crucial for the maintenance of self-tolerance and homeostasis[57], in pathological conditions the expression of immune checkpoint proteins can be dysregulated as a mechanism of immune evasion[58]. One of the most relevant

immune checkpoints is the programmed-death (PD) pathway. PD-1 is an inhibitory receptor expressed by T cells, which interacts with its ligands PD-L1/PD-L2 expressed by mainly antigen presenting cells (APCs) to inhibit T cell effector functions[59–61]. It is known that PCs from healthy subjects express none or very low levels of PD-1 ligands, however, PD-L1/PD-L2 can be found in PCs from myeloma patients[62–65] and in MM cell lines[66]. Moreover, the expression of PD-1 seems to be increased on T cells from MM patients, compared to healthy subjects[67,68]. Together with PD-1/PD-L1, cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) is another immune checkpoint responsible for T cell suppression. Specifically, the binding of CTLA-4 present on T cells to its ligands (CD80/CD86), expressed on APCs, transmits an inhibitory signal to T cells[69]. Some studies have assessed the safety and efficacy of immune checkpoint inhibition as consolidation treatment following ASCT in patients with MM. In CPIT-001 clinical trial ipilimumab [anti-CTLA-4 monoclonal antibody (mAb)] and nivolumab (anti-PD1 mAb) were administered between 14 and 28 days post-ASCT in patients with high-risk MM which had achieved at least stable disease after induction treatment. At 18 months post-ASCT, authors reported a PFS of 71%[70].

1.3.2. B lymphocytes

B lymphocytes are responsible for humoral immunity in the adaptive immune system, which act through antibody secretion[71]. B cell lineage is heavily compromised in MM, with a displaced equilibrium towards a high proliferation of malignant PCs. Indeed, MM patients present both a decrease in $CD19^+$ B cells[72,73] inversely correlating with disease stage[73], and a reduced ability to secrete polyclonal immunoglobulins and to differentiate into antibody-secreting plasma cells[72]. Moreover, the risk of progression to symptomatic MM from presymptomatic stages (MGUS and SMM) has been shown to be directly related to the proportion of normal bone marrow PCs at diagnosis[74]. Furthermore, the number of B regulatory (Bregs) cells with $CD19^+CD24^{high}CD38^{high}$ phenotype, increases in the transition from MGUS to symptomatic MM[75], supporting an immunosuppressive BM milieu by both reducing NK-mediated lysis of MM cells and producing IL-10[76].

1.3.3. NK cells

Natural killer cells (NK cells) are cytotoxic lymphocytes from the innate immune system that take part in the early response to viral antigens as well as in the attack against tumor cells, recognizing and eliminating cells that express stress proteins without needing antigen presentation on major histocompatibility complex (MHC) molecules[77]. In fact, activation of NK cells depends on a balance between signals coming through activating natural cytotoxicity receptors (NCRs), other activating receptors and inhibitory receptors. In humans, NCRs include NKp30, NKp46 and NKp44, other important activating receptors are DNAM-1 and NKG2D, while inhibitory receptors include killing inhibitory receptors (KIRs) and NKG2A, among others[78]. Briefly, NK cells recognize malignant cells and kill them through secretion of granzyme B and perforin or alternatively, through the induction of death signaling pathways in which FasL and TRAIL proteins are involved[79].

In the context of the disease, both MGUS and MM patients present an enrichment in NK cell population in comparison to healthy adults[50,51]. Initially, myeloma cells are sensitive to the lysis induced by NK cells since they express high levels of the stress-induced self-antigen MICA (a ligand of NKG2D receptor). In contrast, as the disease evolves, myeloma cells lose MICA expression through MICA shedding, being this latter phenomenon directly correlated to disease progression[80,81]. Regarding the activating receptors NKG2D and DNAM-1, their expression is reduced on NK cells from myeloma patients with active disease compared to patients in remission or healthy individuals[82,83]. Furthermore, unlike healthy donors, NK cells from myeloma patients express PD-1 molecule which mitigates even more their functionality[68].

1.3.4. Dendritic cells (DCs)

Dendritic cells (DCs) are APCs whose main role is the processing of antigenic material which is then displayed on their cell surface to induce naïve T cell activation. They are classified as plasmacytoid DCs (pDCs), which secrete high levels of type I IFN in response to viral antigens and other stimuli, and myeloid DCs (mDCs) which

are rather involved in antigen presentation and induction of $CD4^+$ and $CD8^+$ T cell responses[84,85]. The role and general status of DCs in MM is not clear yet. Many studies have concluded that DCs from MM patients have impaired T-cell stimulation capacities, whereas contradictory results exist regarding the frequency and phenotype of DCs[86–89].

DCs are concentrated in the BM during MGUS to MM progression and are able to process and cross-present antigens from apoptotic MM cells via CD91, thus activating myeloma-specific $CD8^+$ T cells[90]. Conversely, by using their surface CD80/86 molecules, DCs interact with non-apoptotic PCs via their overexpressed CD28 receptor, inducing the secretion of the immunosuppressive enzyme indoleamine-2,3-dioxygenase (IDO)[91] by DCs which impairs the immune surveillance through different mechanisms:

- Metabolizing and therefore depleting tryptophan from the microenvironment, which is an essential amino acid for T cells, and consequently producing kynurenine, a toxic compound for T and NK cells[92].

- Promoting the development, stabilization and activation of regulatory T lymphocytes (Tregs)[93].

- Polarizing macrophages and DCs towards an immunotolerogenic phenotype[94].

Along with IDO, ADO is also able to increase the number of tolerogenic DCs[56]. Moreover, pDCs and a percentage of mDCs also express high surface levels of PD-L1 in MM[95], thus contributing to the maintenance of the immunosuppressive BM microenvironment. In addition, TGF-β contributes to the altered immune tumor niche since it inhibits the upregulation of critical T-cell costimulatory molecules on the surface of DCs, reducing their antigen-presenting capacity[96].

1.3.5. Tumor associated macrophages (TAMs)

Tumor associated macrophages (TAMs) constitute an abundant component of the myeloma microenvironment that enhances myeloma cell survival and drug resistance through different mechanisms[97]. Within the BM niche, TAMs acquire a secretory profile characterized by the production of IL-6, IL-10, and proangiogenic factors, such as VEGF, metalloproteinases (MMPs) and cyclooxygenase-2 (COX-2)[98], providing an optimal milieu for myeloma cell growth. Moreover, TAMs resemble a M2-like macrophage population, with little cytotoxicity against tumor cells because of their limited production of nitric oxide (NO) and proinflammatory cytokines, as well as a poor antigen-presenting capability[99]. Additionally, ADO further polarizes macrophages towards a M2 phenotype[56].

There has been shown that the number of M2 macrophages is significantly increased in the BM of MM patients compared with MGUS and SMM, as well as with healthy donors, suggesting that malignant plasma cells may be involved in this change to a M2-like phenotype[100]. A recent single-cell RNA sequencing study revealed that mature $CD14^+$ monocytes lose the surface expression of HLA-II molecules as early as in the MGUS stage, resulting in T cell suppression, and indicating that some of these sequential immune alterations begin on an early stage of the disease[51].

1.3.6. Myeloid-derived suppressor cells (MDSCs) and neutrophils

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous group of immature myeloid cells endowed with the capacity to suppress the activation, proliferation and cytotoxic capacity of effector T and NK cells. In humans there are two subsets of MDSCs: granulocytic-MDSCs (G-MDSCs) (also called polymorphonuclear, PMN-MDSCs), and monocytic-MDSCs (M-MDSCs)[101]. In myeloma, G-MDSCs constitutes the predominant MDSC population in BM and PB[102]. MDSCs mainly suppress T cell responses by producing reactive oxygen species (ROS) and high amounts of NO, arginase-1 (Arg-1), and immunosuppressive cytokines such as IL-10. Moreover, it has also been shown that MDSCs from MM patients are able to induce higher Treg differentiation than those from healthy age-matched donors[103]. In addition, MM cells promote the survival of

MDSCs through Mcl-1 upregulation[104], secretion of IL-6 which drives MDSCs expansion by the activation of several molecular cascades such as PI3K/Akt or JAK/STAT3[105,106], and through the accumulation of high levels of ADO by the conversion of NAD^+[56,107].

Neutrophils are myeloid cells that constitute the most abundant white blood cell in PB, and are essential for clearance of extracellular pathogens, both by direct toxicity and by establishing interactions with other immune cells[108]. In the context of MM, neutrophils present functional defects, such as a reduction in lysozyme activity, and an increased secretion of Arg-1 therefore presenting an immunosuppressive behavior[109,110]. In fact, MM patients have increased serum levels of Arg-1[109], which depletes arginine in the tumor microenvironment, an essential amino acid for T and NK cell proliferation[111]. Furthermore, as disease evolves from MGUS to MM, neutrophils progressively activate their JAK-2/STAT3 pathway in response to MM cell exposure, which further supports the immunotolerogenic niche due to the production of pro-inflammatory and survival signals[112].

1.3.7. Regulatory T lymphocytes (Tregs)

Regulatory T lymphocytes (Tregs) are a subpopulation of T cells that modulate the immune system, maintain tolerance to autoantigens and prevent autoimmune reactions. They exert their immunosuppressive activity through cell-to-cell contact, secreting immunosuppressive cytokines such as TGF-β and IL-10[113] or inducing the expression of IDO in DCs, which induces a positive loop since IDO, together with ADO, promotes the expansion of Tregs[56,114]. Furthermore, in BM samples from NDMM patients, CTLA-4 appears to be overexpressed in Tregs, which suggests a local accumulation of Tregs in the tumor microenvironment[115]. In fact, it has been described that CTLA-4 induces Treg expansion and the production of immunosuppressive cytokines in this population[116].

Most authors have reported that MM patients have a higher Treg percentage[51,117–120] in comparison to healthy donors, which suggests that myeloma cells escape from the immune surveillance at least partially through the increase of

this population. Interestingly, Treg levels may be used as a biomarker of disease progression, and patients with higher Treg percentage presented a lower OS[117,121].

1.4. APPROVED DRUGS FOR THE TREATMENT OF MULTIPLE MYELOMA EXCLUDING IMMUNOTHERAPY

Therapy regimens in MM have undergone a marked evolution during the last decades, moving from chemotherapeutic agents to targeted drug therapies, such as proteasome inhibitors (PIs) and immunomodulatory drugs (IMiDs) which more specifically affect the growth and survival mechanisms of myeloma cells. In this sense, alkylators, such as melphalan, and glucocorticoids were the standard of care regimens for over 30 years, providing a median OS of just 2-3 years. However, with the approval of IMiDs or PIs, among others, the median OS raised up to 6 years[122] and it is expected that it could exceed 10 years based on data relative to patients diagnosed after 2015[123].

Hereunder, approved drugs for the treatment of MM will be discussed, excluding immunotherapy, mainly focusing on their mechanisms of action directly exerted on tumor cells. Their immunostimulatory effects or the activities on the BM microenvironment will be reviewed in a later section.

1.4.1. Glucocorticoids

Glucocorticoids, such as prednisone and dexamethasone, are synthetic steroids that have been used to treat MM for over 50 years. They bind cytosolic glucocorticoid receptors which then translocate to the nucleus to modulate gene expression. For instance, glucocorticoids repress target genes through inhibitory interactions with transcription factors including NFκB and activator protein-1 (AP-1)[124], ultimately inducing apoptosis.

Nowadays, glucocorticoids are used as part of combinatorial treatment regimens to promote synergistic effects[125]. In this sense, they have demonstrated to improve

the anti-myeloma effect of IMiDs[126] and PIs[127]. In fact, dexamethasone-free regimens continue to be the exception rather than the rule.

1.4.2. Alkylating agents

Alkylators, together with glucocorticoids, were the first effective myeloma drugs to be used in the clinical setting. Their mechanism of action is based on the addition of an alkyl group to DNA, thereby inhibiting DNA and RNA synthesis, and therefore, leading to cell death[128].

Therapy with melphalan in combination with corticosteroids has been the standard of care for NDMM patients for almost 40 years. Furthermore, melphalan has been combined with PIs[129] and recently has also shown promising results when administered with monoclonal antibodies (mAbs)[130]. Nevertheless, the main current use of melphalan is for the conditioning in autologous stem cell transplantation (ASCT)[131].

Cyclophosphamide, which presents a safer profile in comparison to melphalan, has been used as an alternative to melphalan for years. Nowadays it is a good partner for combination, due to its potential immune stimulating properties, with IMiDs and several other agents[132].

Regarding bendamustine, there are no phase-3 trials in MM with this agent. However, it is known that it is the unique alkylator that does not present overlapping resistance with the others; this is thought to be due to its distinctive cytotoxic mechanism, since it activates the base excision DNA repair pathway instead of alkyltransferase DNA repair mechanism[133].

Melflufen (acronym derived from melphalan flufenamide), is a first-in-class peptide-drug conjugate that is hydrolyzed by aminopeptidases and rapidly releases the alkylating agent into tumor cells inducing irreversible DNA damage, and therefore apoptosis[134]. This drug, which has been recently approved by the United States Food and Drug administration (FDA)[135], has demonstrated durable responses in RRMM patients with an acceptable toxicity profile[136].

1.4.3. Proteasome inhibitors (PIs)

One of the most outbreaking advances in the treatment of MM was the discovery of PIs which demonstrated a stunning efficacy in the clinical setting. The proteasome is a protease complex that exerts the selective hydrolysis of client proteins. This complex is important in the MM context since due to their high immunoglobulin synthesis, myeloma cells hold a high rate of misfolded proteins which ultimately need to be degraded in the proteasome. There are currently three PIs approved for the treatment of MM: bortezomib, carfilzomib and ixazomib. Unlike bortezomib and carfilzomib, which are administered mainly intravenously or subcutaneously (bortezomib), ixazomib is the first approved PI which is orally available[137].

Bortezomib and ixazomib are reversible inhibitors of the β5 catalytic subunit of the proteasome, whereas carfilzomib is an irreversible inhibitor. In general, proteasome inhibition reduces the clearance of misfolded proteins which results in cytotoxicity. Additionally, PIs exert many other different effects which contribute to their anti-myeloma activity including, the accumulation of tumor suppressor proteins, such as p53, the underexpression of adhesion molecules, the inhibition of effectors responsible for DNA repair, the inhibition of angiogenesis and the blockade of NFκB transcription factor[137]. Furthermore, PIs have been reported to have bone anabolic and antiresorptive effects, thus adding a benefit in restraining myeloma-associated osteolytic lesions[138].

In addition to the mentioned direct effects, PIs have recently been shown to exert immunogenic cell death (ICD), which will be reviewed in the next section.

1.4.4. Immunomodulatory drugs (IMiDs)

Together with PIs, IMiDs have notably contributed to the improved outcome of MM patients. IMiDs, which include thalidomide, lenalidomide and pomalidomide, are now part of the backbone therapy for both newly diagnosed and relapsed disease. Thalidomide was used for the first time in the 1990's for the treatment of MM through a compassionate-use protocol[139]. The significant response achieved in subsequent

studies prompted the approval of thalidomide in combination with dexamethasone by the FDA in 2006 for the treatment of NDMM[140,141]. With respect to lenalidomide, FDA approved its use in combination with dexamethasone in 2006 for the treatment of RRMM, and some years later, in 2015, the combination was approved for the treatment of NDMM patients[142]. The other second generation IMiD agent, pomalidomide, was approved in 2013 for the treatment of RRMM patients that had previously received at least two prior regimens including lenalidomide and bortezomib[142].

Cereblon (CRBN), the first described target for IMiDs, is a receptor component of the cullin-4 RING E3 ligase protein complex which mediates the ubiquitination and consequent degradation through the proteasome of target proteins. After CRBN binding to an IMiD, the substrate specificity of the E3 ligase complex is altered, so that other proteins get ubiquitinated and later degraded in the proteasome. Among these new substrates, IKF1 (Ikaros) and IKZF3 (Aiolos) stand out as key PC transcription factors whose expression is greatly downregulated in myeloma cells after treatment with IMiDs. The reduction of IKZF1 and IKZF3 expression leads to a downregulation of the interferon regulatory factor 4 (IRF4) and its target genes *MYC*, *CDK4* and *CASP*, which decreases the survival of myeloma cells[143]. Thus, IMIDs display a wide range of biological activities, many of them relevant from an anti-myeloma perspective. Indeed, IMiDs have anti-angiogenic properties[144], inhibit NFκB, decrease IRF-4 production, activate caspases and increase pro-apoptotic factors while decreasing anti-apoptotic signals[126,145–147]. The effect of IMiDs over the immune system will be reviewed in following sections.

1.4.5. Histone deacetylase inhibitors (HDACi)

As previously mentioned, HDACs are deregulated in MM. In this sense, panobinostat is a-first-in-class pan-HDAC inhibitor that was approved in 2015 by the FDA in combination with bortezomib and dexamethasone for the treatment of MM, in patients who had received at least two prior regimens, including bortezomib and IMiDs[148].

Panobinostat exerts its anti-myeloma efficacy mainly in two different ways: by inhibition of the deacetylation state of histones leading to the transcriptional activation of tumor suppressor genes[149], and by the acetylation of other non-histone proteins such as tubulin. This fact, for example, leads to the inhibition of aggresome formation. The aggresome is an alternative cleavage pathway of misfolded proteins when the proteasome system is not able to eliminate misfolded ubiquitinated proteins. Indeed, this is the basis of the simultaneous combination of bortezomib and panobinostat in the clinic: bortezomib inhibits the proteasome cleavage mechanism alternatively inducing aggresome formation while panobinostat blocks aggresome formation. This double inhibition increases the accumulation of excess proteins eventually leading to cell death[150].

1.4.6. Exportin inhibitors

In 2019, the FDA approved selinexor in combination with dexamethasone for the treatment of RRMM patients who had received at least four prior therapies and whose disease was refractory to at least two PIs, at least two IMiDs, and an anti-CD38 monoclonal antibody[151].

Selinexor is a selective inhibitor of exportin-1 (XPO1), which is a molecule overexpressed in many cancers, including MM[152]. XPO1 is the major nuclear exporter for tumor suppressor proteins, such as p53 or p21, growth regulators and oncoprotein mRNAs (e.g. c-Myc or Bcl-xL). Selective inhibition of XPO1 by selinexor results in accumulation of tumor suppressor proteins in the nucleus, decreased levels of oncoproteins, cell cycle arrest and apoptosis[153].

1.5. IMMUNOTHERAPY IN MULTIPLE MYELOMA

Despite all the different therapeutic approaches that have been mentioned so far, MM is still an incurable disease and patients eventually relapse. In the last years, however, different immunotherapeutic treatments have been developed in order to improve patients' outcome. The main current immunotherapeutic approaches for the

treatment of MM include: (naked) mAbs, antibody–drug conjugates (ADC), bispecific antibodies and chimeric antigen receptor T-cell therapies (CAR-T)[154]. Among them, mAbs have demonstrated high efficacy and good tolerability being three of them approved so far (daratumumab, elotuzumab and isatuximab). Regarding ADCs, one of them (belantamaf mafodotin) has been approved for MM. Also, in March 2021 the first CAR-T cell therapy (idecabtagene vicleucel) was approved for the treatment of RRMM patients and in February 2022 the FDA approved the CAR-T ciltacabtagene autoleucel for the treatment of RRMM patients. Although there are currently no approved bispecific antibodies in MM, some of them are in very advanced phase of clinical development. Next, all six approved immunotherapeutic agents and their mechanisms of action will be described.

1.5.1. Main mechanisms of action of monoclonal antibodies (mAbs)

Antibodies (Abs) are glycoproteins that belong to the immunoglobulin (Ig) superfamily. Abs are formed of two heavy and two light chains and are grouped into different isotypes according to their heavy chain. Specifically, therapeutic mAbs are usually IgG isotype. The hypervariable region of each heavy and light chain combine to form the antigen binding site (Fab; fragment antigen binding domain), whereas the fragment crystallizable (Fc) domain, which is responsible for effector functions, is composed of two constant domains (Figure 5)[155].

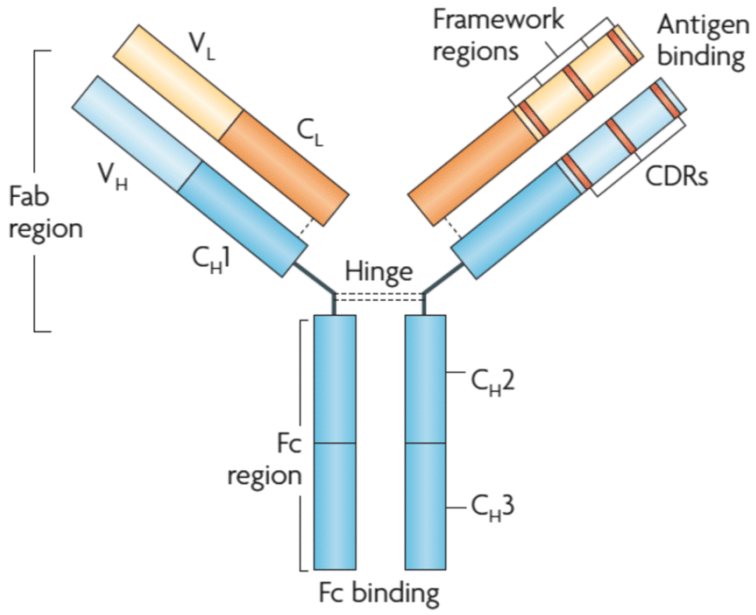

Figure 5. Antibody structure. The heavy chains contain a variable domain (V_H), a hinge region and three constant (C_H1, C_H2 and C_H3) domains. The light chains contain one variable (V_L) and one constant (C_L) domain. The structure can also be divided into the Fragment antigen binding (Fab) region and the fragment crystallizable (Fc) domain. Modified from Buss et al.[156].

The main mechanisms of action typically described for mAbs to exert cytotoxic effects on tumor cells are: induction of apoptosis, complement dependent cytotoxicity (CDC), antibody dependent cellular cytotoxicity (ADCC) and antibody dependent cellular phagocytosis (ADCP).

1.5.1.1. Apoptosis induction

Antibodies can target cell killing by inducing proapoptotic mechanisms or by enhancing the susceptibility of target cells to cytotoxic therapy through the inhibition of antiapoptotic pathways. The mechanisms by which mAbs induce apoptosis are diverse (Figure 6).

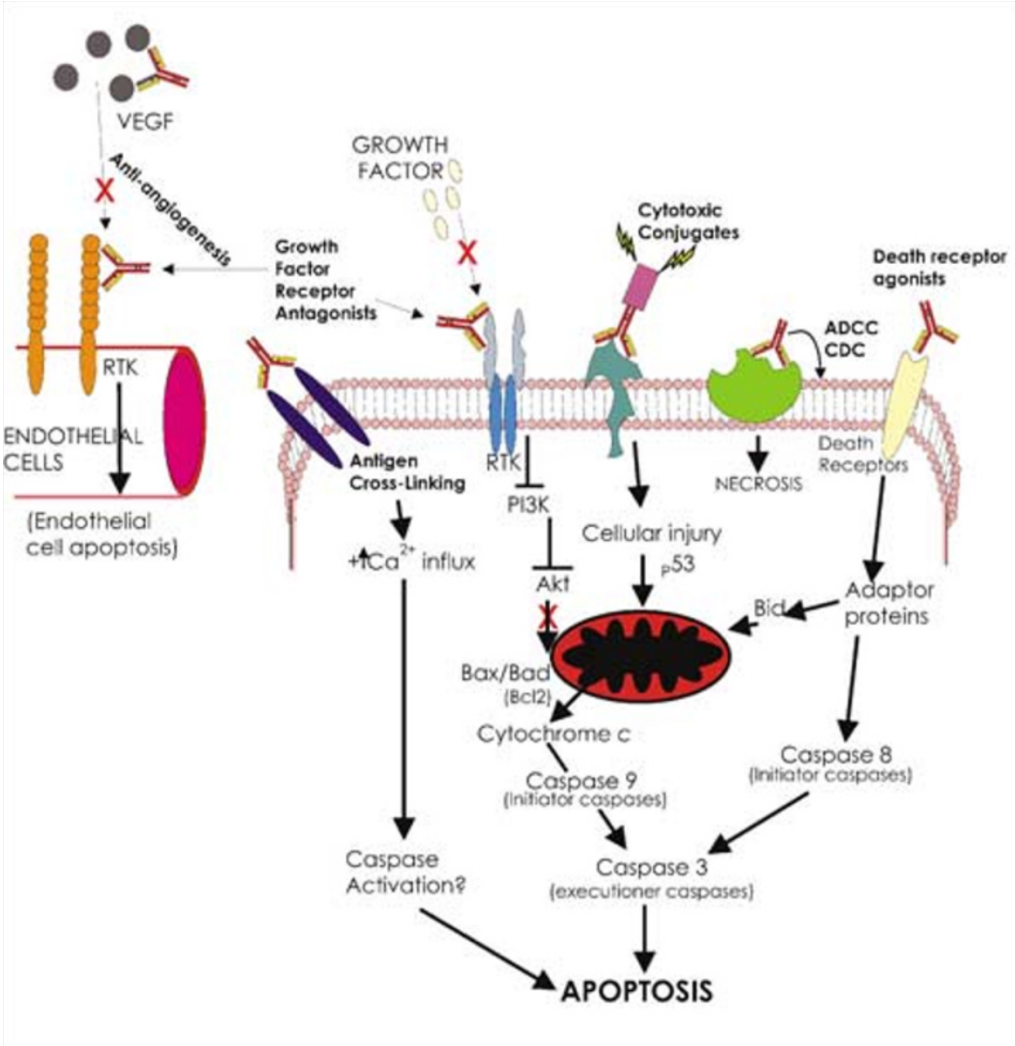

Figure 6. Antibody therapeutic strategies and the signaling mechanisms that can lead to the induction of apoptosis in targeted tumor cells[157].

On the one hand, the interaction between the mAb and the antigen on the tumor cell can induce apoptosis via the extrinsic pathway[158]. Briefly, the apoptotic extrinsic pathway is activated when members of the tumor necrosis factor (TNF) family, such as FASL, TNF-α, or Apo2L (also known as TRAIL), bind to the plasma membrane death receptors (DRs) Fas, TNFR1 and TRAIL-R, respectively. The binding of the DRs with their respective ligands recruits the adapter proteins FADD (Fas-associated Death Domain) and/or TRADD (TNFR1- associated Death Domain), as well as caspase 8, which ultimately, triggers the directed proteolysis of anti-apoptotic proteins or cell cycle regulators, among which Bcl-2 and cyclin E stand out[159]. Therefore, the binding of agonistic mAb targeting the DRs can induce cell death.

Furthermore, antibodies targeting growth factor receptors are able to exert a direct effect on the growth and survival of the tumor cell by antagonizing ligand–receptor signaling. When the mAb binds its antigen, growth factor signaling mediated by receptor tyrosine kinase (RTK) autophosphorylation is inhibited, resulting in the arrest of tumor cell growth. In addition, since growth factor activation may also initiate antiapoptotic signaling, blocking antibodies may therefore reduce tumor cell survival mechanisms[157].

Another example of mAb induced apoptosis are antibodies that can be targeted to cell surface antigens and directly elicit apoptotic signaling. Example of these are antibodies that crosslink targeted surface antigen on tumor cells. Nevertheless, the mechanism by which these antibodies can induce apoptosis may be somewhat atypical since it appears to be insensitive to Bcl-2, is not dependent on Fas, and is only partially blocked by a caspase inhibitor. For instance, regarding the anti-CD20 rituximab, it has been proposed that increases in intracellular Ca^{2+} levels, which occur in response to anti-CD20 crosslinking, may be involved in the apoptotic process, but the precise mechanism remains to be elucidated[157].

1.5.1.2. Complement dependent cytotoxicity (CDC)

CDC is another cell-killing method that can be induced by antibodies. There are three pathways for complement cascade activation: the classical pathway, the alternative pathway and the lectin pathway, being the first the one that is activated after the binding of mAbs to the antigen. In this sense, the formation of antigen-antibody complexes results in the uncloaking of multiple C1q binding sites in proximity to the C_H2 domains of the mAb molecules. The binding of the complement component C1q to its uncloaked binding sites triggers a cascade of events that involves a series of other complement proteins and leads to the proteolytic release of C3b and eventually of C5b. The complement cascade ends up with the formation of a membrane attack complex (MAC), also known as C5b-9, which creates pores in the cell membrane, provoking cell lysis[160]. Specifically, once the MAC complex is formed, Ca^{2+} concentration increases within the cell, although the impact that this may have on the cell death process still awaits clarification. In addition, it has been

recently described that the MAC complex may activate different proteins (RIPK1, RIPK3, MLKL, JNK and Bid) that are responsible for the regulated necrosis also known as necroptosis[161] (Figure 7). Nevertheless, blocking any of these five proteins decreases CDC but not completely, suggesting that this cascade acts in concert with other death-promoting processes, Ca^{2+}-dependent or independent, still pendant of characterization[161].

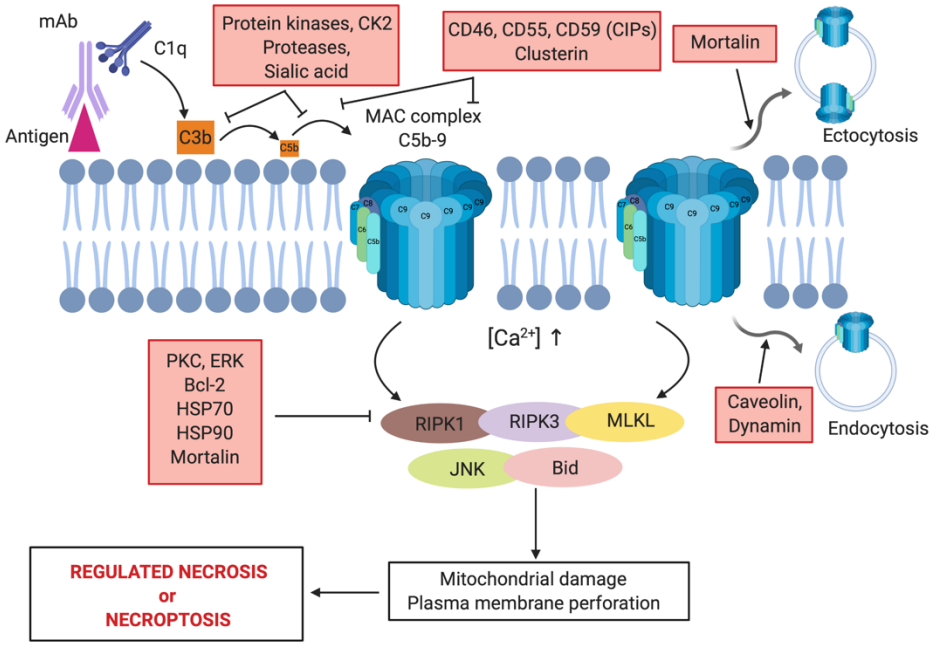

Figure 7. Schematic representation of the cascade of complement dependent cytotoxicity after mAb binding. Red boxes refer to inhibitors of the cascade at different stages. Modified from Fishelson et al.[161], figure made with BioRender tool.

The regulation of the complement-induced cell death mainly occurs at two stages: before the beginning of intracellular signaling cascade and after this event. On the one hand, before the MAC complex is inserted into the plasma membrane and triggers the intracellular signaling cascade, the presence of membrane regulatory proteins like CD55, CD59 or CD46, may inhibit the oligomerization of the MAC complex. Additionally, cell surface proteases, protein kinases and sialic acid are able to degrade the deposited complement proteins. For instance, ecto-protein kinases have been found to phosphorylate C9 inhibiting MAC formation. In line with this, clusterin is a multifunctional soluble protein that participates in the control of the lytic activity of the MAC complex by binding to the C5b-7 complex, preventing the addition

of C8 and C9 and therefore, rendering the MAC complex water soluble and lytically inactive[162,163]. Another additional mechanism to avoid complement attack is to eliminate the MAC complex from the cell surface by either endocytosis or exocytosis in which protein kinase C (PKC) and extracellular signal-regulated kinase (ERK) seem to be involved[164]. Specifically, MAC endocytosis depends on caveolae and dynamin-dependent intracellular release whereas MAC removal by exo-vesiculation requires the expression of the mitochondrial stress protein mortalin/GRP75[165]. In many cases, tumor cells overexpress some of these complement regulatory mechanisms to counteract the MAC-induced effects[161].

On the other hand, protein phosphorylation events involving PKC, mitogen-activated protein kinase kinase (MEK) and ERK support the survival of cancer cells undergoing a MAC attack. Furthermore, proteins of the heat shock protein family (HSPs) have also been described to contribute to intrinsic resistance to CDC[161]. It has been suggested that Bcl-2 could also be involved in the inhibition of CDC mechanism since it inhibits Bid, but the specific mechanisms of this process need to be further explored[161].

1.5.1.3. Antibody dependent cellular cytotoxicity (ADCC)

For ADCC to occur it is essential the interaction of the mAb with an Fc receptor present on the effector cells. In the MM context, much of what we know so far is that NK cells are the main effector cells responsible for ADCC mediated by the approved mAbs. NK cells express on their cell surface the Immunoglobulin Gamma Fc Region Receptor III-A (FcγRIIIA), also known as CD16, which plays a crucial role in the signaling cascade involved in ADCC. Specifically, CD16 contains immunoreceptor tyrosine-based activation motifs (ITAMs), which are consensus sequences containing tyrosine residues susceptible to phosphorylation by Src kinase. Indeed, once the Fc region of the mAb binds to CD16, Src kinase phosphorylates ITAMs which recruit and bind the spleen tyrosine kinase (Syk) protein becoming this latter one activated. This latter activation triggers the three main pathways involved in

ADCC: (i) phospholipase C-gamma pathway (PLC-γ); (ii) phosphatidylinositol 3-kinase (PI-3K) pathway and (iii) Vav/Rho-family G-protein pathway (Figure 8)[166].

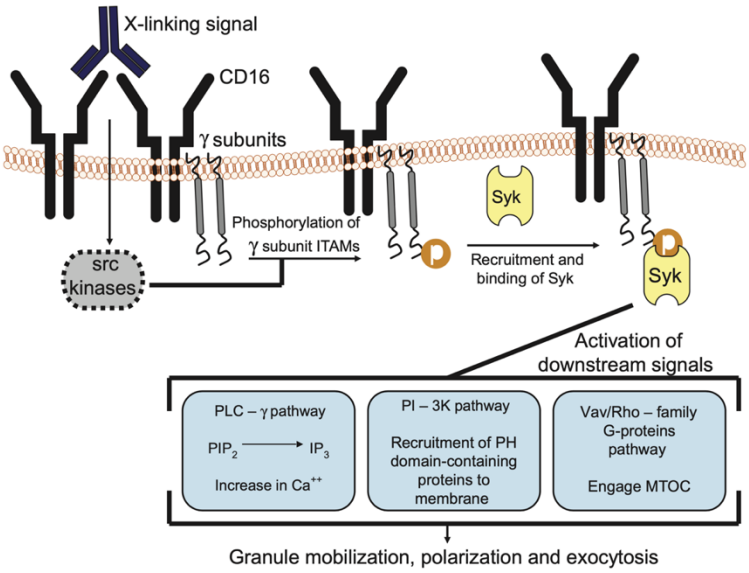

Figure 8. Schematic representation of the signaling pathways underlying ADCC[166].

Due to the activation of the mentioned pathways, NK cells are able to kill target cells through the perforin / granzyme cell death pathway in which granzyme initiates apoptosis mainly through the cleavage of Bid into a truncated form that triggers mitochondrial cytochrome c release, and apoptosome formation leading to caspase activation and manifestation of the apoptosis phenotype[167]. Moreover, NK cells can kill target cells through two other additional mechanisms that have not been so extensively characterized as the previous one: (i) the FAS-L pathway in which NK cells are able to kill target cells that express FAS receptors triggering the already described extrinsic apoptotic pathway; and (ii) the reactive oxygen intermediates/reactive oxygen species (ROI/ROS) or oxidative burst pathway[166].

1.5.1.4. Antibody dependent cellular phagocytosis (ADCP)

The primary immune cell involved in ADCP is the macrophage. Macrophages express FcγRI or CD64 and FcγRIIA or CD32 which, upon binding of the mAb, provokes the activation of the intracellular signaling pathways already mentioned for ADCC mechanism (PLC-γ, PI-3K and Vav/Rho family G-protein pathways), resulting

in the engulfment of the target cell. Then, the phagosome proceeds along a very strictly regulated maturation process characterized by the expression of different molecules such as Rab5 and Lamp-1, as well as a gradual acidification until it fuses with the lysosome, ultimately eliminating the internalized cell[168].

1.5.2. Approved immunotherapeutic treatments in MM

1.5.2.1. Elotuzumab

The first mAb approved by the FDA for the treatment of MM was elotuzumab, a humanized IgG1 anti-SLAMF7 mAb. In particular, elotuzumab was first approved in combination with lenalidomide and dexamethasone for RRMM patients who had received one to three prior therapies[169]. Some years later, the FDA approved it in combination with pomalidomide and dexamethasone for the treatment of adult patients with MM who had received at least two prior therapies, including lenalidomide and a proteasome inhibitor[170]. SLAM Family Member 7 (SLAMF7), also known as CS1, is a cell surface molecule expressed in PCs, $CD8^+$ cytotoxic T lymphocytes, activated B cells, NK cells and mature DCs[171–174]. SLAMF7 is also expressed in both primary malignant PCs and in almost all MM cell lines. In addition, soluble SLAMF7 has been detected in serum of MM patients presenting a direct correlation with disease stage[171,174].

Elotuzumab inhibits MM cell adhesion to BMSCs, which may reverse the protective effect provided by the BM microenvironment to myeloma cells (Figure 9). Additionally, elotuzumab is able to induce ADCC in both MM cell lines and primary plasma cells from myeloma patients (either newly diagnosed or resistant to conventional therapies)[174].

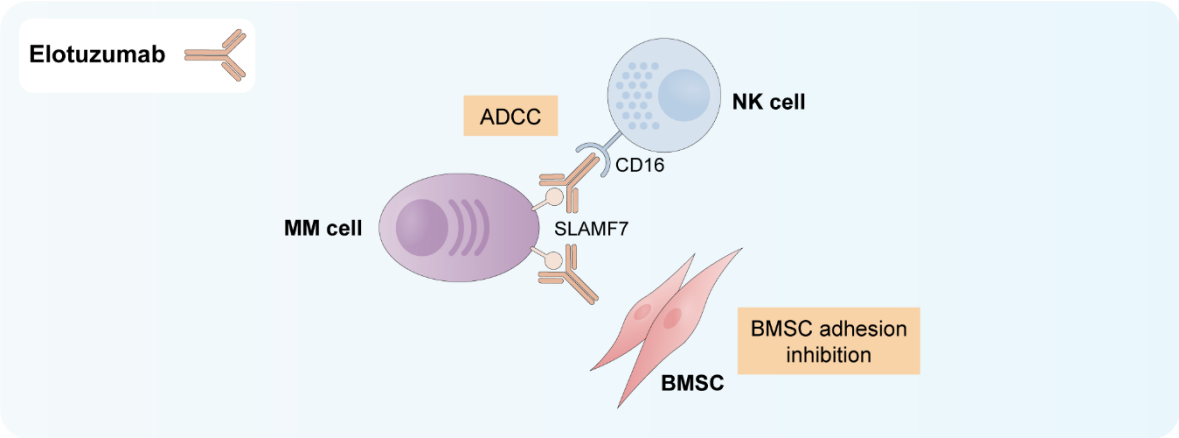

Figure 9. Principal mechanisms of action of elotuzumab in MM. Modified from Díaz-Tejedor et al.[44].

1.5.2.2. Daratumumab

Daratumumab is a humanized IgG1 anti-CD38 mAb that was first approved in 2015 by the FDA for its use in RRMM patients. Since then, it has been approved in different treatment regimens and patients as summarized in Table 1.

Table 1. FDA approval timeline for daratumumab.

Date	FDA approval	Reference
November 2015	In monotherapy for patients who have received at least three prior treatments.	175
November 2016	In combination with lenalidomide and dexamethasone for the treatment of patients who have received at least one prior therapy.	176,177
	In combination with bortezomib and dexamethasone for the treatment of patients who have received at least one prior therapy.	
June 2017	In combination with pomalidomide and dexamethasone for the treatment of patients who have received at least two prior therapies including lenalidomide and a proteasome inhibitor.	178
May 2018	In combination with bortezomib, melphalan and prednisone for the treatment of patients with NDMM who are ineligible for ASCT.	179
June 2019	In combination with lenalidomide and dexamethasone for the treatment of NDMM patients who are ineligible ASCT.	180
September 2019	In combination with bortezomib, thalidomide and dexamethasone for NDMM patients who are eligible for ASCT.	181
August 2020	In combination with carfilzomib plus dexamethasone for the treatment of patients with RRMM who have received one to three previous lines of therapy.	182

CD38 is expressed in different cell subsets from hematopoietic and non-hematopoietic lineages. Noteworthy, $CD138^+$ malignant plasma cells express higher levels of CD38 than other immune subsets and normal plasma cells[183].

Daratumumab was first selected from a panel of 42 human anti-CD38 mAbs for being effective in killing MM cells via CDC and ADCC[184]. Further preclinical studies demonstrated that daratumumab was also able to induce ADCP[185] and apoptosis in the presence of crosslinking agents, both $F(ab)_2$ fragments and Fcγ receptor-expressing cells[186] (Figure 10).

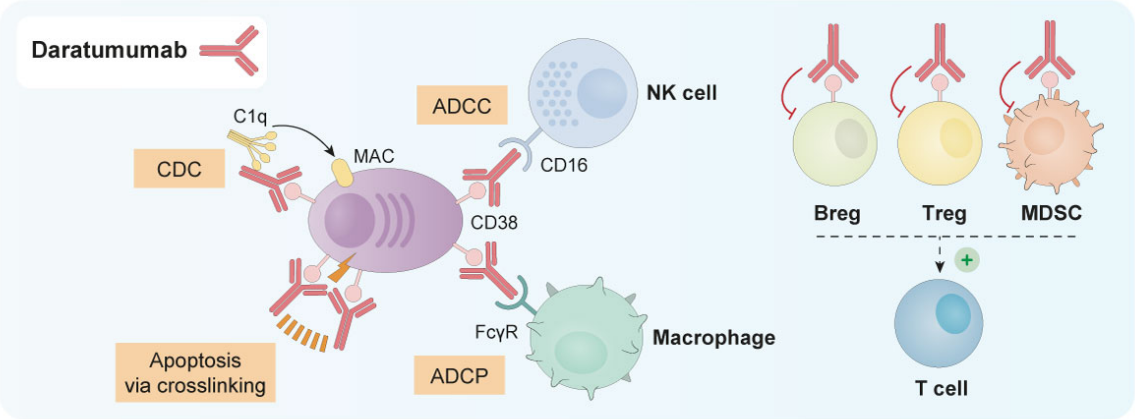

Figure 10. Major mechanisms of action of daratumumab in MM. Modified from Díaz-Tejedor et al.[44].

Given that different immune cell subsets express CD38, daratumumab treatment also has an impact on them. In fact, it has been described that MM patients treated with daratumumab both in monotherapy and in combination with lenalidomide and dexamethasone, show a decrease in NK cells (from 10% to 2%), MDSCs, Bregs and Tregs. On the contrary, other immune populations, such as CD4+ and CD8+ T cells showed increased numbers[120,187–189]. Despite the decrease in NK cell number observed after daratumumab treatment, according to Casneuf et al. the remaining NK cells seemed to be able to contribute to the clinical efficacy of the drug[188]. Furthermore, daratumumab has been reported to induce NK cell activation and degranulation as observed by the upregulation of CD69, CD107a and IFN-γ in this cell subset[190].

1.5.2.3. *Isatuximab*

Isatuximab is a humanized IgG1 anti-CD38 mAb that has been recently approved (March 2020) in combination with either pomalidomide and dexamethasone or carfilzomib and dexamethasone for MM patients who had previously received at least two lines of therapy[191,192]. Isatuximab exerts its anti-myeloma effect through different mechanisms. First, and unlike daratumumab, isatuximab has shown proapoptotic activity against myeloma cells expressing high levels of CD38 without any cross-linking agents[193,194]. Moreover, this mAb also presents immune-mediated cytotoxic effects, such as the induction of CDC, ADCC and ADCP[193,195]. Also, isatuximab completely inhibits the NADase activity of CD38, which may mitigate the immunosuppressive activity of ADO in the BM microenvironment of MM patients[196–198] (Figure 11).

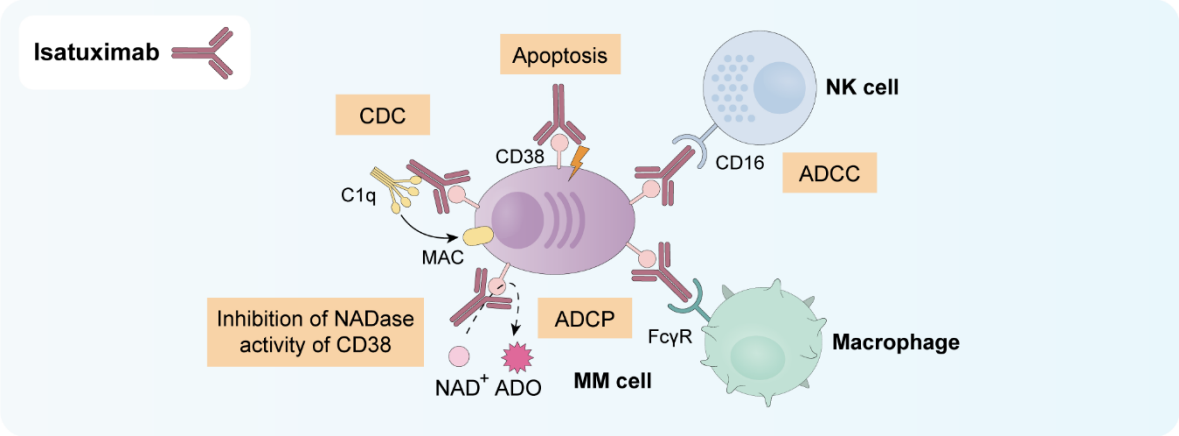

Figure 11. Principal mechanisms of action of isatuximab in MM. Modified from Díaz-Tejedor et al.[44].

1.5.2.4. *Belantamab mafodotin*

Belantamab mafodotin (GSK2857916) is an afucosylated, humanized IgG1 anti-B-cell maturation antigen (BCMA) mAb conjugated with monomethyl auristatin F (MMAF), which is a tubulin polymerization inhibitor[199]. Both parts (anti-BCMA antibody and MMAF toxin) are linked through a non-cleavable maleimidocaproyl linker, which provides better plasma stability of the compound without losing any property and without any nonspecific toxicity[200]. Belantamab mafodotin is the first anti-BCMA ADC approved by the FDA as a single agent for RRMM patients having

received at least four prior therapies[201]. BCMA, also known as TNFRSF-17, is selectively induced during PC differentiation being almost absent on naïve and memory B cells[202,203]. This molecule is also expressed by several myeloma cell lines[204] and high levels of BCMA mRNA are commonly expressed in primary malignant plasma cells[205].

Belantamab mafodotin exerts its anti-myeloma effect through four known mechanisms: (i) ADCC mediated by NK cells; (ii) recruitment of macrophages to promote ADCP; (iii) disruption of microtubules and subsequent G2/M cell-cycle arrest followed by apoptosis after the release of the MMAF toxin in the cytoplasm of myeloma cells[199] and (iv) induction of ICD[200], which is a mechanism characterized by the ability of dying cells to elicit robust adaptive immune responses against altered self-antigens or cancer-derived neo-epitopes[206]. In relation to the latter mechanism, data indicate that treatment of myeloma cells with belantamab mafodotin promotes the exposure of calreticulin (CALR) on their surface and the release of High Mobility Group Box 1 (HMGB1), which subsequently induce the maturation and activation of DCs and eventually the activation of T cells[207] (Figure 12).

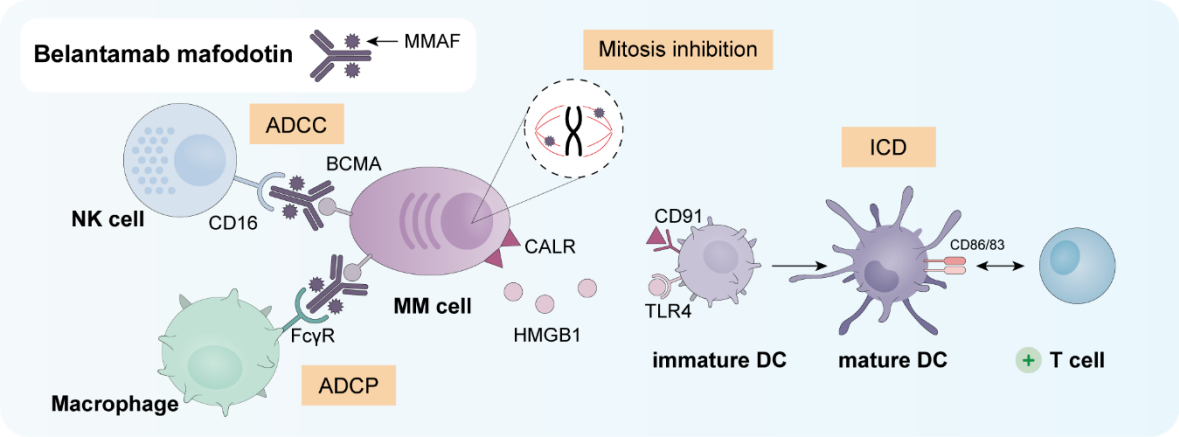

Figure 12. Primary mechanisms of action of belantamab mafodotin in MM. Modified from Díaz-Tejedor et al.[44].

1.5.2.5. CAR-T cell therapy

Idecabtagene vicleucel (ide-cel) is a BCMA–directed CAR T-cell therapy that has been recently approved by the FDA for the treatment of RRMM patients after four or more prior lines of therapy, including an IMiD, a PI and an anti-CD38 mAb.

Importantly, this has been the first FDA-approved cell-based gene therapy for multiple myeloma[208]. Furthermore, in February 2022 the FDA approved ciltacabtagene autoleucel (cilta-cel), another BCMA-directed CAR-T cell therapy, for the treatment of MM patients after four or more prior lines of therapy, including a PI, an IMiD and an anti-CD38 mAb[209].

Briefly, CARs are artificial fusion proteins that consist of an antigen-recognition domain connected to signaling moieties by transmembrane domains. Specifically, antigen-recognition domains consist of the single-chain variable fragment (scFv) derived from antibodies and signaling domains containing both, a co-stimulatory domain such as CD28 or 4-1BB and an activation domain that is usually derived from CD3ζ[210]. As it has already been mentioned, ide-cel targets BCMA in the myeloma cell and it has a 4-1BB molecule as co-stimulatory domain, which allows for better cytokine production and proliferation of the CAR T-cells[211] (Figure 13).

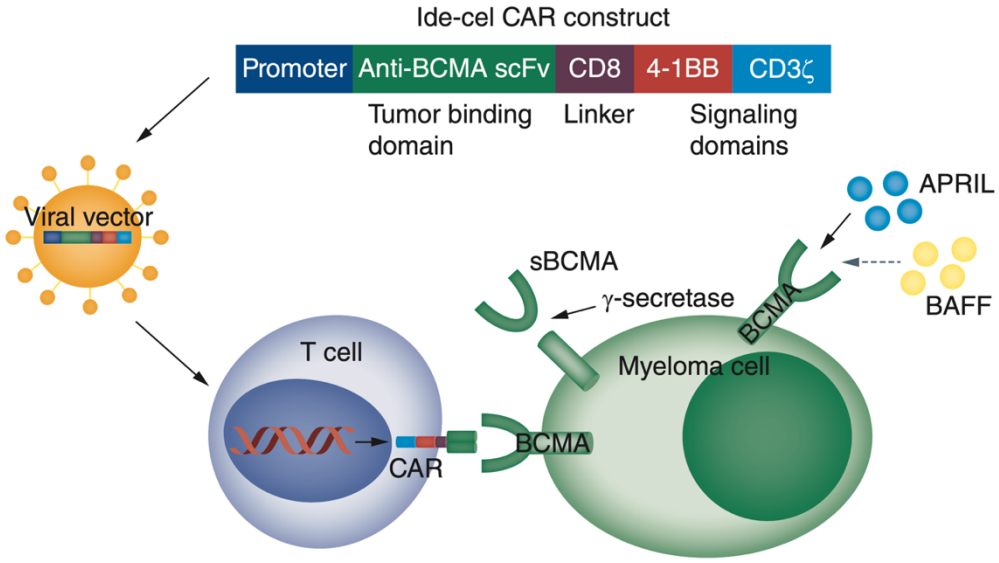

Figure 13. Idecabtagene vicleucel CAR T-cell construct and mechanism[211].

Regarding cilta-cel, T cells from MM patients are genetically modified ex vivo to express a CAR comprising an anti-BCMA targeting domain consisting of two single-domain anti-BCMA antibodies linked to a 4-1BB costimulatory domain and a CD3ζ signaling domain[209].

1.5.3. Drugs with immune-stimulating activity in MM

As described in previous sections, some of the mechanisms of action of mAbs and ADCs require different immune effector cells. Therefore, drugs that either increase the immunogenicity of tumor cells making them more easily recognizable and susceptible to the attack by immune cells, or drugs that activate effector cells could be good partners of these immunotherapies. Indeed, different groups of drugs, such as, alkylating agents, IMiDs, PIs and HDACis, to which some approved drugs for MM treatment belong, have demonstrated immunostimulatory effects as it will be reviewed hereafter (Table 2).

Table 2. Summary of the main immunomodulating effects of alkylating agents, PIs, IMiDs and HDACis. Modified from Díaz-Tejedor et al.[44].

Drug group	Target cell population	Immune Effects
Alkylating agents	Tumor cells	↑ CALR translocation
		↑ release of HMGB1
	DCs	↑ activated phenotype
	MM cells	↑ secretory response
		↓ CD47
	Macrophages	↑ CD64 (↑ ADCP)
Immunomodulatory drugs (IMiDs)	T cells	↑ proliferative responses ↑ IL-2 and IFN-γ
		↑ cytotoxic responses
	NK cells	↑ cytotoxic activity
		↑ Zap-70 phosphorylation
		↑ granzyme-B expression
	DCs	↑ endocytic activity
		↑ MHC Class I and CD86 expression
	MM cells	↑ MICA and PVR expression
		↑ CD38 expression
Proteasome inhibitors (PIs)	Tumor cells	↑ Hsp60 and Hsp90 exposure (↑ ICD induction)
	MM cells	↑ CALR exposure (↑ ICD induction)
Histone deacetylase inhibitors (HDACis)	MM cells	↑ ULPBP2/5/6 and MICA/B expression
		↑ PD-L1 expression
		↑ CD38 expression

1.5.3.1. Alkylating agents

Alkylating agents such as cyclophosphamide have been used in the treatment of MM for over 60 years due to their inhibitory effects on cell division. But, in addition, at low doses, these drugs also present significant immunomodulatory activity[212]. In this sense, treatment of different tumor models with mafosfamide or cyclophosphamide induces CALR translocation to the plasma membrane and the release of HMGB1[213,214], both of them being surrogate ICD markers. Accordingly, cyclophosphamide-treated mice showed an increase in tumor infiltrating DCs with an activated phenotype[213].

In MM there is still no clear evidence of the involvement of the ICD mechanism in the response to cyclophosphamide, although other immune-modulating effects have been observed. Thus, in vitro exposure of MM.1S cells to low doses of cyclophosphamide leads to a secretory response, which, along with downregulation of the "don't eat me" antigen CD47, greatly augments macrophage induced phagocytosis of daratumumab-coated MM cells[215]. Additionally, after cyclophosphamide treatment, macrophages presented increased levels of the CD64 FcγR, possibly further enhancing ADCP[215]. These results have been confirmed in the clinical setting in the CyBorD-DARA trial that combines cyclophosphamide with daratumumab, bortezomib and dexamethasone[216].

1.5.3.2. Immunomodulatory drugs (IMiDs)

Apart from the anti-myeloma effect that has already been mentioned, IMiDs harbor immunomodulatory properties that will be briefly described. Regarding effects over T cells, different authors have reported that this immune cell population increases their cytokine production (i.e. IL-2 and IFN-γ)[217,218] and cytotoxic responses[217,218] after IMiD exposure. IMiDs have also been shown to increase NK cell cytotoxic activity[219–222]. Although this effect was first suggested to occur indirectly via induction of IL-2 production in T cells, the phosphorylation and activation of Zap-70 and the involvement of cereblon have recently been described as two direct and independent mechanisms of pomalidomide-mediated upregulation of granzyme-B

expression and NK cell activity[222]. Interestingly, other authors observed that IMiDs may enhance the susceptibility of myeloma cells to NK cell-mediated recognition and killing by inducing an increased expression of MICA and PVR (the ligand for DNAM-1 receptor) in myeloma cells[223].

It has also been proposed that IMiDs increase anti-tumor immunity by enhancing DC function[224]. Indeed, both pomalidomide and lenalidomide increase the endocytic activity and the expression of MHC Class I and CD86 on DCs. In line with this, when DCs were treated with IMiDs prior to an antigen presentation assay, they effectively increased $CD8^+$ T-cell cross-priming[224].

Finally, since the *CD38* gene seems to be repressed by Ikaros and Aiolos, treatment with lenalidomide increased the surface expression of CD38 in several MM cell lines, leading to higher efficacy of ADCC mediated by daratumumab[225].

1.5.3.3. Proteasome inhibitors (PIs)

As it has already been described, PIs have been incorporated into many combinatorial regimens for the treatment of MM. In addition to their direct effects on tumor cells, PIs can exert ICD. Indeed, treatment with bortezomib in a murine ovarian tumor model resulted in a higher recruitment of $CD8^+$ T lymphocytes into the tumor and higher amounts of tumor-infiltrating IFN-γ^+ T lymphocytes[226]. Moreover, in vitro treatment of ovarian tumor cells with bortezomib led to the surface upregulation of Hsp60 and Hsp90, two ICD markers, which promoted the phagocytosis of these tumor cells by DCs[226]. In MM, the delivery of an activating signal from bortezomib-killed myeloma cells to DCs is mediated by the exposure of Hsp90 on the surface of apoptotic cells[227]. Indeed, DCs pulsed with dying myeloma cells after bortezomib treatment are potent inducers of tumor-specific IFN γ–producing T cells[227]. Furthermore, both bortezomib and carfilzomib promoted the exposure of CALR, another protein marker of ICD, in the plasma membrane in myeloma cell lines[228]. Finally, the combined treatment of carfilzomib and chloroquine (which blocks autophagy) increased both apoptosis and cell surface exposure of CALR, therefore increasing the immunogenic ability of carfilzomib[228].

1.5.3.4. Histone deacetylase inhibitors (HDACis)

HDACis also exert immunogenic effects against MM cells. According to Wu et al. valproic acid (VPA) induced the upregulation of MICA/B and ULBP2 in MM cell lines and patients' myeloma cells, and, consequently, the degranulation and cytotoxic activity of NK cells were enhanced in presence of VPA-pretreated myeloma cells[229]. Additionally, sodium butyrate, another HDACi, upregulates MICA in MM cell lines when combined with a matrix metalloproteinase inhibitor III and phenylarsine oxide, a drug that hinders surface ligand internalization[230]. The pan-HDAC inhibitor panobinostat also upregulates ULPBP2/5/6 and MICA/B in MM cells[231]. Together with panobinostat, entinostat (a class I HDAC-specific inhibitor) and ricolinostat (HDAC6 inhibitor) upregulated PD-L1 in MM cells, probably by histone acetylation of the *PDL1* gene promoter[231].

Interestingly, several HDACi upregulate the expression of CD38[232–234]. In ex vivo cultures, panobinostat and, to a greater extent, ricolinostat upregulated CD38 expression in myeloma cells from both NDMM and RRMM patients, which improved the cytotoxic effects of daratumumab[232,233]. Specifically, the inhibition of HDAC6 by ricolinostat prevents the deacetylation of H3K27 in the *CD38* promoter[233].

Tinostamustine (EDO-S101) is a first-in-class fusion molecule comprising the alkylator bendamustine and the HDAC inhibitor vorinostat, with potent inhibitory activity of both class I and II HDACs[235]. This molecule has shown potent anti-myeloma activity in vitro, in vivo and ex vivo, as a single agent and in combination with bortezomib[236]. Moreover, tinostamustine as a single agent is currently being evaluated in different phase I/II clinical trials for the treatment of advanced solid tumors and relapsed/refractory hematologic malignancies, including RRMM (NCT03345485, NCT02576496). Tinostamustine is also being assessed, in combination with other agents in glioblastoma and advanced melanoma (NCT03452930, NCT03903458). Taking into account that both alkylating agents and HDACis exert immune-stimulating activity, it is likely that the fusion drug tinostamustine may also share this activity therefore potentially being able to

enhance immunotherapies in the context of MM. However, these effects have not been explored yet.

1.6. RESISTANCE MECHANISMS TO MONOCLONAL ANTIBODIES

In general terms, due to the relatively recent introduction of mAbs into the therapeutic armamentarium against MM, the mechanisms of resistance to these drugs are still poorly understood. Nevertheless, resistance mechanisms that have been identified up to date can be divided in the following groups.

1.6.1. Downregulation of target expression

It may seem quite evident that one of the first resistance mechanisms identified for mAbs was the reduced expression of the target molecule. In this regard, it has been broadly described that response to daratumumab is significantly associated with the CD38 basal expression level on tumor cells. Indeed, patients with higher CD38 levels prior to treatment had better responses to the mAb[237–239].

Furthermore, the expression levels of CD38 correlate with daratumumab induced CDC and ADCC in vitro. Specifically, MM cell lines transduced to express higher levels of CD38 exert greater daratumumab-mediated CDC and ADCC than their parental counterpart[237].

A possible cause of acquired resistance to daratumumab is the underexpression of the target after mAb administration; however, the responsible mechanisms are not fully understood. Krejcik et al. reported that CD38 expression on tumor cells is reduced by a process called trogocytosis, whereby CD38-daratumumab complexes are transferred to monocytes and granulocytes[187]. Other authors also reported that daratumumab selectively depleted $CD38^{++}$ MM cells allowing the expansion of $CD38^{-/+}$ MM cells[240]. Moreover, according to Chillemi et al., the binding of daratumumab to its target induces a redistribution of CD38 into polar aggregates in

the plasma membrane which are released to the BM microenvironment through microvesicles[241].

Since ADCs, bispecific mAbs or CAR-Ts also bind specifically to membrane molecules, the underexpression of such molecules could also be involved in resistance to these drugs[242]. In this sense, although there still no evidence relating BCMA basal expression and response to belantamab mafodotin, it is already known that treatment with γ-secretase inhibitors, which inhibit BCMA shedding from the plasma membrane, improves the efficiency of other anti-BCMA therapies, such as, CAR-T[243].

1.6.2. Overexpression of Complement Inhibitory Proteins (CIPs)

The overexpression of the complement inhibitory proteins (CIPs) CD55 and CD59, at the time of disease progression, as compared to the levels before or during daratumumab treatment, has been proposed as another potential mechanism of acquired resistance to daratumumab as reported by Nijhof et al.[238]. Conversely, pretreatment levels of these CIPs on patients' tumor cells did not correlate with the response to daratumumab[238].

1.6.3. Fcγ receptor polymorphisms

As already mentioned, ADCC and ADCP require the presence of FcγRs on effector cells, such as NK cells and macrophages. Polymorphisms in these receptors may also be involved in mAb resistance although results are not conclusive so far.

In a randomized phase II study of elotuzumab + Bd (bortezomib and low dose dexamethasone) versus Bd in RRMM patients, EBd-treated patients homozygous for the high-affinity FcγRIIIA (CD16a) V allele (VV) showed longer PFS than those who were homozygous for the low-affinity FcγRIIIA F allele (FF)[244]. However, a sub-analysis of PFS based on the CD16a genotype showed no significant differences between VV and FF in ELOQUENT-2 clinical trial, which assessed the efficacy of the combination of elotuzumab plus lenalidomide/dexamethasone[245].

Regarding daratumumab, similar results were obtained by Van de Donk et al. since they reported that FcγR polymorphisms only had a modest impact on the response to daratumumab and PFS, but did not significantly affect OS[246].

1.6.4. NK cell count

Since one of the mechanisms of action of all mAbs approved for MM treatment is ADCC, patient NK-cell count prior to treatment could also influence the response. Indeed, high numbers of NK cells prior to elotuzumab administration have been associated with longer PFS[247].

With respect to daratumumab, although preclinical data showed that a lower NK cell:MM cell ratio was associated with decreased ADCC[237], this result was not observed in the clinical setting. Specifically, the decrease in NK cell count is observed at similar levels in responding and non-responding patients and, more importantly, PFS after treatment with daratumumab did not correlate with the amount of NK cell reduction[188].

1.6.5. Immunomodulatory activity

Since daratumumab and isatuximab show immunomodulatory activity, a compensatory upregulation of multiple inhibitory immune checkpoints, may also contribute to development of resistance to the immunomodulatory activities of CD38 antibodies[240]. Indeed, it has been described that T lymphocytes from patients resistant to daratumumab showed an overexpression of the checkpoint inhibitor lymphocyte-activation gene 3 (LAG3) and the T cell immunoreceptor with Ig an ITIM domains (TIGIT) compared to responding patients[248].

HYPOTHESIS AND OBJECTIVES

The introduction of immunotherapeutic approaches into the therapeutic armamentarium of MM has notably improved the response rate, depth of response and survival of patients. In fact, among the different immunotherapeutic treatments, the monoclonal antibody daratumumab has demonstrated efficacy both in monotherapy and in combination therapies. For monoclonal antibodies, the expression of their specific targets by tumor cells, the immunogenic properties of these cells and the immune status of patients influences the efficacy of these treatments. Therefore, drugs able to favor some or all of these aspects are potentially good candidates to be combined with monoclonal antibodies.

Despite the good results obtained with immunotherapy treatments in general, and with monoclonal antibodies in particular, MM is still considered an incurable disease in the vast majority of cases, due, among others, to the presence of resistance (primary or acquired) to the available treatments. However, the mechanisms involved in the acquisition of resistance to monoclonal antibodies are still not well understood. Accordingly, the study of these mechanisms and the search for strategies aimed at overcoming them, constitutes a key field of research in the current therapeutic scenario.

Taking this into account, the specific proposed objectives in our work were:

AIM 1: To analyze the potential role of tinostamustine, an alkylating histone deacetylase inhibitor, in improving the anti-myeloma effect of daratumumab.

1.1. To evaluate the effect of tinostamustine on the surface expression of CD38 in MM cell lines and patients' primary myeloma cells.

1.2. To explore whether treatment of myeloma cells with tinostamustine increases the immunogenicity of these cells through the analysis of surface immune-related molecules, such as ligands for NK cell receptors.

1.3. To determine whether tinostamustine enhances the anti-myeloma effect of daratumumab through its different mechanisms of action using in vitro and ex vivo experiments.

1.4. To evaluate the efficacy of the double combination of tinostamustine and daratumumab through in vivo models.

AIM 2: To identify the molecular causes of the acquired resistance to daratumumab and to explore new strategies to reverse them.

2.1. To generate an in vitro model of acquired resistance to daratumumab-mediated CDC and characterize its immunophenotype and sensitivity profile to additional daratumumab's mechanisms of action.

2.2. To analyze the mechanisms involved in the deregulation of CD38, the target of daratumumab, using the generated resistance model.

2.3. To identify potential genes involved in the acquired resistance to daratumumab-mediated CDC and assess their functional implication.

2.4. To establish new in vitro models of sensitivity to daratumumab-mediated CDC.

MATERIALS AND METHODS

Drugs

Tinostamustine (EDO-S101) was provided by Mundipharma (Basel, Switzerland) and daratumumab and isatuximab were obtained from the Pharmacy Department of the University Hospital of Salamanca. Daratumumab isotype control IgG1 was purchased from Sigma-Aldrich (San Luis, MO, USA).

Cell lines, primary samples and cultures

The human myeloma cell lines MM.1S, U266 and NCI-H929 were purchased from ATCC (Manassas, VA, USA) whereas RPMI-8226, JJN3, KMS12-BM, MOLP-8 and OPM-2 were obtained from DSMZ (Braunschweig, Germany). HEK-293T cells were also acquired from DSMZ. Briefly, MM cell lines were cultured in RPMI-1640 medium supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin and 10% fetal bovine serum (FBS) at 37ºC and 5% CO_2, except for MOLP-8 which was cultured with 20% FBS. HEK-293T cell line was cultured in DMEM medium supplemented as indicated above. Cell culture media, serum, and penicillin-streptomycin were purchased from Invitrogen Corporation (Gaithersburg, MD, USA). Origin and authentication of MM cell lines have been previously reported[249].

BM samples from MM patients used in ex vivo cultures were obtained after approval from the University Hospital of Salamanca Review Board (ethical code: 06/799), and after written informed consent from patients. Research with human samples was conducted in accordance with ethical standards and principles expressed in the Declaration of Helsinki.

Cell viability, apoptosis and cell cycle assays

Viability of MM cell lines (except for the MOLP-8 cell line) was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as

previously explained[250]. Briefly, MTT assay is a colorimetric method that quantitatively measures the metabolic activity of viable cells, which reduce the MTT yellow tetrazolium salt to a dark blue, water-insoluble compound (formazan). This compound is solubilized with a 10% SDS and 0.01 M HCl solution, and subsequently absorbance is measured, being directly proportional to the number of viable cells. Specifically, we measured absorbance in a TECAN plate reader (Tecan, Männedorf, Switzerland), with a reading wavelength of 570 nm and a reference wavelength of 630 nm. For each drug and cell line, 3 independent experiments were performed seeding quadruplicates for each condition.

In experiments to test the viability of the MOLP-8 cell line, Cell Titer Glo (CTG) from Promega (Madison, WI, USA) was used. CTG is a method based on quantification of the ATP present in metabolically active cells. Specifically, after adding the luciferin containing lysis buffer to the culture it results in cell lysis and generation of a luminescent signal, proportional to the amount of ATP present, which is measured in a luminometer. As for MTT assay, for each drug and cell line, 3 independent experiments were performed with technical quadruplicates for each condition.

Apoptosis assays were measured in a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) using an Annexin V / 7AAD kit (Immunostep, Salamanca, Spain), as described elsewhere[251].

Cell cycle analysis was performed by fixing the cells in 70% ethanol for a minimum of 24 hours and subsequently staining with propidium iodide (PI), an intercalating agent that allows DNA content quantification, and therefore, identification of cell cycle phases.

All flow cytometry experiments were analyzed using Infinicyt™ software (Cytognos, Salamanca, Spain).

Flow cytometry antibodies and staining

Mouse anti-human CD138-FITC, CD38-FITC, CD56-PE, CD46-PE, CD59-PE, CD55-PE, CD45-PerCP-Cy5.5, CD19-PE-Cy7, CD38-APC, CD117-APC, CD81-APC-C750, CD138-BV421, CD27-BV510 and the IgG1 κ-APC isotype control were obtained from BD Biosciences. Mouse anti-human MICA-Alexa Fluor 488, MICB-Alexa Fluor 488, ULBP1-PE, ULBP3-PE, CD155-APC (PVR), CD112-APC (Nectin-2) and the corresponding isotype controls IgG2B-Alexa Fluor 488, IgG2A-PE and IgG1 κ-APC were purchased from R&D Systems (Minneapolis, MN, USA). Mouse anti-human unconjugated ULBP-2 was obtained from Sigma-Aldrich. CD38me-FITC (anti-human CD38 multi-epitope) was purchased from Cytognos (Salamanca, Spain) and CD45-APC from Immunostep (Salamanca, Spain).

For surface direct staining, cells were incubated with the corresponding antibodies and 7AAD (Immunostep) for 15 min in the dark. Data were acquired using a FACSCalibur cytometer or a FACSCanto II (BD Bioscience) and analyzed using Infinicyt^{TM} software. The expression of the different surface molecules was determined over the viable cell population (7AAD^{-}). In particular, expression of CD38 in myeloma cells of patients' BM samples was analyzed with the next panel: CD138-FITC / CD56-PE / 7AAD / CD38-APC. Similarly, the expression of MICA and MICB in these cells was determined using the following panel: MICA/B-AF488 / CD56-PE / 7AAD / CD38-APC. Normalized median fluorescence intensity (MFI) was calculated by dividing median fluorescence obtained with the specific antibody by median fluorescence with the corresponding isotype control. Normalized MFI for each treatment condition was expressed as a percentage relative to the control condition as specified in each experiment.

Evaluation of daratumumab binding to myeloma cells

For the evaluation of daratumumab binding to myeloma cells, daratumumab (10 μg/ml) was added to the cells and incubated for 30 minutes. After that time, the cells were washed with PBS twice and then an anti-human-IgG1-AlexaFluor488 (Invitrogen, Carlsbad, CA, USA) was added and incubated during 30 additional

minutes. Cells were acquired in a FACSCalibur cytometer and normalized MFI was calculated as described above.

Ex vivo analysis of treatment efficacy in BM samples from myeloma patients

BM samples from myeloma patients were lysed to remove red blood cells and cultured as previously described[251], in the presence of daratumumab (10 μg/ml), tinostamustine (0.5 – 1 μM), the double combination of daratumumab + tinostamustine or daratumumab's isotype control for 24 hours. After the incubation period, cells were collected in BD Trucount™ Absolute Counting Tubes (Becton Dickinson, Franklin Lakes, NJ) and stained with the corresponding antibodies (CD38me-FITC / CD45-PerCP-Cy5.5 / CD56-PE) for flow cytometry analysis in a FACSCalibur cytometer. The percentage of eliminated cells was calculated following manufacturer's instructions on myelomatous plasma cells ($CD38^{+bright}$, $CD45^{-/low}$, $SSC^{low/intermediate}$, $CD56^{-/+}$) and normal lymphocytes ($CD45^{++}$, SSC^{low}) as described somewhere else[252].

Evaluation of daratumumab's mechanisms of action (MoA)

Apoptosis via cross-linking. MM cell lines were incubated for 24 and 48 hours at 37 ºC in the presence of 1 μg/ml daratumumab[186] or its isotype control, with the addition of 10 μg/ml $F(ab)_2$ fragments (The Jackson Laboratory, Bar Harbor, ME, USA) for cross-linking induction. Apoptosis was evaluated by flow cytometry following Annexin V-FITC / 7AAD staining.

CDC. MM cell lines were incubated in the presence of daratumumab (0.01 – 100 μg/ml) or its isotype control with human serum (10%) as source of complement during 4 hours at 37 ºC. After the incubation time, the percentage of dead cells was evaluated by flow cytometry (Annexin V-FITC / 7AAD).

ADCC. To obtain NK cells, peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation with Lymphoprep™ (Stemcell Technologies, Vancouver, Canada) from healthy donors' buffy coats acquired from *Biobanco del Centro de Hemoterapia y Hemodonación de Castilla y León* (Valladolid, Spain). Afterwards, NK cells were isolated by immunomagnetic negative selection using the NK Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) in an autoMACS® Pro Separator (Miltenyi Biotec) following manufacturer's instructions. NK cells and the corresponding MM cell line were co-cultured (ratio 1:1) in the presence of daratumumab (0.1 – 10 µg/ml) or IgG1 isotype control for 4 hours at 37 ºC. Finally, both NK cell mediated direct cytotoxicity (in absence of daratumumab) and ADCC were evaluated by flow cytometry with the following panel: CD38me-FITC / Annexin V-PE / 7AAD / CD45-APC.

ADCP. PBMCs obtained from healthy donors' buffy coats were used to isolate monocytes by immunomagnetic selection, using the CD14 MicroBeads Kit, human (Miltenyi Biotec). Afterwards, monocytes were differentiated into macrophages in the same culture medium used for MM cell lines supplemented with GM-CSF (10 ng/ml; PeproTech, Rocky Hill, NJ, US) during 7 days. For the evaluation of ADCP, the corresponding MM cell line was stained with PKH26 (Sigma-Aldrich) as per manufacturer's instructions. Co-cultures of macrophages and myeloma cells^{PKH26+} at a 3:1 ratio (effector cell:target cell) were then established and incubated with daratumumab (0.01 – 10 µg/ml) or its isotype control for 4 hours at 37 ºC.

ADCP was evaluated by flow cytometry with the following panel: CD11b-FITC / PKH26 / CD138-APC. Macrophages that had phagocytosed MM cells were double positive ($CD11b^+$ / $PKH26^+$), whereas triple positive events ($CD11b^+$ / $PKH26^+$ / $CD138^+$) were considered as macrophages only adhering to target cells (not engulfing them). ADCP was quantified in two different ways as reported somewhere else[185]. Specifically, first as the percentage of double positive macrophages that was calculated with the following formula:

$$Double\ positive\ macrophages\ (\%) = 100 - [\frac{(100 - treatment\ double\ positive) * 100}{(100 - control\ double\ positive)}]$$

Being “control double positive” the percentage of double positive macrophages within the macrophage population in presence of daratumumab’s isotype control and “treatment double positive” the percentage of double positive macrophages within the macrophage population in presence of daratumumab.

Second, the percentage of elimination of myeloma cells was calculated using the following formula:

$$Eliminated\ myeloma\ cells\ (\%) = 100 - [\frac{(Remaining\ myeloma\ cells\ with\ treatment)}{(Remaining\ myeloma\ cells\ without\ treatment)} * 100]$$

“Remaining myeloma cells without treatment” means the percentage of myeloma cells not phagocytosed (phenotype: $PKH26^{+}CD138^{+}CD11b^{-}$) after 4 hours-incubation with daratumumab’s isotype control, whereas “remaining myeloma cells with treatment” stands for the percentage of myeloma cells not phagocytosed after 4 hours-incubation with daratumumab.

Specifically, for the evaluation of daratumumab’s MoA, in the study entitled “Preclinical evaluation of tinostamustine enhancing the anti-myeloma efficacy of daratumumab in multiple myeloma” (first chapter of the results section), MM cell lines were first preincubated in presence of tinostamustine (2.5 μM) or DMSO for 48 hours. After that time, cells were washed with phosphate buffered saline (PBS) to remove tinostamustine or DMSO from the culture medium, and the corresponding cytotoxic assay was performed.

Immunofluorescence

Immunofluorescence assay was performed, when appropriate, as previously described[250], using an anti-CD38 antibody (Santa Cruz) or daratumumab as primary antibodies. Specifically, immunofluorescence was carried out on slides coated with poly-L-Lysine (Sigma-Aldrich), on which the myeloma cells were left to attach for 30 minutes. Then, the cells were fixed with a 4% formaldehyde solution (Thermo Fisher Scientific, Waltham, MA, USA) in PBS for 15 minutes, and after PBS washes, a 1-

hour block was performed with 5% bovine serum albumin (BSA) in PBS, followed by overnight incubation with the corresponding primary antibody in PBS with 1% BSA. After performing 3 washes with PBS for 5 minutes each, the slides were incubated with a solution containing the secondary antibody (anti-mouse AlexaFluor-488 or anti-human AlexaFluor 488) for 1 hour in the dark. Finally, cell nuclei were stained with the DNA marker DAPI (Cell Signaling Technology, Danvers, MA, USA), and slides mounted with a coverslip using Vectashield® mounting medium (Vector Laboratories, Burlingame, CA, USA). Fluorescent images were acquired with a LEICA SP5 DMI-6000B confocal microscope (Leica, Wetzlar, Germany) with a 63.0x lens zoomed in 6x.

Immunoblotting

Protein lysates were obtained and Western Blot (WB) was performed following standard procedures[253]. Briefly, cells were collected, washed with PBS, and lysed in cold lysis buffer (140 mM NaCl, 50 mM EDTA, 10% glycerol, 1% NP-40, 20 mM Tris HCl, pH 7) with protease and phosphatase inhibitors (Santa Cruz Biotechnology, Dallas, TX, USA). Protein extracts were boiled in loading buffer and subjected to SDS-PAGE at an acrylamide concentration between 6 and 12%. After electrophoresis, the proteins were transferred to PVDF-Immobilon membranes (Merck Millipore, Burlington, MA, USA), which were subsequently blocked for 1 hour with 1% BSA solution in TBST (Tris-HCl buffered saline and Tween-20 at 0.1%). After an overnight incubation of membranes with the primary antibody, they were washed in TBST and incubated with the appropriate secondary antibody conjugated with peroxidase (HRP, Horseradish Peroxidase). Bands were visualized by a luminol-based detection system using Clarity Western ECL Substrate kit (Bio-Rad, Hercules, CA, USA).

All primary antibodies used in WB analysis (anti-Bcl-2, anti-Bcl-xL, anti-CD59, anti-c-Fos, anti-c-Jun, anti-IRF1, anti-NFκB and anti-Mcl-1) were obtained from Cell Signaling Technology, except for anti-β-actin, anti-CD38, anti-clusterin (Abcam,

Cambridge, UK), anti-α-tubulin (Calbiochem, San Louis, MI, USA) and HRP-conjugated anti-mouse and anti-rabbit secondary antibodies (GE Healthcare, Chicago, IL, USA).

Chromatin Immunoprecipitation

This experiment was performed in collaboration with Dr. Ballestar's group at the Josep Carreras Leukaemia Research Institute (IJC, Badalona, Spain). RPMI-8226 and MM.1S cell lines were treated with DMSO or tinostamustine (2.5 μM) for 48 hours. Then, the cells were fixed with Pierce™ fresh methanol-free formaldehyde (ThermoFisher) for 15 minutes and prepared for sonication with the truChIP Chromatin Shearing Kit (Covaris, Woburn, MA, USA), following the manufacturer's instructions and as previously described[40].

For ChIP-qPCR, samples were diluted 1/10, and 4 µL and specific primers (Table 3) were used for each reaction. RT-qPCR was performed in technical triplicates for each biological replicate, using LightCycler® 480 SYBR Green Mix (Roche). The relative amount of immunoprecipitated DNA was compared to the input DNA for each condition.

Table 3. Primers used for ChIP-PCR. *GAPDH* gene was used as a positive control for the histone 3 acetylation (H3Ac), whereas *SAT2* gene was used as a negative control for the same histone modification. F letter stands for forward primer and R letter for reverse.

Gene	Primers used (5' → 3')
CD38	F: CCTCGCTTTCACCGGGAAAT
	R: TGCGGGATTTTGCTATCCCA
GAPDH	F: TACTAGCGGTTTTACGGGCG
	R: TCGAACAGGAGGAGCAGAGAGCGA
SAT2	F: CATCGAATGGAAATGAAAGGAGTC
	R: ACCATTGGATGATTGCGATCAA

In vivo evaluation of the efficacy of the combination of daratumumab + tinostamustine in subcutaneous plasmacytoma models

For the first subcutaneous human plasmacytoma model, NOD.Cg-Prkdc^{scid} Il2rg$^{tm1Wji/SzJ}$ (NSG) mice (16 female mice, 5 to 6-week-old; Servicio de Experimentación Animal O.M.G., USAL, Salamanca, Spain) were subcutaneously inoculated into the right flank with 3 × 10^6 MM.1S cells in 100 µL RPMI-1640 medium plus 100 µL Matrigel (BD Biosciences). When tumors became palpable, mice were randomized into four groups (4 mice/group) to receive: tinostamustine (30 mg/kg, i.v., weekly), daratumumab (8 mg/kg, i.p., weekly), the double combination or vehicle [PBS vehicle solution with 15% 2-hydroxypropyl-β-cyclodextrin (HPBCD), 1.5% acetic acid, and 1.25% $NaHCO_3$]. Tinostamustine was administered 24 hours before daratumumab as appropriate. In addition, all mice were injected with human NK cells isolated from healthy donors' buffy coats as previously explained (1.4 - 2 x 10^6/mouse, depending on NK cells availability in each buffy coat, i.v., weekly) coinciding with daratumumab regimen. Both, human NK cells and the treatments were administered during 4 consecutive weeks. Caliper measurements of the tumor diameters were performed three times a week, and the tumor volume was estimated as the volume of a 3D ellipse using the following formula: $V = 4/3\ \pi \times (a/2) \times (b/2)^2$, where "a" and "b" correspond to the longest and shortest diameter, respectively. Animals were killed when the longest diameter of their tumors reached 2 cm or if distress signs were observed. Statistical differences in tumor volumes between the different groups were evaluated using one-way analysis of variance (ANOVA) and Tukey's HSD post-hoc tests.

For the second subcutaneous human plasmacytoma model, CB17-SCID mice (20 female mice, 5- to 6-week-old; The Jackson Laboratory) were used. The design of the experiment (treatment doses and time schedule) was identical to the one performed with NSG mice except for the inoculation of human NK cells, since CB17-SCID mice have their own functional NK cells[254].

Animal experiments were conducted according to institutional guidelines for the use of laboratory animals, and after granted permission from the University of

Salamanca Animal Ethical Committee for animal experimentation and the Agriculture and Livestock Council of Junta de Castilla y León (Registry Numbers: 0000061 and 292; Registered User Center: ES372740000046).

Gene expression profile studies

Gene expression profile (GEP) studies were carried out at the CIC-IBMCC Genomics Service. For this purpose, 1 x 10^7 cells were frozen in RLT plus (Qiagen, Hilden, Germany) and kept at -80°C until the RNA was extracted using RNeasy Mini columns (Qiagen). Subsequently, the quality of the RNA was analyzed in an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Labeling and hybridizations were carried out according to Affymetrix specifications. Essentially, 100 ng of total RNA was amplified, labeled (WT Plus Reagent Kit, Affymetrix, Santa Clara, CA, USA) and hybridized to Human Gene 2.0 ST microarrays (Affymetrix). Washing and scanning was carried out using the Affimetrix GeneChip system (GeneChip Hybridization Oven 645, GeneChip Fluidics Station 450 and GeneChip Scanner 7G). The fluorescence intensity data obtained from the microarrays were normalized using the RMA (Robust Multichip Average) method[255] implemented in the oligo package (v.1.44.0) in R (v.3.5.0), using a BrainArray custom genetic reference (Custom CDF, Ensembl version 22)[256]. The unsupervised analysis was performed in the statistical software SIMFIT (v.7.4.1), using the Euclidean as a distance measure and the group average as the linkage method. The differentially expressed genes were identified using the SAM method via Shiny (v. 1.1.0) in R (https://github.com/MikeJSeo/SAM). Reactome[257] pathway overrepresentation and enrichment analyses were performed in the Webgestalt suite[258].

Real time quantitative PCR (RT-qPCR)

Total RNA was isolated using the RNeasy Mini Kit (Qiagen), following manufacturer's instructions. Purity and concentration of isolated RNA was

determined by NanoDrop spectrophotometer (Thermo Fisher Scientific). TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) were performed according to the manufacturer's instructions: CD38 (Hs01120071_m1), CD276 (Hs00987207_m1), clusterin (Hs00156548_m1), GAPDH (Hs99999905_m1), HHLA2 (Hs00978112_m1), MICA (Hs00741286_m1) and MICB (Hs00792952_m1). The results were expressed as fold change after subtraction of internal GAPDH housekeeping gene and shown relative to the levels in control cells.

Pyrosequencing of CpG islands

Analysis of gene methylation status was carried out using the pyrosequencing method. First, genomic DNA was extracted with the NZY Tissue gDNA Isolation kit (NZYTech, Lisbon, Portugal) and treated with sodium bisulfite, which transforms unmethylated cytosines (C) into uracils (U). After PCR amplification, the (U) are replaced by thymines (T), which allows to distinguish the methylated and unmethylated C in the original DNA. Pyrosequencing oligos were designed to amplify a region of *CD38* promoter using the UCSC Genome Browser software (UCSC Genomics). The resulting PCR product was purified with GeneJet columns (Thermo Fisher Scientific) and cloned into the plasmid pGEM-T-Easy (Promega) using NotI and EcoRI enzymes. Subsequently, competent DH5α bacteria were transformed with 50 ng of plasmid; they were cultured in LB medium supplemented with 100 µg/ml of ampicillin, 0.5 mM IPTG (Isopropyl-B-D-Thiogalactopyranoside) and 80 µg/ml X-Gal (ß-D-galactopyranoside) from Sigma-Aldrich; and 10 isolated colonies from each sample were sequenced. Sequences were analyzed with BiQ Analyzer v2.0 software (Max Planck Institute for Informatics).

Methylation arrays

Genome-wide global methylation analysis was performed using Illumina Infinium Methylation EPIC array with bisulfite-treated DNA. Methylation at each CpG was

represented as “β values” (ranging from 0 to 1), where 0 is equivalent to 0% methylation and 1 is equivalent to 100% methylation at a given CpG[259]. Statistical analysis was performed on β values. Mean β values were calculated for the different conditions, and the mean Δβ was calculated by subtracting the mean β value of controls from the experiment value. Therefore, a positive Δβ value denotes hypermethylation and a negative Δβ value indicates hypomethylation. Differential methylation analysis was carried out using the limma package (3.46.0). Differentially methylated regions (DMR) were identified by comparing the mean β values in the experimental group with the mean β values in the control group for a particular CpG site. Subsequently, the criteria for DMR were established: an adjusted p-value (FDR)<0.05 and a difference in the β value between groups greater than 0.3 (that is, |Δβ|>0.3). Array data processing and analysis were performed in RStudio (version 1.2.1335) using an R environment (version 3.6.0). For data enrichment and visualization analysis, the online tool GREAT (http://great.stanford.edu) was used.

T lymphocytes activation and proliferation assays

PBMCs obtained from healthy donors’ buffy coats were used to isolate T lymphocytes by immunomagnetic selection, using the Pan T cell Isolation Kit, human (Miltenyi Biotec). T lymphocytes were labelled with Cell Trace CFSE (Thermo Fisher) and co-cultured with MOLP-8 or RMOLP-8 cell line at 4:1 ratio (T lymphocyte:MM cell line) in presence of anti-CD28 antibody (2.5 μg/ml). Before seeding the cells and in order to activate T lymphocytes, the culture dish had been treated to anchor anti-CD3 (5 μg/ml) to the bottom. After 5 days of co-culture at 37ºC, cells were harvested and proliferation and activation of T cells was assessed by flow cytometry with the following panel: CFSE / CD25-PE / CD8- PerCP-Cy5.5 / CD4-APC. Specifically, T lymphocyte proliferation was measured by the loss of CFSE and activation by $CD25^+$ cell population.

Stable protein overexpression

"pMSCV-target" plasmid generation. The backbone used for all protein overexpression assays was CD711B-1_pCDH-MSCV-MCS-EF1α-GFP plasmid (from now on pMSCV plasmid) (System Biosciences, Palo Alto, CA, USA). The protein to be overexpressed in each case was amplified from cDNA obtained from MM cell lines using the primers specified in Table 4.

Table 4. Primers used to amplify the cDNA encoding each of the proteins to be overexpressed. Nucleotides in red indicate the restriction site sequence that allows the subsequent cloning into the plasmid. Nucleotides in lower case are nucleotides that are added for protecting the restriction site in case of primer or amplicon degradation. F letter stands for forward primer and R letter for reverse.

Protein	Cell line from which cDNA was obtained	Primers used (5' → 3')
CD38	MOLP-8	F: cagtTCTAGAATGGCCAACTGCGAG
		R: cagtCGCCGGCGAGTCTAGAGTCTACACG
Clusterin	MM.1S	F: cagtTCTAGAATGATGAAGACTCTGCTGCT
		R: cagtGCGGCCGCTCACTCCTCCCGGTGCTTTT
Bcl-2	JJN3	F: cagtTCTAGAATGGCGCACGCTGGG
		R: ctgaGCGGCCGCTCACTTGTGGCCCAGATAGG
Bcl-xL	MM.1S	F: cagtTCTAGAATGTCTCAGAGCAACCGGGAG
		R: ctgaGCGGCCGCTCATTTCCGACTGAAGAGTGAGCC

After amplification, the cDNA of interest has to be digested with appropriate restriction enzymes with the aim of cloning into the plasmid. Specifically, both the insert and pMSCV-plasmid were digested with NotI and XbaI restriction enzymes (New England Biolabs, Ipswich, MA, USA) and then dephosphorylated and ligated using the T4 DNA Ligase kit (New England Biolabs) so that each insert was cloned within the pMSCV-plasmid.

Afterwards, DH5α bacteria were transformed with each construct and after growth overnight in the presence of ampicillin 100 μg/ml, the plasmid was purified with the GeneJet Plasmid Miniprep kit (Thermo Fisher Scientific). The purified plasmid was then sequenced by the Sanger method to check appropriate sequence incorporation for each gene.

Lentiviral infection. Stable protein overexpression was performed using lentiviral infection. Briefly, 0.5 x 10^6 HEK-293T cells/well were plated in a six-well culture plate in antibiotic-free DMEM medium with 10% FBS. For each well, 9 µl of lipofectamine 2000 (Thermo Fisher Scientific) diluted in 150 µl of Opti-MEM (Thermo Fisher Scientific) were combined with 5 µg of "pMSCV-target" plasmid, in addition to 1.5 µg psPAX2 (packing plasmid; Addgene, Watertown, MA, USA) and 0.4 µg pMD2.G (packing plasmid; Addgene) diluted in another 150 µl Opti-MEM. The lentiviral mixture was incubated for 30 min and was subsequently dispensed dropwise onto HEK-293T cells.

Lentiviral supernatant was collected and filtered using 0.45 µm filters (Millipore) at 24 and 48 hours after transfection and subsequently concentrated using the LentiX concentrator (Takara Bio, Kusatsu, Shiga, Japan) as per manufacturer's instructions. The concentrated virus supernatants were resuspended in PBS and added to the MM cells that had previously been plated at a density of 2 x 10^6 cells/ml in 500 µl in a 24-well plate together with 8 µg/ml of polybrene (Santa Cruz Biotechnology). A spinoculation protocol in the 24-well plates was used for MM cell infection (800 g during 2 hours at 37ºC).

Given that the pMSCV plasmid contained a green fluorescent protein (GFP) coding cassette, both monitorization of transduction efficiency and cell selection were performed by fluorescence-activated cell sorting (FACS) using a FACSAria III cytometer (Becton Dickinson). The overexpression of the target molecule was confirmed by RT-qPCR and/or WB as we have already described.

Stable gene silencing

Stable silencing of clusterin was done using the Human pLKO.1 lentiviral shRNA target gene set kit (PerkinElmer, Waltham, MA, USA) following manufacturer's instructions. Briefly, DH5α bacteria were transformed with each of the shRNAs contained in the kit and the plasmid was purified as already mentioned. RMOLP-8

cell line was transduced with each of the shRNA as explained in “Lentiviral infection” section. Infected cells were selected with puromycin 0.5 μg/ml.

Generation of CD59 knock-out cells using CRISPR/Cas9 technology

To generate a RPMI-8226 CD59 KO cell line by CRISPR/Cas9 editing, a guide RNA (gRNA) was designed taking into account the expression of CD59 gene isoforms, its specificity and efficiency using bioinformatics tools. The upper and lower strands of the selected gRNA were phosphorylated, annealed and cloned into the pLKO5.sgRNA.EFS.tRFP plasmid (Addgene) as previously described[260]. Subsequently, lentiviral particles were generated on the HEK-293T cell line (as described in the previous section).

Lentiviral supernatants were used to transduce the RPMI-8226-Cas9 cell line (a RPMI-8226 cell line previously transduced to stably express the functionally active Cas-9 endonuclease). Single cell separation of RFP positive cells was performed by flow cytometry 72 h after transduction. Once the resulting clones had grown, they were characterized by PCR and Sanger sequencing.

RESULTS

CHAPTER II: EVALUATION OF THE MECHANISMS INVOLVED IN THE ACQUIRED RESISTANCE TO DARATUMUMAB-MEDIATED CDC

1. Generation and characterization of an in vitro resistance model to daratumumab-mediated CDC

The development of a cellular model of resistance to daratumumab-mediated CDC was accomplished through exposure of the sensitive cell line MOLP-8 to increasing doses of daratumumab (0.01 – 100 µg/ml) and 10% human serum, as complement source, for approximately 5 months, until the cells developed resistance (RMOLP-8). Specifically, the cells were exposed to daratumumab + human serum for 4 hours and then maintained in fresh medium without drug for 2 – 3 days until re-exposure to the drug + human serum. Once resistance was reached, the cells were maintained without daratumumab for 2 weeks and resistance was re-checked observing that while 82.75% MOLP-8 cells died in presence of daratumumab (10 µg/ml), only 7.76% RMOLP-8 cells did (Figure 2.1). Thereafter, the cells were continuously cultured without drug for subsequent studies.

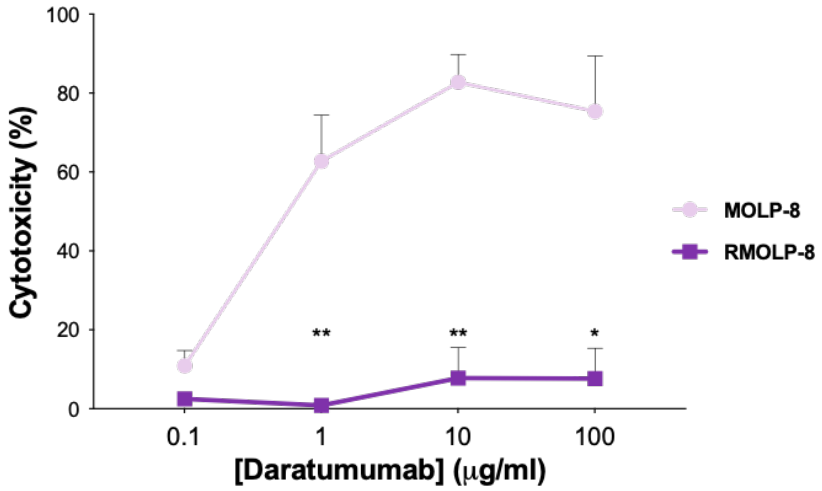

Figure 2.1. Evaluation of the resistance to daratumumab-mediated CDC in RMOLP-8 cells in comparison to MOLP-8 cells. Cells were incubated during 4 hours with daratumumab (0.1 – 100 µg/ml) in the presence of 10% human serum. Dead cells were analyzed by flow cytometry with Annexin V / 7AAD staining and percentage of cytotoxicity was obtained after normalization with dead cells in control condition (cells not treated with daratumumab). Each point shows mean ± SEM (n=3). Statistically significant differences were evaluated by Student's t-test (*p<0.05 and **p<0.01).

Since daratumumab exerts its anti-myeloma activity through the other three classical MoA, we also tested the sensitivity of RMOLP-8 cells to apoptosis via crosslinking, ADCC and ADCP in comparison to the MOLP-8 cell line. Firstly, we

observed that RMOLP-8 cells were completely resistant to apoptosis via crosslinking in comparison to their sensitive counterpart MOLP-8 when the cells were exposed to daratumumab 1 µg/ml during both 24 and 48 hours (Figure 2.2a).

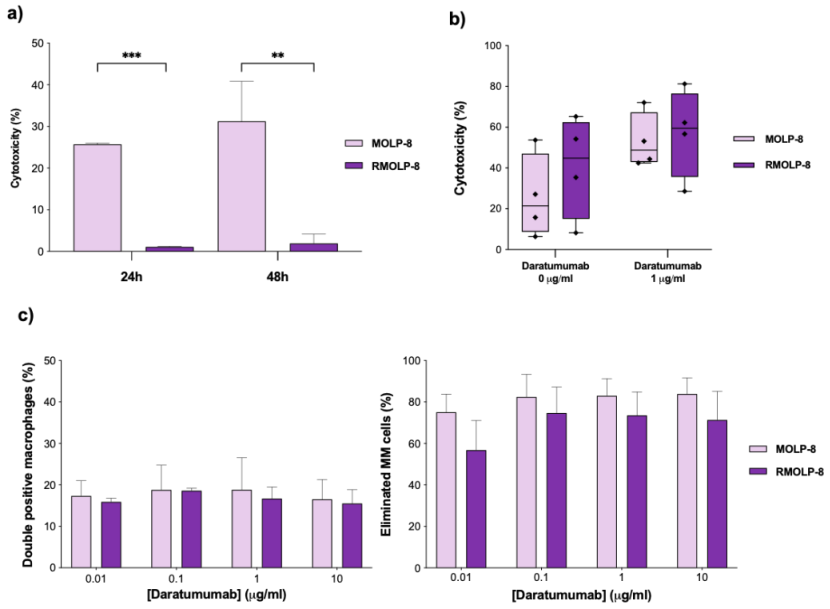

Figure 2.2. Evaluation of the MoA of daratumumab in RMOLP-8 cells in comparison to MOLP-8 cells. a) Study of the apoptosis via crosslinking mediated by daratumumab. MOLP-8 and RMOLP-8 cells were incubated for 24 or 48 hours with daratumumab (1 µg/ml) in the presence of $F(ab)_2$ fragments (10 µg/ml). Apoptosis was analyzed by flow cytometry with Annexin V and 7AAD. Daratumumab-induced apoptosis was normalized with respect to basal death in control cells (cells not treated with daratumumab). Each bar shows mean ± SEM (n=3). Statistically significant differences were evaluated by Student's t-test (**p<0.01 and ***p<0.001). **b)** Evaluation of daratumumab-mediated ADCC. MOLP-8 or RMOLP-8 cells were co-cultured with human NK cells (ratio 1:1, E:T) and incubated for 4 hours in absence or presence of daratumumab (1 µg/ml). Dead cells were analyzed by flow cytometry and cytotoxicity percentage was obtained after normalization with basal death of each cell line. Each box plot shows mean ± SEM (n=4). **c)** Assessment of daratumumab-mediated ADCP. MOLP-8 or RMOLP-8 cells stained with PKH26 were co-cultured with human macrophages (ratio 3:1, E:T) and incubated for 4 hours in absence or presence of daratumumab (0.01 – 10 µg/ml). Phagocytosis was analyzed by flow cytometry, and the percentages of double positive macrophages and eliminated MM cells were calculated as explained in Materials and Methods section. Each bar shows mean ± SEM (n=3). E: effector cells, T: target cells.

Secondly, the sensitivity of both cell lines to daratumumab mediated ADCC was assessed by flow cytometry. In this sense, no differences were observed in daratumumab mediated ADCC when comparing both cell lines; likewise, no differences were found in NK cell mediated direct cytotoxicity, that is, in absence of daratumumab (Figure 2.2b). Finally, RMOLP-8 cells showed similar levels of ADCP in comparison to MOLP-8 cells as evaluated by two different parameters: double

positive macrophages and eliminated MM cells, that were not statistically different between these two cell types (Figure 2.2c).

The sensitivity of RMOLP-8 cells to the CDC mediated by the anti-CD38 isatuximab was also studied. As observed in Figure 2.3, cells with acquired resistance to daratumumab-dependent CDC, also were resistant to the CDC mediated by isatuximab (Figure 2.3).

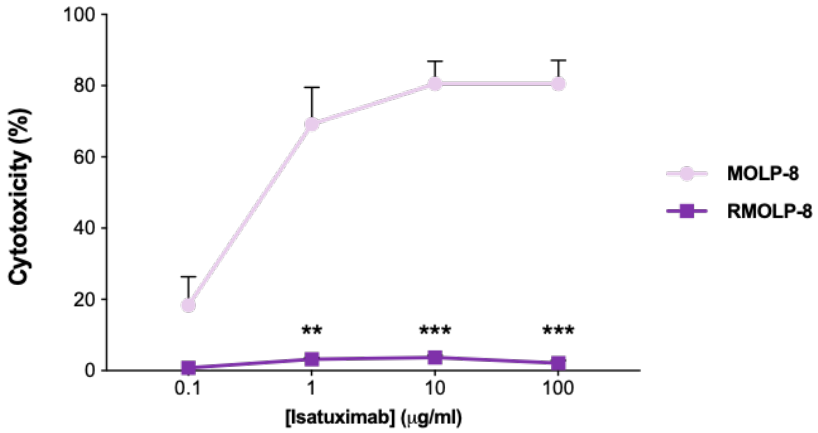

Figure 2.3. Evaluation of the sensitivity to isatuximab-mediated CDC in RMOLP-8 cells in comparison to MOLP-8 cells. Cells were incubated during 4 hours with isatuximab (0.1 – 100 μg/ml) in the presence of 10% human serum. Dead cells were analyzed by flow cytometry by Annexin V / 7AAD satining and the percentage of cytotoxicity was calculated by normalization with basal death of control cells (cells not treated with isatuximab). Each point shows mean ± SEM (n=3). Statistically significant differences were evaluated by Student's t-test (**p<0.01 and ***p<0.001).

In order to characterize in depth the generated resistance cell model, the cell cycle and proliferation were evaluated in both sensitive and resistant cell lines. Regarding cell cycle, it was observed that the RMOLP-8 cell line had a decreased percentage of cells in both G_0 – G_1 and S phases and an increase in the G_2 – M phase in comparison to MOLP-8 cells (Figure 2.4a). Moreover, as shown in Figure 2.4b, RMOLP-8 cells presented a higher proliferation rate in comparison to MOLP-8 cells.

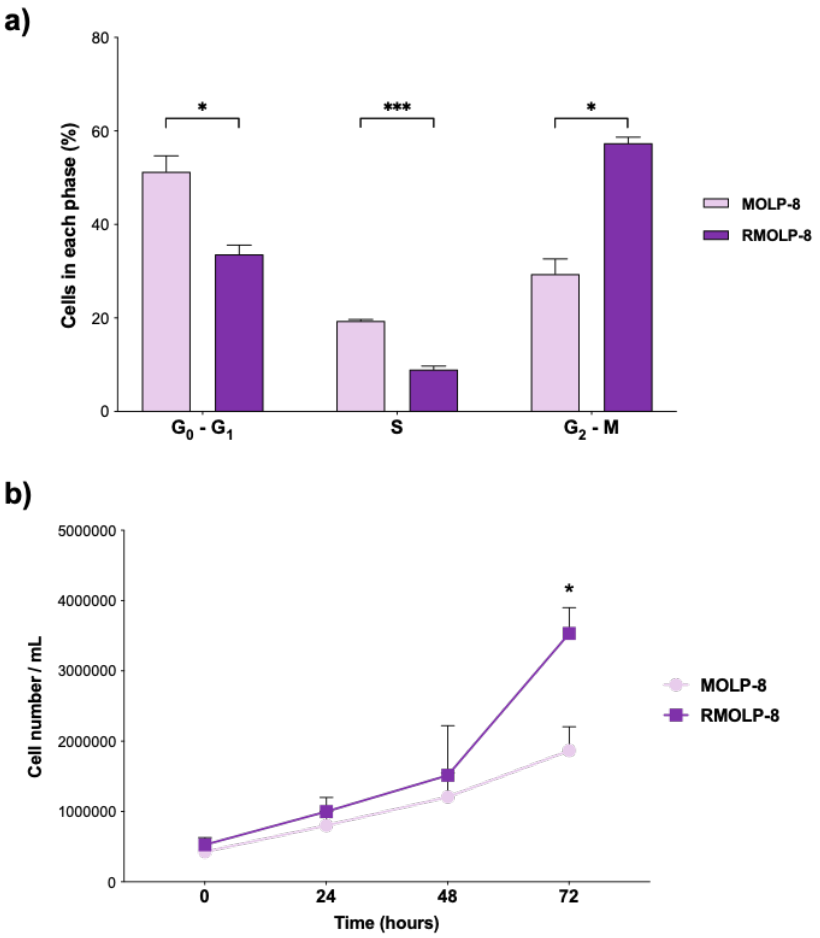

Figure 2.4. Study of the cell cycle and proliferation in RMOLP-8 cells in comparison to MOLP-8 cells. a) Cell cycle profile was examined by flow cytometry after PI staining. Percentages correspond to the non-subG_0 population. **b)** Comparison of MOLP-8 and RMOLP-8 growth curves by counting the number of cells with Neubauer Chamber at different time points after culture. Data represents mean ± SEM (n=3). Statistically significant differences were evaluated by Student's t-test (*p<0.05 and ***p<0.001).

In addition, immunophenotypic markers, routinely used in MM assessment, were studied by flow cytometry in both cell lines, in particular CD38, CD56, CD81, CD138 and CD27 surface markers. RMOLP-8 cells significantly underexpressed CD38, the target of daratumumab (Figure 2.5). Furthermore, CD56 was also downregulated in RMOLP-8 cells compared to MOLP-8 cells, whereas CD81 expression was higher, almost reaching statistical significance (p=0.06).

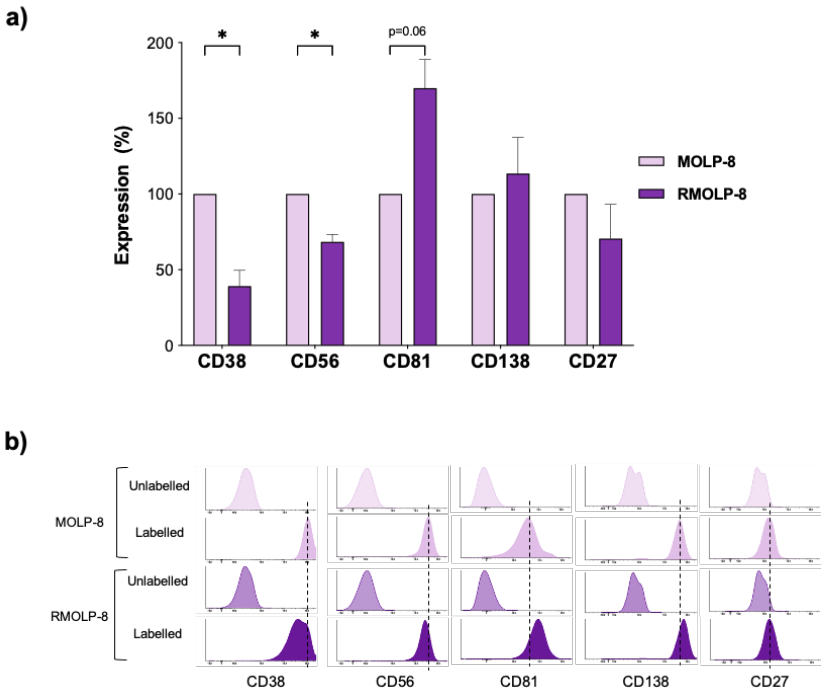

Figure 2.5. Immunophenotypic characterization of RMOLP-8 cells in comparison to MOLP-8 cells by flow cytometry. a) Normalized MFI expression of CD81, CD56, CD27 and CD138 considering the expression in the MOLP-8 cell line as 100%. Each bar shows mean ± SEM (n=2). Statistically significant differences were evaluated by Student's t-test (*p<0.05). **b)** Representative histograms of the analyzed molecules.

In addition, since RMOLP-8 cells are resistant to daratumumab-mediated CDC, the expression of the CIPs CD46, CD55 and CD59 was assessed by flow cytometry. In this sense, we observed that resistant cells expressed similar levels of CD46 and CD55 in comparison with sensitive cells and lower level of CD59 (Figure 2.6).

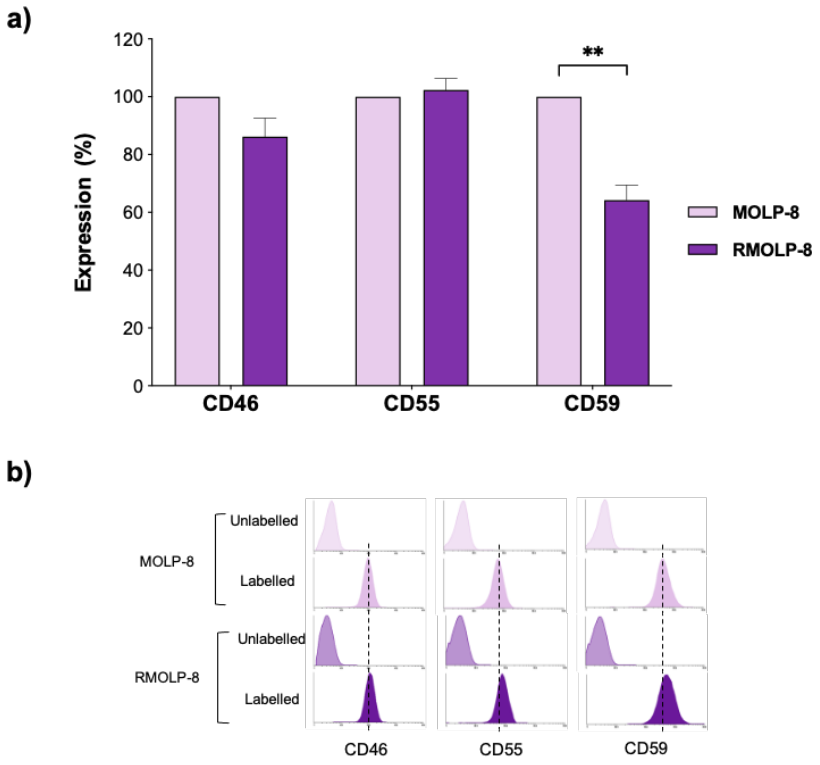

Figure 2.6. Expression of complement inhibitory proteins (CIPs) in MOLP-8 and RMOLP-8 cell lines. a) Normalized MFI expression of CD46, CD55 and CD59 considering MOLP-8 cell line as 100%. Each bar shows mean ± SEM (n=3). Statistically significant differences were evaluated by Student's t-test (**p<0.01). **b)** Representative histograms of the molecules analyzed.

2. Study of the involvement of CD38 expression in the acquired resistance to daratumumab of the generated RMOLP-8 cells

As explained in the previous section, CD38, the target of daratumumab, was down-regulated in RMOLP-8 cells according to flow cytometry data. Here, CD38 expression was analyzed at transcriptional level through RT-qPCR and we observed that RMOLP-8 cells have a diminished expression of this gene in comparison with the MOLP-8 cell line (Figure 2.7a). Moreover, total protein levels of CD38 were lower in RMOLP-8 cells as confirmed by WB and immunofluorescence (Figure 2.7b and Figure 2.7c).

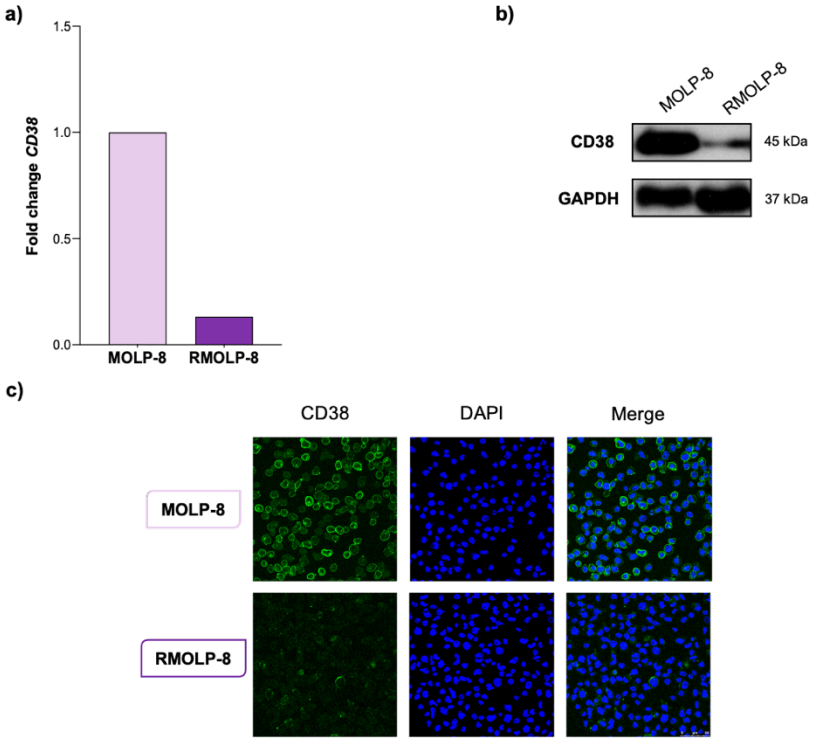

Figure 2.7. Expression of CD38 in RMOLP-8 cells in comparison to MOLP-8 cells. a) CD38 mRNA levels in MOLP-8 and RMOLP-8 cells by RT-qPCR. The results are shown as the fold change referred to sensitive cells after normalization with *GAPDH* and correspond to the average of three experiments. **b)** CD38 expression analyzed by WB in both cell lines. GAPDH was used as loading control. **c)** Analysis of CD38 (green) by immunofluorescence assay in MOLP-8 and RMOLP-8 cells. Cell nuclei were stained with DAPI (blue).

To assess whether the decrease in CD38 expression translated into less daratumumab binding to RMOLP-8 cells, we used daratumumab as a primary anti-CD38 antibody and an anti-human as a secondary antibody to test its binding to both cell lines. Indeed, we observed an evident decrease in daratumumab binding to resistant cells by flow cytometry (Figure 2.8a), which was also confirmed by immunofluorescence staining (Figure 2.8b). In addition, we observed that in those cells binding a significant amount of daratumumab (mainly found in the MOLP-8 cell line), this binding is not uniform along the cell membrane, but rather is patchy (Figure 2.8b). This could be due to the fact that the Fc regions of daratumumab tend to group together.

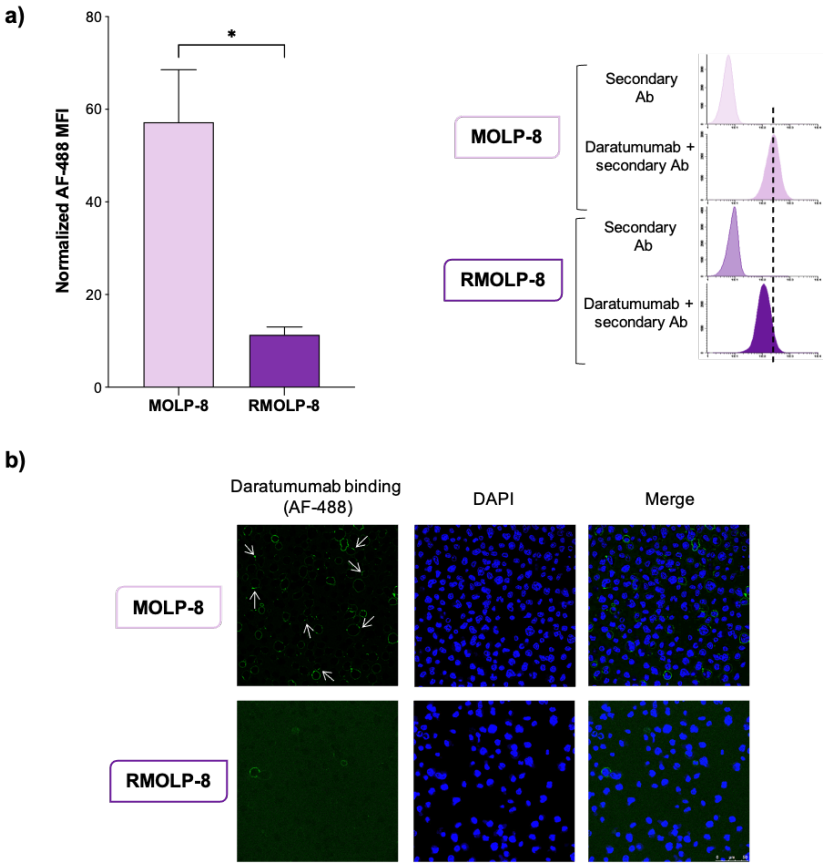

Figure 2.8. Assessment of daratumumab binding to MOLP-8 and RMOLP-8 cells. a) Left panel: normalized MFI of AF-488-anti-human secondary antibody in MOLP-8 and RMOLP-8 cells previously incubated with daratumumab (10 µg/ml) for 30 minutes. Each bar shows mean ± SEM (n=3). Statistically significant differences were evaluated by Student's t-test (*p<0.05). Right panel: Representative flow cytometry histograms of daratumumab binding assay. **b)** Analysis of AF-488-anti-human secondary antibody (green) by immunofluorescence assay in MOLP-8 and RMOLP-8 cells previously incubated with daratumumab (10 µg/ml) for 30 minutes. Cell nuclei were stained with DAPI (blue). Arrows point to patchy fluorescent regions on the plasma membrane.

In order to further understand the importance of CD38 expression in the acquired resistance to daratumumab, *CD38* cDNA was cloned into the pMSCV plasmid (pMSCV-CD38) and RMOLP-8 cell line was transduced with this construct to stably overexpress CD38. Since pMSCV plasmid encodes for the green fluorescent protein (GFP), transduction efficiency was analyzed by flow cytometry being 8.26% for the empty vector and 14.93% for pMSCV-CD38 (Figure 2.9a). Then, GFP^+ cells were sorted by flow cytometry and the overexpression of CD38 was confirmed by flow cytometry (Figure 2.9b).

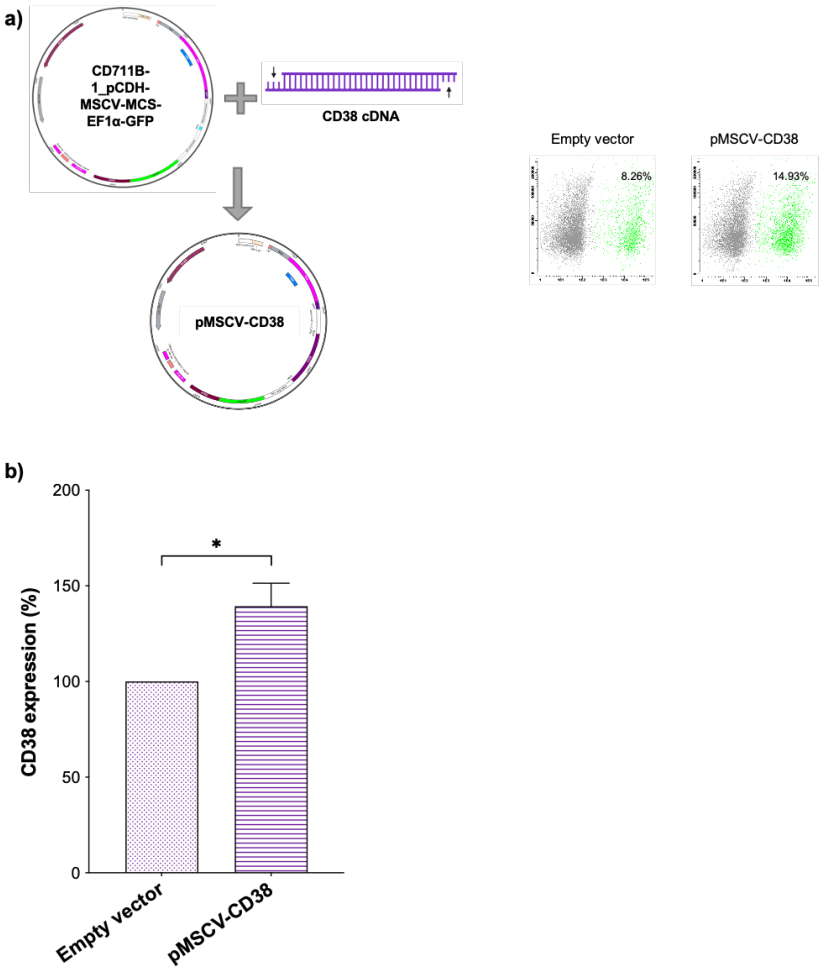

Figure 2.9. Stable overexpression of CD38 in RMOLP-8 cell line using lentiviral transduction. a) *CD38* cDNA was cloned into the pMSCV plasmid as specified in Materials and Methods section. The measurement of GFP expression by flow cytometry allows both evaluation of transduction efficiency and sorting of the transduced cells. **b)** CD38 expression analysis in control cells (empty vector) and in pMSCV-CD38 cells by flow cytometry. Each bar shows mean ± SEM (n=3). Statistically significant differences were evaluated by Student's t-test (*p<0.05).

Next, whether CD38 overexpression in RMOLP-8 cells was translated into a re-sensitization to daratumumab-mediated CDC was tested. As shown in Figure 2.10, a clear tendency to increase the sensitivity to this mechanism was observed with CD38 overexpression in RMOLP-8.

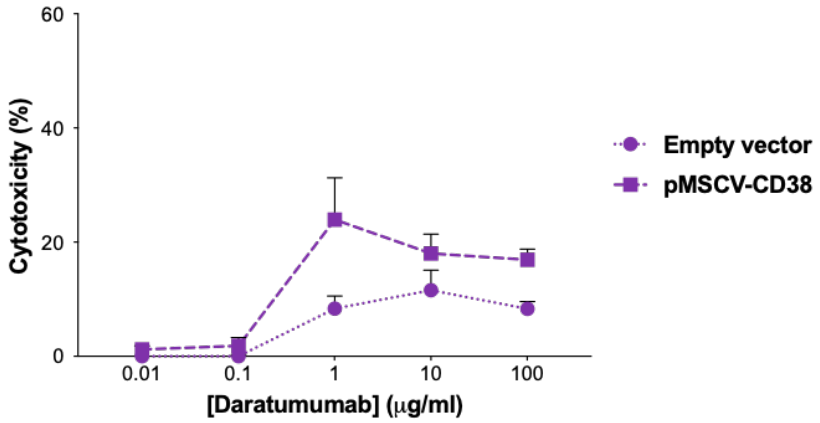

Figure 2.10. Evaluation of the sensitivity to daratumumab-mediated CDC in RMOLP-8 cells with CD38 overexpression. Cells were incubated during 4 hours with daratumumab (0.01 – 100 μg/ml) in the presence of 10% human serum. Dead cells were analyzed by flow cytometry and percentage of cytotoxicity was obtained after normalization with control cells (cells not treated with daratumumab). Each point shows mean ± SEM (n=3).

Furthermore, the effect of tinostamustine on CD38 expression was evaluated by flow cytometry in the RMOLP-8 cell line. Treatment with tinostamustine (1 and 2.5 μM) for 48 hours increased CD38 surface expression in the viable population of RMOLP-8 cells (Figure 2.11).

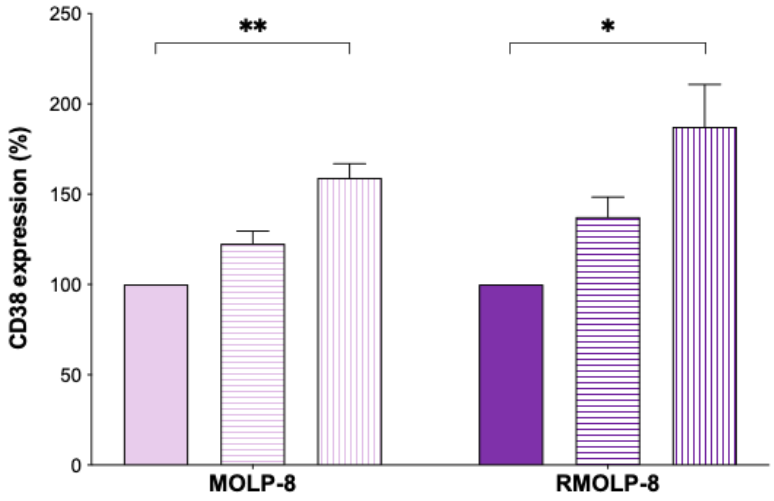

Figure 2.11. Evaluation of the effect of tinostamustine on CD38 surface expression in MOLP-8 and RMOLP-8 cell lines by flow cytometry. Normalized MFI expression of CD38 on the indicated myeloma cell lines after treatment with tinostamustine (1 and 2.5 μM) for 48 hours, considering DMSO-treated cells as 100%. Each bar shows mean ± SEM (n=3). Statistically significant differences were evaluated by one-way ANOVA followed by Tukey's HSD post-hoc test (*$p<0.05$). Solid bars indicate cells treated with DMSO, horizontal striped bars tinostamustine 1 μM and vertical striped bars tinostamustine 2.5 μM.

The effect of tinostamustine in resistant RMOLP-8 cells was further explored. Specifically, we analyzed whether tinostamustine pre-treatment re-sensitized resistant cells to daratumumab-mediated CDC. As shown in figure 2.12, pre-treatment of RMOLP-8 cells with tinostamustine did not affect the sensitivity of these cells to daratumumab.

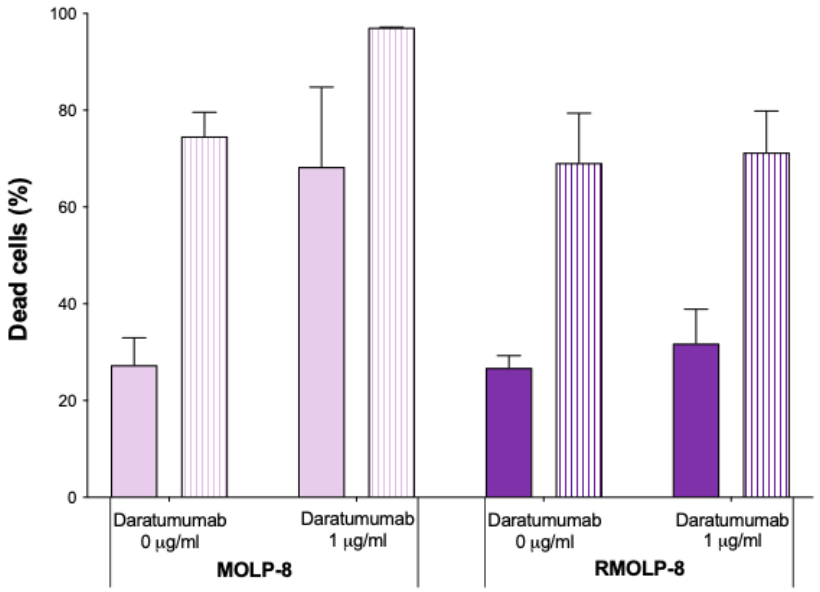

Figure 2.12. Evaluation of tinostamustine pre-treatment on daratumumab-mediated CDC in RMOLP-8 cells. MOLP-8 and RMOLP-8 cell lines were incubated for 48 hours with DMSO or tinostamustine (2.5 μM). After this, tinostamustine or DMSO were removed from the culture medium and tinostamustine or DMSO pre-treated cells were incubated during 4 hours with daratumumab (1 μg/ml) in the presence of 10% human serum. Dead cells were analyzed by flow cytometry (Annexin V / 7AAD staining). Each bar shows mean ± SEM (n=2). Solid bars indicate cells pre-treated with DMSO and vertical striped bars tinostamustine 2.5 μM pre-treated cells.

Additionally, we wondered if the overexpression of CD38 in cell lines with intrinsic resistance to daratumumab-mediated CDC was able to overcome it. Therefore, using the already mentioned pMSCV-CD38 plasmid, four MM cell lines (U266, JJN3, NCI-H929 and RPMI-8226) were transduced with this construct to stably overexpress CD38. As explained before, the GFP cassette made it possible to determine the transduction efficiency and to sort the transduced GFP^+ cells, as shown in Figure 2.13a. The overexpression of CD38 in the transduced cells was confirmed by WB (Figure 2.13b) and flow cytometry (Figure 2.13c).

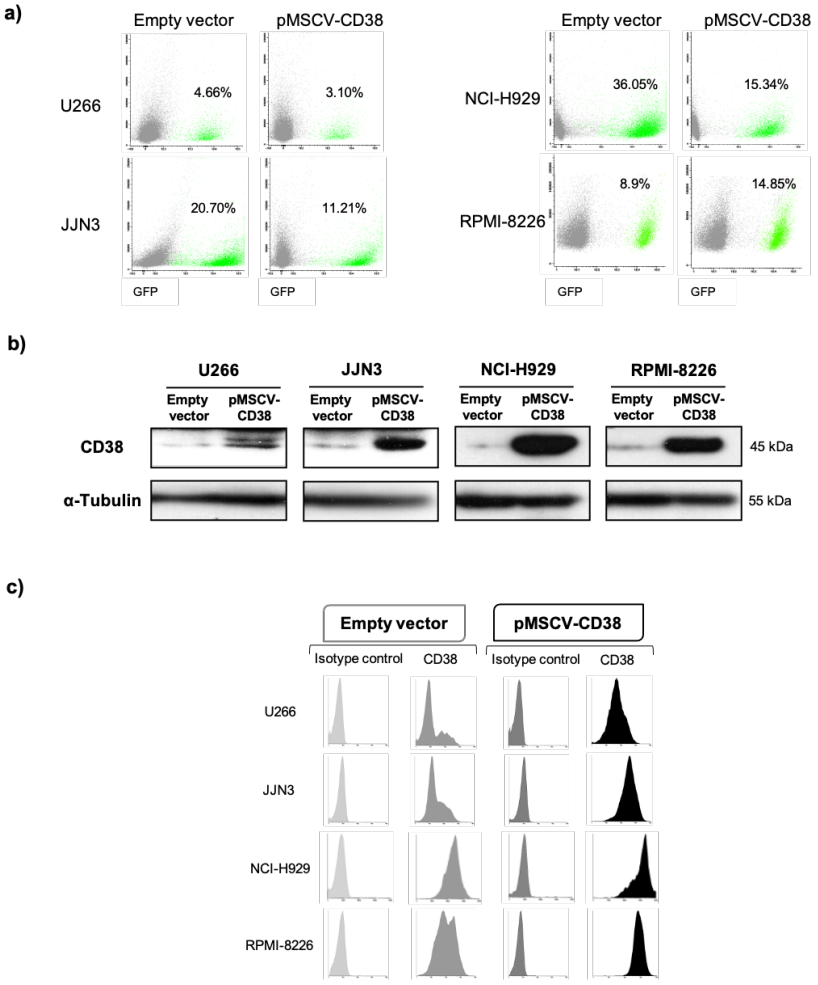

Figure 2.13. Stable overexpression of CD38 in several MM cell lines using lentiviral transduction. a) pMSCV-CD38 plasmid was used for CD38 overexpression. GFP expression by flow cytometry allows both evaluation of transduction efficiency and sorting of the transduced cells. **b)** CD38 expression analysis in control cells (empty vector) and in pMSCV-CD38 cells by WB and **c)** flow cytometry.

We next explored if any of the newly generated CD38-overexpressing cell lines became sensitive to daratumumab-mediated CDC. Data showed that only the NCI-H929 cell line with CD38 overexpression (H929-CD38) seemed to be sensitive to daratumumab in a dose-dependent manner as compared to NCI-H929 transduced with the empty vector or H929-Empty cell line (Figures 2.14a and 2.14b).

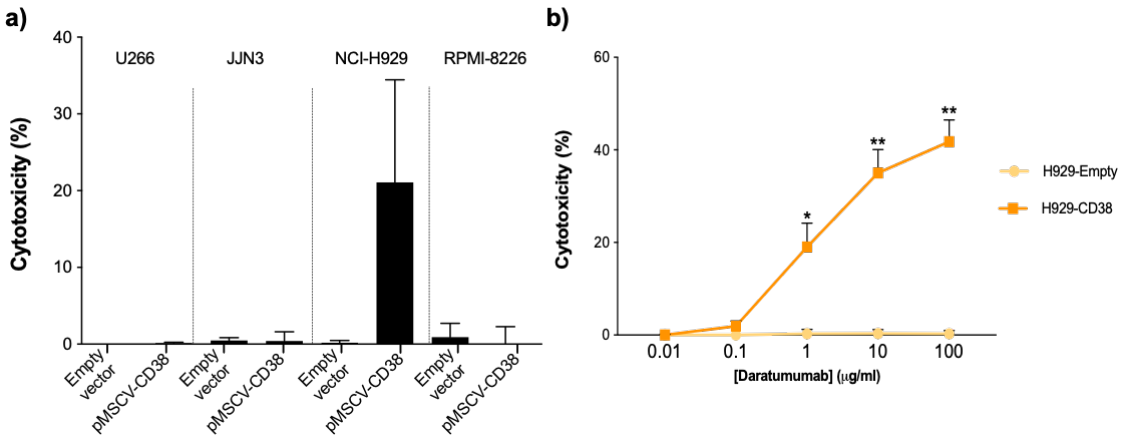

Figure 2.14. Study of the sensitivity of CD38 overexpressing cell lines to daratumumab-mediated CDC. A) Indicated cell lines stably transduced with pMSCV-empty or pMSCV-CD38 were incubated with daratumumab (1 μg/ml) in presence of 10% human serum for 4 hours. The percentage of cytotoxicity, as measured by flow cytometry, was calculated relative to the percentage of dead cells ($7AAD^+$) in the control (cells not treated with daratumumab). Each bar shows mean ± SEM (n=2)**. b)** A similar experiment was carried out with the indicated doses of daratumumab in NCI-H929 cells stably transduced with pMSCV-empty (H929-Empy) or pMSCV-CD38 (H929-CD38). Each point shows mean ± SEM (n=4). Statistical differences were assessed by Student's t test (*p<0.05 and **p<0.01).

2.1. Study of the (epi)genetic mechanisms responsible for CD38 downregulation in RMOLP-8 cells

To try to elucidate the mechanisms by which resistant cells under-express CD38 molecule, several studies were performed. Indeed, Sanger sequencing was performed to check for CD38 gene mutation(s) which might be responsible for the resistant phenotype. No differences were observed either when comparing RMOLP-8 *CD38* sequence to that of MOLP-8 or when comparing it to the reference *CD38* gene. Indeed, according to the EMBL-EBI alignment tool, *CD38* sequence from MOLP-8 and RMOLP-8 cells had a 100% similarity.

Next, the methylation status of CpG islands located in the *CD38* promoter was analyzed. A first study was performed in which the methylation status of 22 CpG islands in the *CD38* promoter was examined using the bisulfite method. We did not find any differences in the methylation level between MOLP-8 and RMOLP-8 cell

lines, finding in both cases a low methylation status for the 22 CpG islands under study (Figure 2.15).

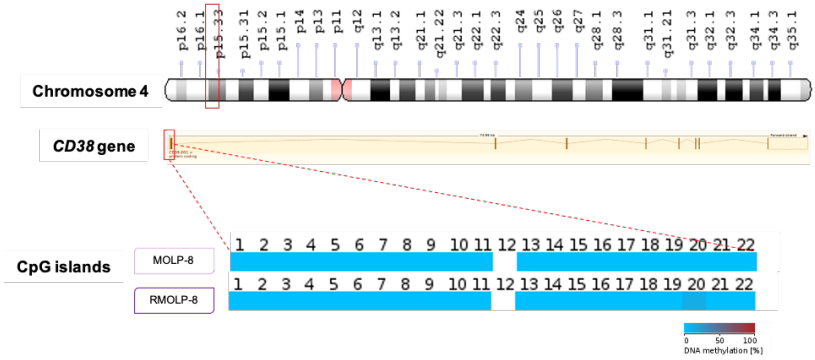

Figure 2.15. Study of *CD38* promoter methylation in MOLP-8 and RMOLP-8 cells. Bisulfite sequencing results of the 22 CpG islands analyzed.

Since we did not observe methylation differences in these studies, a methylation array was performed. Specifically, the “Infinium methylation EPIC array” allows the interrogation of methylation patterns at the genome-wide level, covering more than 850.000 methylation sites. In this sense, after data normalization we obtained results from 812.181 DNA methylation positions, of which 394 promoter regions were hypomethylated in RMOLP-8 cells in comparison to MOLP-8 cells, while 249 were hypermethylated. Regarding methylation sites at gene coding sequence, 474 were hypomethylated in resistant cells whereas 582 were hypermethylated. Furthermore, the EPIC array contained 27 CpG islands associated to the *CD38* gene, 9 of which were associated to the promoter region and the remaining 18 to the body of the gene. Interestingly, the promoter region of *CD38* in resistant cells presented a major methylation status in comparison to sensitive cells (Figure 2.16a) while differences in the methylation status of *CD38* gene body were not observed between both cell models (Figure 2.16b).

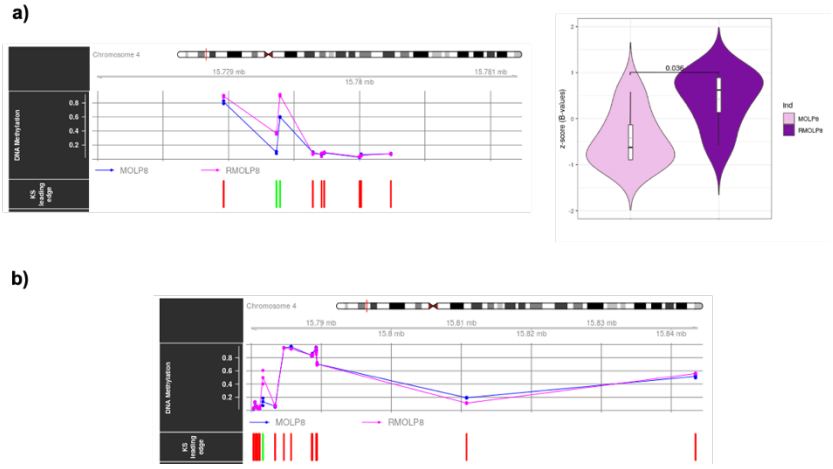

Figure 2.16. Results from the methylation array corresponding to the *CD38* gene. a) Left panel: Methylation status of CpG islands covered in the array that correspond to the *CD38* gene promoter. Right panel: Statistical analysis of the differences observed in the left panel following the procedure explained in Materials and Methods section. **b)** Methylation status of CpG islands covered in the array that correspond to *CD38* gene body.

Noteworthy, the bisulfite method study and the methylation array only had in common 1 CpG island (GGCACTG[CG]GGGACAG). No differences in methylation were observed by both methodologies in sensitive and resistant cells in this specific island.

3. Transcriptomic characterization of the acquired resistance model to daratumumab mediated CDC

Differences in the gene expression profile (GEP) between MOLP-8 and RMOLP-8 cells were evaluated using the Human Gene 2.0 ST RNA microarrays. After applying the filter values (adjusted p-value or q-value < 0.05), 546 differentially expressed genes between the two cell lines were obtained, of which 182 were underexpressed and 364 overexpressed in the resistance model in comparison to its sensitive counterpart (Figure 2.17).

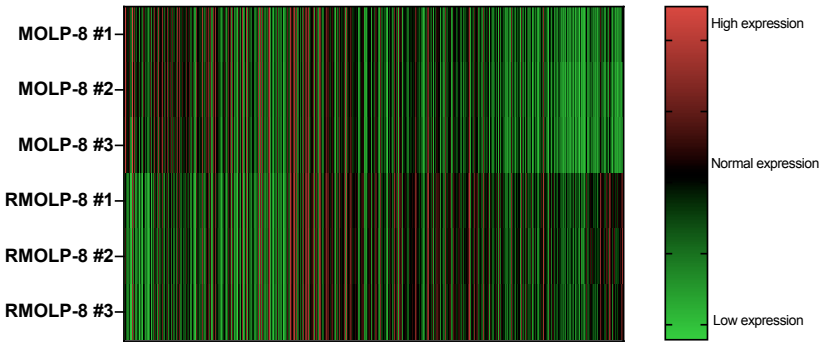

Figure 2.17. Significantly deregulated genes in daratumumab-resistant cells in comparison to their sensitive counterpart. Heat map of the deregulated genes in both cell lines. Results from three different samples for each cell line are shown.

The top-10 of the overexpressed and underexpressed genes in RMOLP-8 cells in comparison to MOLP-8 cells are listed in Table 2.1. The whole information about significantly deregulated genes can be found on the Appendix section (Supplementary Table 1).

Table 2.1. Top-10 of significantly underexpressed and overexpressed genes in daratumumab-resistant cells in comparison to their sensitive counterpart. In green: underexpressed genes in RMOLP-8 cells; in red: overexpressed genes in RMOLP-8 cells.

Gene	Score	Fold Change	q value (%)
TENM1	-7.5433859	0.078748	0
LINC00632	-12.132038	0.079267	0
TMEM47	-17.096345	0.167870	0
ITM2A	-15.957792	0.208208	0
CDR1	-9.9106124	0.210661	0
FGFR2	-12.826855	0.223028	0
LMO3	-6.9580591	0.225133	0
SLITRK4	-4.9992532	0.256175	0.85322913
PROX1	-4.0609772	0.264420	2.77992522
CD38	-6.4556382	0.265209	0
AZGP1	4.1664865	5.061123	1.44744227
COBLL1	11.9525514	5.173809	0
KIAA1024L	3.62714505	5.616566	1.70446473
LPCAT2	9.59789583	5.719166	0
CD9	5.31820348	7.049557	0

MAGEB1	8.37442881	7.801585	0
TPD52L1	8.73863435	9.858854	0
GJA1	5.41176258	12.490549	0
SPRR2A	3.50917778	18.248015	2.77992522
HHLA2	3.76288489	27.932568	1.70446473

In addition, gene set enrichment analysis (GSEA) was performed in order to identify significantly altered biological processes in resistant cells. As shown in Figure 2.18a, three biological processes were enriched in resistant cells in comparison to sensitive cells: *reelin signaling pathway*, *metabolism of lipids* and *immune system*. Specifically, *reelin signaling pathway* was the most enriched one with an enrichment ratio of 18.62 ($p<0.001$) mainly because 3 out of the 5 genes on the gene set were deregulated in resistant cells (Figure 2.18b). Regarding *metabolism of lipids* and *immune system*, they were enriched in resistant cells with a ratio of 1.70 ($p<0.001$) and 1.5 ($p<0.001$), respectively.

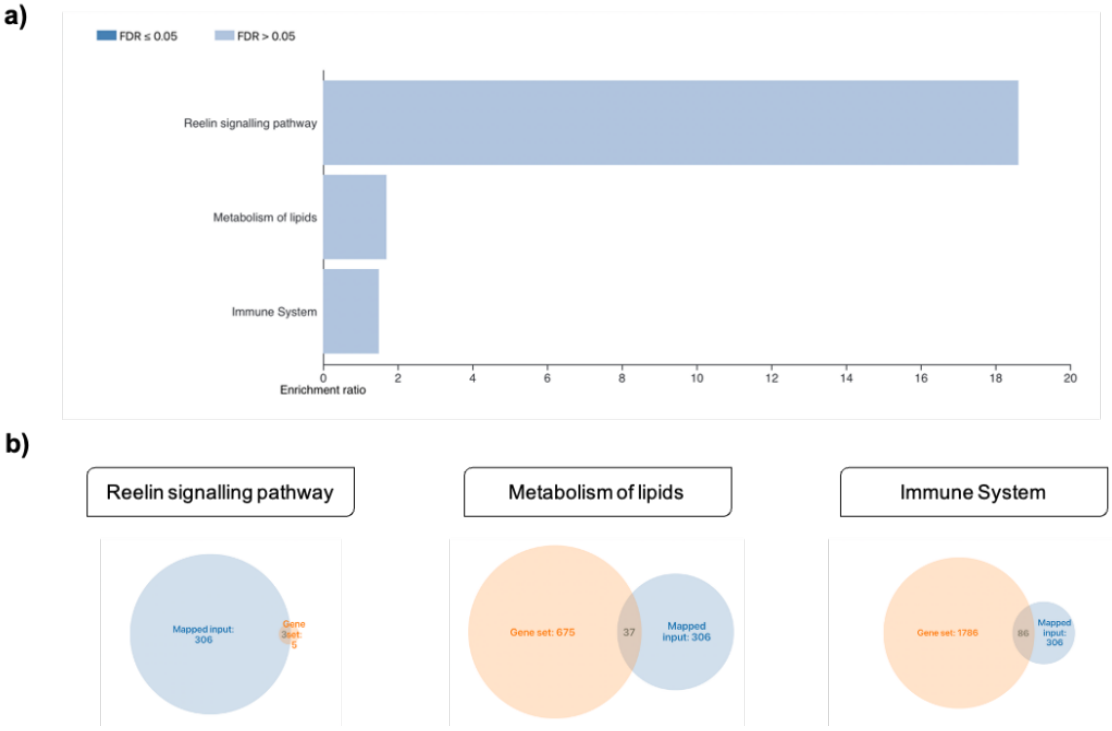

Figure 2.18. Significantly deregulated biological processes in daratumumab-resistant cells in comparison to their sensitive counterpart. a) Graph showing the significantly deregulated biological states in RMOLP-8 cells in comparison to MOLP-8 cells. **b)** Venn diagram of the number of genes contained in each of the REACTOME gene sets and the genes in the mapped input.

Of the three biological processes mentioned, *immune system* drew our attention, with 86 significatively deregulated genes (Appendix section; Supplementary Table 2). Among them and confirming our previous data, *NCAM1* (CD56) and *CD81* appeared to be significantly deregulated in resistant cells in comparison to sensitive cells, being down-regulated (fold change: 0.683; q-value: 1.45) and up-regulated (fold change: 1.41; q-value: 4.85), respectively. Moreover, the antiapoptotic molecule Bcl-2 was also up-regulated (fold change: 2.47; q-value: 1.7) in RMOLP-8 cells in comparison to MOLP-8. Furthermore, the inhibitor of the C5b-9 complex involved in the complement cascade, Clusterin (*CLU*), was overexpressed (fold change: 3.94; q-value: 1.45) in resistant cells in comparison to sensitive cells.

Additional deregulated genes not included in the *immune system* biological process, such as *HHLA2, CD276* and *CD38* were also considered relevant. Specifically, *HHLA2* (B7-H7) and *CD276* (B7-H3) were up-regulated in RMOLP-8 cells in comparison to MOLP-8 (fold change: 27.93, q-value: 1.70; fold change: 1.38, q-value: 3.32, respectively), both of them with T cell modulatory functions. With respect to *CD38*, and confirming results presented in previous sections, it was the tenth gene more significantly down-regulated in resistant cells in comparison to sensitive cells with a fold change of 0.265 and a q-value of 0.

Hereunder, functional studies performed in relation to these genes will be described. Specifically, for *BCL2* and *CLU,* the potential direct implication that their deregulation may have in the acquired resistance to daratumumab-mediated CDC will be explored.

3.1. Study of the potential role of RMOLP-8 cells in inhibiting the functionality of T lymphocytes

The regulation and activation of T lymphocytes depends on signaling by the T cell receptor (TCR) and by coinhibitory and costimulatory receptors that deliver negative or positive signals, respectively (Figure 2.19). The amplitude and quality of the

immune response exerted by T cells is controlled by an equilibrium between these costimulatory signals and their coinhibitory counterparts called immune checkpoints.

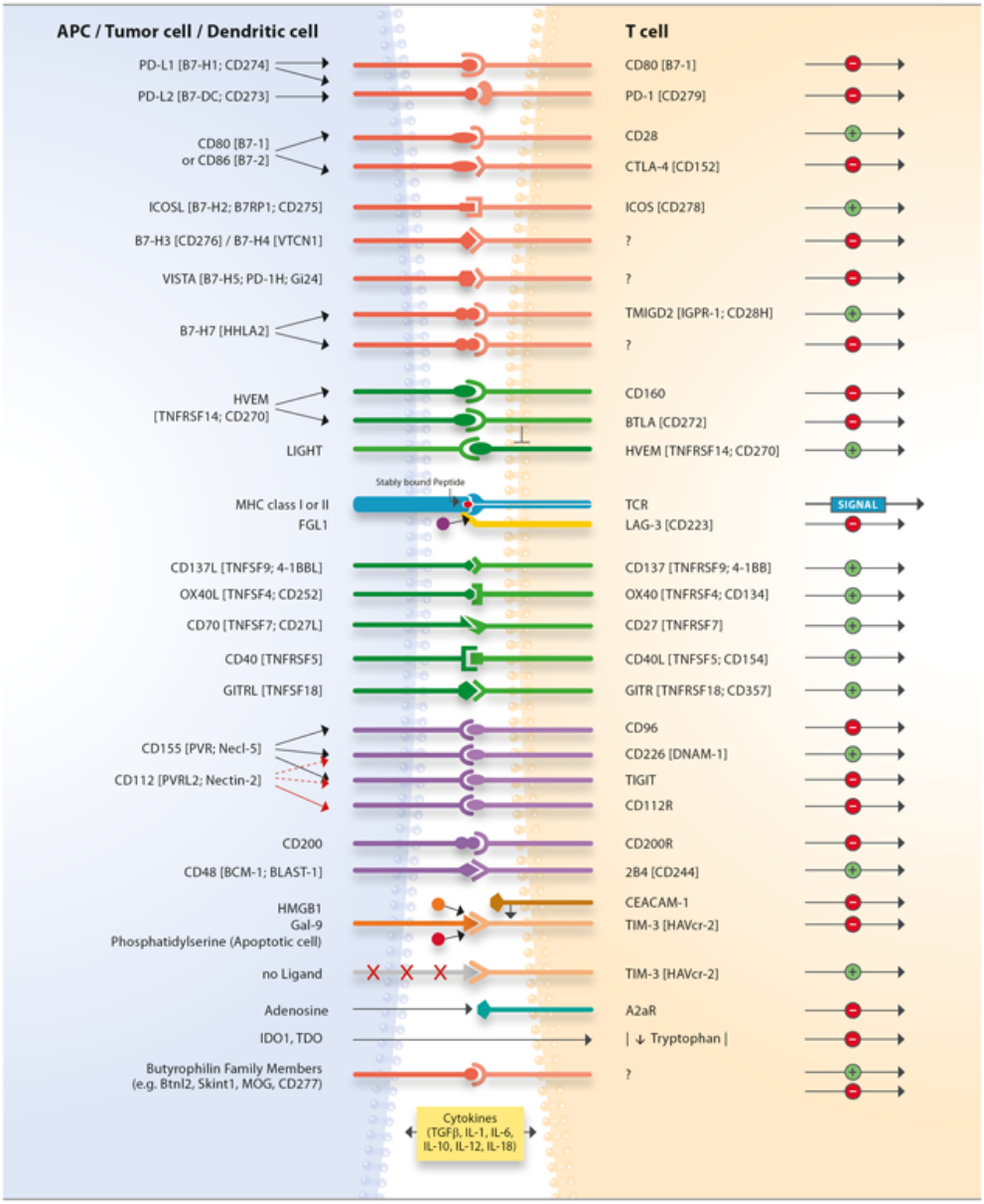

Figure 2.19. Coinhibitory and costimulatory receptors of T lymphocytes and their corresponding ligands[263].

RT-qPCR data showed that the genes with T cell modulatory functions, *HHLA2*, and *CD276* were approximately 25-fold and 2.5-fold overexpressed in RMOLP-8 cells vs MOLP-8 cells (Figure 2.20).

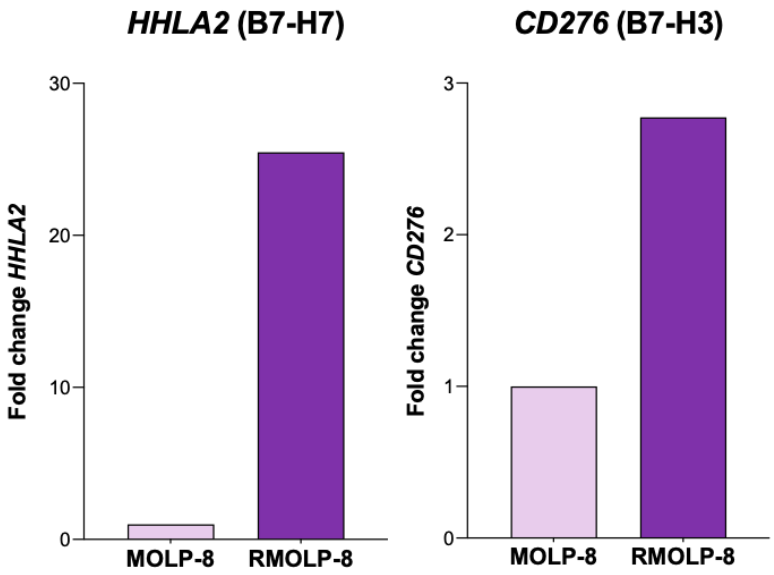

Figure 2.20. Assessment of the expression of T cell modulator molecules in RMOLP-8 cells in comparison to MOLP-8 cells by RT-qPCR. *HHLA2* and *CD276* mRNA levels on MOLP-8 and RMOLP-8 cells assessed by RT-qPCR. The results are shown as the fold change referred to sensitive cells after normalization with *GAPDH* and correspond to the average of three experiments.

Taking these data into account, a T cell activation/proliferation assay was performed in co-culture with MOLP-8 or RMOLP-8 cells. Thus, T lymphocytes that had been in co-culture with resistant cells had lower proliferation and activation rates compared to T lymphocytes that had been co-cultured with sensitive cells, as measured by CFSE loss and CD25 expression, respectively (Figure 2.21).

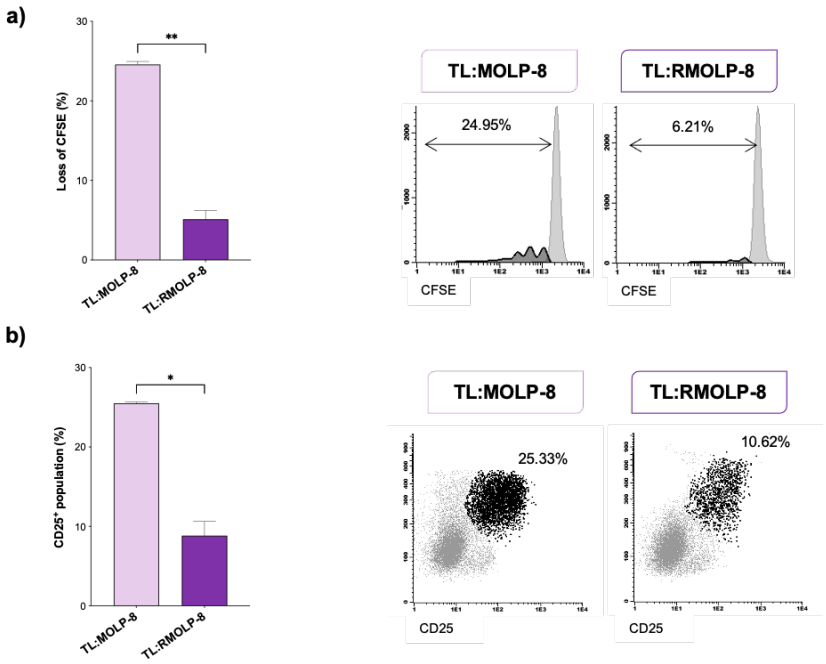

Figure 2.21. Study of the proliferation and activation of T lymphocytes co-cultured either with RMOLP-8 or MOLP-8 cells. T lymphocytes were labelled with CFSE and co-cultured with MOLP-8 or RMOLP-8 cells (ratio 4:1, TL:MOLP-8 or TL:RMOLP-8) in presence of anti-CD3 and anti-CD28 for 5 days. **a)** The loss of CFSE was evaluated by flow cytometry in the T cell population, as a measure of proliferation. **b)** The percentage of $CD25^+$ T lymphocytes was analyzed by flow cytometry, as a measure of activation. Each bar represents mean ± SEM (n=2). Statistically significant differences were evaluated by Student's t-test (*$p<0.05$ and **$p<0.01$). TL: T lymphocyte.

3.2. Evaluation of the involvement of the antiapoptotic molecule Bcl-2 in the resistance to daratumumab-mediated CDC

The up-regulation of *BCL2* in RMOLP-8 vs MOLP-8 identified in the GEP study was confirmed by both RT-qPCR and WB as shown in Figure 2.22. Moreover, other antiapoptotic proteins (Bcl-xL and Mcl-1) were evaluated in both cell lines, being Bcl-xL also overexpressed in RMOLP-8 cells but not Mcl-1 (Figure 2.22b).

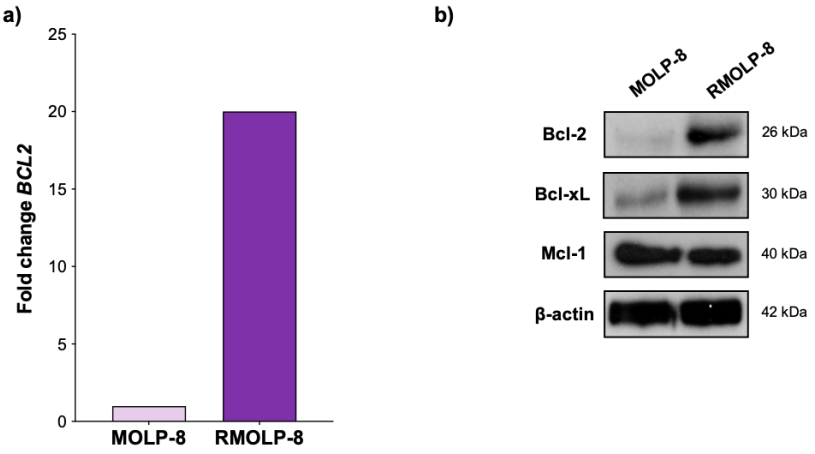

Figure 2.22. Confirmation of the overexpression of the antiapoptotic molecule Bcl-2 in RMOLP-8 cells in comparison with MOLP-8 cells. a) Normalized *BCL2* mRNA levels in MOLP-8 and RMOLP-8 cells as assessed by RT-qPCR. The results are shown as the fold change referred to sensitive cells after normalization with *GAPDH* and correspond to the average of three experiments. **b)** Basal Bcl-2, Bcl-xL and Mcl-1 protein level in MOLP-8 and RMOLP-8 cell lines analyzed by WB. β-actin was used as loading control.

The expression of these antiapoptotic proteins was also analyzed in sensitive cells under daratumumab treatment observing that Bcl-2, Bcl-xL and Mcl-1 increased in presence of the mAb (Figure 2.23), which suggests an attempt of MOLP-8 cells to escape from death induced by daratumumab.

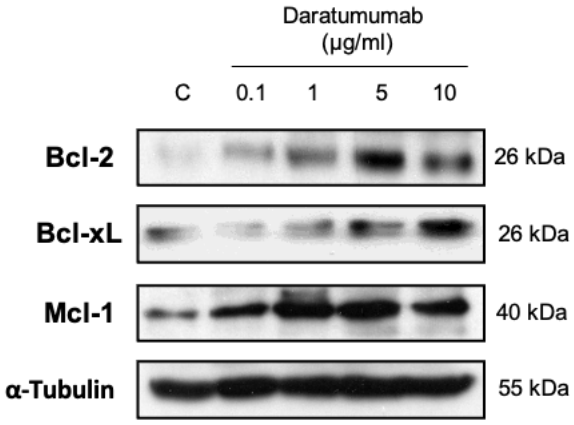

Figure 2.23. Effect of daratumumab on the expression of antiapoptotic proteins in MOLP-8 cell line. Bcl-2, Bcl-xL and Mcl-1 protein levels analyzed by WB after the exposure to daratumumab at the indicated doses + 10% human serum during 1 hour in MOLP-8 cell line. C: control.

Due to the upregulation of Bcl-2 observed in daratumumab-resistant cells, it was assessed whether this increase was translated into a greater sensitivity to the Bcl-2 inhibitor venetoclax. Thus, as shown in Figure 2.24, RMOLP-8 cells have a

significant higher sensitivity to venetoclax at 48 hours of treatment compared to MOLP-8 cells.

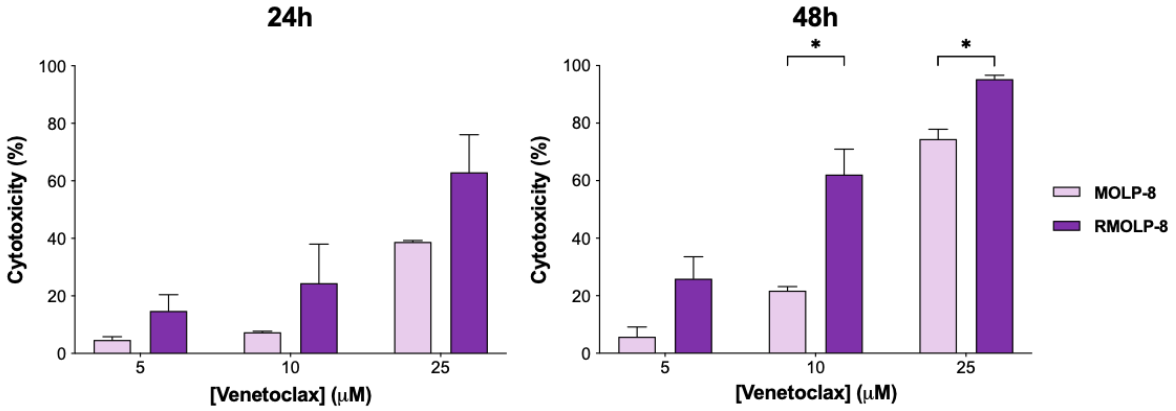

Figure 2.24. Assessment of the cytotoxicity of the Bcl-2 inhibitor venetoclax in MOLP-8 and RMOLP-8 cell lines. Both cell lines were incubated with the indicated concentrations of venetoclax for 24 and 48 hours. The percentage of cytotoxicity, as measured by flow cytometry (Annexin V / 7AAD), is shown relative to the percentage of dead cells in the control condition (cells treated with DMSO). Each bar shows mean ± SEM (n=2). Statistically significant differences were evaluated by Student's t-test (*p<0.05).

Due to the overexpression of Bcl-2 by RMOLP-8 cell line, the effect of pre-treatment with venetoclax on the potential sensitization of RMOLP-8 to daratumumab-mediated CDC was assessed. In this sense, we observed that venetoclax pre-treatment was not clearly able to reverse the acquired resistance to daratumumab (Figure 2.25).

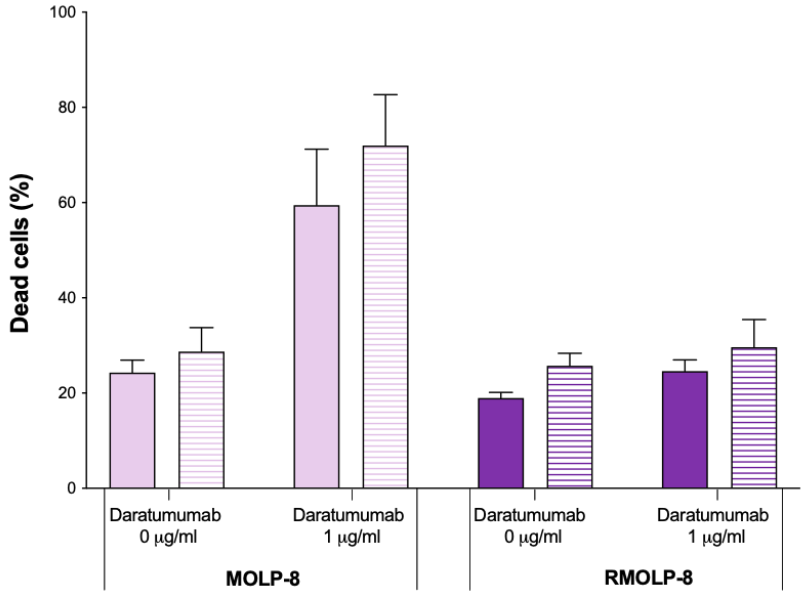

Figure 2.25. Evaluation of the effect of venetoclax pre-treatment on daratumumab-mediated CDC in vitro. The indicated cell lines were incubated for 24 hours with DMSO or venetoclax (10 µM). After the pre-treatment, venetoclax or DMSO were removed from the culture medium and pre-treated cells were incubated during 4 hours with daratumumab (1 µg/ml) in the presence of 10% human serum. Dead cells were analyzed by flow cytometry (Annexin V / 7AAD). Solid bars indicate cells pre-treated with DMSO and horizontal striped bars venetoclax pre-treated cells.

Nevertheless, since the overexpression of Bcl-2 and Bcl-xL in RMOLP-8 cells suggested a potential role of these proteins in the acquired resistance to daratumumab, the effect of stable overexpression of Bcl-2 and Bcl-xL on the sensitivity of the MOLP-8 cell line to daratumumab-mediated CDC was evaluated. To do so, MOLP-8 cells were transduced with a plasmid encoding *BCL2* (pMSCV-Bcl2), *BCLXL* (pMSCV-BclxL) or the empty vector as a control (pMSCV). The transduction efficiency was analyzed by flow cytometry being 12.29% for the empty vector, 12.06% for pMSCV-Bcl2 and 68.11% for pMSCV-BclxL (Figure 2.26a). Then, GFP^+ cells were sorted by flow cytometry and the overexpression of both proteins was confirmed by WB (Figure 2.26b).

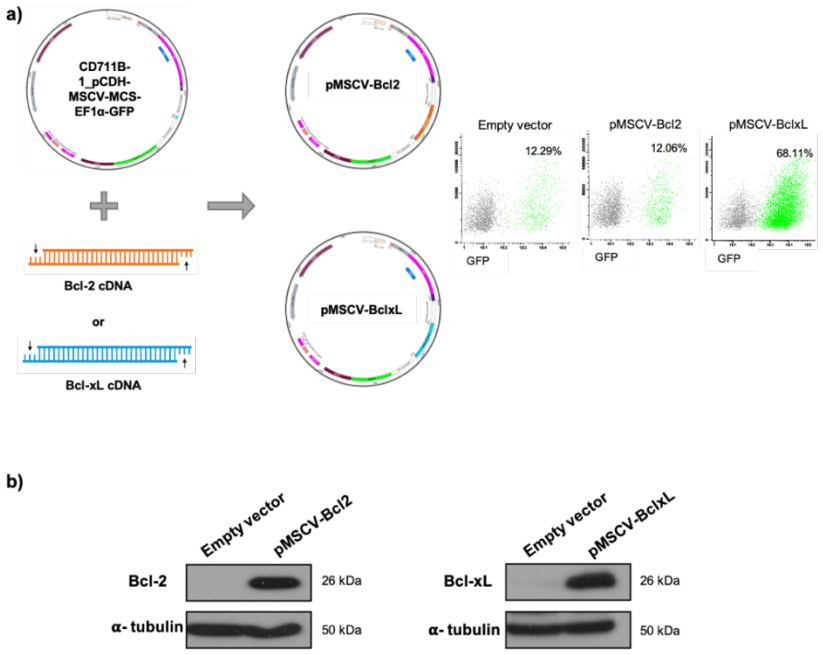

Figure 2.26. Stable overexpression of Bcl-2 and Bcl-xL in the MOLP-8 cell line using lentiviral transduction. a) *BCL2* and *BCLXL* cDNAs were cloned into pMSCV plasmid as specified in Materials and Methods section. GFP expression by flow cytometry allows both evaluation of transduction efficiency and sorting of the transduced cells. **b)** Bcl-2 and Bcl-xL expression analysis in control cells (empty vector) and in MOLP-8-pMSCV-Bcl2 and MOLP-8-pMSCV-BclxL cells by WB. α-tubulin was used as loading control.

After confirming the overexpression of both molecules in the transduced cells, their sensitivity to daratumumab-mediated CDC was tested. In this sense, cells overexpressing Bcl-2 (pMSCV-Bcl2) and cells overexpressing Bcl-xL (pMSCV-BclxL) were as sensitive as the control cells (empty vector) to the CDC mediated by daratumumab (Figure 2.27).

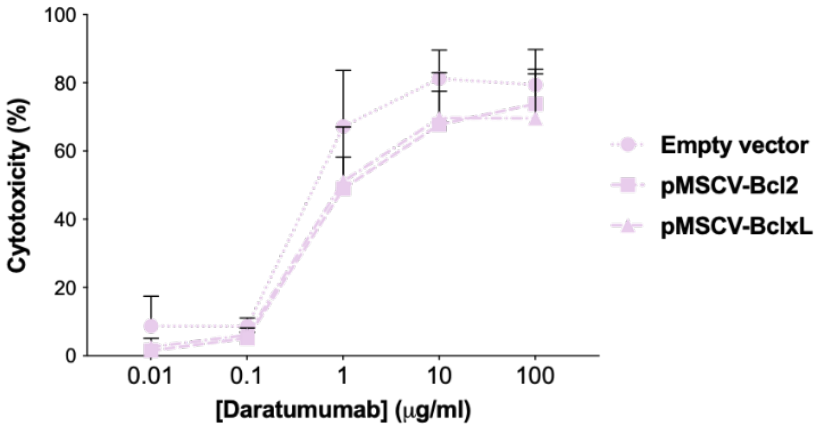

Figure 2.27. Study of the sensitivity of Bcl-2 and Bcl-xL overexpressing MOLP-8 cell lines to daratumumab-mediated CDC. Cell lines stably transduced with pMSCV (empty vector), pMSCV-Bcl2 or pMSCV-BclxL were incubated with increasing doses of daratumumab + 10% human serum for 4 hours. The percentage of cytotoxicity, as measured by flow cytometry, was calculated relative to the percentage of dead cells ($7AAD^+$) in the control (cells treated with isotype control). Each point shows mean ± SEM (n=3).

3.3. Study of the implication of the C5b-9 complex inhibitor clusterin in the resistance to daratumumab-mediated CDC

The upregulation of *CLU* expression in daratumumab-resistant cells that was observed in the microarrays´ study was confirmed by both RT-qPCR and WB (Figure 2.28).

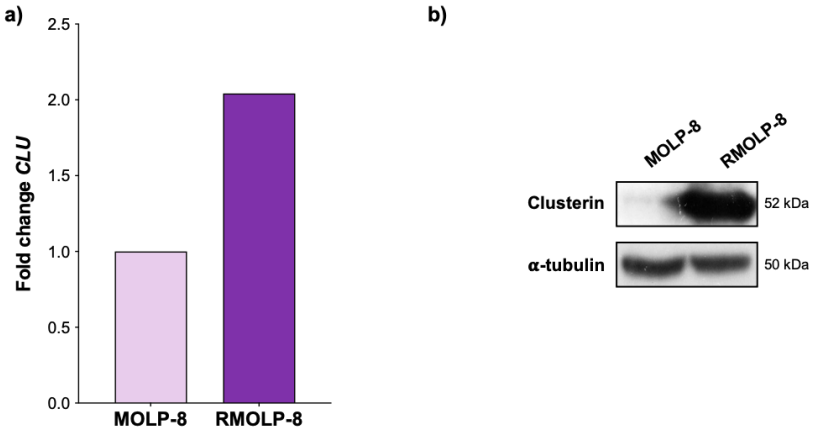

Figure 2.28. Confirmation of the overexpression of the C5b-9 complex inhibitor Clusterin in RMOLP-8 cells in comparison with MOLP-8 cells. a) Normalized *CLU* mRNA levels in MOLP-8 and RMOLP-8 cells by RT-qPCR. The results are shown as the fold change referred to sensitive cells after normalization with *GAPDH* and correspond to the average of at least two samples per cell line. **b)** Basal Clusterin protein level in MOLP-8 and RMOLP-8 cell lines analyzed by WB. α-tubulin was used as loading control.

In order to elucidate the involvement of Clusterin in the acquired resistance to daratumumab-mediated CDC, MOLP-8 cells were transduced to stably overexpress this protein. In this sense, cDNA encoding Clusterin was cloned into pMSCV plasmid (pMSCV-Clu) and MOLP-8 cells were transduced. As shown in Figure 2.29a, transduction efficiency was 0.89% for pMSCV-Clu cells. GFP^+ cells were sorted by flow cytometry and the overexpression of Clusterin was confirmed by RT-qPCR and WB in comparison with MOLP-8 cells transduced with the empty vector as shown in the previous section (Figures 2.29b and 2.29c).

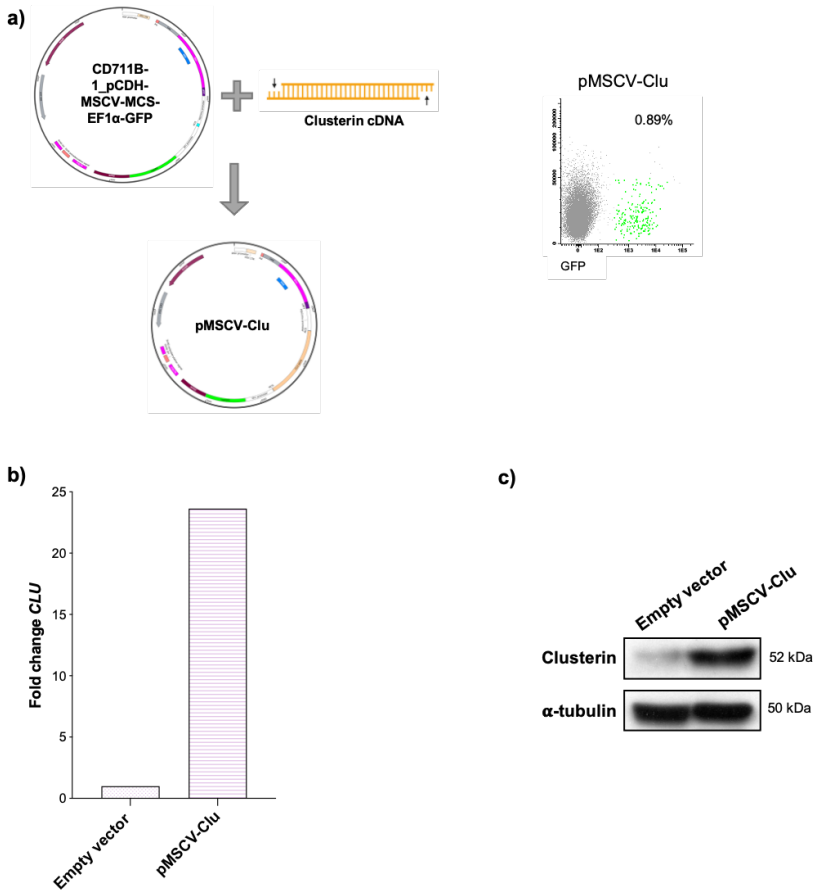

Figure 2.29. Stable overexpression of Clusterin in MOLP-8 cell line using lentiviral transduction. a) *CLU* cDNA was cloned into the pMSCV plasmid as specified in Materials and Methods section. The measurement of GFP expression by flow cytometry allows both evaluation of transduction efficiency and sorting of the transduced cells. **b)** Normalized *CLU* mRNA level in control cells (empty vector) and pMSCV-Clu cells by RT-qPCR. The results are shown as the fold change referred to sensitive cells after normalization with *GAPDH* and correspond to the average of three samples per cell line. **c)** Clusterin expression analysis in control cells (empty vector) and in pMSCV-Clu cells by WB. α-tubulin was used as loading control.

The sensitivity to daratumumab of the new generated cell lines was tested. It is shown that MOLP-8 cells with stable overexpression of Clusterin were significantly more resistant to daratumumab-mediated CDC in comparison to the cells transduced with the empty vector (Figure 2.30).

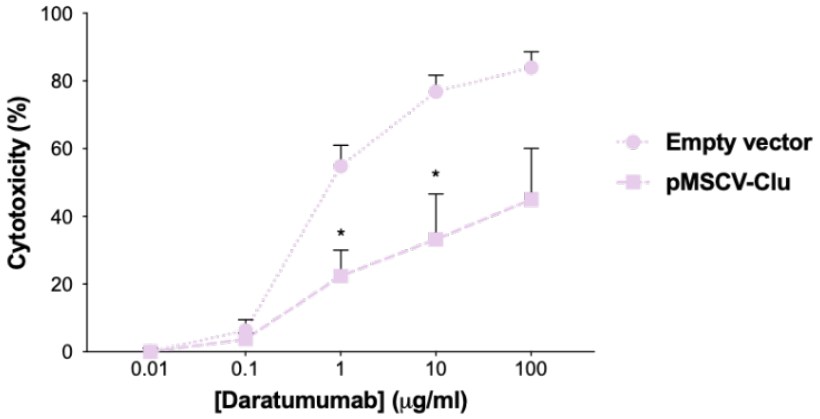

Figure 2.30. Study of the sensitivity of Clusterin overexpressing MOLP-8 cell line to daratumumab-mediated CDC. Cell lines stably transduced with pMSCV (empty vector) or pMSCV-Clu were incubated with increasing doses of daratumumab + 10% human serum for 4 hours. The percentage of cytotoxicity, as measured by flow cytometry ($7AAD^+$), was calculated relative to the percentage of dead cells in the control (cells not treated with daratumumab). Each point shows mean ± SEM (n=3). Statistical differences were assessed by Student's t test (*p<0.05).

Interestingly, MOLP-8 cells with Clusterin overexpression were also more resistant to isatuximab-mediated CDC as shown in Figure 2.31.

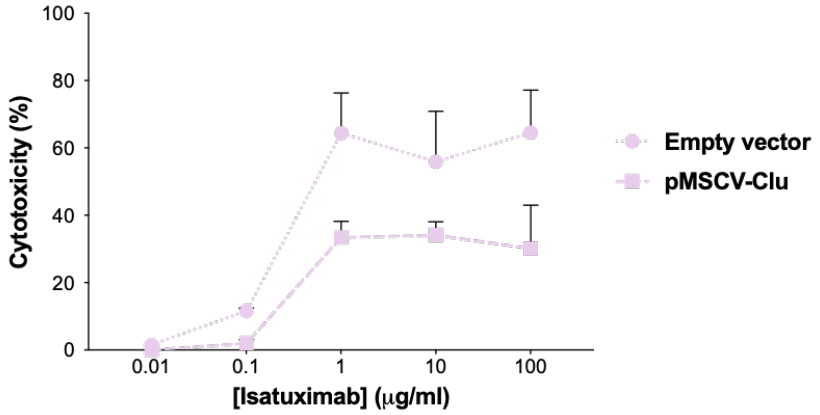

Figure 2.31. Study of the sensitivity of Clusterin overexpressing MOLP-8 cell line to isatuximab-mediated CDC. Cell lines stably transduced with pMSCV (empty vector) or pMSCV-Clu were incubated with increasing doses of isatuximab + 10% human serum for 4 hours. The percentage of cytotoxicity, as measured by flow cytometry ($7AAD^+$), was calculated relative to the percentage of dead cells in the control (cells not treated with isatuximab). Each point shows mean ± SEM (n=3).

On the other hand, Clusterin was stably knockdown in RMOLP-8 cells using several shRNAs. In this sense, silencing of Clusterin reversed the resistance to daratumumab-mediated CDC partially with #509 and #603 shRNAs and completely with #853 shRNA (Figure 2.32).

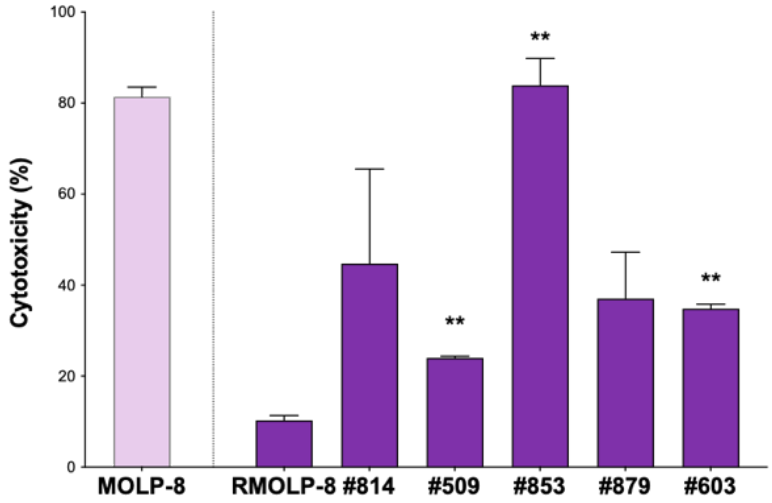

Figure 2.32. Assessment of the effect of Clusterin knockdown on the sensitivity to daratumumab. RMOLP-8 cell line was stably transduced with different shRNAs targeting Clusterin. Specified cell lines were incubated with daratumumab 10 μg/ml + 10% human serum for 4 hours. The percentage of cytotoxicity, as measured by flow cytometry (7AAD+), was calculated relative to the percentage of dead cells in the control (cells not treated with daratumumab). Each point shows mean ± SEM (n=2). Statistical differences were assessed by Student's t test (**p<0.01).

4. Generation of new cell models sensitive to daratumumab-mediated CDC

Taking into account that the availability of MM cell lines sensitive to daratumumab-mediated CDC is very limited[238], we attempted to generate new MM cell lines sensitive to this mechanism. In this sense, in a previous work Nijhof et al. found that daratumumab-sensitive cell lines had lower expression of the CIPs CD59 and CD55, when compared to CDC-resistant cell lines[238]. In fact, when CD55 and CD59 were removed from the cell surface with phospholipase-C, this rendered cell lines more sensitive to daratumumab-mediated CDC[238].

These previous data supported the idea of using CIP knockout cell lines as a potential strategy for stablishing a sensitive cell model to daratumumab-mediated CDC. In this sense, CRISPR/Cas9 technology was used to knock out CD55 and CD59 in RPMI-8226, a myeloma cell line with intermediate to high levels of CD38 in comparison to other myeloma cell lines, but intrinsically resistant to daratumumab-mediated CDC[238].

For this purpose, we first generated a RPMI-8226 cell line with constitutive expression of the Cas9 endonuclease (RPMI-Cas9) by transduction with the lentiCas9-Blast plasmid (Addgene, #52962). The functionality of the Cas9 endonuclease was tested by transduction with a plasmid that induces GFP expression and a gRNA to guide the Cas9 endonuclease on GFP DNA (px011). Therefore, if cells express a functional Cas9 endonuclease, GFP expression should disappear over time. In fact, results indicated that RPMI-Cas9 cells harbored a functional endonuclease since, even if the efficiency of the transduction was not very high, GFP^+ cells percentage decreased in RPMI-Cas9 transduced with px011, whereas it did not change in RPMI-8226 cells transduced with the same plasmid but not expressing Cas9 (Figure 2.33).

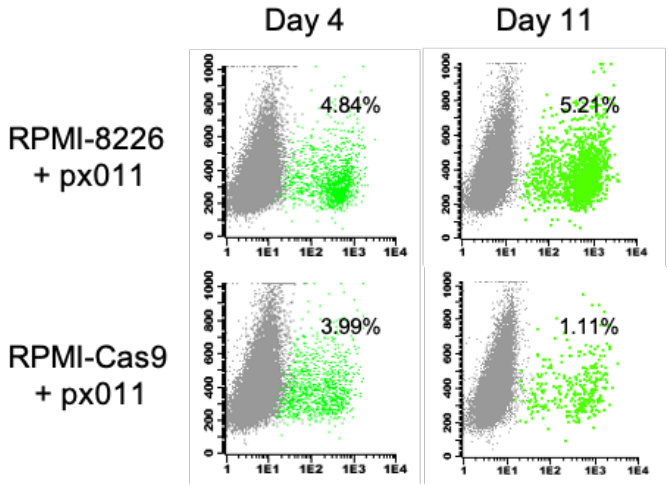

Figure 2.33. Assessment of Cas9 endonuclease functionality. RPMI-8226 and RPMI-Cas9 cell lines were transduced with the px011 plasmid. The percentage of cells positive for GFP was assessed over the time by flow cytometry.

Once Cas9 functionality was confirmed, RPMI-Cas9 cell line was stably transduced with gRNAs against the CIPs CD55 or CD59. The efficiency of the edition was analyzed by amplifying the target region by PCR, sequencing and comparing the DNA from the knockout cell pool and that of cells transduced with the empty vector using the TIDE software[264]. We obtained a 5.3% of efficiency for the CD55 gRNA and 29.1% for the CD59 gRNA (Figure 2.34).

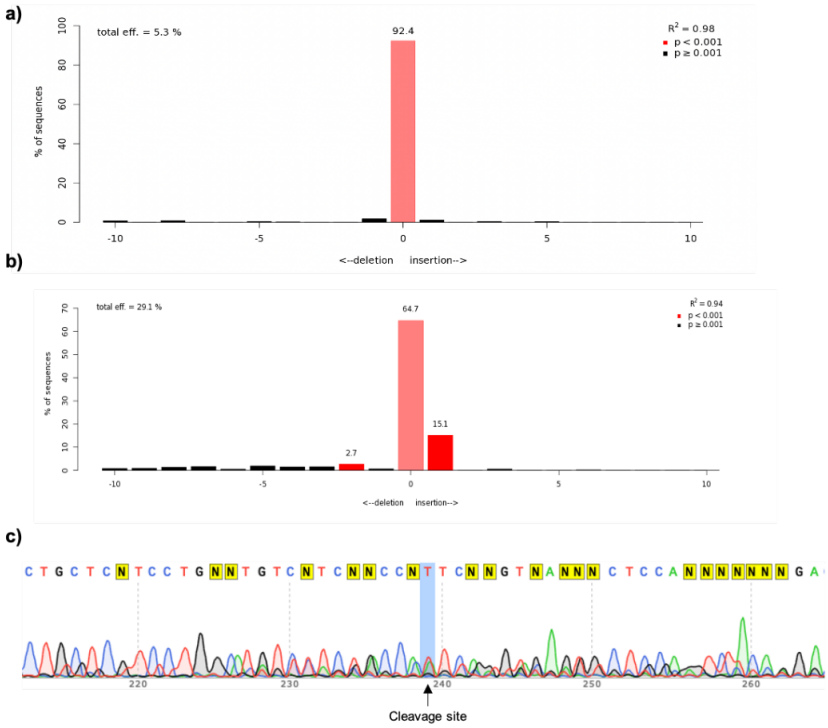

Figure 2.34. Assessment of the edition of *CD59* gene in the RPMI-8226 cell line. gRNA editing efficiency of **a)** CD55 gene and **b)** CD59 gene. The y-axis indicates the percentage of sequences presenting a specific alteration, whereas the x-axis represents the identified deletion (<0) or insertion (>0) indicating the number of base pairs in the insertion or deletion. For instance, in letter b 15.1% of the sequences showed a one base pair insertion, being the total efficiency 29.1%. **c)** Cleavage site of the CD59 gRNA in the DNA by Sanger sequencing.

Since we were not able to obtain CD55 KO cells, we decided to focus on the characterization of the CD59 edited cells. After single cell dilution, 13 homogeneous CD59 KO clones were obtained, which were characterized for CD59 expression by both WB and flow cytometry (Figure 2.35).

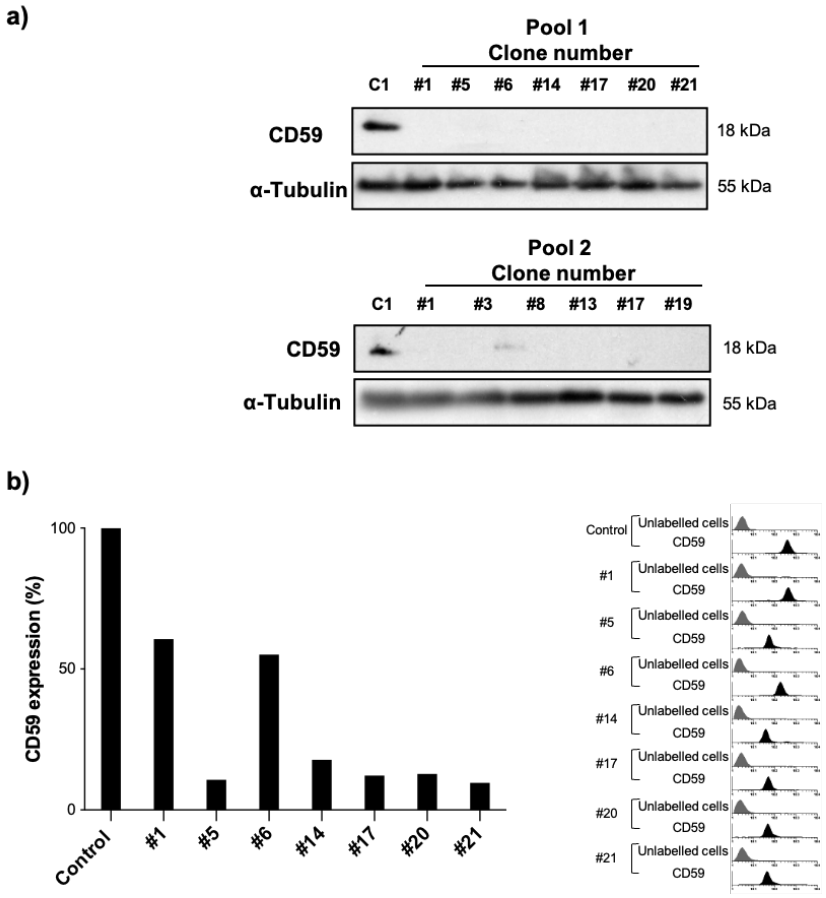

Figure 2.35. Confirmation of CD59 knockout (KO) clones. Single cell dilution was performed separately in two different days (pool 1 and pool 2). **a)** CD59 expression analysis in control cells (transduced with empty vector; C1) and in CD59 gRNA-transduced cells by WB. α-tubulin was used as loading control. **b)** Left: Normalized CD59 MFI expression for the indicated CD59 KO clones, considering control cells as 100%. Right: Histograms corresponding to data shown on the left graph.

The sensitivity to daratumumab-mediated CDC of the CD59-KO clones was tested, observing that 5 clones from pool 1 (#5, #14, #17, #20, #21) and 3 from pool 2 (#8, #13, #19) became sensitive to this mechanism of daratumumab in comparison to the control cells (Figure 2.36).

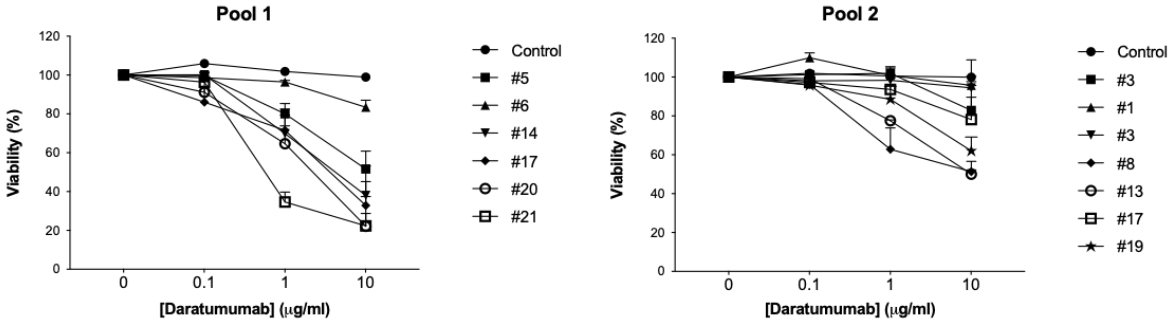

Figure 2.36. Sensitivity of CD59 KO clones to daratumumab-mediated CDC. The single cell derived CD59 KO clones were incubated with increasing doses of daratumumab in presence of 10% human serum for 4 hours. Cell viability was obtained from the average absorbance relative to the percentage of the control. Each point shows mean ± SEM (n=3).

The obtained CD59 KO clones, together with the RMOLP-8 model, will be mainly used in future experiments to delve into the exact role of Clusterin in the acquired resistance to daratumumab.

DISCUSSION

MM is one of the neoplasms that has experienced the highest progress in the last two decades, regarding both biological knowledge of the disease and development of new therapies. Importantly, the improvement of myeloma treatment with the introduction of new therapeutic strategies, including immunotherapy, has resulted in better responses leading to longer survival, and even enabling a large proportion of patients to achieve negative measurable residual disease.

In the context of immunotherapy, the anti-CD38 mAb daratumumab has reported remarkable results in myeloma patients; in fact, it has shown 31.1% ORR as single agent in heavily pretreated RRMM patients[265] and deep responses and prolonged PFS when combined with standards of care[266,267]. However, despite the well-established clinical efficacy of this mAb, there is substantial heterogeneity in quality and duration of response among patients[240] and, what is more, eventually almost all patients become refractory to daratumumab[268] due to resistance mechanisms. Regarding the first point, it is known that response to daratumumab is significantly associated with CD38 expression levels on tumor cells[238,269], therefore drugs with the capacity of modulating this protein may improve the efficacy of daratumumab[269–271] and eventually improve the clinical outcome of patients receiving this mAb. With respect to the second point, the detailed characterization of the mechanisms involved in resistance might lead to the development of strategies aimed at avoiding or reversing its appearance in the clinic.

Tinostamustine, a first-in-class alkylating histone deacetylase inhibitor, has shown anti-myeloma activity in preclinical models as reported in a previous work from our group[236]. Here, we have assessed the effect of tinostamustine in increasing the expression of CD38, the target of daratumumab, and demonstrated that this drug significantly increases the surface expression of CD38 in most of the evaluated MM cell lines and in myeloma cells of 30% of the patients analyzed. Moreover, in this study we have also demonstrated that tinostamustine increases the effect of daratumumab in vitro in MM cell lines and, more importantly, ex vivo in primary myeloma cells as well as in vivo, in murine models. Considering that part of tinostamustine molecule is composed of an epigenetic drug (vorinostat), our data

are consistent with the previous observations that these type of drugs such as DNMT or HDAC inhibitors increase CD38 expression in myeloma cells[233,270,271].

The increase of CD38 triggered by tinostamustine seems to be, at least in part, transcriptionally regulated since in 3 out of 4 evaluated myeloma cell lines (JJN3, MM.1S and RPMI-8226) *CD38* mRNA levels increase after treatment with this drug. This effect could be due to the increase of Histone 3 acetylation in the *CD38* region observed in MM.1S and RPMI-8226 cell lines after treatment with tinostamustine, which would allow an open conformation of the DNA favoring *CD38* transcription[272]. This finding is in line with García-Guerrero et al. who suggested that the inhibition of HDAC6 by ricolinostat prevented the deacetylation of the *CD38* promoter, and hence, activation of *CD38* transcription[233]. Furthermore, in MM.1S and RPMI-8226 cell lines, exposure to tinostamustine enhances the expression of c-Jun and c-Fos, subunits of the transcription factor AP-1 which has been previously reported to mediate the expression of CD38 in other types of cells such as osteoblasts and osteoclasts[273]. Therefore, our results suggest a potential implication of the AP-1 transcription factor in tinostamustine-mediated CD38 increase in these cell lines. On the contrary, tinostamustine did not seem to increase the expression of c-Jun and c-Fos in MOLP-8 and JJN3 cell lines, but this drug did augment the expression of other transcription factors potentially involved in the regulation of CD38 expression, specifically IRF-1 in MOLP-8 and NFκB in JJN3[262,274]. Taken together, these results suggest different transcriptional mechanisms that could be involved in the increased expression of CD38 promoted by tinostamustine. In fact, a similar conclusion was reported by Bat-Erdene et al. who observed an IRF1-dependent and independent CD38 upregulation by IFN-α in myeloma cells and suggested a role of AP-1 activation in the IRF1-independent CD38 upregulation by IFN-α[234].

In addition to the increase of CD38 expression, the upregulation of ligands for NK cell activating receptors on MM cells promoted by tinostamustine may also be beneficial when combined with immunotherapy treatments. Accordingly, we have demonstrated that tinostamustine increases the expression of several NKG2D and DNAM-1 ligands, especially MICA and MICB, in myeloma cell lines and primary

myeloma cells. This finding is consistent with previous works in which HDACis, such as panobinostat or valproic acid (VPA), contributed to increase the NK-cell-mediated immunity against both solid and hematologic tumors[229,275–277]. Therefore, tinostamustine may be of benefit not only when combined with mAb therapy whose efficacy depends in part on the activity of NK cells, but also with other immunotherapies such as adoptive therapy with NK cells, for example the NKG2D-CAR-NK-cell therapy[278,279]. It is also important to mention that, in addition to NK cells, γδ T cells and CD8 T cells also express NKG2D[277] suggesting that myeloma cells that survive the direct induction of cell death by tinostamustine might become the target of these populations.

Despite the growing development of new pharmacological combinations for MM, the appearance of resistance continues to be an Achilles' heel in the treatment of patients with this disease. That is why the identification of the mechanisms involved in drug resistance is essential to eventually reverse it. In this PhD work some of the potential mechanisms involved in the acquired resistance to daratumumab have been identified.

As previously reported, target expression is crucial for the efficacy of therapeutic mAbs. Indeed, patients with higher CD38 levels before daratumumab administration showed better responses to the therapy[238,239,269]. On the contrary, MM cells from patients at the time of progression during daratumumab therapy have low CD38 expression levels[238]. Thus, the downregulation of CD38 observed in the resistance model generated in the present work is in line with the few published data on daratumumab resistance. As reviewed in the Introduction section, several mechanisms have been described to explain CD38 downregulation, being trogocytosis, depletion of $CD38^{++}$ MM cells and release of CD38 via extracellular vesicles the described mechanisms so far[187,240,241]. In the present work we show that epigenetic modifications of the *CD38* gene promoter may also be involved in its downregulated expression and subsequent diminished protein levels. Specifically, the hypermethylation observed in the promoter region of *CD38* in daratumumab-resistant cells may be responsible, at least in part, for these effects. Although

changes in the methylation pattern of CD38 have not been described in daratumumab-resistance context, Mottahedeh et al. reported that CD38 expression was repressed by methylation in prostate cancer[280]. In addition to hypermethylation, other epigenetic mechanisms could also be involved in CD38 regulation. In particular, the upregulated expression of CD38 promoted by tinostamustine in RMOLP-8 cells suggests that the histone acetylation state might repress or activate the transcription of the *CD38* gene.

Several studies have correlated the expression levels of a specific mAb target to the ability of that mAb to induce CDC, but not ADCC. In fact, Meerten et al. showed that the sensitivity to rituximab-induced CDC, but not ADCC, correlated well with the level of CD20 expression in the used cell lines; thus, cells with high CD20 expression were sensitive to rituximab-mediated CDC, whereas no correlation was observed between CD20 expression level and ADCC sensitivity[281]. With respect to daratumumab, in a panel of 14 MM and 19 non-Hodgkin's lymphoma cell lines it was shown that only 6 cell lines were sensitive to daratumumab-mediated CDC, having the sensitive cell lines significant higher CD38 expression levels compared with the resistant ones[238]. On the contrary, in another study, the great majority of CD38-expressing MM cell lines, including those that were resistant to daratumumab-mediated CDC, were found to be sensitive to ADCC[282]. In addition, different studies have demonstrated that overexpressing CD38 in MM cell lines would sensitize them to the CDC mediated by anti-CD38 antibodies[187,238,283]. The fact that RMOLP-8 cells are resistant to the CDC mechanism but not to ADCC confirms that the lower CD38 expression found in this cell line in comparison to the parental counterpart (MOLP-8 cell line) is enough to make them resistant to daratumumab mediated CDC but no to ADCC. Moreover, among the four MM cell lines (U266, JJN3, NCI-H929 and RPMI-8226) used in our work to overexpress *CD38*, NCI-H929 cell line was the only one that regained sensitiveness to daratumumab-mediated CDC and also the one with the highest CD38 levels, further confirming the importance of CD38 expression threshold to trigger this mechanism of action. Nevertheless, CD38 overexpression in RMOLP-8 cells either by exogenous expression or by tinostamustine treatment, was not able to re-sensitize them to daratumumab. Whether this is because the

necessary CD38 expression threshold was not reached or because additional mechanisms are involved needs further investigation. Regarding the other mechanisms of action of daratumumab, RMOLP-8 cell line is not resistant to ADCP. This is consistent with previous observations by Overdijk et al. regarding daratumumab-mediated ADCP even in cell lines with relatively low CD38 levels, finding it difficult to define a threshold level of CD38 expression that allowed efficient daratumumab dependent phagocytosis and suggesting that additional factors were likely to determine the efficacy of this mechanism[185]. However, RMOLP-8 cells are almost completely resistant to apoptosis via cross-linking mediated by daratumumab, suggesting that a high CD38 threshold is necessary to induce this mechanism of action. In fact, authors who first described the apoptosis via cross-linking activity of daratumumab used CD38-transduced myeloma cell lines for their studies[186].

In addition to CD38 downregulation, the large number of deregulated genes in RMOLP-8 cell line vs MOLP-8 cell line suggests that other mechanisms may be involved in daratumumab resistance. In this sense, we studied the potential implication of the antiapoptotic proteins Bcl-2 and Bcl-xL in daratumumab resistance. Considering that both proteins are overexpressed in RMOLP-8 vs MOLP-8, and taking into account that this type of proteins are not only general survival factors[284] but also, they have been proposed as modulators of the cytotoxicity induced by mAbs[285–287]. Moreover, we have observed that the expression of Bcl-2, Bcl-xL and Mcl-1 increased in sensitive MOLP-8 cells under daratumumab treatment, presumably as an escape mechanism from the activity of daratumumab. Despite this initial hypothesis, neither Bcl-2 nor Bcl-xL stable exogenous expression made MOLP-8 cells more resistant to daratumumab. Moreover, pretreatment of RMOLP-8 cells with the Bcl-2 inhibitor venetoclax did not sensitize them to daratumumab. Therefore, a direct implication of Bcl-2 family of proteins in daratumumab resistance has not been confirmed in our model. However, one aspect that is interesting is that RMOLP-8 cells were more sensitive to venetoclax than MOLP-8 cells.

Resistance mechanisms to mAbs involving complement cascade molecules, in particular the membrane-bound CIPs CD55, CD59 and CD46, have also been reported both in solid tumors and in hematological malignancies. For instance, the specific inhibition of CIPs in breast cancer cell lines increased the CDC mediated by trastuzumab from 10% to 80%[288]. In another work, cells from non-Hodgkin lymphoma patients who did not respond to rituximab treatment, had higher CD46 and CD59 levels in comparison to patients achieving complete response[289]. Similarly, rituximab-resistant B-lymphoma cell lines had an increased expression of CD55 and CD59[290]. In line with this, Nijhof et al. observed that CD55 and CD59 were significantly increased on PCs from MM patients treated with daratumumab at the time of progression[238]. Moreover, the sensitization of our RPMI-8226 cell line observed after knocking-out CD59 supports the involvement of this protein in daratumumab-mediated CDC resistance.

Despite the above data, other protection mechanisms employed by tumor cells to escape complement attack have also been described. For example, inhibition of CIPs with specific mAbs did not affect complement-mediated lysis of some glioma cell lines, which express high levels of CD46, CD55 and CD59[291]. Furthermore, in the present work, our daratumumab-mediated CDC resistance model did not overexpress any of the already mentioned CIPs, but it did overexpress Clusterin, another complement inhibitory molecule. Clusterin is a soluble complement inhibitor that prevents the association of C5b-9 complexes with the membrane[161]. In agreement with this, silencing of this molecule in RMOLP-8 cells or overexpressing it in MOLP-8 cells re-sensitized cells to daratumumab-mediated CDC or induced resistance to this mechanism, respectively, corroborating the involvement of Clusterin in the mechanism of resistance. As far as we know, the present work is the first to study Clusterin in MM and to specifically correlate Clusterin overexpression with daratumumab resistance. Nevertheless, the role of Clusterin in other types of cancer such as gastric and pancreatic cancer, hepatocellular carcinoma, osteosarcoma or acute myeloid leukemia has been previously described. In particular, this protein has been found to be involved in chemotherapy resistance, proliferation and invasion[292–296]. Interestingly, custirsen (OGX-011), a second

generation 2'-methoxyethyl gapmer antisense oligonucleotide complementary to Clusterin mRNA that inhibits its expression, has been shown to potentiate the anti-tumoral activity of different therapies (e.g. trastuzumab, radiation, chemotherapy or zoledronic acid) in preclinical models of solid tumors[297–300]. More importantly, custirsen is under evaluation in some clinical trials in patients with prostate cancer (NCT00054106, NCT00258388)[301,302], metastatic or locally recurrent solid tumors (NCT00471432)[303], breast cancer (NCT00258375)[304] and non-small cell lung cancer (NCT01630733)[305]. However, custirsen has not been tested yet neither in hematological malignancies in general, nor in MM in particular.

Finally, the characterization of the resistance model generated in the present work has enabled to identify the upregulation of a couple of genes belonging to the B7 family of immune checkpoints, specifically *CD276* (B7-H3) and *HHLA2* (B7-H7). These two proteins together with other B7 family members such as PD-L1 (B7-H1 or CD274) and PD-L2 (B7-DC or CD273) are implicated in the regulation of T-cell function[306]. Although some authors have proposed that CD276 has a dual role in the immune system with both costimulatory and coinhibitory functions[307], other authors observed its broad overexpression by multiple human cancers as well as its vital role in tumor progression[308]. In addition to its immunomodulatory functions, CD276 also promotes proliferation and drug resistance, as it has been shown in preclinical myeloma models[309], in line with its overexpression in daratumumab-resistant RMOLP-8 cells. In addition to CD276, RMOLP-8 cells also overexpress HHLA2, for which co-stimulatory and co-inhibitory properties have been reported. In fact, there are studies supporting that a high expression of HHLA2 is related to a favorable prognosis in cancer[310,311], while others consider HHLA2 as a human immunosuppressive protein and a potential therapeutic target[312,313]. Although the specific role of HHLA2 in myeloma context needs to be elucidated, the inhibition of T lymphocytes' proliferation and activation exerted by RMOLP-8 cells, in which a 25-fold increase of *HHLA2* was observed relative to MOLP-8, suggests that, together with CD276, this molecule may have an immunosuppressive role.

Overall, the data collected in this dissertation support the effect of tinostamustine in improving the efficacy of daratumumab, partly due to the enhanced expression of CD38 as well as MICA and MICB, two ligands for NK cell activating receptors. Indeed, these results suggest that tinostamustine could be an appropriate candidate to improve the anti-myeloma efficacy of anti-CD38 mAbs, such as daratumumab, in the clinic. Furthermore, through the characterization of a cellular model of acquired resistance to daratumumab, our work provides an overview of potential molecules that could be involved in the mechanism of resistance to this mAb. Specifically, functional studies point to the implication of Clusterin overexpression in resistance to daratumumab-mediated CDC. We hope that these results may contribute in the future to improve the outcome of MM patients treated with daratumumab.

CONCLUDING REMARKS

CHAPTER II: EVALUATION OF THE MECHANISMS INVOLVED IN THE ACQUIRED RESISTANCE TO DARATUMUMAB-MEDIATED CDC

1. An in vitro model of acquired resistance to daratumumab-mediated complement dependent cytotoxicity, namely RMOLP-8 cell line, was obtained by long-term exposure of the parental sensitive MOLP-8 myeloma cell line to increasing doses of daratumumab and human serum.
2. RMOLP-8 cells are also resistant to daratumumab-induced apoptosis via cross-linking, but not to antibody-dependent cellular cytotoxicity nor to antibody-dependent cellular phagocytosis promoted by this monoclonal antibody.
3. RMOLP-8 cell line is cross-resistant to isatuximab-mediated complement dependent cytotoxicity.
4. The higher proliferation rate and the immunophenotype observed in RMOLP-8 cells, specifically CD56 loss and CD81 increase, indicates a more aggressive phenotype than MOLP-8 cells consistent with poor prognosis myeloma.
5. Resistant cells have lower CD38 expression at both mRNA and protein level in comparison to their sensitive counterpart, in line with the hypermethylation observed of the CD38 gene promoter. Consequently, daratumumab binding to RMOLP-8 cells is lower in comparison to MOLP-8 cells.
6. Tinostamustine increases CD38 expression in RMOLP-8 cells, suggesting that deacetylation mechanisms might also be involved in CD38 downregulation. Nevertheless, treatment with tinostamustine does not sensitize RMOLP-8 cells to daratumumab.
7. RMOLP-8 cells overexpress the anti-apoptotic proteins Bcl-2 and Bcl-xL. Although RMOLP-8 cells are more sensitive to venetoclax than MOLP-8 cells, pre-treatment with this Bcl-2 inhibitor is not able to reverse the acquired resistance to daratumumab.
8. Cells with acquired resistance to daratumumab show increased expression of Clusterin, a soluble complement lysis inhibitor. The knocking down of Clusterin in these cells sensitizes them to daratumumab corroborating its involvement in the mechanism of resistance.

9. The presence of RMOLP-8 cells inhibits the activation and proliferation of T cells. The overexpression of genes with T lymphocyte modulatory functions by RMOLP-8 cells, specifically HHLA2 and CD276, may be involved in these effects.

RESUMEN EN CASTELLANO

INTRODUCCIÓN

1.1. MIELOMA MÚLTIPLE

El mieloma múltiple (MM) es una neoplasia hematológica caracterizada por la expansión clonal de células plasmáticas (CP) en la médula ósea (MO). El MM es la segunda neoplasia hematológica más común y representa el 0,9% de todos los diagnósticos de cáncer en 2018 según las estadísticas del Observatorio Global del Cáncer (GLOBOCAN)[1]. La mediana de edad de los pacientes con MM en el momento del diagnóstico es de alrededor de 65 años, siendo ligeramente más común en hombres que en mujeres y dos veces más común en afroamericanos en comparación con los caucásicos[1,2] (Figura 1).

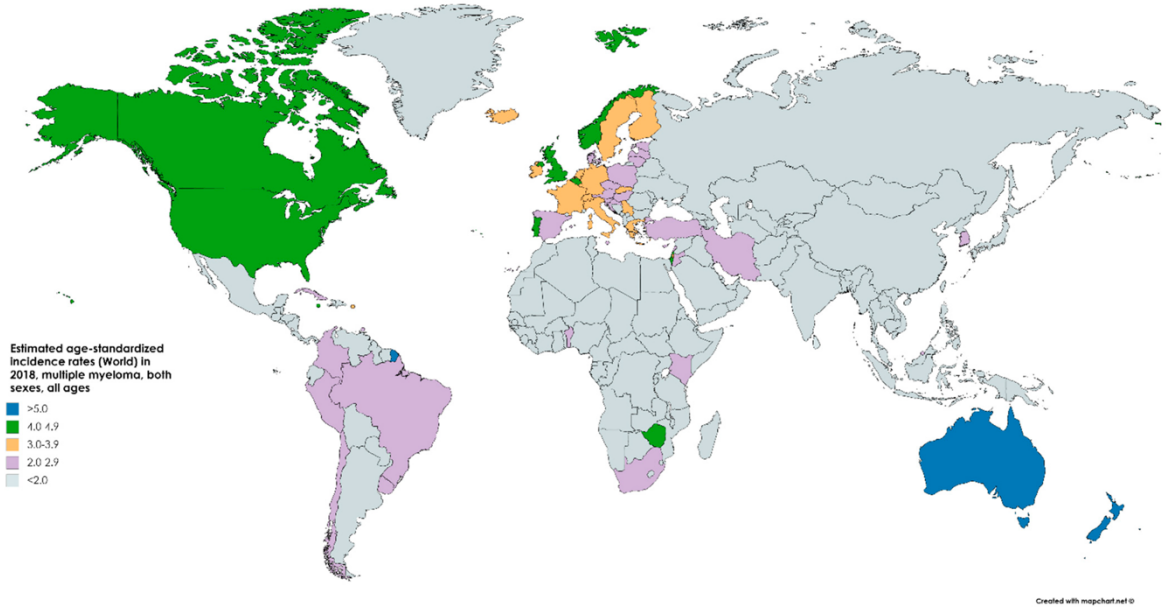

Figure 1. Tasa de incidencia global estimada estandarizada por edad por cada 100.000 habitantes (en todo el mundo) de MM para ambos sexos y todas las edades[1].

Según el Grupo Internacional de Mieloma (IMWG), el diagnóstico de la enfermedad se basa tanto en la infiltración de CPs en la MO (≥10 % de celularidad en el examen morfológico de la MO o un plasmocitoma extramedular comprobado por biopsia) como en la presencia de uno o más eventos definitorios de mielomas (MDE). Los MDE consisten en: (i) daño de órgano terminal definido por las características CRAB (acrónimo derivado de las manifestaciones clínicas de la enfermedad: hipercalcemia, insuficiencia renal, anemia y enfermedad ósea lítica);

(ii) porcentaje de células plasmáticas clonales en la MO ≥60%; (iii) proporción de cadenas ligeras libres (FLC) séricas involucradas:no involucradas ≥100; y (iv) la presencia de una o más lesiones osteolíticas en la radiografía esquelética, tomografía computarizada (TC) o tomografía por emisión de positrones-tomografía computarizada (PET-CT)[3,4].

Casi todos los pacientes con MM evolucionan desde un estadio premaligno asintomático denominado gammapatía monoclonal de significado incierto (MGUS)[5,6], que progresa a MM a una tasa del 1% anual[7]. Dado que el MGUS es asintomático, más del 50% de las personas a las que se les diagnostica han tenido la afección durante más de 10 años antes del diagnóstico clínico[8]. Según el IMWG, el MGUS se define por la presencia de proteína monoclonal sérica (tipo IgM o no IgM) <3 g/dL, <10% de células plasmáticas en la MO y ausencia de características CRAB[3]. Los principales factores asociados a la progresión a MM son: la presencia de más del 5% de CPs malignas en la MO, el aumento de la concentración de proteína M sérica, el tipo de proteína M (pacientes con isotipos IgA e IgM presentan un mayor riesgo de progresión que aquellos con isotipo IgG), y la proporción anormal de FLC Kappa-Lambda en suero[7].

En algunos pacientes, se puede diagnosticar una etapa premaligna intermedia asintomática pero más avanzada denominada mieloma múltiple latente (SMM). El SMM se define por la presencia de proteína monoclonal sérica de ≥3 g/dl o proteína monoclonal urinaria ≥500 mg durante 24 h y/o 10% a 60% de CPs clonales en la MO sin evidencia de ningún otro MDE[9]. El SMM progresa a un ratio de 10% al año durante los primeros 5 años tras el diagnóstico y disminuye a partir de ese momento[10]. Los factores de riesgo de progresión incluyen: alta concentración de proteína M sérica, isotipo IgA, alta infiltración de CPs en la MO, alta proporción de FLC en suero, inmunoparesia y la presencia de más del 95% de células plasmáticas malignas en la MO[11].

1.2. FISIOPATOLOGÍA DEL MIELOMA MÚLTIPLE

El MM es una enfermedad heterogénea que resulta de múltiples eventos genómicos que conducen al desarrollo y progresión del tumor. Junto con las anomalías genómicas, las alteraciones epigenéticas y la interacción de las células plasmáticas con el microambiente de la MO juegan un papel fundamental en la progresión de la enfermedad y la aparición de resistencia a los tratamientos.

1.2.1. Alteraciones genéticas

Los estudios de citogenética molecular han demostrado que casi todos los pacientes con MM albergan alteraciones citogenéticas que conducen a la inestabilidad genómica que caracteriza a esta enfermedad. Principalmente, la patogénesis genética del MM se puede dividir en dos grupos: eventos genéticos primarios y eventos genéticos secundarios (Figura 2).

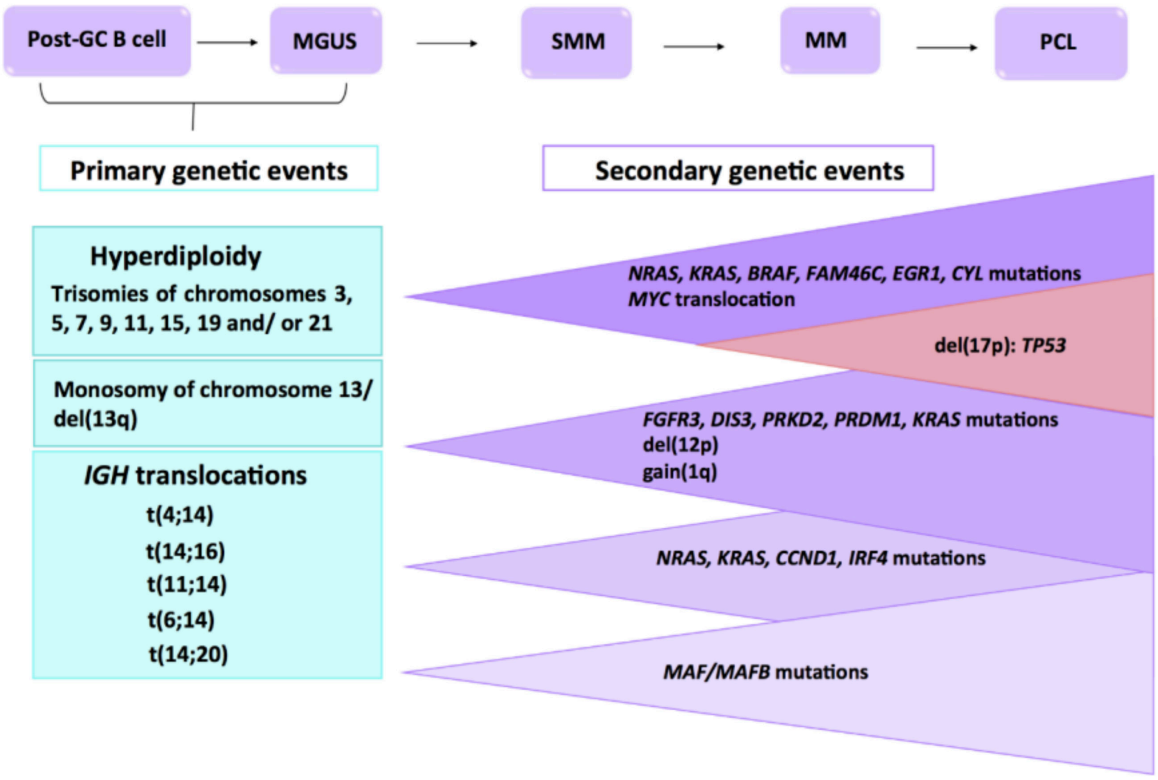

Figura 2. Eventos genéticos primarios y secundarios involucrados en la transformación y progresión del MM[12].

1.2.1.1. Alteraciones genéticas primarias

Los eventos genéticos primarios generalmente se dividen en subtipos hiperdiploides (HRD) y no HRD. Casi la mitad de los tumores MM son del subtipo HRD preferentemente con trisomías de algunos o todos los cromosomas 3, 5, 7, 9, 11, 15, 19 y 21[13].

Entre los tumores no HRD, la deleción del brazo corto del cromosoma 13 [del(13q)] se puede encontrar en el 45-50% de los pacientes con MM y también está presente en la etapa MGUS[14]. Los pacientes con del(13q) tienen niveles más bajos del gen supresor de tumores Retinoblastoma (*RB1*), lo que permite la progresión del ciclo celular[15].

Finalmente, una de las características centrales de los tumores no HRD son las translocaciones cromosómicas que están presentes hasta en el 50% de los pacientes. De hecho, la gran mayoría (>90%) de las translocaciones afectan al cromosoma 14, específicamente al locus de la cadena pesada de inmunoglobulina (*IGH*)[14]. Debido a estas translocaciones, el producto de fusión resultante termina bajo el control del potenciador *IGH*, que es uno de los más activos en las células plasmáticas[16]:

- Las translocaciones t(11;14) y t(6;14) yuxtaponen el potenciador de *IGH* con los genes de ciclina D (*CCND1* y *CCND3*, respectivamente), inactivando posteriormente el gen *RB1*[12]. Los pacientes con estas translocaciones se clasifican como de riesgo estándar[17].

- La translocación t(4;14) se observa en el 15% de los pacientes y conduce a la sobreexpresión simultánea de dos genes: el receptor 3 del factor de crecimiento de fibroblastos (*FGFR3*) y la proteína 2 del dominio SET de unión al receptor nuclear (*NSD2*), comúnmente conocida como *MMSET*[14]. La sobreexpresión de estos dos genes contribuye a una mayor proliferación tumoral y a una alta tumorigenicidad. Esta translocación se asocia con mal pronóstico y los pacientes se clasifican en la categoría de alto riesgo[12].

- Las translocaciones t(14;16) y t(14;20) desregulan los genes *MAF* y *MAFB*, respectivamente. La sobreexpresión resultante induce la proliferación y protege a las células de la apoptosis inducida por fármacos. Los pacientes con estas translocaciones se consideran pacientes de alto riesgo[12].

1.2.1.2. Alteraciones genéticas secundarias

El desarrollo de la secuenciación de masiva (NGS) ha permitido la detección e identificación de muchas mutaciones genéticas diferentes que podrían desempeñar un papel en la progresión de la MM. A pesar de la heterogeneidad mutacional que caracteriza al MM, los genes mutados más recurrentes pueden agruparse en vías de señalización específicas. De hecho, las mutaciones que afectan a la vía de la proteína quinasa activada por mitógenos (MAPK) (es decir, mutaciones en los genes *KRAS*, *NRAS*, *BRAF*, *EGR1* y *FGFR3*) son las más frecuentes[14]. Además, la vía de reparación del ADN está mutada en el 15% de los pacientes observándose mutaciones en genes como *TP53*, *ATR*, *ATM* y *ZFHX4*[18]. También se han descrito mutaciones en genes pertenecientes a la vía del factor nuclear kappa B (NFκB), como *TRAF3*, *LTB* o *CYLD*[19].

Además de las mutaciones, las translocaciones que afectan a *MYC* también se consideran eventos genéticos secundarios, ya que se encuentran con bajas frecuencias en pacientes con MGUS o SMM (3-4%), mientras que en pacientes con MM de nuevo diagnóstico (NDMM) la frecuencia sube a 15-20%[20] y al 50% en mieloma múltiple resistente y/o refractario (RRMM)[21]. Se ha descrito que *MYC* se yuxtapone a diferentes loci, pero en todas las situaciones, la translocación conduce a la sobreexpresión de *MYC*, lo que aumenta el daño al ADN y las especies reactivas de oxígeno (ROS)[22]. Es importante destacar que el reordenamiento de *MYC* se ha convertido en un evento central de MM desde que se identificó en la transición de condiciones premalignas a MM sintomático[12].

Finalmente, otras alteraciones genéticas secundarias son:

- Deleción del brazo corto del cromosoma 17 [del(17p)]. Esto se ha descrito como un evento secundario ya que aunque solo el 10% de los pacientes con NDMM presentan esta deleción, llega hasta el 80% en etapas posteriores de la enfermedad[23]. Esta alteración citogenética conlleva la pérdida del gen *TP53* que es un gen supresor de tumores responsable del control del ciclo celular y la respuesta al daño del ADN[12]. Por lo tanto, del(17p) es un factor de mal pronóstico para la supervivencia global (OS) en MM[14].

- La deleción del brazo corto del cromosoma 12 [del(12p)] también podría ser un evento secundario en la progresión de la enfermedad. De hecho, según el IMWG, esta deleción podría ser un marcador pronóstico de riesgo intermedio-alto.[24].

- La ganancia del brazo largo del cromosoma 1 [gain(1q)] ocurre en casi el 50% de los pacientes con NDMM y alrededor del 68% en los RRMM[12]. Esta alteración se asocia a mal pronóstico. De hecho, la alteración gain(1q) forma parte del concepto de mieloma de "doble impacto" junto con el estadio 3 del sistema de estadificación internacional (ISS). Se considera que los pacientes con "doble impacto" tienen una enfermedad de riesgo ultra-alto[25]. Sin embargo, el gen o los genes responsables del mal pronóstico de gain(1q) siguen siendo desconocidos[13].

1.2.2. Alteraciones epigenéticas

Además de las alteraciones genéticas ya descritas, datos recientes sugieren que las aberraciones epigenéticas también podrían desempeñar un papel importante en la patogénesis del MM. Las principales alteraciones epigenéticas descritas en las células cancerosas son la metilación aberrante del ADN, las modificaciones de histonas y la expresión anormal de micro-ARN (miARN).

1.2.2.1. Metilación del ADN

La metilación del ADN implica la adición covalente de un grupo metilo al anillo aromático de un residuo de citosina del dinucleótido CpG. Este proceso está

regulado por una familia de ADN metiltransferasas (DNMT) que incluye DNMT1, DNMT3a y DNMT3b, entre otras. Esta modificación del ADN generalmente se ha relacionado con el silenciamiento transcripcional.

En el contexto del MM, como en otros tipos de cáncer, se ha descrito una hipometilación global a lo largo del genoma, debido a la rápida proliferación celular en el centro germinal[26]. A pesar de la extensa hipometilación general, las células mielomatosas frecuentemente presentan hipermetilación en regiones concretas del ADN. Este es el caso del promotor de los genes *CDKN2B* y *CDKN2A* que conducen a un silenciamiento transcripcional de estos genes y por tanto a la progresión del ciclo celular[27]. Además, también se ha encontrado hipermetilación del promotor en algunos miembros de la vía de señalización WNT, así como en componentes de la vía de señalización JAK/STAT[26]. En ambas vías, el silenciamiento transcripcional de los genes puede desempeñar un papel importante en la progresión del MM al desencadenar las dos vías de señalización.

1.2.2.2. Modificaciones postraduccionales de histonas

Las histonas son proteínas que junto con el ADN constituyen la cromatina. La cromatina está organizada en nucleosomas que están formados por un octámero de histonas alrededor del cual está envuelto el ADN. El dominio N-terminal de las histonas puede estar sujeto a diferentes modificaciones postraduccionales como la metilación o la acetilación[28].

Como ya se ha mencionado, uno de los genes más comúnmente translocados en MM es *MMSET* [t(4;14)]. Este gen codifica para la metiltransferasa H3K36, por lo que la translocación de este gen al potenciador *IGH* induce la marca H3K36me2 y en consecuencia un aumento de la accesibilidad a la cromatina[29]. Además de t(4;14), se han identificado mutaciones en varios genes con un papel relevante en la regulación epigenética. De hecho, recientemente se han descrito mutaciones en los genes *IDH1* e *IDH2*[30]. Estos genes codifican isocitrato deshidrogenasas que normalmente producen α-cetoglutarato, pero cuando mutan conducen a la acumulación de 2-hidroxiglutarato, un sustrato que inhibe las enzimas histona

desmetilasa. Estas mutaciones conducen, en última instancia, a una función alterada de factores de transcripción como c-Myc y MAX, que son sensibles al estado de metilación del ADN[31].

Además de estar metiladas, las histonas también pueden estar acetiladas. La acetilación de histonas es una marca general de activación transcripcional que está regulada por histonas acetiltransferasas (HAT) y desacetilasas (HDAC). En MM, la sobreexpresión de HDACs contribuye a que el acetiloma aberrante desempeñe un papel en el fenotipo maligno de MM[28]. Por ejemplo, la sobreexpresión de HDAC1 podría dar lugar a la proliferación a través de la represión de los inhibidores de la quinasa dependiente de ciclina supresora de tumores, y la angiogénesis mediante la represión de p53 y la inducción del factor inducible de hipoxia (HIF-1) y genes sensibles a la hipoxia como VEGF[32].

1.2.2.3. Micro-ARNs (miARNs)

Los micro-ARN (miARN) son ARN reguladores, cortos, monocatenarios y no codificantes que controlan la expresión génica a través del silenciamiento postranscripcional de sus genes diana[26]. Se estima que la expresión de un tercio de los genes podría estar regulada por miARNs.

El desarrollo de la secuenciación de ARN en los últimos años ha permitido la identificación de diferentes grupos importantes de miARNs en el contexto del MM. De hecho, según lo revisado por Amodio et al. firmas distintivas de miARNs, como el aumento de los "miARNs oncogénicos" miR-18, miR-21, miR-125a-5p, miR-32, miR-92a, entre otros, y la regulación a la baja de miR-29b, miR -125b, miR-199a-5p, miR-15a y miR-16 y miR-370 han sido descritos[26]. Además, la sobreexpresión de *MMSET* que ocurre con t(4; 14) reduce los niveles de miR-126, lo que finalmente favorece la regulación positiva de c-Myc[33]. Además, se ha demostrado que la expresión de miARNs circulantes puede tener valor pronóstico en MM[34].

Con respecto a la progresión de la enfermedad, los estudios del perfil de expresión de miARN realizados con CPs normales, y de pacientes de MGUS y MM

revelaron diferentes firmas de miARNs para cada una de estas etapas. De hecho, Pichiorri et al. identificaron miR-21, miR-106b/-25 cluster y miR-181a/b sobreexpresados en CPs de pacientes con MM y MGUS en comparación con donantes sanos. Además, la regulación positiva de miR-32 y miR-17/92 solo se detectó en el contexto del MM[35].

1.2.3. Interacción de las CPs con el microambiente de la MO

Junto con las alteraciones (epi)genéticas ya mencionadas, la interacción entre las CPs de mieloma y su microambiente también juega un papel en la patogénesis del MM. El microambiente de la MO está constituido por diferentes componentes, como células inmunitarias (es decir, células T, células NK, células dendríticas, etc.), células no inmunitarias [es decir, células del estroma de la médula ósea (BMSCs), osteoblastos, osteoclastos, etc.], proteínas de la matriz (es decir, fibronectina, laminina, etc.) y factores solubles secretados (es decir, citoquinas, factores de crecimiento, etc.). La mayoría de los estudios que describen el microambiente de MM se centran en la interacción entre las células tumorales y las BMSCs, que puede ocurrir a través del contacto directo de célula a célula, factores solubles y, como se ha descrito recientemente, a través de vesículas extracelulares que contribuyen a la interacción por la transferencia de proteínas, ARNs no codificantes e incluso lípidos. Estas interacciones desempeñan un papel en diferentes procesos biológicos en el contexto del MM, incluidos, entre otros, el crecimiento y la supervivencia del tumor, la inhibición de la diferenciación de osteoblastos, la resistencia a los fármacos, la diseminación y la localización y el entorno inmunosupresor y proinflamatorio.

La interacción entre las células de MM y el microambiente de la MO contribuye activamente a la patogénesis de la enfermedad, según lo revisado por Maiso et al.[36] (Figura 3). La interacción de las células de mieloma con el nicho de la MO se produce a través de un conjunto de moléculas de adhesión, incluidas las integrinas α y β1 (VLA-4, VLA-5 y VLA-6) en las células de mieloma, con la molécula de adhesión intercelular ICAM-1, así como la molécula de adhesión VCAM-1 en

BMSC[37]. Además, las BMSCs producen factores como SDF-1, que se une al receptor CXCR4 en las células de MM, induciendo una mayor expresión de moléculas de adhesión[37]. Las moléculas expresadas en células mielomatosas como CD138, CD38, CD44 y CD106 facilitan esta interacción con las BMSCs[36].

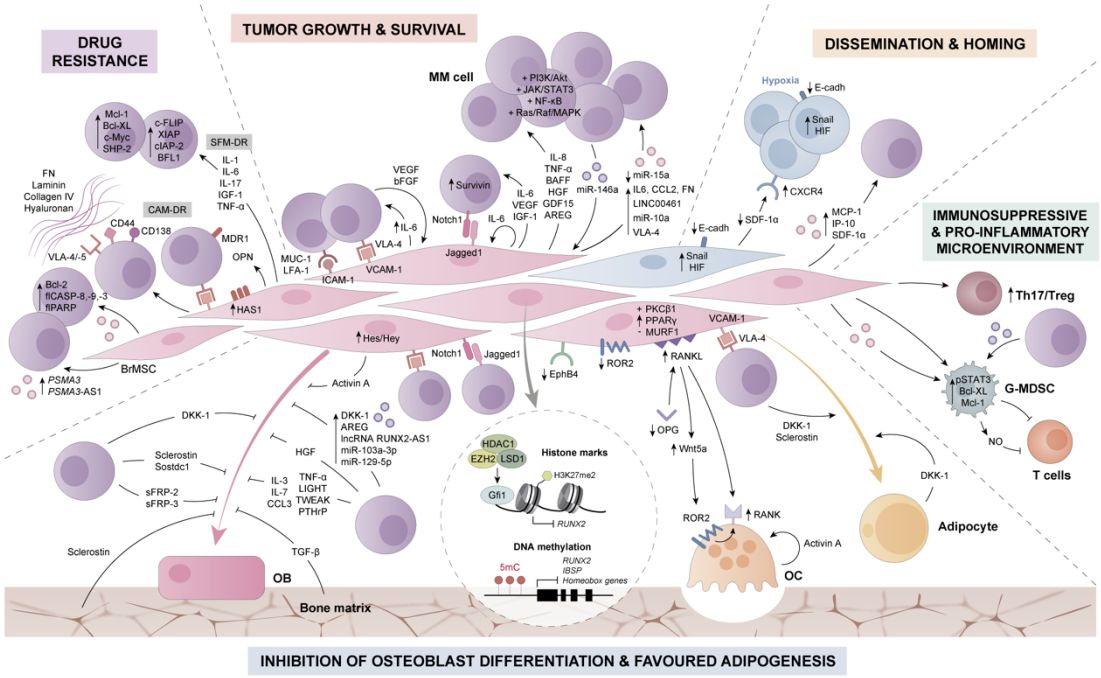

Figura 3. Actividad biológica mediada por BMSCs en el microambiente medular de MM[36].

La producción y secreción de citoquinas por las células del microambiente y por las propias células del mieloma, como TNF-α, IL6, IL21, IGF1, SDF-1α y VEGF entre otros, activan múltiples cascadas de señalización en las células del mieloma (MAPKs, PI3K/AKT, NFκB y JAK/STAT), que, a su vez, inducen la inhibición de la apoptosis y la supervivencia y proliferación de las células tumorales[38].

Además, las células de MM también interactúan en la MO con osteoclastos (OCs), osteoblastos (OBs), células endoteliales y células del sistema inmunitario. En resumen, la interacción de las células de MM con las BMSCs cambia el perfil transcriptómico e induce cambios epigenéticos, reduciendo finalmente la capacidad de formación de hueso de los OBs y favoreciendo tanto la diferenciación como la actividad de reabsorción de los OCs, dando lugar a las lesiones osteolíticas características del MM[39,40].

En cuanto a la implicación del microambiente en la resistencia a fármacos, se sabe que tanto los factores solubles como IL-6, IGF-1, IL-1, IL-17 y TNF-α; y las moléculas de adhesión que incluyen fibronectina, laminina, hialuronano y colágeno IV son responsables de ello[41]. Por un lado, la IL-6 es uno de los mediadores de la resistencia a la dexametasona en las células de mieloma a través de PTPN11[42]. Al igual que la IL-6, el IGF-1 es otro mediador importante de la farmacorresistencia en el contexto del MM. De hecho, IGF-1 induce la activación de múltiples vías de señalización en las células de mieloma (PI3K/AKT, MAPK y NFκB) que tienen como resultado un aumento de la actividad de la telomerasa y una regulación positiva de las moléculas antiapoptóticas[43]. Por otro lado, se ha demostrado que la unión de las células de mieloma a los componentes extracelulares a través de moléculas de adhesión contribuye aún más a la resistencia a fármacos. Específicamente, la unión de fibronectina a través de $\alpha 4\beta 1$ o $\alpha 5\beta 1$ aumenta p27, induce la activación de NFκB y se ha demostrado que altera la expresión de 469 genes en células MM. Además, también se ha descrito una mayor producción de osteopontina (OPN) y hialuronano sintasa 1 (Has1) por parte de las BMSCs que confieren resistencia a las células de MM[36].

Además, la progresión de MM implica una movilización continua de células de MM hacia la sangre periférica (SP) y regreso a la MO. Las CPs malignas dependen del microentorno de la MO para sobrevivir y expandirse; sin embargo, a medida que progresa el mieloma, tanto las células del mieloma como el microambiente se vuelven hipóxicos, lo que lleva a la movilización de las células tumorales a la SP. Como se muestra en la Figura 3, la posterior adaptación de las células MM a la MO depende de las quimioquinas, como SDF-1α, que regulan la adhesión de MM a las BMSCs. Se ha demostrado que la reducción de CXCL12 o la regulación al alza de CXCR4 por hipoxia induce la movilización de CPs fuera de la MO[36].

La participación del microambiente tumoral en la contribución a un nicho tumoral inmunosupresor se revisará en la siguiente sección.

1.3. ALTERACIONES DEL SISTEMA INMUNE EN EL MIELOMA MÚLTIPLE

Los pacientes con gammapatías monoclonales, incluido el MM, han mostrado un estado de inmunosupresión general caracterizado por la alteración de diferentes poblaciones inmunes y perfil de citoquinas. Esta alteración es el resultado tanto del contacto célula a célula como de la presencia de diferentes componentes extracelulares que finalmente inhiben las células citotóxicas y reclutan y activan poblaciones inmunosupresoras[44]. A continuación, discutiremos las alteraciones que se han descrito en el número y función de las principales poblaciones inmunes, así como de las principales moléculas involucradas (Figura 4).

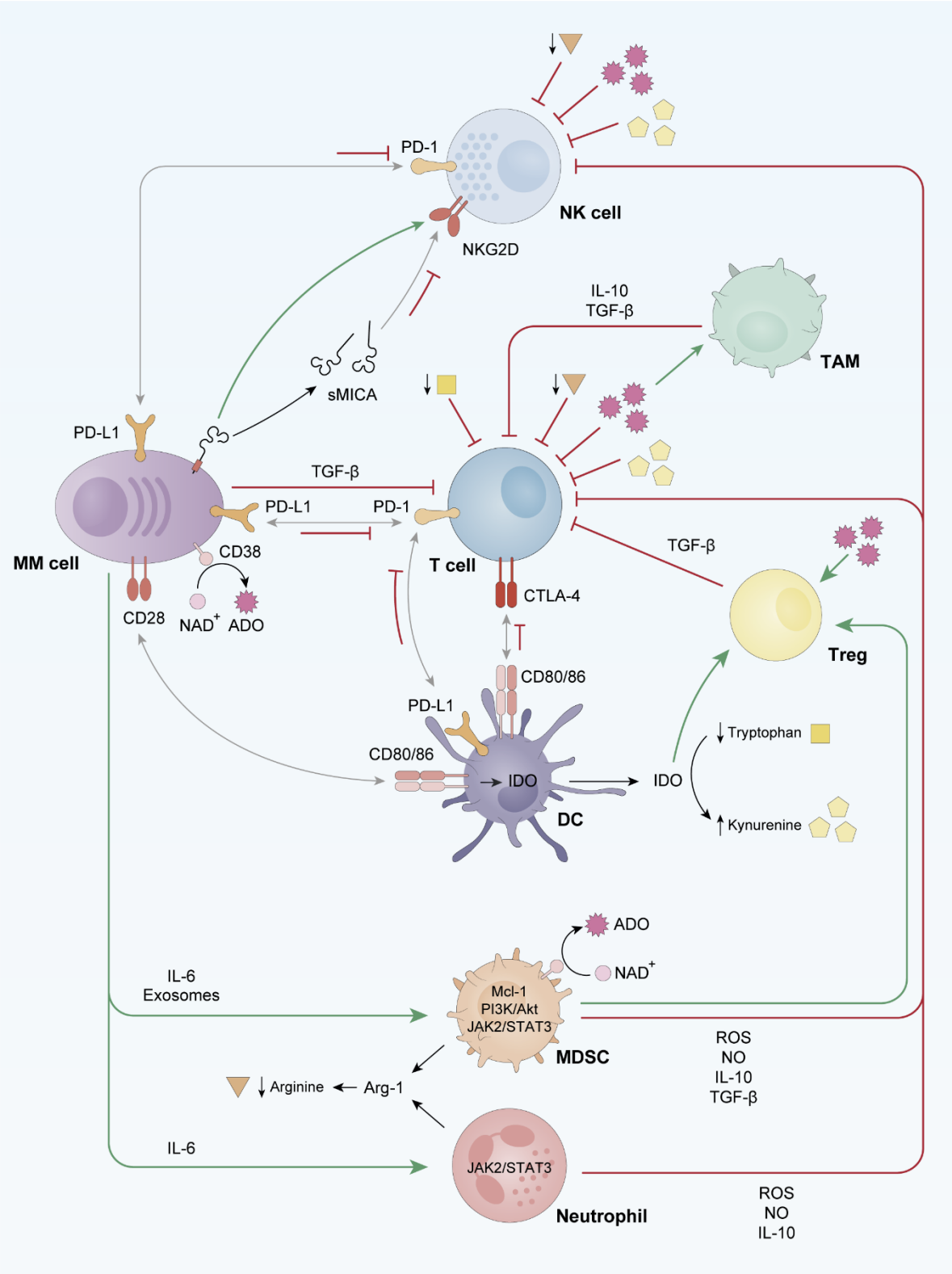

Figura 4. Representación esquemática de las principales alteraciones del sistema inmune descritas en pacientes con MM[44].

1.3.1. Linfocitos T

Los linfocitos T, tanto los T $CD4^+$ como los T $CD8^+$ citotóxicos, son componentes principales del sistema inmunitario adaptativo que actúan como coordinadores y efectores de la inmunidad[45]. Ambas subpoblaciones juegan un papel crucial en la inmunidad antitumoral. En cuanto a las alteraciones encontradas en estas poblaciones en pacientes con MM, la descrita con mayor frecuencia es la disminución de los recuentos de células $CD4^+$, que se asocia con una menor supervivencia libre de progresión (PFS) y OS y una mayor probabilidad de recaída[46,47]. Además, la producción de TGF-β e IL-6 a partir de células MM y BMSCs induce la diferenciación de células T naive a células Th17[48], uns subpoblación de células T $CD4^+$ inmunosupresoras que, como un circuito de retroalimentación positiva, secreta diferentes citoquinas que promueven la expansión de las células MM[49].

Con respecto a la población de células T citotóxicas, se ha descrito un aumento en el número total de estas células tanto en MGUS como en MM sintomático en comparación con donantes sanos[50]. Más recientemente, Zavidij et al. han demostrado que durante la progresión de la enfermedad se observa un agotamiento de la subpoblación de células T $CD8^+$ de memoria, por lo que son más abundantes en individuos con MGUS en comparación con SMM y MM activo[51]. Se ha observado que los factores solubles modulan la actividad de las células T citotóxicas. En este sentido, el TGF-β inhibe la proliferación y maduración de las células T dependiente de IL-2 y evita que las células T naive adquieran funciones efectoras[52,53]. La actividad de los linfocitos T efectores también es inhibida por el nucleósido inmunosupresor adenosina (ADO), que se deriva de ATP o NAD^+ después de reacciones catalíticas secuenciales iniciadas, entre otras, por la molécula de superficie CD38 en células MM[54–56].

La activación de las células T se inicia a través del reconocimiento del antígeno por parte del receptor de células T (TCR), y luego es regulada por un equilibrio entre señales coestimuladoras y coinhibitorias denominadas puntos de control

inmunitarios[57]. Aunque los puntos de control inmunitarios son cruciales para el mantenimiento de la autotolerancia y la homeostasis[57] en condiciones patológicas, la expresión de las proteínas del punto de control inmunitario puede desregularse como un mecanismo de evasión inmunitaria[58]. Uno de los puntos de control inmunológico más relevantes es la vía de muerte programada (PD). PD-1 es un receptor inhibidor expresado por las células T, que interactúa con sus ligandos PD-L1/PD-L2 expresados principalmente por células presentadoras de antígenos (APC) para inhibir las funciones efectoras de las células T[59–61]. Se sabe que las CPs de sujetos sanos no expresan ligandos PD-1, sin embargo, PD-L1/PD-L2 se pueden encontrar en CPs de pacientes con mieloma[62–65] y en líneas celulares de MM[66]. Además, la expresión de PD-1 parece aumentar en las células T de pacientes con MM, en comparación con sujetos sanos[67,68]. Junto con PD-1/PD-L1, el antígeno 4 de linfocitos T citotóxicos (CTLA-4) es otro punto de control inmunitario responsable de la supresión de células T. En concreto, la unión de CTLA-4 presente en las células T a sus ligandos (CD80/CD86), expresados en las APC, transmite una señal inhibitoria a las células T[69]. Algunos estudios han evaluado la seguridad y eficacia de la inhibición de CTLA-4 como tratamiento de consolidación después de ASCT en pacientes con MM. En el ensayo clínico CPIT-001, se administraron ipilimumab [anticuerpo monoclonal (AcMo) anti-CTLA-4] y nivolumab (AcMo anti-PD1) entre 14 y 28 días después del ASCT en pacientes con MM de alto riesgo que habían alcanzado al menos una enfermedad estable después del tratamiento de inducción. A los 18 meses posteriores al ASCT, los autores informaron una PFS del 71%[70].

1.3.2. Linfocitos B

Los linfocitos B son los componentes responsables de la inmunidad humoral en el sistema inmunitario adaptativo, que actúan a través de la secreción de anticuerpos[71]. El linaje de células B está muy comprometido en MM, con un equilibrio desplazado hacia una alta proliferación de CPs malignas. De hecho, los pacientes con MM presentan tanto una disminución de las células B $CD19^{+}$[72,73] que se correlaciona de manera inversa con el estado de la enfermedad[73], como una capacidad reducida para secretar inmunoglobulinas policlonales y para

diferenciarse en células plasmáticas secretoras de anticuerpos[72]. Además, se ha demostrado que el riesgo de progresión a MM sintomático desde estadios presintomáticos (MGUS y SMM) está directamente relacionado con la proporción de CPs normales en la médula ósea en el momento del diagnóstico[74]. Adicionalmente, el número de células B reguladoras (Bregs) con fenotipo $CD19^{+}CD24^{high}CD38^{high}$ aumenta en la transición de MGUS a MM sintomático[75], favoreciendo un entorno inmunosupresor en la MO al reducir la lisis mediada por NK de las células de MM y producir IL-10[76].

1.3.3. Células NK

Las células natural killer (células NK) son linfocitos citotóxicos del sistema inmunitario innato que intervienen en la respuesta temprana a antígenos virales así como en el ataque a células tumorales, reconociendo y eliminando células que expresan proteínas de estrés sin necesidad de presentación de antígenos a través del complejo mayor de histocompatibilidad (MHC)[**77**]. De hecho, la activación de las células NK depende de un equilibrio entre las señales que llegan a través de la activación de los receptores naturales de citotoxicidad (NCR) y los receptores inhibitorios. En humanos, los receptores activadores incluyen NKp30, NKp46, NKp44, DNAM-1 y NKG2D, entre otros, mientras que los receptores inhibidores incluyen los receptores inhibidores asesinos (KIR) y NKG2A, entre otros[**78**]. Brevemente, las células NK reconocen las células malignas y las matan a través de la secreción de granzima B y perforina o, alternativamente, a través de la inducción de vías de señalización de muerte en las que están involucradas las proteínas FasL y TRAIL[**79**].

En el contexto de la enfermedad, tanto los pacientes con MGUS como con MM activo presentan un enriquecimiento en la población de células NK en comparación con los adultos sanos[*50,51*]. Inicialmente, las células de mieloma son sensibles a la lisis inducida por las células NK ya que expresan altos niveles de MICA (un ligando del receptor NKG2D). Por el contrario, a medida que la enfermedad evoluciona, las células de mieloma pierden la expresión de MICA a través del desprendimiento de

MICA, estando este último fenómeno directamente relacionado con la progresión de la enfermedad[80,81]. En cuanto al receptor activador DNAM-1, su expresión está reducida en las células NK de pacientes con mieloma con enfermedad activa en comparación con pacientes en remisión o individuos sanos[82]. Además, a diferencia de los donantes sanos, las células NK de pacientes con mieloma expresan la molécula PD-1 que mitiga aún más su funcionalidad[68].

1.3.4. Células dendríticas (DCs)

Las células dendríticas (DCs) son APCs cuya función principal es el procesamiento de material antigénico que luego se muestra en su superficie celular para inducir la activación de células T naive. Se clasifican como DC plasmocitoides (pDC), que secretan altos niveles de IFN tipo I en respuesta a antígenos virales y otros estímulos, y DC mieloides (mDC), que están bastante involucradas en la presentación de antígenos y la inducción de respuestas de células T $CD4^+$ y $CD8^+$[84,85]. El papel y el estado general de las DCs en MM aún no están claros. Muchos estudios han concluido que las DC de pacientes con MM tienen las capacidades de estimulación de células T disminuidas, mientras que existen resultados contradictorios con respecto a la frecuencia y el fenotipo de las DC[86–89].

Las DCs se concentran en la MO durante la progresión de MGUS a MM y pueden procesar y presentar antígenos de células MM apoptóticas a través de CD91, activando así las células T $CD8^+$ específicas del mieloma[90]. Por el contrario, al usar sus moléculas CD80/86 de superficie, las DCs interactúan con las CPs no apoptóticas a través de su receptor CD28 sobreexpresado, lo que induce la secreción de la enzima inmunosupresora indoleamina-2,3-dioxigenasa (IDO)[91] por las DCs lo que perjudica al sistema inmune a través de diferentes mecanismos:

- Metabolizando y, por lo tanto, agotando el triptófano del microambiente, que es un aminoácido esencial para las células T y, en consecuencia, produciendo quinurenina, un compuesto tóxico para las células T y NK[92].

- Favoreciendo el desarrollo, estabilización y activación de los linfocitos T reguladores (Tregs)[93].

- Polarizando macrófagos y DCs hacia un fenotipo inmunotolerogénico[94].

Junto con IDO, ADO también puede aumentar la cantidad de DCs tolerogénicas[56]. Además, las pDCs y un porcentaje de mDCs también expresan altos niveles superficiales de PD-L1 en MM [95], contribuyendo así al mantenimiento del microambiente inmunosupresor de la MO. Además, TGF-β contribuye al nicho tumoral inmune alterado ya que inhibe la regulación positiva de moléculas coestimuladoras de células T críticas en la superficie de las DCs, reduciendo su capacidad de presentación de antígenos[96].

1.3.5. Macrófagos asociados al tumor (TAMs)

Los macrófagos asociados al tumor (TAMs) constituyen un componente abundante del microambiente del mieloma que mejora la supervivencia de las células del mieloma y la resistencia a los fármacos a través de diferentes mecanismos[97]. Dentro del nicho de la MO, los TAMs adquieren un perfil secretor caracterizado por la producción de IL-6, IL-10 y factores proangiogénicos, como VEGF, metaloproteinasas (MMP) y ciclooxigenasa-2 (COX-2)[98], proporcionando un entorno óptimo para el crecimiento de células de mieloma. Además, los TAMs se asemejan a una población de macrófagos similares a M2, con poca citotoxicidad contra las células tumorales debido a su producción limitada de óxido nítrico (NO) y citoquinas proinflamatorias, así como a una pobre capacidad de presentación de antígenos[99]. Además, ADO polariza aún más los macrófagos hacia un fenotipo M2[56].

Se ha demostrado que la cantidad de macrófagos M2 aumenta significativamente en la MO de pacientes con MM en comparación con MGUS y SMM, así como con donantes sanos, lo que sugiere que las células plasmáticas malignas pueden estar involucradas en este cambio a un fenotipo similar a M2[100]. Un estudio reciente de secuenciación de ARN reveló que los monocitos $CD14^+$ maduros pierden la expresión superficial de las moléculas HLA-II ya en la etapa de MGUS, lo que da

como resultado la supresión de las células T e indica que algunas de estas alteraciones inmunitarias secuenciales comienzan en una etapa temprana de la enfermedad[51].

1.3.6. Células mieloide supresoras (MDSCs) y neutrófilos

Las células mieloide supresoras (MDSCs) son un grupo heterogéneo de células mieloides inmaduras dotadas de la capacidad de suprimir la activación, proliferación y capacidad citotóxica de las células T y NK. En los seres humanos, hay dos subconjuntos de MDSC: MDSC granulocíticas (G-MDSCs) (también llamadas polimorfonucleares, PMN-MDSCs) y MDSC monocíticas (M-MDSCs)[101]. En mieloma, las G-MDSCs constituyen la población predominante de MDSCs en MO y SP[102]. Las MDSCs suprimen principalmente las respuestas de las células T mediante la producción de especies reactivas de oxígeno (ROS) y grandes cantidades de NO, arginasa-1 (Arg-1) y citoquinas inmunosupresoras como la IL-10. Además, también se ha demostrado que las MDSCs de pacientes con MM pueden inducir una mayor diferenciación de Treg que las de donantes sanos de la misma edad[103]. Además, las células de MM promueven la supervivencia de las MDSCs a través de la regulación positiva de Mcl-1[104], secreción de IL-6 que impulsa la expansión de MDSCs mediante la activación de varias cascadas moleculares como PI3K/Akt o JAK/STAT3[105,106], y a través de la acumulación de altos niveles de ADO por la conversión de NAD^+[56,107].

Los neutrófilos son células mieloides que constituyen el leucocito más abundante en la SP, y son esenciales para la eliminación de patógenos extracelulares, tanto por toxicidad directa como por establecimiento de interacciones con otras células inmunitarias[108]. En el contexto del MM, los neutrófilos presentan defectos funcionales, como una reducción de la actividad de la lisozima y un aumento de la secreción de Arg-1, por lo que presentan un comportamiento inmunosupresor[109,110]. De hecho, los pacientes con MM tienen niveles séricos elevados de Arg-1[109], que agota la arginina en el microambiente tumoral, un aminoácido esencial para la proliferación de células T y NK[111]. Además, a medida que la enfermedad evoluciona de MGUS a MM, los neutrófilos activan progresivamente su vía JAK/STAT3 en

respuesta a la exposición de células MM, lo que respalda aún más el nicho inmunotolerogénico debido a la producción de señales proinflamatorias y de supervivencia[112].

1.3.7. Linfocitos T reguladores (Tregs)

Los linfocitos T reguladores (Tregs) son una subpoblación de células T que modulan el sistema inmunológico, mantienen la tolerancia a los autoantígenos y previenen las reacciones autoinmunes. Ejercen su actividad inmunosupresora a través del contacto célula a célula, secretando citoquinas inmunosupresoras como TGF-β e IL-10[113] o induciendo la expresión de IDO en DCs, lo que a su vez induce un bucle positivo ya que IDO, junto con ADO, promueve la expansión de Tregs[56,114]. Además, en muestras de MO de pacientes de nuevo diagnóstico, CTLA-4 parece estar sobreexpresado en Tregs, lo que sugiere una acumulación local de Tregs en el microambiente del tumor[115]. De hecho, se ha descrito que CTLA-4 induce la expansión de Tregs y la producción de citoquinas inmunosupresoras en esta población[116].

La mayoría de los autores han informado que los pacientes con MM tienen un mayor porcentaje de Tregs[51,117–120] en comparación con donantes sanos, lo que sugiere que las células de mieloma escapan de la vigilancia inmunológica, al menos parcialmente, a través del aumento de esta población. Curiosamente, los niveles de Tregs pueden usarse como un biomarcador de la progresión de la enfermedad, y los pacientes con un porcentaje de Tregs más alto presentaron una OS más baja[117,121].

1.4. FÁRMACOS APROBADOS PARA EL TRATAMIENTO DEL MIELOMA MÚLTIPLE EXCLUYENDO LA INMUNOTERAPIA

Los regímenes terapéuticos en MM han experimentado una marcada evolución durante las últimas décadas, pasando de agentes quimioterapéuticos sin dianas específicas a terapias farmacológicas dirigidas, como los inhibidores del proteasoma (IP) y los fármacos inmunomoduladores (IMiDs), que afectan más

específicamente a los mecanismos de crecimiento y supervivencia de células de mieloma. En este sentido, los alquilantes, como el melfalán, y los glucocorticoides fueron los regímenes de atención estándar durante más de 30 años, proporcionando una mediana de OS de solo 2-3 años. Sin embargo, con la aprobación de IMiDs o PIs, entre otros, la mediana de OS se elevó hasta los 6 años[122] y se espera que pueda superar los 10 años en base a datos relativos a pacientes diagnosticados después de 2015[123].

A continuación, se discutirán los fármacos aprobados para el tratamiento del MM, excluyendo la inmunoterapia, centrándose principalmente en sus mecanismos de acción ejercidos directamente sobre las células tumorales. Los efectos inmunoestimuladores u otras actividades sobre el microambiente de la MO descritos para estos fármacos se revisarán en una sección posterior.

1.4.1. Glucocorticoides

Los glucocorticoides, como la prednisona y la dexametasona, son esteroides sintéticos que se han utilizado para tratar el MM durante más de 50 años. Se unen a los receptores de glucocorticoides citosólicos que luego se trasladan al núcleo para modular la expresión génica. Por ejemplo, los glucocorticoides reprimen los genes diana a través de interacciones inhibitorias con factores de transcripción, incluidos NFκB y AP-1[124], induciendo apoptosis.

Hoy en día, los glucocorticoides se utilizan como parte de regímenes de tratamiento combinados para promover efectos sinérgicos[125]. En este sentido, han demostrado mejorar el efecto antimieloma de los IMiDs[126] e IPs[127]. De hecho, los regímenes sin dexametasona continúan siendo la excepción más que la regla.

1.4.2. Agentes alquilantes

Los alquilantes, junto con los glucocorticoides, fueron los primeros medicamentos efectivos para el mieloma que se usaron en el entorno clínico. Su mecanismo de acción se basa en la adición de un grupo alquilo al ADN, lo que inhibe la síntesis de ADN y ARN y, por lo tanto, conduce a la muerte celular[128].

La terapia con melfalán en combinación con corticosteroides ha sido el estándar de atención para pacientes de nuevo diagnóstico durante casi 40 años y sigue siendo el tratamiento de elección para pacientes de edad avanzada. Además, el melfalán se ha combinado con IPs[129] y recientemente también ha mostrado resultados prometedores cuando se administra con anticuerpos monoclonales (AcMos)[130]. Sin embargo, el principal uso actual de melfalán es para el acondicionamiento en el trasplante autólogo de células madre (ASCT)[131].

La ciclofosfamida, que presenta un perfil más seguro en comparación con el melfalán, se ha utilizado como alternativa al melfalán durante años. Actualmente se está estudiando en el contexto de varios ensayos clínicos, debido a sus potenciales propiedades inmunoestimulantes cuando se combina con IMiDs[132].

Se sabe poco sobre la eficacia de la bendamustina debido a la falta de ensayos de fase 3 en el contexto de MM. Sin embargo, se sabe que es el único alquilante que no presenta resistencia superpuesta con los demás; se cree que esto se debe a su mecanismo citotóxico distintivo, ya que activa la vía de reparación del ADN por escisión de bases en lugar del mecanismo de reparación del ADN por alquiltransferasa[133].

Melflufen (acrónimo derivado de melfalán flufenamida), es un conjugado de fármaco y péptido de primera clase que es hidrolizado por aminopeptidasas y libera rápidamente el agente alquilante en las células tumorales, lo que induce daño irreversible en el ADN y, por lo tanto, apoptosis[134]. Este fármaco, que ha sido aprobado recientemente por la FDA[135], ha demostrado respuestas duraderas en pacientes con RRMM con un perfil de toxicidad aceptable[136].

1.4.3. Inhibidores del proteasoma (IPs)

Uno de los avances más destacados en el tratamiento del MM fue el descubrimiento de los IPs, que demostraron una eficacia sorprendente en el entorno clínico. El proteasoma es un complejo de proteasas que ejerce la hidrólisis selectiva de las proteínas cliente. Este complejo es importante en el contexto de MM ya que

debido a su alta síntesis de inmunoglobulinas, las células de mieloma contienen una alta tasa de proteínas mal plegadas que finalmente deben degradarse en el proteasoma. Actualmente hay tres IP aprobados para el tratamiento del MM: bortezomib, carfilzomib e ixazomib. A diferencia de bortezomib y carfilzomib, que se administran principalmente por vía intravenosa o subcutánea (bortezomib), ixazomib es el primer IP aprobado disponible por vía oral[137].

Bortezomib e ixazomib son inhibidores reversibles de la subunidad catalítica β5 del proteasoma, mientras que carfilzomib es un inhibidor irreversible. En general, la inhibición del proteasoma reduce la eliminación de proteínas mal plegadas, lo que da como resultado citotoxicidad. Además, los IPs ejercen muchos otros efectos diferentes que contribuyen a su actividad antimieloma, incluida la acumulación de proteínas supresoras de tumores, como p53, la infraexpresión de moléculas de adhesión, la inhibición de los efectores responsables de la reparación del ADN, la inhibición de la angiogénesis y la bloqueo del factor de transcripción NFκB[137]. Además, se ha sabe que los IPs tienen efectos anabólicos y antirresortivos óseos, lo que agrega un beneficia las lesiones osteolíticas asociadas al mieloma[138].

Además de los efectos directos mencionados, recientemente se ha demostrado que los IPs ejercen la muerte celular inmunogénica (ICD). En este sentido, la entrega de una señal de activación de las células de mieloma moribundas a las DCs está mediada por la exposición de Hsp90, un marcador de ICD, en la superficie de las células apoptóticas. De hecho, las DCs activadas con células de mieloma moribundas después del tratamiento con bortezomib son potentes inductoras de células T productoras de IFN específicas de tumores[227]. Tanto bortezomib como carfilzomib promueven la exposición de CALR en líneas celulares de mieloma, otra proteína marcadora de ICD[228].

1.4.4. Fármacos inmunomoduladores (IMiDs)

Junto con los IPs, los IMiDs han contribuido notablemente a mejorar la evolución de los pacientes con MM. Los IMiDs, que incluyen talidomida, lenalidomida y pomalidomida, ahora forman parte de la terapia principal para la enfermedad tanto

recién diagnosticada como en recaída. La talidomida se utilizó por primera vez en la década de 1990 para el tratamiento del MM a través de un protocolo de uso compasivo[139]. La importante respuesta lograda en estudios posteriores impulsó la aprobación de talidomida en combinación con dexametasona por parte de la FDA en 2006 para el tratamiento de MM de nuevo diagnóstico[140,141]. Con respecto a la lenalidomida, la FDA aprobó su uso en combinación con dexametasona en 2006 para el tratamiento de RRMM, y unos años después, en 2015, se aprobó la combinación para el tratamiento de pacientes con NDMM[142]. El otro IMiD de segunda generación, pomalidomida, fue aprobado en 2013 para el tratamiento de pacientes con RRMM que habían recibido al menos dos regímenes previos que incluían lenalidomida y bortezomib[142].

Cereblon (CRBN), la primera diana descrita para los IMiDs, es un componente del receptor del complejo de proteína ligasa cullin-4 RING E3 que media la ubiquitinación y la consiguiente degradación a través del proteasoma de las proteínas diana. Después de la unión de CRBN a un IMiD, la especificidad de sustrato del complejo E3 ligasa se altera, de modo que otras proteínas se ubiquitinan y luego se degradan en el proteasoma. Entre estos nuevos sustratos, IKF1 (Ikaros) e IKZF3 (Aiolos) destacan como factores clave de transcripción de CPs cuya expresión está muy disminuida en las células de mieloma después del tratamiento con IMiDs. La reducción de la expresión de IKZF1 e IKZF3 conduce a una regulación a la baja del factor regulador de interferón 4 (IRF4) y sus genes diana *MYC*, *CDK4* y *CASP*, lo que disminuye la supervivencia de las células de mieloma[143]. Por lo tanto, los IMiDs desencadenan diversas actividades biológicas, muchas de ellas relevantes desde una perspectiva antimieloma. De hecho, los IMiDs tienen propiedades antiangiogénicas[144], inhiben NFκB, disminuyen la producción de IRF-4, activan las caspasas e incrementan factores proapoptóticos mientras que reducen las señales antiapoptóticas[126,145–147]. El efecto de los IMiDs sobre el sistema inmune se revisará en la siguiente sección.

1.4.5. Inhibidores de histona desacetilasa (HDACi)

Como se mencionó anteriormente, las HDAC están desreguladas en MM. En este sentido, panobinostat es un inhibidor pan-HDAC de primera clase que fue aprobado en 2015 por la FDA en combinación con bortezomib y dexametasona para el tratamiento del MM, en pacientes que habían recibido al menos dos regímenes previos, incluidos bortezomib e IMiDs[148].

Panobinostat ejerce su eficacia contra el mieloma principalmente de dos maneras diferentes: mediante la inhibición de la desacetilación de las histonas que conduce a la activación transcripcional de los genes supresores de tumores[149], y por la inhibición de la formación de agresomas a través de la acetilación de proteínas no histonas como la tubulina. El agresoma es una vía de escisión alternativa de proteínas mal plegadas cuando el sistema del proteosoma no es capaz de eliminar las proteínas ubiquitinadas mal plegadas. De hecho, esta es la base de la combinación simultánea de bortezomib y panobinostat en la clínica: bortezomib inhibe el mecanismo de escisión del proteasoma induciendo alternativamente la formación de agresomas, mientras que panobinostat bloquea la formación de agresomas. Esta doble inhibición aumenta la acumulación de proteínas mal plegadas, lo que eventualmente conduce a la muerte celular[150].

1.4.6. Inhibidores de exportina

En 2019, la FDA aprobó selinexor en combinación con bortezomib y dexametasona para el tratamiento de pacientes en recaída o refractarios que habían recibido al menos cuatro terapias previas y cuya enfermedad era refractaria al menos dos IPs, al menos dos IMiDs y un anticuerpo monoclonal anti-CD38[151].

Selinexor es un inhibidor selectivo de la exportina-1 (XPO1), que es una molécula sobreexpresada en muchos tipos de cáncer, incluido el MM[152]. XPO1 es el principal exportador nuclear de proteínas supresoras de tumores, como p53 o p21, reguladores del crecimiento y ARNm de oncoproteínas (p. ej., c-Myc o Bcl-xL). La inhibición selectiva de XPO1 por selinexor da como resultado la acumulación de proteínas supresoras de tumores en el núcleo, niveles reducidos de oncoproteínas, detención del ciclo celular y apoptosis[153].

1.5. INMMUNOTERAPIA EN MIELOMA MÚLTIPLE

A pesar de los diferentes enfoques terapéuticos que se han mencionado hasta ahora, el MM sigue siendo una enfermedad incurable y los pacientes eventualmente recaen. En los últimos años, sin embargo, se han desarrollado diferentes tratamientos inmunoterapéuticos para mejorar la evolución de los pacientes. Los principales enfoques inmunoterapéuticos actuales para el tratamiento del MM incluyen: AcMo (desnudos), conjugados de anticuerpo-fármaco (ADC), anticuerpos biespecíficos y terapias de células T con receptor de antígeno quimérico (CAR-T)[154]. Entre ellos, los AcMo han demostrado alta eficacia y buena tolerabilidad, estando tres de ellos aprobados hasta el momento (daratumumab, elotuzumab e isatuximab). En cuanto a los ADC, uno de ellos (belantamaf mafodotin) ha sido aprobado para MM. Además, en marzo de 2021 se aprobó la primera terapia con células CAR-T (idecabtagene vicleucel) para el tratamiento de pacientes con RRMM. Aunque actualmente no existen anticuerpos biespecíficos aprobados en MM, algunos de ellos se encuentran en una fase muy avanzada de desarrollo clínico. A continuación, se describirán los cinco agentes inmunoterapéuticos aprobados y sus mecanismos de acción.

1.5.1. Mecanismos de acción principales de los AcMo

Los anticuerpos (Acs) son glicoproteínas que pertenecen a la superfamilia de las inmunoglobulinas (Ig). Los Acs están formados por dos cadenas pesadas y dos ligeras y se agrupan en diferentes isotipos según su cadena pesada. Específicamente, los AcMo terapéuticos suelen ser de isotipo IgG. La región hipervariable de cada cadena pesada y ligera se combina para formar el sitio de unión al antígeno (Fab; dominio de unión al antígeno del fragmento), mientras que el dominio del fragmento cristalizable (Fc), que es responsable de las funciones efectoras, se compone de dos dominios constantes (Figura 5)[155].

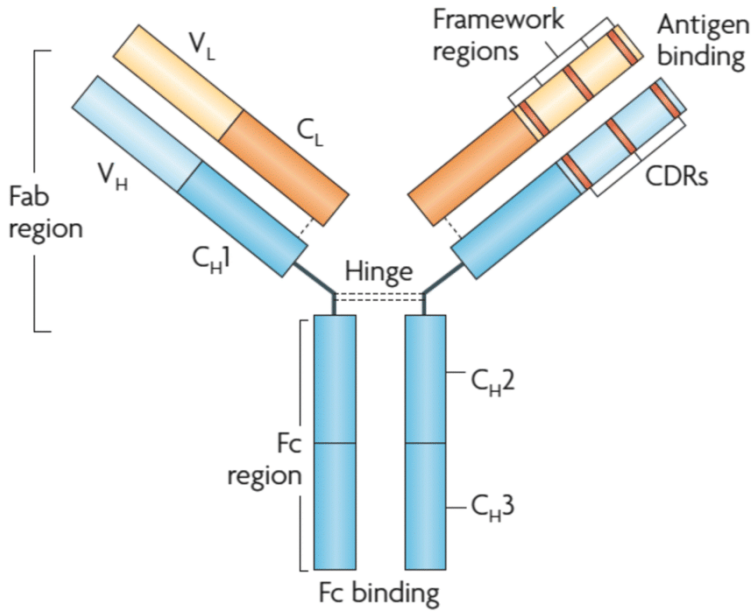

Figura 5. Estructura del anticuerpo. Las cadenas pesadas contienen un dominio variable (V_H), una región bisagra y tres dominios constantes (C_H1, C_H2 y C_H3). Las cadenas ligeras contienen un dominio variable (V_L) y uno constante (C_L). La estructura también se puede dividir en la región de unión al antígeno del fragmento (Fab) y el dominio cristalizable del fragmento (Fc). Modificado de Buss et al.[156].

Los principales mecanismos de acción típicamente descritos para que los AcMos ejerzan efectos citotóxicos sobre las células tumorales son: inducción de apoptosis, citotoxicidad dependiente del complemento (CDC), citotoxicidad celular dependiente de anticuerpo (ADCC) y fagocitosis celular dependiente de anticuerpo (ADCP).

1.5.1.1. Inducción de apoptosis

Los anticuerpos pueden matar las células tumorales induciendo mecanismos proapoptóticos o aumentando la susceptibilidad de las células diana a la terapia citotóxica a través de la inhibición de las vías antiapoptóticas. Los mecanismos por los que los AcMos inducen la apoptosis son diversos (Figura 6).

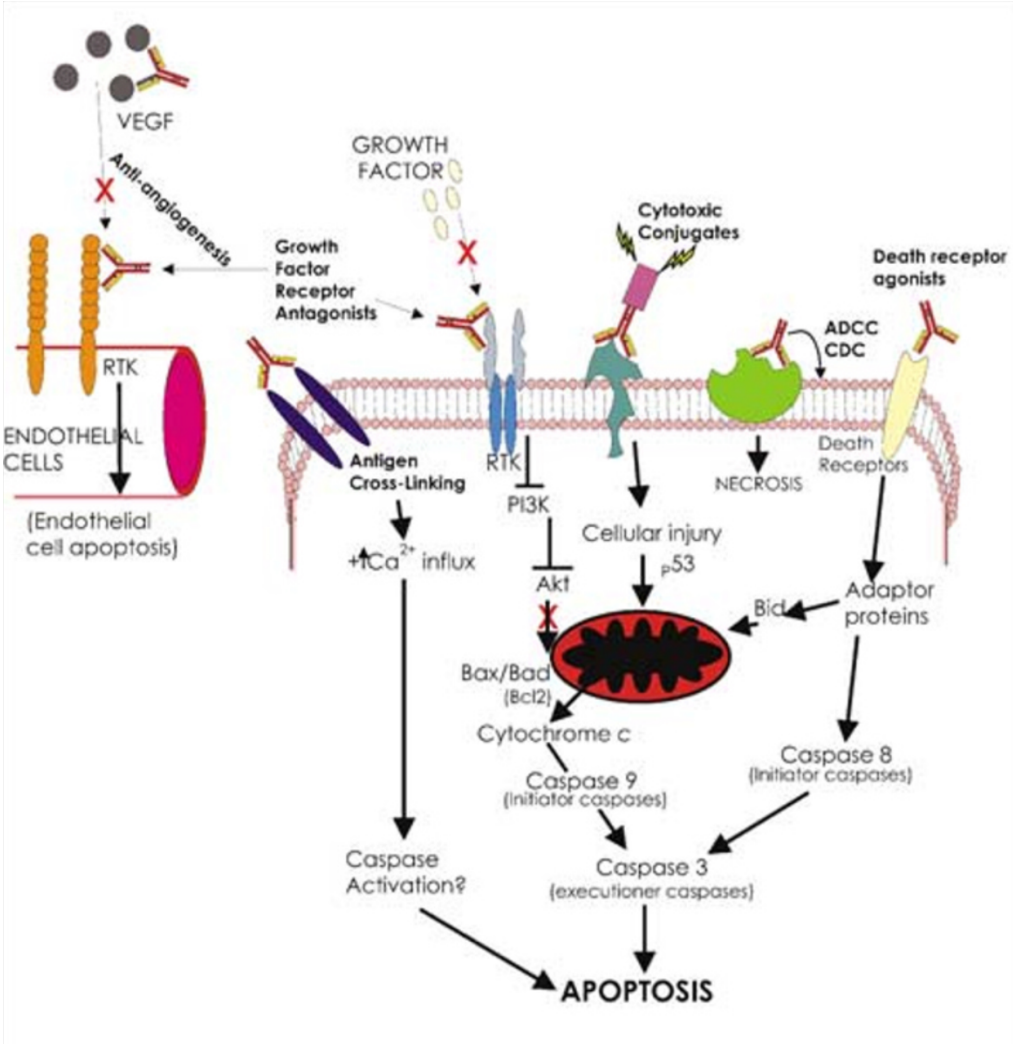

Figura 6. Estrategias terapéuticas de anticuerpos y mecanismos de señalización que pueden conducir a la inducción de apoptosis en células tumorales diana[157].

Por un lado, la interacción entre el AcMo y el antígeno en la célula tumoral puede inducir la apoptosis por la vía extrínseca[158]. Brevemente, la vía extrínseca apoptótica se activa cuando los miembros de la familia del factor de necrosis tumoral (TNF), como FASL, TNF-α o Apo2L (también conocido como TRAIL), se unen a los receptores de muerte (DR) de la membrana plasmática Fas, TNFR1 y TRAIL-R, respectivamente. La unión de los DRs con sus respectivos ligandos recluta las proteínas adaptadoras FADD y/o TRADD, así como la caspasa 8, que en última instancia desencadena la proteólisis dirigida de proteínas antiapoptóticas o reguladores del ciclo celular, entre los que destacan la Bcl-2 y la ciclina E[159]. Por lo tanto, la unión del AcMo a los DRs puede inducir la muerte celular.

Además, los anticuerpos que se dirigen a los receptores del factor de crecimiento pueden ejercer un efecto directo sobre el crecimiento y la supervivencia de la célula tumoral al antagonizar la señalización del ligando-receptor. Cuando el AcMo se une al antígeno, se inhibe la señalización del factor de crecimiento mediada por la autofosforilación de la tirosina quinasa del receptor (RTK), lo que da como resultado la detención del crecimiento de las células tumorales. Además, dado que la activación del factor de crecimiento también puede iniciar la señalización antiapoptótica, los anticuerpos bloqueadores pueden reducir los mecanismos de supervivencia de las células tumorales[157].

Otro ejemplo de apoptosis inducida por AcMos son los anticuerpos que pueden dirigirse a los antígenos de la superficie celular y provocar directamente la señalización apoptótica. Un ejemplo de estos son los anticuerpos que entrecruzan el antígeno de superficie diana en las células tumorales. Sin embargo, el mecanismo por el cual estos anticuerpos pueden inducir la apoptosis puede ser algo atípico, ya que parece ser insensible a Bcl-2, no depende de Fas y solo está parcialmente bloqueado por un inhibidor de caspasa. Por ejemplo, con respecto al anti-CD20 rituximab, se ha propuesto que el aumento en los niveles intracelulares de Ca^{2+}, que ocurre en respuesta al entrecruzamiento de anti-CD20, puede estar involucrado en el proceso apoptótico, pero el mecanismo preciso aún no se ha dilucidado[157].

1.5.1.2. Citotoxicidad dependiente de complemento (CDC)

La CDC es otro método de eliminación de células que puede ser desencadenado por anticuerpos. Existen tres vías para la activación de la cascada del complemento: la vía alternativa, la vía de las lectinas y la vía clásica, siendo esta última la que se activa tras la unión de los AcMos al antígeno. En este sentido, la formación de complejos antígeno-anticuerpo da como resultado el desencubrimiento de múltiples sitios de unión de C1q en la proximidad de los dominios CH2 de los AcMo. La unión del componente C1q del complemento a sus sitios de unión desencadena una cascada de eventos que involucra una serie de otras proteínas del complemento y conduce a la liberación proteolítica de C3b y eventualmente de C5b. La cascada del complemento termina con la formación del complejo de ataque a la membrana

(MAC), también conocido como C5b-9, que crea poros en la membrana celular, provocando la lisis celular[160]. En concreto, una vez que se forma el complejo MAC, la concentración de Ca^{2+} aumenta dentro de la célula, aunque el impacto que esto puede tener en el proceso de muerte celular aún está pendiente de aclaración. Además, recientemente se ha descrito que el complejo MAC puede activar diferentes proteínas (RIPK1, RIPK3, MLKL, JNK y Bid) que son las responsables de la necrosis regulada, también conocida como necroptosis[161] (Figura 7). Sin embargo, el bloqueo de cualquiera de estas cinco proteínas disminuye la CDC pero no completamente, lo que sugiere que esta cascada actúa en concierto con otros procesos promotores de muerte, dependientes o independientes de Ca^{2+}, aún pendientes de caracterización[161].

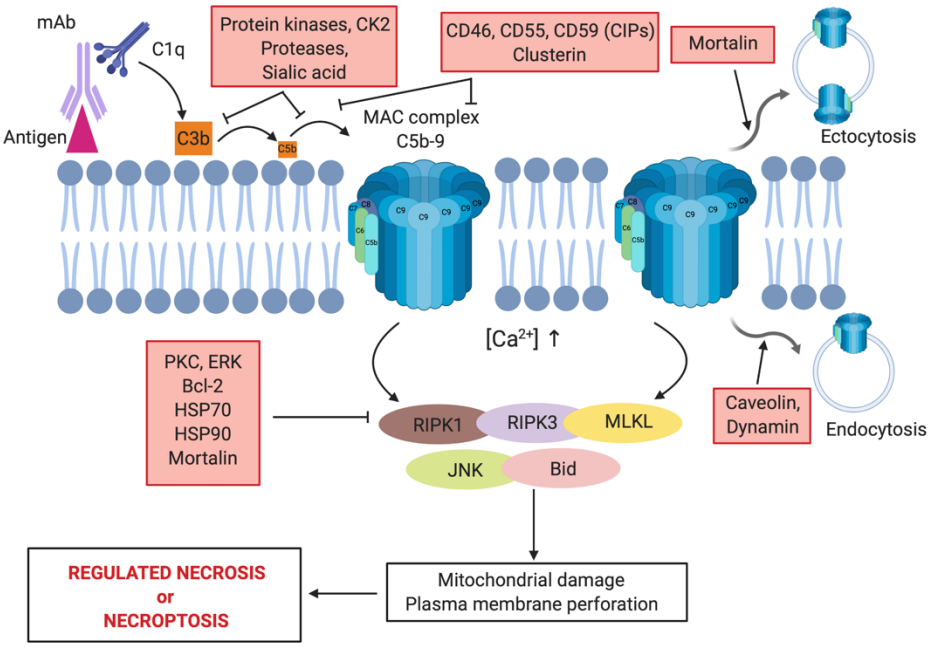

Figura 7. Representación esquemática de la cascada de citotoxicidad dependiente del complemento después de la unión del AcMo. Los cuadros rojos se refieren a los inhibidores de la cascada en diferentes etapas. Modificado de Fishelson et al.[161], figura realizada con la herramienta BioRender.

La regulación de la muerte celular inducida por el complemento ocurre principalmente en dos etapas: antes del comienzo de la cascada de señalización intracelular y después de este evento. Por un lado, antes de que el complejo MAC se inserte en la membrana plasmática y desencadene la cascada de señalización intracelular, la presencia de proteínas reguladoras de membrana como CD55, CD59

o CD46 pueden inhibir la oligomerización del complejo MAC. Además, las proteasas de la superficie celular, las quinasas y el ácido siálico pueden degradar las proteínas del complemento depositadas. Por ejemplo, se ha descubierto que las ectoproteínas quinasas fosforilan C9 inhibiendo la formación de MAC. En consonancia con esto, la clusterina es una proteína soluble multifuncional que participa en el control de la actividad lítica del complejo MAC uniéndose al complejo C5b-7, impidiendo la adición de C8 y C9 y por tanto, haciendo que el complejo MAC sea hidrosoluble y líticamente inactivo[162,163]. Otro mecanismo adicional para evitar el ataque del complemento es eliminar el complejo MAC de la superficie celular por endocitosis o exocitosis en la que parecen estar involucradas la proteína quinasa C (PKC) y la quinasa regulada por señales extracelulares (ERK)[164]. Específicamente, la endocitosis de MAC depende de la liberación intracelular dependiente de caveolas y dinamina, mientras que la eliminación de MAC por exovesiculación requiere la expresión de la proteína de estrés mitocondrial mortalina/GRP75[165]. En muchos casos, las células tumorales sobreexpresan algunos de estos mecanismos reguladores del complemento para contrarrestar los efectos inducidos por MAC[161].

Por otro lado, los eventos de fosforilación de proteínas que involucran PKC, MEK y ERK respaldan la supervivencia de las células cancerosas que sufren un ataque MAC. Además, también se ha descrito que las proteínas HSP contribuyen a la resistencia intrínseca a CDC[161]. Se ha sugerido que Bcl-2 podría estar involucrada en la inhibición de este mecanismo ya que inhibe a Bid, pero los mecanismos específicos de este proceso deben explorarse más a fondo[161].

1.5.1.3. Citotoxicidad celular dependiente de anticuerpo (ADCC)

Para que se produzca la ADCC es esencial la interacción del AcMo con el receptor Fc presente en las células efectoras. En el contexto del MM, gran parte de lo que sabemos hasta ahora es que las células NK son las principales células efectoras responsables de la ADCC mediada por los AcMos aprobados. Las células NK expresan en su superficie celular el receptor FcγRIIIA, también conocido como CD16a, que juega un papel crucial en la cascada de señalización involucrada en la

ADCC. Específicamente, CD16 contiene motivos de activación ITAMs, que son secuencias de consenso que contienen residuos de tirosina susceptibles de fosforilación por la quinasa Src. De hecho, una vez que la región Fc del AcMo se une a CD16, la quinasa Src fosforila los ITAMs que, a su vez, reclutan y se unen a la proteína tirosina quinasa del bazo (Syk) activándose esta última. Esta última activación desencadena las tres vías principales implicadas en la ADCC: (i) la vía de la fosfolipasa C-gamma (PLC-γ); (ii) vía de la fosfatidilinositol 3-quinasa (PI-3K) y (iii) vía de la proteína G de la familia Vav/Rho (Figura 8)[166].

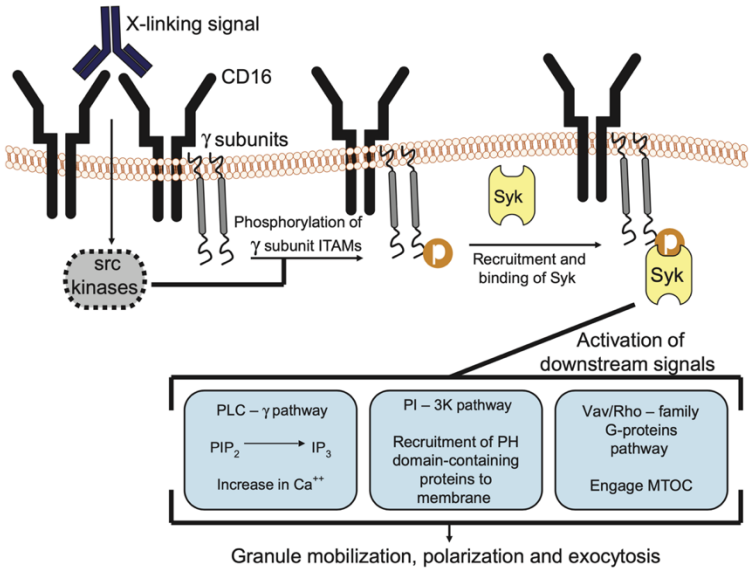

Figura 8. Representación esquemática de las cascadas de señalización subyacentes a la ADCC[166].

Debido a la activación de las vías mencionadas, las células NK pueden destruir las células diana a través de la vía de muerte celular perforina/granzima, en la que la granzima inicia la apoptosis principalmente a través de la escisión de Bid en una forma truncada que desencadena la liberación de citocromo c mitocondrial y la formación de apoptosomas. Esto conduce a la activación de la caspasa y la manifestación del fenotipo de apoptosis[167]. Además, las células NK pueden matar células diana a través de otros dos mecanismos adicionales que no han sido tan ampliamente caracterizados como el anterior: (i) la vía FAS-L en la que las células NK son capaces de matar células que expresan receptores FAS desencadenando la ya descrita vía apoptótica extrínseca; y (ii) la vía del estrés oxidativo[166].

1.5.1.4. Fagocitosis celular dependiente de anticuerpo (ADCP)

La principal célula inmunitaria implicada en la ADCP es el macrófago. Los macrófagos expresan FcγRI o CD64 y FcγRIIA o CD32 que, al unirse al AcMo, provoca la activación de las vías de señalización intracelular ya mencionadas para el mecanismo de ADCC (PLC-γ, PI-3K y las vías de las proteínas G Vav/Rho), lo que resulta en la fagocitosis de la célula diana. Luego, el fagosoma procede a un proceso de maduración muy estrictamente regulado caracterizado por la expresión de diferentes moléculas como Rab5 y Lamp-1, así como una acidificación gradual hasta fusionarse con el lisosoma, eliminando finalmente la célula interiorizada[168].

1.5.2. Tratamientos inmunoterápicos aprobados en MM

1.5.2.1. Elotuzumab

El primer AcMo aprobado por la FDA para el tratamiento del MM fue elotuzumab, un AcMo IgG1 anti-SLAMF7 humanizado. En particular, elotuzumab se aprobó por primera vez en combinación con lenalidomida y dexametasona para pacientes con RRMM que habían recibido de una a tres terapias previas[169]. Algunos años más tarde, la FDA lo aprobó en combinación con pomalidomida y dexametasona para el tratamiento de pacientes adultos con MM que habían recibido al menos dos terapias previas, incluida la lenalidomida y un inhibidor del proteasoma[170]. SLAMF7, también conocido como CS1, es una molécula de superficie celular expresada en CPs, linfocitos T citotóxicos $CD8^+$, células B activadas, células NK y DCs maduras[171–174]. SLAMF7 también se expresa tanto en CPs malignas primarias como en casi todas las líneas celulares de MM. Además, se ha detectado SLAMF7 soluble en suero de pacientes con MM que presenta una correlación directa con el estadio de la enfermedad[171,174].

Elotuzumab inhibe la adhesión de las células de MM a las BMSCs, lo que puede revertir el efecto protector proporcionado por el microambiente de la MO a las células de mieloma (Figura 9). Además, elotuzumab puede inducir ADCC tanto en líneas celulares de MM como en células plasmáticas primarias de pacientes con

mieloma (ya sea recién diagnosticados o resistentes a las terapias convencionales)[174].

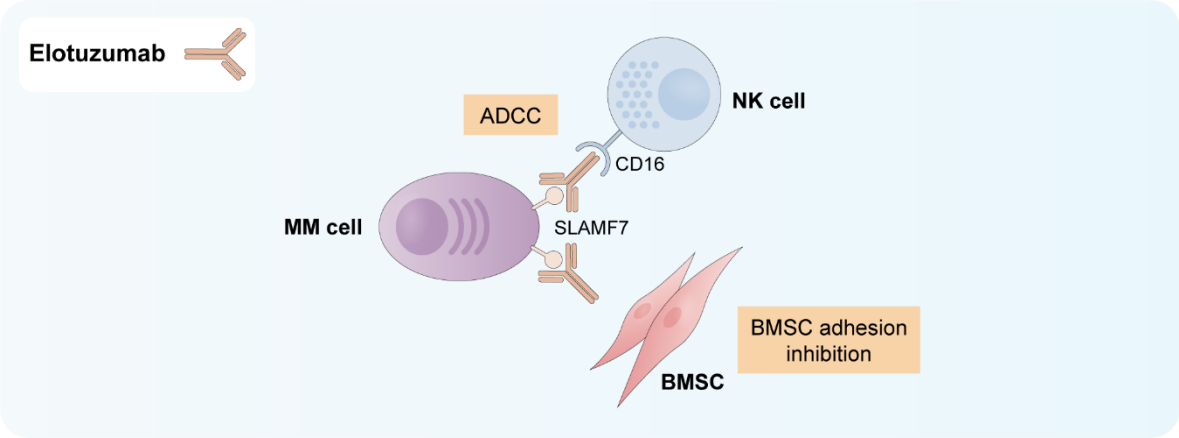

Figura 9. Principales mecanismos de acción de elotuzumab en MM. Modificado de Díaz-Tejedor et al.[44].

1.5.2.2. Daratumumab

Daratumumab es un AcMo IgG1 anti-CD38 humanizado que fue aprobado por primera vez en 2015 por la FDA para su uso en pacientes con RRMM. Desde entonces, ha sido aprobado en diferentes regímenes de tratamiento y pacientes, como se resume en la Tabla 1.

Tabla 1. Calendario de aprobación de la FDA para daratumumab.

Fecha	Aprobación de la FDA	Referencia
Noviembre 2015	En monoterapia para pacientes que hayan recibido al menos tres tratamientos previos.	175
Noviembre 2016	En combinación con lenalidomida y dexametasona para el tratamiento de pacientes que han recibido al menos un tratamiento previo.	176,177
	En combinación con bortezomib y dexametasona para el tratamiento de pacientes que han recibido al menos un tratamiento previo.	
Junio 2017	En combinación con pomalidomida y dexametasona para el tratamiento de pacientes que han recibido al menos dos terapias previas que incluyen lenalidomida y un inhibidor del proteasoma.	178
Mayo 2018	En combinación con bortezomib, melfalán y prednisona para el tratamiento de pacientes con NDMM que no son elegibles para ASCT.	179

Junio 2019	En combinación con lenalidomida y dexametasona para el tratamiento de pacientes con NDMM que no son elegibles para el ASCT.	180
Septiembre 2019	En combinación con bortezomib, talidomida y dexametasona para pacientes con NDMM que son elegibles para ASCT.	181
Agosto 2020	En combinación con carfilzomib y dexametasona para el tratamiento de pacientes con RRMM que han recibido de una a tres líneas previas.	182

CD38 se expresa en diferentes subpoblaciones de células hematopoyéticas y no hematopoyéticas. Cabe destacar que las células plasmáticas malignas $CD138^+$ expresan niveles más altos de CD38 que otras subpoblaciones inmunitarias y células plasmáticas normales[183].

Daratumumab se seleccionó por primera vez de un panel de 42 AcMos humanos anti-CD38 por su eficacia en la eliminación de células MM a través de CDC y ADCC[184]. Otros estudios preclínicos demostraron que daratumumab también podía inducir ADCP[185] y apoptosis en presencia de agentes de entrecruzamiento, tanto fragmentos $F(ab)_2$ como células que expresan el receptor Fcγ[186] (Figura 10).

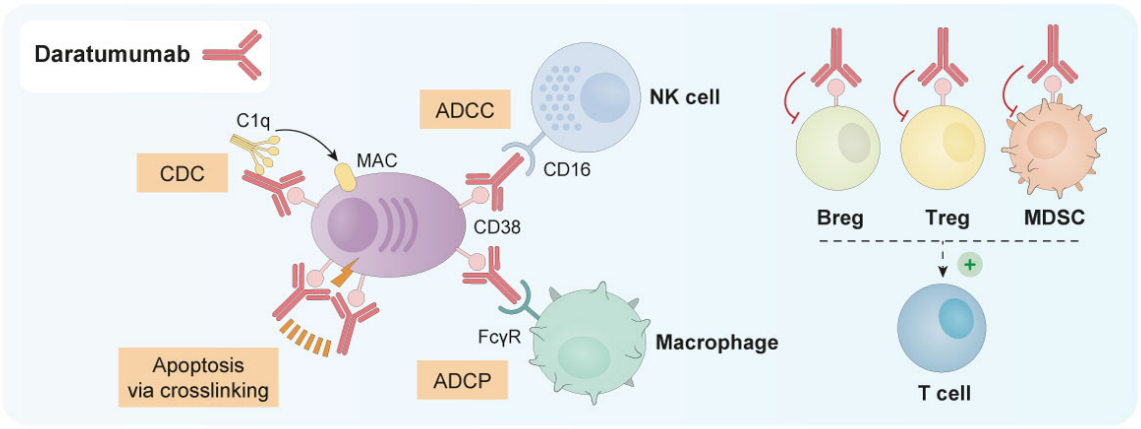

Figura 10. Principales mecanismos de acción de daratumumab en MM. Modificado de Díaz-Tejedor et al.[44].

Dado que diferentes poblaciones de células inmunes expresan CD38, el tratamiento con daratumumab también tiene un impacto sobre ellos. De hecho, se ha descrito que los pacientes con MM tratados con daratumumab tanto en monoterapia como en combinación con lenalidomida y dexametasona, presentan

una disminución de células NK (del 10% al 2%), MDSCs, Bregs y Tregs. Por el contrario, otras poblaciones inmunitarias, como las células T $CD4^+$ y $CD8^+$, mostraron números aumentados[120,187–189]. A pesar de la disminución del número de células NK observada tras el tratamiento con daratumumab, según Casneuf et al. las células NK restantes parecían poder contribuir a la eficacia clínica del fármaco[188]. Además, se ha descrito que daratumumab induce la activación y degranulación de las células NK como se observa por la sobreexpresión de CD69, CD107a e IFN-γ en estas células[190].

1.5.2.3. Isatuximab

Isatuximab es un AcMo IgG1 anti-CD38 humanizado que se ha aprobado recientemente (marzo de 2020) en combinación con pomalidomida y dexametasona para pacientes con MM que habían recibido previamente al menos dos líneas de terapia[191]. Isatuximab ejerce su actividad antimieloma a través de diferentes mecanismos. En primer lugar, y a diferencia de daratumumab, isatuximab ha mostrado actividad proapoptótica frente a células de mieloma que expresan altos niveles de CD38 sin ningún agente de entrecruzamiento[193,194]. Además, este AcMo también presenta efectos citotóxicos mediados por componentes del sistema inmune, como la inducción de CDC, ADCC y ADCP[193,195]. Además, isatuximab inhibe por completo la actividad NADasa de CD38, lo que puede mitigar la actividad inmunosupresora de ADO en el microambiente de la MO de los pacientes con MM[196–198] (Figura 11).

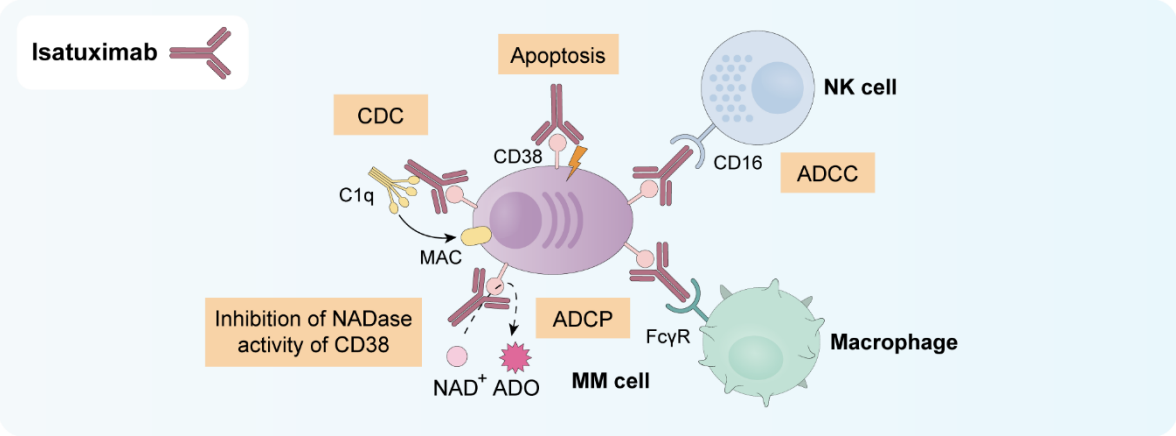

Figura 11. Principales mecanismos de acción de isatuximab en MM. Modificado de Díaz-Tejedor et al.[44].

1.5.2.4. Belantamab Mafodotin

Belantamab mafodotin (GSK2857916) es un AcMo humanizado IgG1 anti- BCMA afucosilado conjugado con monometil auristatina F (MMAF), que es un inhibidor de la polimerización de tubulina[199]. Ambas partes (anticuerpo y toxina) están unidas a través de un conector de maleimidocaproilo no escindible, lo que proporciona una mejor estabilidad plasmática del compuesto sin perder ninguna propiedad y sin toxicidad inespecífica[200]. Belantamab mafodotin es el primer ADC anti-BCMA aprobado por la FDA como agente único para pacientes con RRMM que han recibido al menos cuatro terapias previas[201]. BCMA, también conocido como TNFRSF-17, se induce selectivamente durante la diferenciación de CPs y está casi ausente en las células B inmaduras y de memoria[202,203]. Esta molécula también se expresa en líneas celulares de mieloma[204] y se pueden detectar altos niveles de ARNm de BCMA en células plasmáticas malignas primarias [205].

Belantamab mafodotin ejerce su efecto antimieloma a través de cuatro mecanismos conocidos: (i) ADCC mediada por células NK; (ii) reclutamiento de macrófagos para promover ADCP; (iii) alteración de los microtúbulos y posterior detención del ciclo celular en fase G2/M seguida de apoptosis después de la liberación de la toxina MMAF en el citoplasma de las células de mieloma[199] e (iv) inducción de ICD[200], que es un mecanismo caracterizado por la capacidad de las células moribundas para provocar respuestas inmunitarias adaptativas sólidas contra autoantígenos alterados o neoepítopos derivados del cáncer[206]. En relación con este último mecanismo, los datos indican que el tratamiento de células de mieloma con belantamab mafodotin promueve la exposición de calreticulina (CALR) en su superficie y la liberación de HMGB1, que posteriormente inducen la maduración y activación de DCs y eventualmente la activación de las células T[207] (Figura 12).

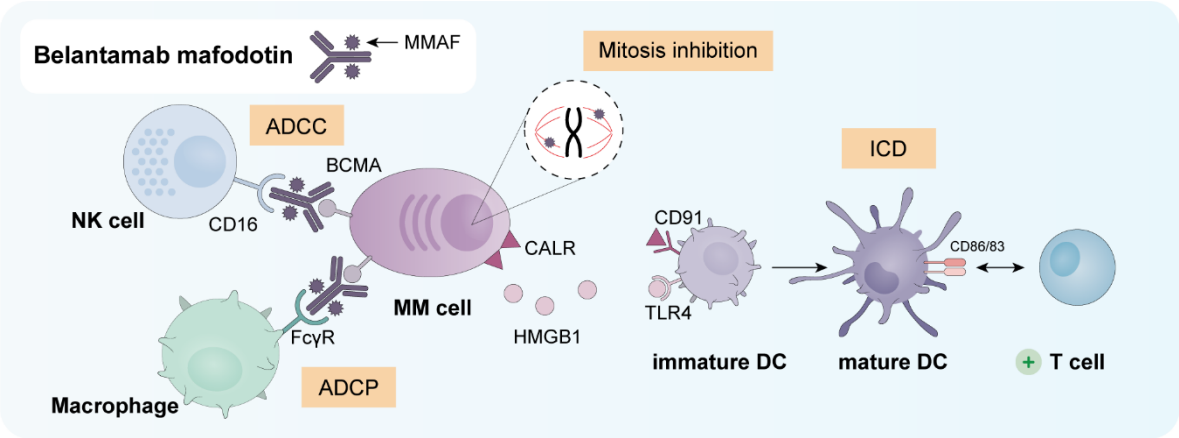

Figura 12. Mecanismos primarios de acción de belantamab mafodotin en MM. Modificado de Díaz-Tejedor et al.[44].

1.5.2.5. Terapia con células CAR-T

Idecabtagene vicleucel (ide-cel) es una terapia de células CAR-T anti-BCMA que ha sido aprobada recientemente por la FDA para el tratamiento de pacientes con RRMM después de cuatro o más líneas de terapia anteriores, que incluyen un IMiD, un IP y un AcMo anti-CD38. Es importante destacar que esta ha sido la primera terapia génica basada en células aprobada por la FDA para el mieloma múltiple[208]. Además, en febrero de 2022, la FDA aprobó ciltacabtagene autoleucel (cilta-cel), otra terapia de células CAR-T dirigida frente a BCMA, para el tratamiento de pacientes con MM después de cuatro líneas o más de terapia previas, incluido un IP, un IMiD y un AcMo anti-CD38[209].

Brevemente, los CAR son proteínas de fusión artificiales que consisten en un dominio de reconocimiento de antígeno conectado a fracciones de señalización mediante dominios transmembrana. Específicamente, los dominios de reconocimiento de antígenos consisten en el fragmento variable de cadena única (scFv) derivado de anticuerpos y los dominios de señalización contienen un dominio coestimulador como CD28 o 4-1BB y un dominio de activación que generalmente se deriva de CD3ζ[210]. Como ya se ha mencionado, ide-cel se dirige a BCMA en la célula de mieloma y tiene una molécula 4-1BB como dominio coestimulador, lo que permite una mejor producción de citoquinas y proliferación de las células CAR T[211] (Figura 13).

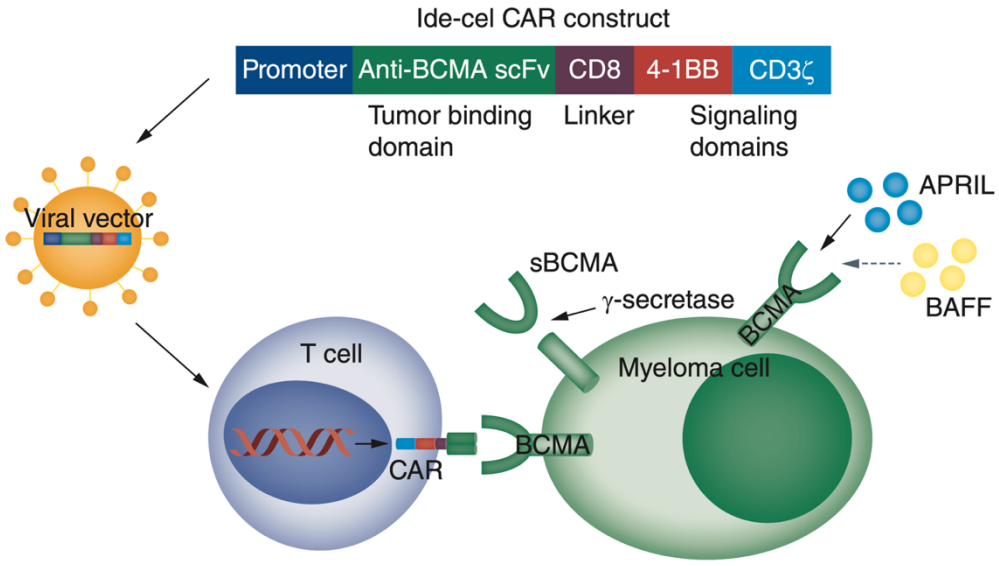

Figura 13. Estructura del CAR-T idecabtagene vicleucel y su mecanismo[211].

Con respecto a cilta-cel, las células T de pacientes con MM se modifican genéticamente ex vivo para expresar un CAR que comprende un dominio de direccionamiento anti-BCMA que consta de dos anticuerpos anti-BCMA de un solo dominio unidos a un dominio coestimulador 4-1BB y un dominio de señalización CD3ζ[209].

1.5.3. Fármacos con actividad inmunoestimulante en MM

Como se describió en secciones anteriores, algunos de los mecanismos de acción de los AcMos y ADCs requieren diferentes células efectoras inmunitarias. Por lo tanto, los fármacos que aumentan la inmunogenicidad de las células tumorales haciéndolas más fácilmente reconocibles y susceptibles al ataque de las células inmunitarias, o los fármacos que activan las células efectoras, podrían ser buenos aliados de estas inmunoterapias. De hecho, diferentes grupos de fármacos, como los agentes alquilantes, los IMiDs, los IPs y los HDACis, a los que pertenecen algunos fármacos aprobados para el tratamiento del MM, han demostrado efectos inmunoestimuladores, como se revisará más adelante (Tabla 2).

Tabla 2. Resumen de los principales efectos inmunomoduladores de los agentes alquilantes, IPs, IMiDs y HDACis. Modificado de Díaz-Tejedor et al.[44].

Grupo de fármaco	Población celular diana	Efectos inmunes
Agentes alquilantes	Células tumorales	translocación CALR ↑
		secreción de HMGB1 ↑
	DCs	fenotipo de activación ↑
	Células de MM	respuesta secretora ↑
		CD47 ↓
	Macrófagos	CD64 ↑ (ADCP ↑)
Fármacos inmunomoduladores (IMiDs)	Células T	respuestas proliferativas ↑ IL-2 e IFN-γ ↑
		respuestas citotóxicas ↑
	Células NK	actividad citotóxica ↑
		fosforilación de Zap-70 ↑
		expresión de granzima-B ↑
	DCs	actividad endocítica ↑
		expression de MHC-I y CD86 ↑
	Células de MM	expresión de MICA y PVR ↑
		expresión de CD38 ↑
Inhibidores del proteasoma (PIs)	Células tumorales	exposición de Hsp60 y Hsp90 ↑ (inducción de ICD ↑)
	Células de MM	exposición de CALR ↑ (inducción de ICD ↑)
Inhibidores de histona desacetilasa (HDACis)	Células de MM	expression de ULPBP2/5/6 y MICA/B ↑
		expresión de PD-L1 ↑
		expresión de CD38 ↑

1.5.3.1. Agentes alquilantes

Los agentes alquilantes como la ciclofosfamida se han utilizado en el tratamiento del MM durante más de 60 años debido a sus efectos inhibidores sobre la división celular. Pero, además, a dosis bajas, estos fármacos también presentan una importante actividad inmunomoduladora[212]. En este sentido, el tratamiento de diferentes modelos tumorales con mafosfamida o ciclofosfamida induce la translocación de CALR a la membrana plasmática y la liberación de HMGB1[213,214], siendo ambos marcadores de ICD. En consecuencia, los ratones tratados con ciclofosfamida mostraron un aumento en las DCs infiltrantes de tumores con un fenotipo activado[213].

En el MM aún no hay evidencia clara de la implicación del mecanismo de ICD en la respuesta a la ciclofosfamida, aunque se han observado otros efectos

inmunomoduladores. De hecho, la exposición in vitro de células MM.1S a dosis bajas de ciclofosfamida conduce a una respuesta secretora que, junto con la regulación a la baja del antígeno CD47, aumenta en gran medida la fagocitosis inducida por macrófagos de células MM recubiertas de daratumumab[215]. Además, después del tratamiento con ciclofosfamida, los macrófagos presentaron niveles elevados de CD64, lo que posiblemente mejoró aún más la ADCP[215]. Estos resultados se han confirmado en la clínica en el ensayo CyBorD-DARA que combina ciclofosfamida con daratumumab, bortezomib y dexametasona[216].

1.5.3.2. Fármacos inmunomoduladores (IMiDs)

Además del efecto antimieloma que ya se ha mencionado, los IMiDs tienen propiedades inmunomoduladoras que se describirán brevemente. En cuanto a los efectos sobre las células T, diferentes autores han informado que esta población de células inmunes aumenta su producción de citoquinas[217,218] y respuestas citotóxicas[217,218] tras la exposición a IMiDs. También se ha demostrado que los IMiDs aumentan la actividad citotóxica de las células NK[219–222]. Aunque se sugirió por primera vez que este efecto ocurría indirectamente a través de la inducción de la producción de IL-2 en las células T, la fosforilación y activación de Zap-70 y la participación de cereblon se han descrito recientemente como dos mecanismos directos e independientes de la regulación al alza de la granzima mediada por pomalidomida[222]. Curiosamente, otros autores observaron que los IMiDs pueden aumentar la susceptibilidad de las células de mieloma al reconocimiento y destrucción mediado por células NK al inducir una mayor expresión de MICA y PVR (el ligando del receptor DNAM-1) en las células de mieloma[223].

También se ha propuesto que los IMiDs aumentan la inmunidad antitumoral al mejorar la función de DCs[224]. De hecho, tanto la pomalidomida como la lenalidomida aumentan la actividad endocítica y la expresión de MHC-I y CD86 en las DCs. De acuerdo con esto, cuando las DCs se trataron con IMiDs antes de la presentación de antígeno, aumentaron las células T $CD8^{+}$[224].

Finalmente, dado que el gen *CD38* parece estar reprimido por Ikaros y Aiolos, el tratamiento con lenalidomida aumentó la expresión superficial de CD38 en varias líneas celulares de MM, lo que conllevó una mayor ADCC mediada por daratumumab[225].

1.5.3.3. Inhibidores del proteasoma (IPs)

Como ya se ha descrito, los IPs se han incorporado a muchos regímenes combinatorios para el tratamiento del MM. Además de sus efectos directos sobre las células tumorales, los IPs pueden ejercer ICD. De hecho, el tratamiento con bortezomib en un modelo de tumor de ovario murino dio como resultado un mayor reclutamiento de linfocitos T $CD8^+$ en el tumor y mayores cantidades de linfocitos T IFN-γ^+ que infiltran el tumor[226]. Además, el tratamiento in vitro de células tumorales de ovario con bortezomib condujo a la sobreexpresión de Hsp60 y Hsp90 en superficie, dos marcadores de ICD, que promovieron la fagocitosis de estas células tumorales por parte de las DCs[226]. En MM, la señal de activación de las células de mieloma muertas con bortezomib a las DCs está mediada por la exposición de Hsp90 en la superficie de las células apoptóticas[227]. De hecho, las DCs pulsadas con células de mieloma muertas por bortezomib son potentes inductores de células T productoras de IFN-γ específico de tumor[227]. Además, tanto bortezomib como carfilzomib promovieron la exposición de CALR en la membrana plasmática en líneas celulares de MM[228]. Finalmente, el tratamiento combinado de carfilzomib y cloroquina (que bloquea la autofagia) aumentó tanto la apoptosis como la exposición de la superficie celular de CALR, aumentando así la capacidad inmunogénica de carfilzomib[228].

1.5.3.4. Inhibidores de histona desactilasa (HDACis)

Los HDACis también ejercen efectos inmunogénicos contra las células de MM. Según Wu et al. el ácido valproico (VPA) indujo la regulación positiva de MICA/B y ULBP2 tanto en líneas celulares de MM como en células de mieloma de pacientes y, en consecuencia, la desgranulación y la actividad citotóxica de las células NK aumentaron en presencia de células de mieloma tratadas previamente con VPA[229].

Además, el butirato de sodio, otro HDACi, incrementa la expression de MICA en líneas celulares de MM cuando se combina con un inhibidor de la metaloproteinasa de la matriz III y óxido de fenilarsina, un fármaco que dificulta la internalización del ligando de superficie[230]. El pan-HDACi panobinostat también aumenta la expresión de ULPBP2/5/6 y MICA/B en células MM[231]. Junto con panobinostat, entinostat (un inhibidor específico de HDAC de clase I) y ricolinostat (inhibidor de HDAC6) aumentaron PD-L1 en células MM, probablemente por acetilación de histonas del promotor del gen *PDL1*[231].

Curiosamente, varios HDACi inducen la sobreexpresión de CD38[232–234]. En cultivos ex vivo, panobinostat y, en mayor medida, ricolinostat aumentaron la expresión de CD38 en células de mieloma de pacientes de MM, lo que mejoró la eficacia de daratumumab[232,233]. En concreto, la inhibición de HDAC6 por ricolinostat evita la desacetilación de H3K27 en el promotor de *CD38*[233].

La tinostamustina (EDO-S101) es una molécula de fusión primera en su clase que combina el agente alquilante bendamustina y el inhibidor de HDAC vorinostat, con una potente actividad inhibidora de las HDAC de clase I y II[235]. Esta molécula ha mostrado una potente actividad antimieloma in vitro, in vivo y ex vivo, como agente único y en combinación con bortezomib[236]. Además, la tinostamustina como agente único se está evaluando actualmente en diferentes ensayos clínicos de fase I/II para el tratamiento de tumores sólidos avanzados y neoplasias malignas hematológicas, incluido el RRMM (NCT03345485, NCT02576496). También se está evaluando la tinostamustina, en combinación con otros agentes en glioblastoma y melanoma avanzado (NCT03452930, NCT03903458). Teniendo en cuenta que tanto los agentes alquilantes como los HDACis ejercen una actividad inmunoestimulante, es probable que el fármaco de fusión tinostamustina también comparta esta actividad, por lo que podría potenciar las inmunoterapias en el contexto del MM. Sin embargo, estos efectos aún no han sido explorados.

1.6. MECANISMOS DE RESISTENCIA A AcMos

En términos generales, debido a la introducción relativamente reciente de los AcMos en el armamento terapéutico contra el MM, los mecanismos de resistencia a estos fármacos aún son poco conocidos. No obstante, los mecanismos de resistencia identificados hasta el momento se pueden dividir en los siguientes grupos.

1.6.1. Infraexpresión de la diana

Puede parecer bastante evidente que uno de los primeros mecanismos de resistencia identificados para los AcMos fue la expresión reducida de la molécula diana. En este sentido, se ha descrito ampliamente que la respuesta a daratumumab se asocia significativamente con el nivel de expresión basal de CD38 en las células tumorales. De hecho, los pacientes con niveles más altos de CD38 antes del tratamiento respondieron mejor al AcMo[237–239].

Además, los niveles de expresión de CD38 se correlacionan con la CDC y ADCC inducidas por daratumumab in vitro. Específicamente, las líneas celulares de MM transducidas para expresar niveles más altos de CD38 ejercen una mayor CDC y ADCC mediada por daratumumab que la línea parental[237].

Una posible causa de la resistencia adquirida es la infraexpresión de la diana tras la administración del AcMo, cuyos mecanismos no se conocen del todo. Krejcik et al. informaron que la expresión de CD38 en las células tumorales se reduce mediante un proceso llamado trogocitosis, mediante el cual los complejos de CD38-daratumumab se transfieren a monocitos y granulocitos[187]. Otros autores también informaron que daratumumab deplecionó selectivamente las células MM $CD38^{++}$, permitiendo así la expansión de las células de MM $CD38^{-/+}$[240]. Además, según Chillemi et al., la unión de daratumumab a su diana induce una redistribución de CD38 en agregados polares en la membrana plasmática que son liberados al microambiente de la MO a través de microvesículas[241].

Dado que los ADCs también se unen específicamente a las moléculas de membrana, su subexpresión también podría estar involucrada en la resistencia a

estos fármacos[242]. En este sentido, aunque todavía no hay evidencia que relacione la expresión basal de BCMA y la respuesta a belantamab mafodotin, ya se sabe que el tratamiento con inhibidores de la γ-secretasa, que inhiben la escisión de BCMA de la membrana plasmática, mejora la eficacia de otras terapias anti-BCMA, tales como CAR-T[243].

1.6.2. Sobreexpresión de las proteínas inhibidoras del complemento (CIPs)

La sobreexpresión de las proteínas inhibidoras del complemento (CIPs) CD55 y CD59, en el momento de la progresión de la enfermedad, en comparación con los niveles antes o durante el tratamiento con daratumumab, se ha propuesto como otro potencial mecanismo de resistencia adquirida a daratumumab, según Nijhof et al.[238]. Por el contrario, los niveles previos al tratamiento de estos CIPs en las células tumorales de los pacientes no se correlaciona con la respuesta a daratumumab[238].

1.6.3. Polimorfismos del receptor Fcγ

Como ya se ha mencionado, la ADCC y ADCP requieren la presencia de FcγR en las células NK y los macrófagos. Los polimorfismos en estos receptores también pueden estar involucrados en la resistencia a mAb, aunque los resultados no son concluyentes hasta el momento.

En un estudio aleatorizado de fase II de elotuzumab + Bd (bortezomib y dosis bajas de dexametasona) vs Bd en pacientes con RRMM, los pacientes tratados con EBd homocigotos para el alelo V (VV) de alta afinidad FcγRIIIA (CD16a) mostraron una PFS más larga que aquellos que eran homocigotos para el alelo F de FcγRIIIA F de baja afinidad (FF)[244]. Sin embargo, un subanálisis de PFS basado en el genotipo de CD16a no mostró diferencias significativas entre VV y FF en el ensayo clínico ELOQUENT-2, que evaluó la eficacia de la combinación de elotuzumab más lenalidomida/dexametasona[245].

Con respecto a daratumumab, Van de Donk et al. obtuvieron resultados similares ya que según los autores, los polimorfismos de FcγR solo tuvieron un impacto modesto en la respuesta a daratumumab y en la PFS, pero no afectaron significativamente a la OS[246].

1.6.4. Número de células NK

Dado que uno de los mecanismos de acción de todos los AcMos aprobados para el tratamiento del MM es la ADCC, el recuento de células NK del paciente antes del tratamiento también podría influir en la respuesta. De hecho, un alto número de células NK antes de la administración de elotuzumab se ha asociado con una PFS más prolongada[247].

Con respecto a daratumumab, aunque los datos preclínicos mostraron que una proporción más baja de células NK:células MM se asoció con una disminución de la ADCC[237], este resultado no se observó en el entorno clínico. Específicamente, la disminución en el recuento de células NK se observa de igual manera en pacientes que responden y que no responden y, lo que es más importante, la PFS después del tratamiento con daratumumab no se correlacionó con la cantidad de reducción de células NK[188].

1.6.5. Actividad inmunomoduladora

Dado que daratumumab e isatuximab muestran actividad inmunomoduladora, una regulación ascendente compensatoria de múltiples puntos de control inmunitarios inhibidores también puede contribuir al desarrollo de resistencia a las actividades inmunomoduladoras de los anticuerpos CD38[240]. De hecho, se ha descrito que los pacientes resistentes a daratumumab mostraron una sobreexpresión del gen *LAG3* y *TIGIT* en comparación con los pacientes respondedores[248].

HIPÓTESIS Y OBJETIVOS

La introducción de enfoques inmunoterapéuticos en el arsenal terapéutico del MM ha mejorado notablemente la tasa y profundidad de respuesta, así como la supervivencia de los pacientes. De hecho, entre los diferentes tratamientos inmunoterapéuticos, el anticuerpo monoclonal daratumumab ha demostrado eficacia tanto en monoterapia como en terapias combinadas. En el caso de los anticuerpos monoclonales, la expresión de sus dianas específicas por parte de las células tumorales, las propiedades inmunogénicas de estas células y el estado inmunitario de los pacientes influyen en la eficacia de estos tratamientos. Por tanto, los fármacos capaces de favorecer alguno o todos estos aspectos son potencialmente buenos candidatos para ser combinados con anticuerpos monoclonales.

A pesar de los buenos resultados obtenidos con los tratamientos de inmunoterapia en general, y con los anticuerpos monoclonales en particular, el MM sigue considerándose una enfermedad incurable en la gran mayoría de los casos, debido, entre otros, a la presencia de resistencia (primaria o adquirida) a los fármacos disponibles. Sin embargo, los mecanismos implicados en la adquisición de resistencia a los anticuerpos monoclonales aún no se conocen bien. Por tanto, el estudio de estos mecanismos y la búsqueda de estrategias encaminadas a superarlos, constituye un campo de investigación clave en el escenario terapéutico actual.

Teniendo esto en cuenta, los objetivos específicos propuestos en nuestro trabajo fueron:

OBJETIVO 1: Analizar el posible papel de tinostamustina, un inhibidor de histona desacetilasa alquilante, en la mejora del efecto antimieloma de daratumumab.

1.1. Evaluar el efecto de tinostamustina sobre la expresión de membrana de CD38 en líneas celulares de MM y en células primarias de pacientes con MM.

1.2. Explorar si el tratamiento de las células de mieloma con tinostamustina aumenta la inmunogenicidad de estas células a través del análisis de moléculas de superficie relacionadas con el sistema inmunitario, como los ligandos de los receptores de células NK.

1.3. Determinar si tinostamustina potencia el efecto antimieloma de daratumumab a través de sus diferentes mecanismos de acción mediante experimentos in vitro y ex vivo.

1.4. Evaluar la eficacia de la doble combinación de tinostamustina y daratumumab en modelos in vivo.

OBJETIVO 2: Identificar las causas moleculares de la resistencia adquirida a daratumumab y explorar nuevas estrategias para revertirla.

2.1. Generar un modelo in vitro de resistencia adquirida a la CDC mediada por daratumumab y caracterizar su inmunofenotipo y perfil de sensibilidad a los mecanismos de acción adicionales de daratumumab.

2.2. Analizar los mecanismos implicados en la desregulación de CD38, diana de daratumumab, utilizando el modelo de resistencia generado.

2.3. Identificar los genes implicados en la resistencia adquirida a la CDC mediada por daratumumab y evaluar su implicación funcional.

2.4. Establecer nuevos modelos in vitro de sensibilidad a CDC mediada por daratumumab.

RESULTADOS CAPÍTULO II: EVALUACIÓN DE LOS MECANISMOS IMPLICADOS EN LA RESISTENCIA ADQUIRIDA A LA CDC MEDIADA POR DARATUMUMAB

1. Establecimiento y caracterización de un modelo de resistencia in vitro a la CDC mediada por daratumumab

El desarrollo de un modelo celular de resistencia a la CDC mediada por daratumumab se logró mediante la exposición de la línea celular sensible MOLP-8 a dosis crecientes de daratumumab (0,01 – 100 µg/ml) y 10 % de suero humano, como fuente de complemento, durante aproximadamente 5 meses, hasta que las células desarrollaron resistencia (RMOLP-8). Una vez alcanzada la resistencia, las células se mantuvieron sin daratumumab durante 2 semanas y se volvió a comprobar la resistencia observándose que mientras el 82,75% de las células MOLP-8 morían en presencia de daratumumab (10 µg/ml), solo el 7,76% de las RMOLP-8 las células lo hacían.

Dado que daratumumab ejerce su actividad antimieloma a través de los otros tres mecanismos de acción clásicos, también probamos la sensibilidad de las células RMOLP-8 a la apoptosis via cross-linking, ADCC y ADCP en comparación con la línea celular MOLP-8. Las células RMOLP-8 eran completamente resistentes a la apoptosis vía cross-linking en comparación con su equivalente sensible MOLP-8. Sin embargo, no se observaron diferencias ni en la ADCC ni en la ADCP mediada por daratumumab al comparar ambas líneas celulares. Además, las células con resistencia adquirida a daratumumab, también fueron resistentes a la CDC mediada por isatuximab.

Para caracterizar el modelo celular de resistencia generado, se evaluó el ciclo celular y la proliferación tanto en líneas celulares sensibles como resistentes. En cuanto al ciclo celular, se observó que la línea celular RMOLP-8 tuvo un porcentaje disminuido de células tanto en la fase G0 – G1 como S y un aumento en la fase G2 – M en comparación con las células MOLP-8. Además, las células RMOLP-8

presentaron una mayor tasa de proliferación en comparación con las células MOLP-8.

Además, se estudiaron los marcadores inmunofenotípicos utilizados habitualmente en la evaluación de MM mediante citometría de flujo en ambas líneas celulares. Así, las células RMOLP-8 infraexpresaron significativamente CD38, diana de daratumumab. Además, CD56 también se reguló negativamente en las células RMOLP-8 en comparación con las células MOLP-8, mientras que la expresión de CD81 fue mayor.

2. Estudio de la implicación de la expresión de CD38 en la resistencia adquirida a daratumumab de las células RMOLP-8

Como se explicó en la sección anterior, las células RMOLP-8 infraexpresaban CD38. Aquí, la expresión de CD38 se analizó a nivel transcripcional a través de RT-qPCR; observamos que las células RMOLP-8 tienen una expresión disminuida de esta molécula en comparación con la línea celular MOLP-8. Además, los niveles de proteína total de CD38 fueron más bajos en las células RMOLP-8 según lo confirmado por WB e inmunofluorescencia. Dicha infraexpresión de CD38 se tradujo en una menor unión de daratumumab a las células RMOLP-8.

Para comprender mejor la importancia de la expresión de CD38 en la resistencia adquirida a daratumumab, se clonó ADNc de CD38 en el plásmido pMSCV (pMSCV-CD38) y se transdujo la línea celular RMOLP-8 con esta construcción para sobreexpresar CD38 de manera estable para analizar si dicha sobreexpresión se traducía en una resensibilización a CDC mediada por daratumumab. Observamos que existía una clara tendencia a aumentar la sensibilidad a este mecanismo con la sobreexpresión de CD38 en RMOLP-8, aunque no fue capaz de revertir completamente la resistencia a CDC mediada por daratumumab.

Además, se evaluó el efecto de tinostamustina en la expresión de CD38 mediante citometría de flujo en la línea celular RMOLP-8. El tratamiento con tinostamustina (1 y 2,5 μM) durante 48 horas aumentó la expresión superficial de CD38 en la

población viable de células RMOLP-8. Posteriormente, analizamos si el pretratamiento con tinostamustina re-sensibilizaba las células resistentes al CDC d mediado por daratumumab, observando que el pretratamiento de células RMOLP-8 con tinostamustina no afectó la sensibilidad de estas células a daratumumab.

Por otro lado, nos preguntamos si la sobreexpresión de CD38 en líneas celulares con resistencia intrínseca a la CDC mediada por daratumumab era capaz de revertirla. Por lo tanto, utilizando el plásmido pMSCV-CD38 ya mencionado, se transdujeron cuatro líneas celulares MM (U266, JJN3, NCI-H929 y RPMI-8226) con esta construcción para sobreexpresar CD38 de forma estable. De las líneas celulares generadas con sobreexpresión de CD38, solo la línea celular NCI-H929 con sobreexpresión de CD38 (H929-CD38) parecía ser sensible a daratumumab de manera dosis-dependiente en comparación con la línea parental transducida con el vector vacío.

2.1. Estudio de los mecanismos (epi)genéticos responsables de la infraexpresión de CD38 en células RMOLP-8

Para tratar de dilucidar los mecanismos por los cuales las células resistentes infraexpresan la molécula CD38, se realizaron varios estudios. La secuenciación Sanger se realizó para comprobar si había mutaciones en el gen CD38 que podrían ser responsables del fenotipo de las células resistentes. No se observaron diferencias ni al comparar la secuencia de CD38 de RMOLP-8 con la de MOLP-8 ni al compararla con el gen *CD38* de referencia. De hecho, según la herramienta de alineación EMBL-EBI, la secuencia de *CD38* de las células MOLP-8 y RMOLP-8 tenía una similitud del 100%.

A continuación, se analizó el estado de metilación de las islas CpG ubicadas en el promotor de *CD38*. Se realizó un primer estudio en el que se examinó el estado de metilación de 22 islas CpG en el promotor de *CD38* mediante el método del bisulfito. No encontramos diferencias en el nivel de metilación entre las líneas celulares MOLP-8 y RMOLP-8, encontrando en ambos casos un estado de metilación bajo para las 22 islas CpG estudiadas.

Dado que no observamos diferencias de metilación en estos estudios, se realizó un array de metilación. Específicamente, el "Infinium methylation EPIC array" que permite la interrogación de los patrones de metilación a nivel de todo el genoma, cubriendo más de 850.000 sitios de metilación. En este sentido, después de la normalización de datos obtuvimos resultados de 812.181 posiciones de metilación del ADN, de las cuales 394 regiones promotoras estaban hipometiladas en células RMOLP-8 en comparación con células MOLP-8, mientras que 249 estaban hipermetiladas. Con respecto a los sitios de metilación en la secuencia de codificación del gen, 474 estaban hipometilados en células resistentes, mientras que 582 estaban hipermetilados. Además, la matriz EPIC contenía 27 islas CpG asociadas al gen *CD38*, 9 de las cuales estaban asociadas a la región promotora y las 18 restantes al cuerpo génico. Curiosamente, la región promotora de *CD38* en células resistentes presentó un estado de metilación mayor en comparación con las células sensibles, mientras que no se observaron diferencias en el estado de metilación del cuerpo del gen CD38 entre ambos modelos celulares.

3. Caracterización transcriptómica del modelo de resistencia adquirida a la CDC mediada por daratumumab

Las diferencias en el perfil de expresión génica (GEP) entre las células MOLP-8 y RMOLP-8 se evaluaron utilizando los microarrays Human Gene 2.0 ST RNA. Después de aplicar los valores de filtro, se obtuvieron 546 genes expresados diferencialmente entre las dos líneas celulares, de los cuales 182 estaban infraexpresados y 364 sobreexpresados en el modelo de resistencia en comparación con su contraparte sensible.

Además, se realizó un análisis de enriquecimiento GSEA para identificar procesos biológicos significativamente alterados en células resistentes. Tres procesos biológicos se enriquecieron en las células resistentes en comparación con las células sensibles: la vía de señalización de la reelina, el metabolismo de los lípidos y el sistema inmunitario. Específicamente, la vía de señalización de la reelina fue la más enriquecida con un Fold change de 18,62. En cuanto al metabolismo de

lípidos y sistema inmunológico, se enriquecieron en células resistentes con un fold change de 1,70 y de 1,5, respectivamente.

De los tres procesos biológicos mencionados, el de sistema inmune llamó nuestra atención, con 86 genes significativamente desregulados. Entre ellos y confirmando nuestros datos anteriores, *NCAM1* (CD56) y *CD81* parecían estar significativamente desregulados en las células resistentes en comparación con las células sensibles. Además, la molécula antiapoptótica Bcl-2 también se reguló positivamente en células RMOLP-8 en comparación con MOLP-8. Además, el inhibidor del complejo C5b-9 implicado en la cascada del complemento, Clusterina (*CLU*), se sobreexpresó en células resistentes en comparación con las células sensibles. También se consideraron relevantes genes desregulados adicionales no incluidos en el proceso biológico del Sistema Inmune, como *HHLA2*, *CD276* y *CD38*. Específicamente, *HHLA2* (B7-H7) y *CD276* (B7-H3) aumentaron en células RMOLP-8 en comparación con MOLP-8, ambos con funciones moduladoras de células T.

3.1. Estudio del papel de las células RMOLP-8 en la inhibición de la funcionalidad de los linfocitos T

Los datos de RT-qPCR mostraron que los genes con funciones moduladoras de células T, *HHLA2* y *CD276* se sobreexpresaron aproximadamente 25 veces y 2,5 veces en células RMOLP-8 frente a células MOLP-8.

Teniendo en cuenta estos datos, se realizó un ensayo de activación/proliferación de células T en cocultivo con células MOLP-8 o RMOLP-8. ASí, los linfocitos T que habían estado en cultivo conjunto con células resistentes tenían tasas más bajas de proliferación y activación en comparación con los linfocitos T que habían estado en cultivo con células sensibles.

3.2. Evaluación de la implicación de la molécula antiapoptótica Bcl-2 en la resistencia a la CDC mediada por daratumumab

La sobreexpresión de *BCL2* en RMOLP-8 frente a MOLP-8 identificada en el estudio GEP fue confirmada tanto por RT-qPCR como por WB. Además, se

evaluaron otras proteínas antiapoptóticas (Bcl-xL y Mcl-1) en ambas líneas celulares, estando Bcl-xL también sobreexpresada en células RMOLP-8 pero no Mcl-1. La expresión de estas proteínas antiapoptóticas también se analizó en células sensibles tras el tratamiento con daratumumab y se observó que Bcl-2, Bcl-xL y Mcl-1 aumentaron en presencia del fármaco, lo que sugiere un intento de escape de las células MOLP-8 de la muerte inducida por daratumumab.

Debido a la sobreexpresión de Bcl-2 observada en células resistentes a daratumumab, se evaluó si este aumento se traducía en una mayor sensibilidad al inhibidor de Bcl-2 venetoclax. Las células RMOLP-8 tienen una sensibilidad significativamente mayor al venetoclax a las 48 horas de tratamiento en comparación con las células MOLP-8. Sin embargo, el pretratamiento con venetoclax no fue capaz de revertir la resistencia adquirida a daratumumab.

Dado que la sobreexpresión de Bcl-2 y Bcl-xL en células RMOLP-8 sugirió un papel de estas proteínas en la resistencia adquirida a daratumumab, se evaluó el efecto de la sobreexpresión estable de Bcl-2 y Bcl-xL sobre la sensibilidad de la línea celular MOLP-8 a la CDC mediada por daratumumab. Para ello, las células MOLP-8 se transdujeron con los plásmidos pMSCV-Bcl2, pMSCV-BclxL o el vector vacío como control. Tras confirmar la sobreexpresión de ambas moléculas en las células transducidas, se probó su sensibilidad a CDC mediada por daratumumab. En este sentido, las células que sobreexpresan Bcl-2 y las células que sobreexpresan Bcl-xL fueron tan sensibles como las células control a la CDC mediada por daratumumab.

3.3. Estudio de la implicación del inhibidor del complejo C5b-9 clusterina en la resistencia a daratumumab

La sobreexpresión de *CLU* en células resistentes a daratumumab que se observó en el estudio de los microarrays se confirmó tanto por RT-qPCR como por WB. Con el fin de dilucidar la participación de Clusterin en la resistencia adquirida a CDC mediada por daratumumab, se transdujeron células MOLP-8 para sobreexpresar de

forma estable esta proteína. La sobreexpresión de clusterina se confirmó mediante RT-qPCR y WB en comparación con las células control.

Se probó la sensibilidad a daratumumab de las nuevas líneas celulares generadas. Se muestra que las células MOLP-8 con sobreexpresión estable de Clusterina fueron significativamente más resistentes a la CDC mediada por daratumumab en comparación con las células transducidas con el vector vacío. Además, las células con sobreexpresión de Clusterina también fueron más resistentes a la CDC mediada por isatuximab. Por otro lado, Clusterina fue silenciado de manera estable en células RMOLP-8 usando varios shRNA. En este sentido, el silenciamiento de Clusterina revirtió la resistencia a la CDC mediada por daratumumab.

4. Generación de nuevos modelos celulares sensibles a la CDC mediada por daratumumab

Se empleó la tecnología CRISPR/Cas9 para eliminar CD55 y CD59 en RPMI-8226, una línea celular de mieloma con niveles de intermedios a altos de CD38 en comparación con otras líneas celulares de mieloma, pero intrínsecamente resistente a la CDC mediada por daratumumab. Para ello, primero generamos una línea celular RPMI-8226 con expresión constitutiva de la endonucleasa Cas9 (RPMI-Cas9). Una vez que se confirmó la funcionalidad de la Cas9, la línea celular RPMI-Cas9 se transdujo de manera estable con ARNg contra las proteínas inhibidoras del complemento CD55 o CD59.

No pudimos obtener células CD55 KO, por lo que decidimos centrarnos en la caracterización de las células editadas con CD59. Después de la dilución de células individuales, se obtuvieron 13 clones CD59 KO homogéneos, que se caracterizaron por la expresión de CD59 tanto por WB como por citometría de flujo. Se probó la sensibilidad a CDC mediada por daratumumab de los clones CD59-KO, observándose que 5 clones del pool 1 (#5, #14, #17, #20, #21) y 3 del pool 2 (#8, #13, #19) se volvieron sensibles a este mecanismo de daratumumab en comparación con las células control.

CONCLUSIONES

CAPÍTULO II: EVALUACIÓN DE LOS MECANISMOS IMPLICADOS EN LA RESISTENCIA ADQUIRIDA A LA CDC MEDIADA POR DARATUMUMAB

1. Se obtuvo un modelo in vitro de resistencia adquirida a la citotoxicidad dependiente de complemento mediada por daratumumab, línea celular RMOLP-8, mediante la exposición prolongada de la línea celular parental sensible MOLP-8 a dosis crecientes de daratumumab y suero humano.
2. Las células RMOLP-8 también son resistentes a la apoptosis vía cross-linking inducida por daratumumab, pero no a la citotoxicidad celular dependiente de anticuerpos ni a la fagocitosis celular dependiente de anticuerpos promovida por este AcMo.
3. La línea celular RMOLP-8 tiene resistencia cruzada a la citotoxicidad dependiente de complemento mediada por isatuximab.
4. La tasa de proliferación más alta y el inmunofenotipo observado en las células RMOLP-8, específicamente la pérdida de CD56 y el aumento de CD81, indican un fenotipo más agresivo que las células MOLP-8 consistente con un mieloma de mal pronóstico.
5. Las células resistentes tienen una menor expresión de CD38 tanto a nivel de mRNA como de proteína en comparación con su contrapartida sensible, en línea con la hipermetilación observada del promotor del gen CD38. En consecuencia, la unión de daratumumab a las células RMOLP-8 es menor en comparación con las células MOLP-8.
6. Tinostamustina aumenta la expresión de CD38 en las células RMOLP-8, lo que sugiere que los mecanismos de desacetilación también podrían estar involucrados en la infraexpresión de CD38. Sin embargo, el tratamiento con tinostamustina no sensibiliza las células RMOLP-8 a daratumumab.
7. Las células RMOLP-8 sobreexpresan las proteínas antiapoptóticas Bcl-2 y Bcl-xL. Aunque las células RMOLP-8 son más sensibles a venetoclax que las células MOLP-8, el tratamiento previo con este inhibidor de Bcl-2 no es capaz de revertir la resistencia adquirida a daratumumab.

8. Las células con resistencia adquirida a daratumumab muestran una mayor expresión de Clusterina, un inhibidor de la lisis del complemento soluble. El silenciamiento de Clusterina en estas células las sensibiliza a daratumumab corroborando su participación en el mecanismo de resistencia.
9. Las células RMOLP-8 inhiben la activación y proliferación de células T. La sobreexpresión de genes con funciones moduladoras de linfocitos T por parte de las células RMOLP-8, en concreto *HHLA2* y CD276, puede estar implicada en estos efectos.

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SUPPLEMENTARY MATERIAL

Supplementary Table 1. List of the diferentially deregulated genes in RMOLP-8 cells in comparison to MOLP-8 cells ordered by Fold Change. Gene identification and q-value are given. 182 genes were underexpressed (green) and 364 overexpressed (red).

Gene symbol	Fold Change	q-value(%)
TENM1	0.078748	0
LINC00632	0.079267	0
TMEM47	0.167870	0
ITM2A	0.208208	0
CDR1	0.210661	0
FGFR2	0.223028	0
LMO3	0.225133	0
SLITRK4	0.256175	0.85322913
PROX1	0.264420	2.77992522
CD38	0.265209	0
SLCO3A1	0.274450	0
TMEM108	0.276355	4.85318878
IGSF11	0.293543	0
BEX3	0.298700	0
ID2	0.310910	0.85322913
SULF1	0.311946	0
STXBP6	0.320920	0
ATRNL1	0.320945	4.85318878
WLS	0.334914	0
SLC16A14	0.338668	0
ITGBL1	0.355541	0
PTPN13	0.356550	1.70446473
DPYD	0.363798	1.70446473
SORCS1	0.363837	0
GPX1	0.376139	0
TSHZ2	0.378090	0.85322913
ABI3BP	0.391910	0
MME	0.395214	0
ZNF732	0.399969	1.44744227
DAPK1	0.404128	0
SLC16A7	0.406393	0
MAGEC2	0.407605	0
CCR2	0.408439	0
FBH1	0.412486	0
FYN	0.421369	3.32375632
MEI1	0.432255	3.32375632
H1F0	0.435247	2.77992522
GAB1	0.436656	2.77992522
FRMPD3	0.437636	3.32375632
TMSB15A	0.438446	2.77992522
RARRES3	0.438827	0.85322913
ATP5ME	0.446250	1.44744227
METTL7A	0.450102	1.70446473
LBH	0.450231	0
ADAM19	0.466272	2.77992522
KLF2	0.480278	3.32375632
TRIM2	0.483030	0
UTRN	0.486054	1.44744227
KIAA1217	0.486913	1.44744227
AUTS2	0.494087	0.85322913
CTBP1	0.495733	2.77992522
CASTOR2	0.498550	0.85322913
GALC	0.500414	1.44744227
SOS1	0.501076	3.32375632
PCSK5	0.501836	4.85318878
SYNE1	0.511572	3.32375632
GSTP1	0.512490	3.32375632
AGAP1	0.514190	0
TSHR	0.514500	4.85318878
GAS2	0.517647	2.77992522
GPR160	0.520209	4.85318878
ANKRD16	0.523814	3.32375632
BACH2	0.524443	4.85318878
BTBD3	0.525929	4.85318878
PTPRG	0.527217	3.32375632
KDM2B	0.529215	3.32375632
LCOR	0.529674	1.44744227
CALHM2	0.532358	3.32375632
CASTOR3	0.538328	3.32375632
TMEM175	0.542655	2.77992522
VAV1	0.542744	3.32375632
ATG9A	0.544148	3.32375632
CRYL1	0.544452	3.32375632
PIGG	0.545630	4.85318878

Gene symbol	Fold Change	q-value(%)
KANSL1	0.555704	2.77992522
MED13L	0.557588	2.77992522
SSX9P	0.557672	2.77992522
ATP8A1	0.558836	3.32375632
EPHA4	0.559465	2.77992522
CHST15	0.561964	4.85318878
LARGE1	0.562423	1.31799621
CCM2	0.564632	2.77992522
ACVR2A	0.564969	3.32375632
RCN3	0.567605	4.85318878
HS3ST3B1	0.568145	3.32375632
DCC	0.569237	4.85318878
ACSL1	0.569389	0.85322913
SUN2	0.571888	4.85318878
ELAVL2	0.572883	3.32375632
TRIO	0.581011	3.32375632
GBF1	0.583336	2.77992522
MYCBP2	0.594225	2.77992522
SLC44A1	0.598770	0.85322913
TNFAIP8L1	0.603969	4.85318878
NCOA3	0.609361	3.32375632
CLIP4	0.612392	3.32375632
CSRP2	0.615795	4.85318878
ERN1	0.616407	3.32375632
SPAG4	0.616559	0
DHTKD1	0.617014	0
TXNDC11	0.617367	1.31799621
ACTR1A	0.621079	3.32375632
ROBO1	0.621300	3.32375632
GAREM1	0.622212	4.85318878
TPO	0.622705	0.85322913
KYAT1	0.630374	1.31799621
TBX15	0.635546	4.85318878
TRRAP	0.636458	4.85318878
DTYMK	0.638526	3.32375632
HUWE1	0.640035	1.31799621
TIRAP	0.644215	4.85318878
INTS1	0.650706	4.85318878
BEND4	0.651086	0
TP53I3	0.654740	2.77992522

Gene symbol	Fold Change	q-value(%)
SMAD3	0.655553	3.32375632
IAH1	0.655942	3.32375632
SERF2	0.656329	3.32375632
FMR1NB	0.664473	1.70446473
RPS14	0.664473	4.85318878
PAN2	0.665793	3.32375632
OTUD1	0.666433	4.85318878
RBFOX1	0.666475	0
CUX1	0.667299	2.77992522
MAP3K3	0.668533	1.31799621
LAMB1	0.668576	3.32375632
SAMD4A	0.669782	2.77992522
CWF19L1	0.671212	4.85318878
ADCY9	0.676530	0
DECR1	0.679575	2.77992522
NCAM1	0.683194	1.44744227
RUBCNL	0.686049	2.77992522
ADAM11	0.686887	1.70446473
HERC4	0.687855	2.77992522
ATP5MC2	0.688362	3.32375632
KLHL24	0.691993	4.85318878
SPOCK3	0.692792	1.44744227
PLA2G16	0.694663	4.85318878
POLR3A	0.695241	4.85318878
IL27RA	0.697810	4.85318878
SNX29	0.701092	4.85318878
SIDT2	0.704045	2.77992522
IFT122	0.705110	4.85318878
HIP1	0.707011	2.77992522
SOWAHD	0.708259	3.32375632
OGFOD3	0.708399	3.32375632
PACSIN3	0.713268	3.32375632
JUP	0.719597	3.32375632
HIPK2	0.724233	3.32375632
RELN	0.726473	4.85318878
ATRAID	0.726773	4.85318878
TNFRSF19	0.727133	3.32375632
APH1A	0.729992	3.32375632
HPN-AS1	0.731863	1.31799621
EPG5	0.732165	2.77992522

Gene symbol	Fold Change	q-value(%)
SPANXB1	0.732239	3.32375632
0	0.733221	3.32375632
ACP4	0.735323	4.85318878
MYEOV	0.736286	2.77992522
GBA	0.737134	3.32375632
FNDC3B	0.739072	4.85318878
MARCO	0.739658	2.77992522
MFSD2A	0.741630	2.77992522
NAV2	0.745121	4.85318878
DNAJB8	0.745676	3.32375632
EEF2K	0.748576	3.32375632
IQGAP3	0.751913	4.85318878
METRN	0.752957	4.85318878
GPC6	0.755939	4.85318878
CTSK	0.756882	4.85318878
YIPF1	0.761213	1.70446473
SLC35G1	0.765524	3.32375632
PIGV	0.767888	1.70446473
PHF21B	0.771153	4.85318878
KANSL3	0.772175	4.85318878
GPR50	0.773275	2.77992522
C22orf34	0.775405	3.32375632
LRRC46	0.788152	1.70446473
ZNF251	0.788555	3.32375632
NDUFB11	0.790257	4.85318878
SFTPD	0.794763	4.85318878
ASB16	0.801711	4.85318878
TIAL1	0.811519	4.85318878
COX5A	1.198850	4.85318878
MAGEA3	1.212769	4.85318878
RPP38	1.225366	4.85318878
YWHAB	1.232130	2.77992522
MBD2	1.235500	3.32375632
SEPHS2	1.257485	1.70446473
CERS6	1.259196	3.32375632
GSAP	1.260724	4.85318878
AJAP1	1.261345	3.32375632
SENP2	1.264264	3.32375632
CIB1	1.268088	3.32375632
MMADHC	1.276567	3.32375632

Gene symbol	Fold Change	q-value(%)
APMAP	1.277385	3.32375632
NAXD	1.279626	2.77992522
NARS	1.280472	4.85318878
YRDC	1.280772	2.77992522
NOL7	1.284615	3.32375632
SEC11A	1.287764	4.85318878
LGMN	1.287962	3.32375632
HSPA9	1.292349	4.85318878
MTRF1	1.292791	4.85318878
GNPTAB	1.307638	4.85318878
RNF20	1.314871	2.77992522
SNAP29	1.317662	4.85318878
SMARCAD1	1.321781	4.85318878
RAB5A	1.324196	2.77992522
ERVW-1	1.324461	1.44744227
TTC1	1.324757	4.85318878
SLC39A6	1.331282	2.77992522
TBCC	1.332206	3.32375632
ADAMTS1	1.338547	2.77992522
HSP90AA1	1.338760	3.32375632
EIF3M	1.340346	4.85318878
PBX1	1.341646	1.70446473
G3BP2	1.343477	1.31799621
SEMA7A	1.346269	4.85318878
ZBTB43	1.347461	4.85318878
AAGAB	1.347988	2.77992522
CDH18	1.348773	2.77992522
DEGS1	1.349446	1.70446473
TRMT6	1.356416	4.85318878
VEGFA	1.359696	1.70446473
ZNF595	1.359754	2.77992522
TMEM182	1.360109	4.85318878
OSBPL9	1.360809	2.77992522
TNPO1	1.361916	2.77992522
DNTTIP2	1.371029	3.32375632
RRS1	1.375508	4.85318878
CD276	1.377927	3.32375632
CCDC171	1.379403	3.32375632
PLRG1	1.380859	2.77992522
TRMT10A	1.381047	1.70446473

Gene symbol	Fold Change	q-value(%)
MORC3	1.382334	4.85318878
PRKAA1	1.383291	4.85318878
TENT2	1.385972	3.32375632
ALKBH1	1.386120	1.70446473
LRRC28	1.389404	1.70446473
RNMT	1.390002	3.32375632
SEH1L	1.394845	3.32375632
EIF2S1	1.398881	4.85318878
ZNF470	1.401394	1.31799621
UCHL1	1.403040	0
CLYBL	1.405180	1.44744227
PSMB9	1.407108	4.85318878
NOL10	1.407711	4.85318878
CD81	1.407773	4.85318878
LRRC8D	1.408080	4.85318878
CDK7	1.410177	1.44744227
ABRACL	1.411853	3.32375632
NDRG1	1.413674	4.85318878
ETF1	1.414369	0.85322913
PES1	1.415362	4.85318878
CCDC112	1.415657	4.85318878
SLC9A3R1	1.420417	2.77992522
MTM1	1.420633	2.77992522
HLA-DPA1	1.420666	4.85318878
TMEM154	1.421491	1.44744227
MPI	1.421852	3.32375632
NSUN2	1.422154	3.32375632
MRFAP1L1	1.423044	4.85318878
C1orf198	1.428908	4.85318878
ZBTB18	1.430023	4.85318878
HSPA4	1.430943	1.70446473
VSTM4	1.432571	3.32375632
IL17RC	1.433880	2.77992522
PRXL2C	1.436027	4.85318878
VRK2	1.436045	4.85318878
HCCS	1.446810	2.77992522
SLC22A5	1.447694	4.85318878
RBM18	1.453170	1.70446473
NEO1	1.453840	1.70446473
SHANK2	1.454393	1.44744227

Gene symbol	Fold Change	q-value(%)
UTP3	1.462736	1.44744227
ETFA	1.466182	1.70446473
ODR4	1.467417	3.32375632
MSANTD4	1.467662	4.85318878
PAK1	1.469229	4.85318878
VASH2	1.470052	1.44744227
IMPAD1	1.470697	3.32375632
CYP2R1	1.472005	0
NOL4L	1.473457	1.44744227
RYBP	1.477849	0
ZNF568	1.479789	1.70446473
MGLL	1.487119	1.44744227
KIR3DL2	1.487507	3.32375632
FLVCR1	1.487741	3.32375632
PPP4R4	1.492258	4.85318878
CEBPG	1.492737	4.85318878
FBXO45	1.492886	3.32375632
HTATSF1	1.497488	1.44744227
BAG5	1.498676	4.85318878
PARP11	1.499510	2.77992522
HOXB7	1.501456	1.44744227
LTA4H	1.505134	4.85318878
CPNE8	1.507230	1.70446473
UBAP2	1.510377	2.77992522
PCYOX1	1.512493	2.77992522
MALT1	1.512630	2.77992522
COX7B2	1.516859	1.70446473
SACM1L	1.517603	3.32375632
CWC22	1.520862	4.85318878
ASAH1	1.522477	4.85318878
DPYSL3	1.523114	3.32375632
MPZL3	1.524607	4.85318878
PLCG2	1.524904	4.85318878
GALNT14	1.527827	1.70446473
FASTKD2	1.528575	2.77992522
PRMT6	1.529218	1.44744227
CMTM4	1.531207	4.85318878
ZBTB5	1.531590	4.85318878
GATC	1.531804	4.85318878
NAPG	1.532961	4.85318878

Gene symbol	Fold Change	q-value(%)
CDC40	1.533147	4.85318878
COG2	1.535851	1.70446473
OTUD6B	1.536710	1.70446473
MAP7D3	1.540400	1.70446473
DNAJA1	1.541948	3.32375632
ALG13	1.545800	2.77992522
UEVLD	1.547067	3.32375632
WDR89	1.548756	2.77992522
GTF3C6	1.551386	2.77992522
PUM3	1.551649	0
ZNF114	1.551905	4.85318878
TMEM206	1.551967	4.85318878
CLGN	1.554260	4.85318878
GRAMD1C	1.554616	1.70446473
ZNF583	1.555509	0
STX18	1.558606	0.85322913
APOBEC3G	1.559079	1.31799621
WIPF1	1.560669	0
IDH3A	1.561315	2.77992522
VPS4B	1.561526	4.85318878
ARID3B	1.561884	4.85318878
PUS3	1.562489	2.77992522
ZNF426	1.562824	2.77992522
MAX	1.568543	4.85318878
SLC16A9	1.570396	4.85318878
ESF1	1.576498	4.85318878
HENMT1	1.578783	4.85318878
MTMR6	1.578921	1.31799621
P2RY6	1.580615	3.32375632
ATF1	1.586700	2.77992522
SH3KBP1	1.587065	2.77992522
BACE2	1.589108	1.31799621
UBLCP1	1.593561	1.44744227
ZBTB11	1.593661	1.70446473
PDCD2L	1.597905	2.77992522
FGD6	1.598145	2.77992522
SLC25A43	1.604334	2.77992522
HIF1A	1.605081	1.31799621
KIR3DX1	1.606449	2.77992522
GCLM	1.610620	3.32375632

Gene symbol	Fold Change	q-value(%)
STK39	1.613289	4.85318878
PNO1	1.613745	2.77992522
MRPL18	1.615758	4.85318878
ABHD17C	1.616206	2.77992522
TCEAL8	1.616641	3.32375632
ARHGEF3	1.620644	1.70446473
IMP3	1.621737	4.85318878
CAB39	1.627646	4.85318878
COQ10B	1.639849	4.85318878
ZBTB6	1.642607	3.32375632
LPGAT1	1.647117	1.44744227
GADD45B	1.649431	2.77992522
SIN3A	1.656269	2.77992522
PDGFRL	1.657281	0
IL2RB	1.658114	2.77992522
UTP14A	1.658353	1.44744227
GABRB3	1.665369	1.70446473
NECTIN2	1.665517	3.32375632
GCLC	1.667282	4.85318878
SLC35E4	1.670720	3.32375632
SHQ1	1.674159	1.44744227
RRN3	1.676386	1.70446473
RPF1	1.678824	2.77992522
PKM	1.682425	1.70446473
CHI3L1	1.690488	1.44744227
SNUPN	1.693662	2.77992522
RANBP6	1.695129	1.44744227
SMPDL3B	1.700024	0
PSMB8	1.700852	0
BAG2	1.703798	3.32375632
PANX1	1.705691	1.31799621
TKTL1	1.706921	1.44744227
RAB18	1.707424	2.77992522
CHMP4C	1.711238	2.77992522
FAH	1.711781	1.70446473
ERBIN	1.714126	4.85318878
PKIG	1.720512	4.85318878
CYYR1	1.721669	1.70446473
BTBD1	1.724980	1.44744227
NR1D1	1.726165	4.85318878

Gene symbol	Fold Change	q-value(%)
CCL5	1.733686	0
GTPBP8	1.739186	4.85318878
EPDR1	1.747603	1.44744227
PARP14	1.752459	4.85318878
LYRM2	1.752853	0
STAT4	1.755104	2.77992522
MGAT5	1.757221	0.85322913
KCNN4	1.757570	1.31799621
FNBP1L	1.760946	1.44744227
WDR61	1.761264	1.31799621
ZNF227	1.764329	3.32375632
PAGE2	1.766892	1.31799621
HMG20A	1.771805	0.85322913
NT5DC1	1.772178	3.32375632
FANCF	1.772365	2.77992522
LURAP1L	1.774322	1.70446473
LMNA	1.776359	1.31799621
MBOAT2	1.777567	4.85318878
QTRT2	1.784733	2.77992522
CRTC3	1.789398	2.77992522
DTWD2	1.795973	1.44744227
NKIRAS1	1.800679	4.85318878
GRK3	1.816745	1.31799621
MYO1B	1.817493	1.31799621
ZNF486	1.823615	3.32375632
NMI	1.827420	2.77992522
MYOM2	1.836042	4.85318878
PRSS2	1.836587	4.85318878
NECTIN1	1.841808	2.77992522
CHSY1	1.846819	4.85318878
HES1	1.849766	0
IMPA1	1.851267	4.85318878
EPB41L4B	1.863641	1.44744227
PPFIBP2	1.864639	3.32375632
OSTM1	1.866290	3.32375632
PYROXD1	1.866712	2.77992522
RAB8B	1.868664	1.70446473
DOCK2	1.870344	0
TNFRSF1B	1.874797	3.32375632
GCA	1.878922	4.85318878

Gene symbol	Fold Change	q-value(%)
EBAG9	1.895860	0.85322913
C15orf39	1.902382	1.31799621
PRSS16	1.931847	1.31799621
RPP40	1.941078	1.70446473
MAOA	1.941268	1.44744227
UNC93B1	1.954818	1.70446473
UBE2Q2	1.955464	0
GNAI1	1.959775	0
MDFIC	1.960520	4.85318878
FSTL4	1.962023	1.44744227
ITGB1BP1	1.966595	1.31799621
FIGNL1	1.968456	1.31799621
TGFBR2	1.968936	4.85318878
LRRC38	1.978491	1.44744227
NAMPT	1.986722	1.70446473
KAZN	2.003077	1.70446473
TARSL2	2.007099	0
TMEM200A	2.011444	2.77992522
BAIAP2L1	2.016256	2.77992522
PDPN	2.017459	0.85322913
ADAM23	2.025184	1.44744227
SH3RF2	2.040156	1.44744227
VANGL1	2.046818	2.77992522
COTL1	2.079072	0
S100A11	2.081469	2.77992522
MEOX2	2.103958	1.70446473
ICA1	2.111861	3.32375632
LIG4	2.114517	1.44744227
PCDHB3	2.119303	1.31799621
STARD13	2.128567	0
TTC22	2.139568	4.85318878
CD99	2.139774	0
ENO3	2.150548	3.32375632
STEAP2	2.161099	2.77992522
CLU	2.190935	1.44744227
GALNT1	2.212570	0
LIPH	2.234486	0
MAP3K21	2.242252	0
PALLD	2.273465	2.77992522
IFIH1	2.289796	0

Gene symbol	Fold Change	q-value(%)
TNFRSF8	2.290176	1.31799621
NDRG4	2.311942	3.32375632
MID1IP1	2.327363	1.44744227
MANEAL	2.337724	3.32375632
LONRF3	2.338034	0
SAMD9L	2.369011	1.44744227
GULP1	2.380583	0
NOVA1	2.398892	0
PABPC5	2.403610	1.31799621
SP110	2.404041	2.77992522
FARP1	2.441587	1.44744227
UBXN8	2.441921	0
ZNF516	2.447392	1.31799621
GLIPR1	2.455838	2.77992522
SMPDL3A	2.463085	1.70446473
BCL2	2.470961	1.70446473
TPBG	2.472780	0
SNX10	2.475058	1.70446473
OSBPL1A	2.486182	1.44744227
CTSH	2.491969	1.31799621
LHX8	2.499374	0
CLIC2	2.505934	1.31799621
PRKAA2	2.508837	1.70446473
FAM107B	2.549638	0
NIPAL2	2.554784	3.32375632
ARHGAP28	2.569977	1.70446473
IL21R	2.614542	1.31799621
GCSAML	2.634467	2.77992522
SEMA3E	2.676816	1.70446473
LINC01879	2.727626	4.85318878
CD163L1	2.876464	1.31799621
ISLR	2.985913	0
ZXDA	3.029142	0
TLR3	3.065938	0
SELL	3.073255	0
SNAI2	3.136715	1.31799621
FAM174A	3.167243	1.31799621
IL32	3.180425	0.85322913
CCDC3	3.198608	3.32375632
PPM1H	3.207874	1.31799621

Gene symbol	Fold Change	q-value(%)
PLAUR	3.210419	0.85322913
MCTP1	3.268773	0
MAGEB2	3.324233	0
CCR1	3.330562	1.70446473
IL5RA	3.330658	1.31799621
SLC27A2	3.556028	0
FBXO32	3.586862	1.44744227
BMP7	3.613460	0
SMOC2	3.622241	0
EMP1	3.642799	4.85318878
NES	3.949297	1.31799621
DTNA	3.997575	0
PRKN	4.221713	0
MAGEC1	4.373040	0
TOX	4.385743	0
PTGFR	4.393044	0
OLFML3	4.399154	0
FAT1	4.491326	0.85322913
ZDHHC15	4.510015	0
GBP1	4.555031	3.32375632
NFE2L3	4.574092	0
BASP1	4.925341	0
AZGP1	5.061123	1.44744227
COBLL1	5.173809	0
KIAA1024L	5.616566	1.70446473
LPCAT2	5.719166	0
CD9	7.049557	0
MAGEB1	7.801585	0
TPD52L1	9.858854	0
GJA1	12.490549	0
SPRR2A	18.248015	2.77992522
HHLA2	27.932568	1.70446473

Supplementary Table 2. List of the 86 deregulated genes in *immune system* biological process after the GSEA analysis. The genes are arranged alphabetically according to "gene symbol".

Gene Symbol	Gene ID
ACTR1A	10121
ASAH1	427
ASB16	92591
ATF1	466
ATP8A1	10396
BCL2	596
BTBD1	53339
CAB39	51719
CCL5	6352
CCR1	1230
CCR2	729230
CD81	975
CD99	4267
CHI3L1	1116
CLU	1191
COTL1	23406
CTSH	1512
CTSK	1513
DEGS1	8560
DOCK2	1794
FBXO32	114907
FRMPD3	84443
FYN	2534
GBP1	2633
GCA	25801
GLIPR1	11010
GSTP1	2950
HERC4	26091
HIF1A	3091

Gene Symbol	Gene ID
HLA-DPA1	3113
HSP90AA1	3320
HSPA9	3313
HUWE1	10075
IFIH1	64135
IL17RC	84818
IL21R	50615
IL27RA	9466
IL2RB	3560
IL32	9235
IL5RA	3568
JUP	3728
KIR3DL2	3812
LGMN	5641
LTA4H	4048
MALT1	10892
MAOA	4128
MAP3K3	4215
METTL7A	25840
MME	4311
NCAM1	4684
NECTIN2	5819
NKIRAS1	28512
OSBPL1A	114876
PAK1	5058
PANX1	24145
PKM	5315
PLAUR	5329
PLCG2	5336

Gene Symbol	Gene ID
POLR3A	11128
PRKN	5071
PRSS2	5645
PSMB8	5696
PSMB9	5698
PTPN13	5783
RAB18	22931
S100A11	6282
SEH1L	81929
SELL	6402
SFTPD	6441
SH3KBP1	30011
SLC27A2	11001
SMAD3	4088
SNAP29	9342
SOS1	6654
STAT4	6775
TIRAP	114609
TLR3	7098
TNFRSF1B	7133
TNFRSF8	943
TRIM2	23321
UBE2Q2	92912
UNC93B1	81622
VAV1	7409
VEGFA	7422
WIPF1	7456
YWHAB	7529

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Review

Immune System Alterations in Multiple Myeloma: Molecular Mechanisms and Therapeutic Strategies to Reverse Immunosuppression

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Simple Summary: A common characteristic of multiple myeloma (MM) is the dysfunction of patients' immune system, a condition termed immunosuppression. This state is mainly due to alterations in the number and functionality of the principal immune populations. In this setting, immunotherapy has acquired high relevance in the last years and the investigation of agents that boost the immune system represent a field of interest. In the present review, we will summarize the main cellular and molecular alterations observed in MM patients' immune system. Furthermore, we will describe the mechanisms of action of the four immunotherapeutic drugs approved so far for the treatment of MM, which are part of the group of monoclonal antibodies (mAbs). Finally, the immune-stimulating effects of several therapeutic agents are described due to their potential role in reversing immunosuppression and, therefore, in favoring the efficacy of immunotherapy drugs, such as mAbs, as part of future pharmacological combinations.

Abstract: Immunosuppression is a common feature of multiple myeloma (MM) patients and has been associated with disease evolution from its precursor stages. MM cells promote immunosuppressive effects due to both the secretion of soluble factors, which inhibit the function of immune effector cells, and the recruitment of immunosuppressive populations. Alterations in the expression of surface molecules are also responsible for immunosuppression. In this scenario, immunotherapy, as is the case of immunotherapeutic monoclonal antibodies (mAbs), aims to boost the immune system against tumor cells. In fact, mAbs exert part of their cytotoxic effects through different cellular and soluble immune components and, therefore, patients' immunosuppressive status could reduce their efficacy. Here, we will expose the alterations observed in symptomatic MM, as compared to its precursor stages and healthy subjects, in the main immune populations, especially the inhibition of effector cells and the activation of immunosuppressive populations. Additionally, we will revise the mechanisms responsible for all these alterations, including the interplay between MM cells and immune cells and the interactions among immune cells themselves. We will also summarize the main mechanisms of action of the four mAbs approved so far for the treatment of MM. Finally, we will discuss the potential immune-stimulating effects of non-immunotherapeutic drugs, which could enhance the efficacy of immunotherapeutic treatments.

Keywords: multiple myeloma; immune system; immunosuppression; monoclonal antibodies; immune stimulating drugs

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1. Introduction

Multiple myeloma (MM), the second most common hematological malignancy, is characterized by the accumulation of malignant plasma cells in the bone marrow (BM) leading to hypercalcemia, bone destruction, anemia and renal failure [1]. Although novel treatments have improved the outcome of MM patients, the disease remains incurable due to continuous relapses increasingly resistant to treatments [2]. Taking into account that MM is strongly influenced by the BM microenvironment [3], treatments should ideally have a dual role, not only on the myeloma cell but also on the microenvironment. In fact, immunomodulatory drugs (IMiDs) have been shown not only to directly attack the tumor, but also to stimulate the immune system [4] through different mechanisms that will be further reviewed.

Although the BM microenvironment is constituted by different components such as, immune cells (i.e., T cells, natural killer (NK) cells, dendritic cells, etc.), non-immune cells (i.e., bone marrow stromal cells (BMSCs), osteoblasts, osteoclasts, etc.), matrix proteins (i.e., fibronectin, laminin, etc.) and secreted soluble factors (i.e., cytokines, growth factors, etc.), the present review will be focused on the major alterations described for immune components in patients with MM. Specifically, we will review how different immune populations together with soluble factors are altered in the context of MM and its precursor stages, leading altogether to an immunosuppressive microenvironment. Finally, we will further address how immunotherapy and immune-stimulating drugs are able to revert this state.

2. General Alterations of the Immune System in the Context of Monoclonal Gammopathies

A general alteration of different immune populations and the cytokine profile has been described in patients with monoclonal gammopathies. It is broadly known that in the context of MM, both cell-to-cell contacts, in which myeloma cells, immune cells and other cells from the BM microenvironment are involved, and the presence of different extracellular factors lead to a general immunosuppressive status, which inhibits effector populations and recruits and activates immunosuppressive populations [5].

Hereunder, we will discuss the alterations described in the number and function of the major immune populations and the molecular factors involved in these variations. These aspects are schematized in Figure 1.

2.1. T Cells

T lymphocytes, both T helper $CD4^+$ and cytotoxic $CD8^+$ cells, are the components of the adaptive immune system that act as coordinators and effectors of immunity [6]. Both subsets play a crucial role in the antitumoral immunity. Regarding MM, the most frequently described alteration in patients is the decrease in $CD4^+$ T lymphocyte counts, which is associated with a lower progression free survival (PFS) and overall survival (OS) and a higher relapsing probability [5,7]. Preliminary data from our group show higher percentages of $CD4^+$ T cells in the BM of patients with newly diagnosed MM (NDMM) and high risk smoldering MM as compared to healthy subjects, but this was not found in patients with monoclonal gammopathy of undetermined significance (MGUS) [8]. Depending on the immunological context, $CD4^+$ T cells can acquire phenotypes with pro or anti-inflammatory functions, named Th1 and Th2, respectively, being the balance of these two cell types important for an efficient immune response [9]. In MM, both increase or decrease in the Th1/Th2 ratio have been observed, therefore making it difficult to elucidate the clinical implications of these findings [10–12]. Additionally, the production of TGF- β and IL-6 from MM cells and BMSCs induces the differentiation of naïve T cells to Th17 cells [13], an immunosuppressive $CD4^+$ T cell subset, which, as a positive feedback loop, secretes different cytokines promoting MM cell expansion [14].

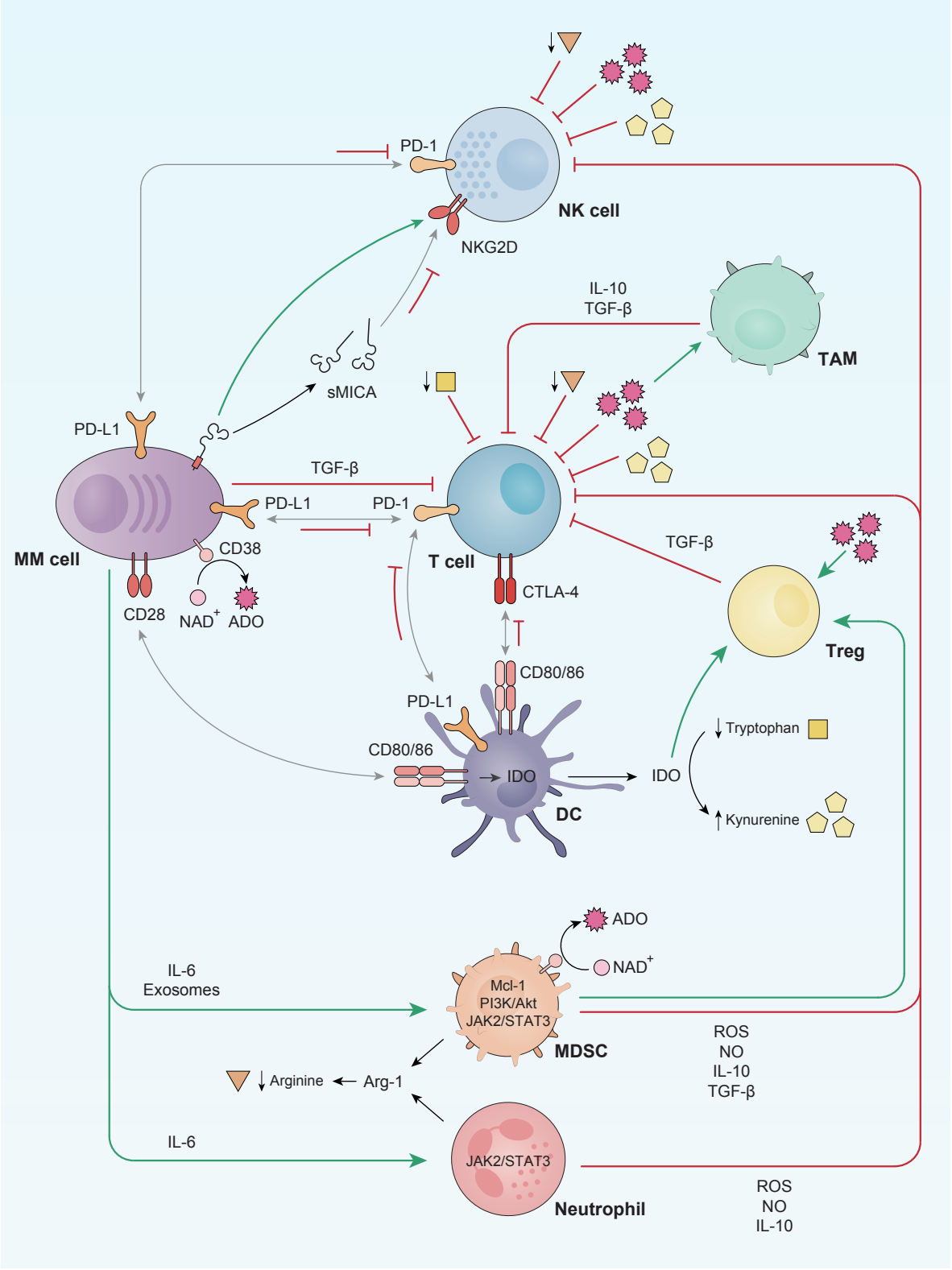

Figure 1. Schematic representation of the main immune system alterations described in multiple myeloma (MM) patients. Briefly, T and natural killer (NK) cells are inhibited through both soluble factors and cell-to-cell contacts with either myeloma cells or other immune cell populations. Grey arrows refer to receptor-ligand union, green arrows stand for activation whereas red bar-headed lines indicate inhibition. Regarding soluble factors, squares refer to tryptophan, pentagons to kynurenine, spiked circles to adenosine (ADO) and triangles to arginine. TAM: tumor associated macrophages; Treg: regulatory T lymphocytes; MDSC: myeloid derived suppressor cells; IDO: indoleamine-2,3-dioxygenase.

Regarding cytotoxic T cells, our group has reported an increase in the total number of T $CD8^+$ cells in both MGUS and symptomatic MM with respect to healthy donors [15]. More recently Zavidij et al. have found that during disease progression a depletion of memory $CD8^+$ T cell subset is observed, thus, being more abundant in healthy and MGUS individuals in comparison to smoldering myeloma (SMM) and active MM [16]. Additionally, an impaired response against viral antigens in MM patients has been observed [17], which may be associated to the increased expression of suppressor of cytokine signaling 1 (SOCS1) by T $CD8^+$ subset, which, in turn, inhibits IL-2, IL-6 and IFN-γ production in these same cells, attenuating Th1 and cytotoxic T lymphocytes (CTL)-mediated responses [18]. Soluble factors have also been observed to modulate the activity of cytotoxic T cells. In this sense, TGF-β inhibits IL-2-dependent proliferation and maturation of T cells and prevents naïve T cells from acquiring effector functions [19,20]. The activity of effector T lymphocytes is also inhibited by the immunosuppressive nucleoside adenosine (ADO), derived from ATP or NAD^+ after sequential catalytic reactions initiated by the surface molecule CD38 in MM cells [21–23].

The activation of T cells is initiated through antigen recognition by the T cell receptor (TCR), and then regulated by a balance between costimulatory and coinhibitory signals denominated immune checkpoints [24]. Although immune checkpoints are crucial for the maintenance of self-tolerance and homeostasis [24], the expression of immune checkpoint proteins can be dysregulated by tumors as a mechanism of immune evasion [25]. One of the most relevant immune checkpoints is the programmed-death (PD) pathway. PD-1 is an inhibitory receptor expressed by T cells, which interacts with its ligands PD-L1/PD-L2 expressed by antigen presenting cells (APCs) to inhibit T cell effector functions [26–28]. It is known that plasma cells from healthy subjects do not express PD-1 ligands, however, PD-L1/PD-L2 can be found in plasma cells from myeloma patients [29–32] and in MM cell lines [33]. In addition, our group reported that PD-L1 is also expressed by BMSCs [30]. Moreover, the expression of PD-1 seems to be increased on T cells from MM patients, compared to healthy subjects [34,35], particularly in the setting of relapsed/refractory disease [30,35]. The soluble form of PD-L1, which is released from tumor cells' surface is also thought to exert immunosuppressive activity [36]. Indeed, high serum soluble PD-L1 levels are associated with poor prognosis in MM patients [37,38] and PD-$L1^+$ MM cells show greater drug resistance [39] and higher levels of antiapoptotic proteins [31]. Considering all these data, three main mAbs targeting PD-1 (nivolumab, pembrolizumab and pidilizumab) have been evaluated in MM. Although preclinical murine models showed that PD-1 blockade inhibited tumor growth, both in monotherapy [30,35,40] and in combination [34], data from clinical trials indicate no benefit when used in monotherapy. In fact, our group tested the use of pembrolizumab as consolidation in patients achieving at least a very good partial response (VGPR) but with persistent measurable disease after a first or second line treatment; nevertheless, no upgrades in the quality of the baseline responses could be documented [41]. The combination of pembrolizumab with either pomalidomide or lenalidomide was tested in phase II trials with promising results [42,43]. However, phase III trials of pembrolizumab in combination with the same agents (KEYNOTE-183 (NCT02576977) and KEYNOTE-185 (NCT02579863)) had to be prematurely stopped due to a survival imbalance disfavoring patients receiving the mAb [44,45]. Together with PD-1/PD-L1 pathway, cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) is another immune checkpoint responsible of T cell suppression. The binding of CTLA-4 present in T cells to its ligands (CD80/CD86), expressed on APCs, transmits an inhibitory signal to T cells [46]. In the clinical setting, some studies have assessed the safety and efficacy of CTLA-4 inhibition as consolidation following ASCT in patients with MM. One of them administered ipilimumab and nivolumab between 14 and 28 days post-ASCT in patients with high-risk MM achieving at least stable disease after induction treatment. At 18 months post-ASCT, authors reported a PFS of 71% [47]. The definite niche of checkpoint inhibitors in the treatment of MM patients will be based on the results of clinical trials testing their optimal partners and times of administration.

2.2. NK Cells

Natural killer cells (NK cells) are cytotoxic lymphocytes from the innate immune system that take part in the early response to viral antigens and in attacking tumor cells, recognizing and eliminating cells that express stress proteins without needing antigen presentation on major histocompatibility complex (MHC-I) molecules [48]. In fact, activation of NK cells depends on a balance between activating natural cytotoxicity receptors (NCRs) and inhibitory receptors. In humans, activating receptors include NKp30, NKp46, NKp44, DNAM-1 and NKG2D, among others, while inhibitory receptors include killing inhibitory receptors (KIRs) and NKG2A, among others [18]. Briefly, NK cells recognize malignant cells and kill them through secretion of granzyme B and perforin or alternatively, through death signaling pathways in which FasL and TRAIL proteins are involved [49].

In the context of the disease, both MGUS and MM patients present an enrichment in NK cell population in comparison to healthy adults [15,16]. Initially, myeloma cells are sensitive to the lysis induced by NK cells since they express high levels of the stress-induced self-antigen MICA (the ligand of NKG2D receptor). In contrast, as the disease evolves, myeloma cells lose MICA expression and MICA shedding occurs, this latter phenomenon being directly correlated to disease progression [50,51]. In addition, some authors have reported that NKG2D expression was lowered in NK cells from MM patients [51–53], while others have not found any differential expression in comparison to healthy donors [54]. Regarding DNAM-1, its expression is reduced on NK cells from myeloma patients with active disease compared to patients in remission or healthy individuals [55]. Furthermore, unlike healthy donors, NK cells from myeloma patients express the PD-1 molecule, which mitigates their functionality even more [35]. It has been reported that NK cell functionality is inhibited by immunosuppressive cytokines found in the tumor milieu, such as ADO, which inhibits NK cell lytic activity [23], or TGF-β, which inhibits the differentiation of functional $CD16^+$ NK cells from its $CD16^-$ counterparts [56]. Although specific data confirming these findings in the context of MM have not been published, it is possible that similar effects occur since both ADO and TGF-β are increased in the MM microenvironment.

In addition to cytokines, extracellular vesicles (EVs) also have an impact on the behavior of NK cells. In fact, under some circumstances, such as under treatment with sublethal doses of melphalan or doxorubicin, myeloma cells have been found to produce exosomes capable of activating IFN-γ production by NK cells [57] or augmenting NK proliferation and activation [58], thus enhancing NK-cell immune surveillance. However, tumor-derived exosomes have been observed to contain TGF-β, MICA/B, ULBP3, PI-9 and miR-1245 and to contribute to impairment of NK function [59]. In fact, it has been reported that exosomes from myeloma cell lines contain TGF-β and ligands for NK activating receptors, and negatively regulate NK cytolytic ability against MM [60]. In line with this, our group has found that exosomes from BM plasma of myeloma patients similarly reduced the cytolytic activity of normal NKs on myeloma cells, thus contributing to myeloma immunosuppression (unpublished data from our group).

2.3. B Cells

B lymphocytes are the components of humoral immunity in the adaptive immune system, which act through antibody secretion [61]. B cell lineage is heavily compromised in MM, with a displaced equilibrium towards a high proliferation of malignant plasma cells. Indeed, MM patients present both a decrease in $CD19^+$ B cells [62,63], inversely correlated with disease stage [63], and a reduced ability to secrete polyclonal immunoglobulins and to differentiate into antibody-secreting plasma cells [62]. Moreover, the risk of progression to symptomatic MM from presymptomatic stages (MGUS and SMM) is directly related to the proportion of normal bone marrow plasma cells at diagnosis [64]. Furthermore, the number of B regulatory (Bregs) cells with $CD19^+CD24^{high}CD38^{high}$ phenotype, increases in the transition from MGUS to symptomatic MM [65], supporting the bone marrow milieu by both reducing NK-mediated lysis of MM cells and producing IL-10 [66].

2.4. Dendritic Cells (DCs)

Dendritic cells (DCs) are APCs whose main role is the processing of antigenic material, which is then displayed on their cell surface to induce naïve T cell activation. They are classified as plasmacytoid DCs (pDCs), which secrete high levels of type I IFN in response to viral antigens and other stimuli, and myeloid DCs (mDCs) rather involved in antigen presenting and inducing T $CD4^+$ and $CD8^+$ cell responses [67,68]. The role and general status of DCs in MM is not clear yet. Many studies concluded that DCs from MM patients have impaired T-cell stimulation capacities, whereas contradictory results exist regarding the frequency and phenotype of DCs [69–72]. Our group demonstrated that the number of BM DCs differed significantly between MM patients with long-term disease control and those with symptomatic disease, with a trend to cell count recovering in the former cohort towards levels similar to those found in healthy adults [15].

DCs are concentrated in the BM during MGUS to MM progression and are able to process and cross-present antigens from apoptotic MM cells via CD91, thus activating myeloma-specific $CD8^+$ T cells [73]. Besides, by using their surface CD80/86 molecules, DCs interact with nonapoptotic plasma cells via the overexpressed CD28 receptor, provoking the production of the immunosuppressive enzyme indoleamine-2,3-dioxygenase (IDO) [74], which impairs the immune surveillance through different mechanisms: (i) metabolizing and, therefore depleting, tryptophan from the microenvironment, which is an essential amino acid for T cells, and consequently, producing kynurenine, a toxic compound for T and NK cells [75]; (ii) promoting the development, stabilization and activation of Tregs [76] and (iii) polarizing macrophages and DCs towards an immunotolerogenic phenotype [77]. Along with IDO, ADO is also able to increase the number of tolerogenic DCs [23]. Moreover, pDCs and a percentage of mDCs also express high surface levels of PD-L1 [78], participating in the maintenance of the immunosuppressive bone marrow microenvironment. In addition, TGF-β contributes to the altered immune tumor niche since it inhibits the upregulation of critical T-cell costimulatory molecules on the surface of DCs, reducing their antigen-presenting capacity [79].

2.5. Tumor Associated Macrophages (TAMs)

Tumor associated macrophages (TAMs) constitute an abundant component of the myeloma microenvironment that enhances myeloma cell survival and drug resistance through different mechanisms [80]. Within the BM niche, TAMs acquire a secretory profile characterized by a great production of IL-6, IL-10 and proangiogenic factors, such as vascular endothelial growth factor (VEGF), metalloproteinases (MMPs) and cyclooxygenase-2 (COX-2) [81], providing an optimal milieu for myeloma cell growth. Moreover, TAMs resemble a M2-like macrophage population, with little cytotoxicity against tumor cells because of their limited production of nitric oxide (NO) and proinflammatory cytokines, and a poor antigen-presenting capability [82]. Additionally, ADO further polarizes macrophages towards a M2 phenotype [23].

There are several reports describing an association between macrophage infiltration, vascularity and disease prognosis. Suyani et al. showed increased numbers of M2 macrophages in the BM of 68 MM patients, which was associated with unfavorable prognosis and increased microvessel density [83]. Two different studies also reported a negative correlation between CD163 and CD206 expression, which are M2–macrophage markers, and OS in patients with MM [84,85]. Further studies in MM patients confirmed that TAM infiltration in the BM was associated with poor prognosis and drug resistance [86].

There has been shown that the number of M2 macrophages was significantly increased in the BM of MM patients compared with MGUS and SMM, and with healthy donors, suggesting that the malignant plasma cell may be involved in this change to a M2-like phenotype [87]. A very recent single-cell RNA sequencing study revealed that mature $CD14^+$ monocytes lose the surface expression of HLA-II molecules as early as in the MGUS stage, resulting in T cell suppression, and suggesting that some of these sequential immune alterations begin on an early stage of the disease [16].

2.6. Myeloid-Derived Suppressor Cells (MDSCs) and Neutrophils

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous group of immature myeloid cells endowed with the capacity to suppress the activation, proliferation and cytotoxic capacity of effector T and NK cells. In humans there are two subsets of MDSCs; granulocytic-MDSCs (G-MDSCs) (also called polymorphonuclear, PMN-MDSCs) and monocytic-MDSCs (M-MDSCs) [88]. In myeloma the G-MDSC constitutes the predominant MDSC population in BM and peripheral blood (PB) as opposed to M-MDSC [89]. MDSCs mainly suppress T cell responses by producing reactive oxygen species (ROS) and high amounts of NO, arginase-1 (Arg-1), and immunosuppressive cytokines such as IL-10. There is an increase of G-MDSCs in both PB [90,91] and BM [89,92] of patients with active MM, compared with samples from MGUS or healthy donors. Moreover, it has also been shown that MDSCs from MM patients were able to induce higher Treg differentiation than those from healthy age-matched donors [89]. In vitro data indicate that MDSCs support MM progression by inhibiting effector cells, enhancing Treg development [93], and even by differentiating into osteoclasts [94]. In addition, MM cells promote the survival of MDSCs through Mcl-1 upregulation [95], secretion of IL-6, which drives MDSCs expansion by the activation of several molecular cascades such as PI3K/Akt or JAK/STAT3 [96,97], and through the accumulation of high levels of ADO by the conversion of NAD^+ [23,98]. Together with cytokines, Wang et al. showed that both BMSC-derived and myeloma-derived exosomes promoted the proliferation and survival of MDSCs [99]; upon incorporation of exosomes, MDSCs also increased their NO production, thus contributing to T cell inhibition [100].

Neutrophils are the most abundant white blood cell in PB, and are essential for clearance of extracellular pathogens, both by direct toxicity and by establishing interactions with other immune cells [101]. In the context of MM, neutrophils present functional defects, such as a reduction in lysozyme activity and an increased secretion of Arg-1 therefore presenting an immunosuppressive behavior [102,103]. In fact, MM patients have increased serum levels of Arg-1 [102], which depletes arginine on tumor microenvironment, an essential amino acid for T and NK cell proliferation [104]. Furthermore, as disease evolves from MGUS to MM, neutrophils progressively activate the JAK-2/STAT3 pathway in response to MM cell exposure, which further supports the immunotolerogenic niche due to the production of proinflammatory and survival signals [105]. Interestingly, the neutrophil to lymphocyte ratio (NLR) at diagnosis is able to predict both the outcome in NDMM patients treated with novel agents [106], and the prognosis in patients at day +100 post stem cell autologous transplant [107].

2.7. Regulatory T Lymphocytes (Tregs)

Regulatory T lymphocytes (Tregs) are a subpopulation of T cells that modulate the immune system, maintain tolerance to autoantigens and prevent autoimmune reactions. They present a $CD4^+CD25^+$ phenotype and an increase in FOXP3 factor, which is determinant for the development of this subpopulation [108]. They exert their immunosuppressive activity through cell-to-cell contact, secreting immunosuppressive cytokines such as TGF-β and IL-10 [109] or inducing the expression of IDO in DCs, which induces a positive loop since IDO promotes the expansion of Tregs [110]. In addition to IDO, ADO also promotes the expansion of Tregs [23]. Furthermore, in BM samples from NDMM patients, CTLA-4 appears to be overexpressed (along with FOXP3) in Tregs, which suggests a local accumulation of Tregs in the tumor microenvironment [111]. In fact, it has been described that CTLA-4 induces Treg expansion and induction of immunosuppressive cytokines in this population [46].

Most authors have reported that MM patients have a higher Treg percentage [16,112–115] in comparison to healthy donors, suggesting that myeloma cells escape from the immune system at least partially through the increase of this population. Indeed, recent work suggests that this immune scape occurs early in disease development, since it has already been described in patients with SMM [16]. Interestingly, our group reported that

the number of Tregs was lower in patients with long-term disease control than in those with symptomatic MM [15]. In addition, Treg number can be used as a biomarker of disease progression, since patients with higher Treg percentage presented a lower OS [112,116].

3. Currently Approved Immunotherapeutic Treatments in MM

Monoclonal antibodies (mAbs) have emerged as a backbone therapy for many B-cell tumors, due to their high efficacy and good tolerability. However, the development of effective mAbs for the treatment of MM has been tough, since the discovery of target molecules unique for all MM cells resulted challenging. Up to date, there are three naked mAbs and one antibody–drug conjugate (ADC) approved for the treatment of MM. Their main mechanisms of action can be found in Figure 2.

3.1. Elotuzumab

Elotuzumab (anti-SLAMF7) was the first mAb approved by the US Food and Drug Administration (FDA) for the treatment of MM. In particular, elotuzumab was first approved in combination with lenalidomide and dexamethasone for relapsed/refractory myeloma patients who had received one to three prior therapies [117]. SLAMF7, also known as CS1, is a cell surface molecule expressed in plasma cells, $CD8^+$ cytotoxic T lymphocytes, activated B cells, NK cells and mature DCs [118–121]. In the context of MM, SLAMF7 is expressed in both primary malignant plasma cells and in almost all MM cell lines. In addition, soluble SLAMF7 has been detected in serum of MM patients presenting a direct correlation with disease stage [118,121].

Elotuzumab is a humanized IgG1 mAb that inhibits MM cell adhesion to BMSCs, which may reverse the protective effect provided by the bone marrow microenvironment to myeloma cells. Additionally, elotuzumab is able to induce antibody dependent cellular cytotoxicity (ADCC) mediated by NK cells in both MM cell lines and primary plasma cells from myeloma patients (either newly diagnosed or resistant to conventional therapies) [121].

Since NK cells and a small subset of activated lymphocytes express SLAMF7, elotuzumab is able to activate ex vivo different subsets of peripheral blood mononuclear cells (PBMCs) from myeloma patients and healthy donors. Indeed, elotuzumab selectively activated the subpopulation of $CD56^{dim}$ NK cells, upregulating CD69, CD11b and CD54 and downregulating CD16 expression and resulting in the killing of myeloma cells via a CD16-independent mechanism [122]. Moreover, elotuzumab also activated monocytes as evidenced by the up-regulation of SLAMF7, HLA-DR and CD54 [123].

3.2. Daratumumab

Daratumumab is an anti-CD38 mAb that was approved in 2015 by the FDA for MM patients who had received at least three prior lines of therapy or for patients double refractory to proteasome inhibitors and immunomodulatory agents [124,125]. Besides, it has been recently approved for NDMM patients ineligible for stem-cell transplantation [126]. CD38 is expressed in different cell subsets from hematopoietic and non-hematopoietic lineages. Regarding the first, CD38 is expressed in Tregs, circulating monocytes, $CD4^+$ and $CD8^+$ T cells, NK cells, granulocytes/neutrophils, B cell precursors and in terminally differentiated plasma cells from healthy donors [127–129]. In the context of MM, $CD138^+$ malignant plasma cells express higher levels of CD38 than other immune subsets and normal plasma cells [130]. Moreover, CD38 is also expressed by osteoclasts in the tumor niche [131].

Daratumumab was first selected from a panel of 42 human anti-CD38 mAbs for being effective in killing MM cells via complement dependent cytotoxicity (CDC) and ADCC [132]. Further studies in vitro, ex vivo and in vivo demonstrated that daratumumab was also able to induce programmed cell death in the presence of crosslinking agents (both $F(ab)_2$ fragments and Fcγ receptor-expressing cells) [133], and antibody dependent cellular phagocytosis (ADCP) [134].

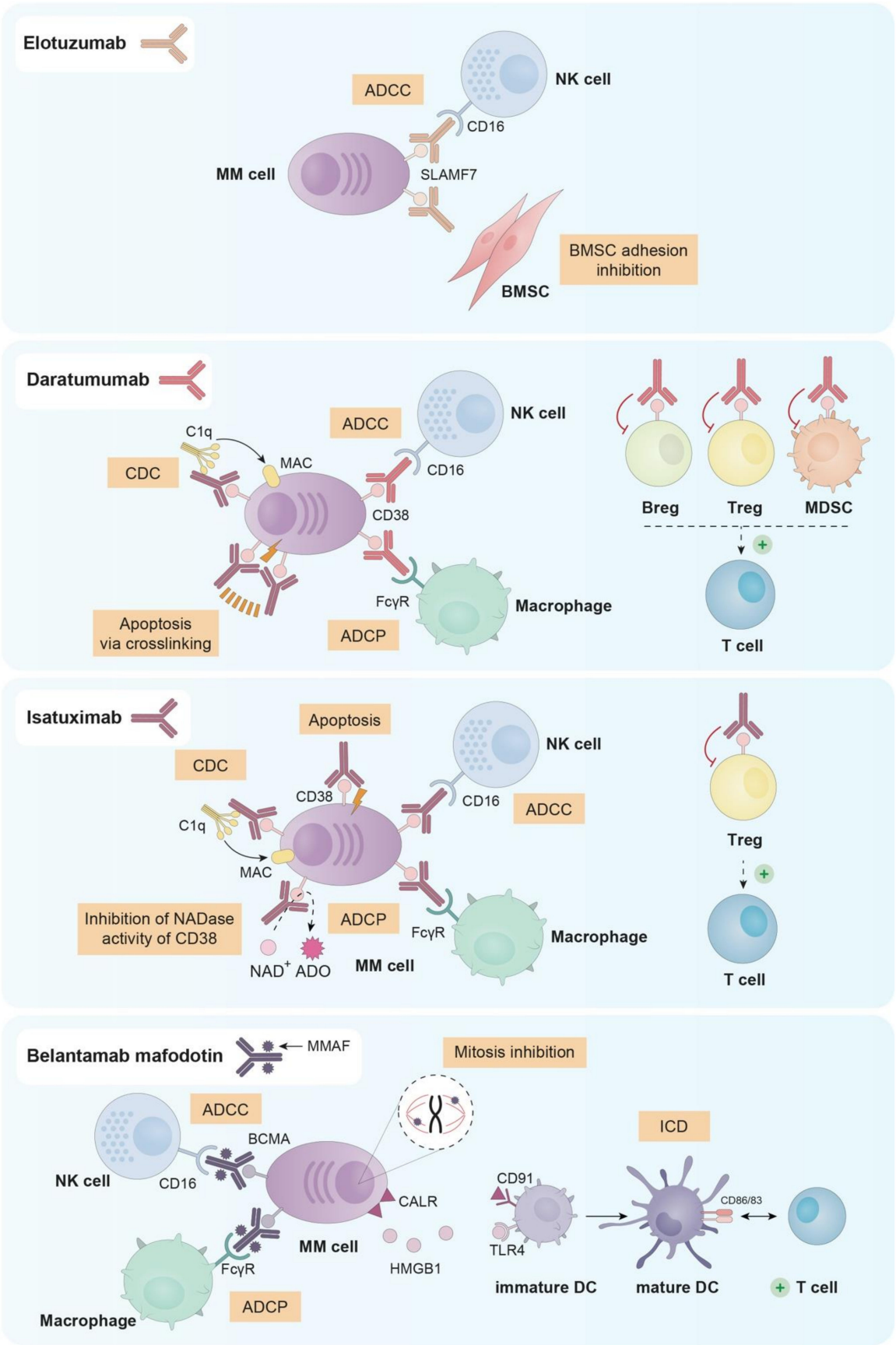

Figure 2. Principal mechanisms of action of the three naked mAbs (elotuzumab, daratumumab and isatuximab) and the ADC (belantamab mafodotin) approved for the treatment of MM.

Given that different immune cell subsets express CD38, daratumumab treatment has an impact on them. In fact, it has been described that MM patients treated with daratumumab both in monotherapy and in combination with lenalidomide and dexamethasone, present a decrease in absolute cell count in NK cells (from 10% to 2%), MDSCs, Bregs and Tregs. On the contrary, other immune populations, such as $CD4^+$ and $CD8^+$ T cells showed increased numbers [135–138]. Despite the decrease in NK cell number observed after daratumumab treatment, according to Casneuf et al. the remaining NK cells seemed to be able to contribute to the clinical efficacy of the drug [137]. Furthermore, daratumumab has been reported to induce NK cell activation and degranulation as observed by the upregulation of CD69, CD107a and IFN-γ in this cell subset [139].

3.3. Isatuximab

Isatuximab is a humanized IgG1 anti-CD38 mAb that has been recently approved (March 2020) in combination with pomalidomide and dexamethasone for MM patients who had previously received at least two lines of therapy [140]. Isatuximab exerts its antimyeloma effect through different mechanisms. First, and unlike daratumumab, isatuximab has shown proapoptotic activity against myeloma cells expressing high levels of CD38 without any cross-linking agents [141,142]. Moreover, isatuximab also presents immune-mediated cytotoxic effects, such as, the induction of strong CDC, potent ADCC and ADCP [141,143]. In contrast to daratumumab, isatuximab completely inhibits the NADase activity of CD38, which may mitigate the immunosuppressive microenvironment in the bone marrow of MM patients [144–146].

As observed with daratumumab, isatuximab is able to suppress Tregs. In fact, in PBMCs from both healthy donors and MM patients treated with isatuximab in vitro, the percentage of Tregs was reduced while the percentage of effector T cells increased. This reduction of Treg frequency was more significant in cells from MM patients than from healthy donors, probably due to the higher expression of CD38 observed in patients' Tregs [127]. Furthermore, isatuximab upregulated the activation molecules CD107a and IFNγ in monocytes, $CD8^+$ T cells and NK cells not only from healthy donors but also from myeloma patients, augmenting the cytotoxic functions of these three cell subsets both in the presence and in the absence of $CD38^+$ target cells [127,129]. Additionally, Moreno et al. observed that isatuximab depleted in vitro $CD38^{high}$ B-lymphocyte precursors, basophils and NK cells [143]. In fact, the NK cell depletion observed after isatuximab treatment seems to be generated through activation followed by exhaustion of these cells [143].

3.4. Belantamab Mafodotin

Belantamab mafodotin (GSK2857916) is an afucosylated, humanized IgG1 anti-B-cell maturation antigen (BCMA) mAb conjugated with monomethyl auristatin F (MMAF), which is a tubulin polymerization inhibitor [147]. Both parts (anti-BCMA antibody and MMAF toxin) are linked through a non-cleavable maleimidocaproyl linker, which provides better plasma stability of the compound without losing any property and without any nonspecific toxicity [148]. Belantamab mafodotin is the first anti-BCMA ADC approved by the FDA as a single agent for relapsed/refractory multiple myeloma (RRMM) patients who have received at least four prior therapies [149]. BCMA, also known as TNFRSF-17, is selectively induced during plasma cell differentiation being almost absent on naïve and memory B cells [150,151]. BCMA is expressed by several myeloma cell lines [152] and BCMA mRNA is commonly expressed at high levels in primary malignant plasma cells [153].

Belantamab mafodotin exerts its antimyeloma effect through four known mechanisms: (i) ADCC mediated by NK cells; (ii) recruitment of macrophages to promote ADCP; (iii) disruption of microtubules and subsequent G_2/M cell-cycle arrest followed by apoptosis after the release of the MMAF toxin in the cytoplasm of myeloma cells [147] and (iv) induction of immunogenic cell death (ICD) [148], which is a mechanism characterized by the ability of dying cells to elicit robust adaptive immune responses against altered

self-antigens or cancer-derived neo-epitopes [154]. In relation to the latter mechanism, preliminary data indicates that treatment of myeloma cells with belantamab mafodotin promotes the exposure of calreticulin (CALR) on their surface and the release of HMGB1, which subsequently induce the maturation and activation of DCs and eventually the activation of T cells [148].

4. Drugs with Immune-Stimulating Activity in Multiple Myeloma

As explained before, some of the mechanisms of action of mAbs require the presence of different immune effector cell subsets. Therefore, agents with immune-stimulating effects could be good partners of mAbs. Next, we will explain the effects on the immune system of different drugs that are currently combined with mAbs in the clinic and some others, which could be postulated as appropriate candidates in new combinations with mAbs. A summary of these effects can be found in Table 1.

Table 1. Summary of the main immunomodulating effects of immunomodulatory drugs (IMiDs), proteasome inhibitors, histone deacetylase inhibitors, cyclophosphamide, arginase inhibitors and IDO inhibitors.

Drug Group	Drug Name	Target Cell Population	Immune Effects/Molecular Mechanisms	Reference
Immunomodulatory drugs (IMiDs)	Thalidomide	T cells	↑ cytotoxic responses	[155]
			↑ proliferative responses ↑ IL-2 and IFN-γ	[155,156]
		NK cells	↑ cytotoxic activity	[156]
	Lenalidomide	T cells	↑ IL-2 production	[157,158]
			↑ AP-1 transcriptional activity	[157]
			↑ activation; ↓ Ikaros and Aiolos	[159]
		NK cells	↑ cytotoxic activity	[158]
		PBMCs	↑ cytotoxic activity	[159,160]
			↑ ADCC	[158]
		MM cells	↑ MICA and PVR expression	[161]
			↓ PD-L1 expression	[35,162]
			No change in PD-L1 expression	[163]
		DCs	↑ endocytic activity ↑ MHC Class I and CD86 expression	[164]
		Treg	↓ proliferation	[165]
			↓ differentiation	[166]
	Pomalidomide	T cells	↑ IL-2 production	[157,158]
			↑ AP-1 transcriptional activity	[157]
			Nuclear translocation of AP-1 and NFAT2	[158]
		NK cells	↑ granzyme-B expression	[167]
			↑ cytotoxic activity	[158,167]
			↑ Zap-70 phosphorylation	[167]
		PBMCs	↑ ADCC	[158]
		MM cells	↑ MICA and PVR expression	[161]
			↑ CD38 expression	[168]
		DCs	↑ endocytic activity ↑ MHC Class I and CD86 expression	[164]
		Treg	↓ proliferation	[165]

Table 1. *Cont.*

Drug Group	Drug Name	Target Cell Population	Immune Effects/Molecular Mechanisms	Reference
Proteasome inhibitors(PIs)	Bortezomib	Tumor cells	↑ Hsp60 and Hsp90 exposure (↑ ICD induction)	[169,170]
		MM cells	↑ CALR exposure (↑ ICD induction)	[171]
	Carfilzomib	MM cells	↑ CALR exposure (↑ ICD induction)	[171]
Histone deacetylase inhibitors (HDACi)	MGCD0103 (mocetinostat)	MM cells	↑ susceptibility to lysis by MAGE-A3-specific CTLs	[172]
	Valproic acid (VPA)	MM cells	↑ MICA/B and ULBP2 expression	[173]
	Sodium butyrate	MM cells	↑ MICA expression	[174]
	Panobinostat	MM cells	↑ ULPBP2/5/6 and MICA/B expression	[163]
			↑ PD-L1 expression	[163]
			↑ CD38 expression	[175]
		DCs	Impairment of DCs function to stimulate antigen-specific immune responses	[176]
	ACY-241	DCs	↓ PD-L1 expression (pDCs) ↑ CD80, CD86 and MHC molecules (Class I and II) expression	[177,178]
		MM cells	↓ PD-L1 expression ↑ CD80, CD86 and MHC molecules (Class I and II) expression	[178]
			↑ CD38 expression	[179]
		Treg	↓ PD-L1 expression	[178]
		T cells	↓ PD-1 expression	[178]
	Entinostat	MM cells	↑ PD-L1 expression	[163]
			↑ CD38 expression	[180]
	Ricolinostat	MM cells	↑ PD-L1 expression	[163]
			↑ CD38 expression	[179]
	WT-161	MM cells	↑ CD38 expression	[179]
Alkylating agents	Cyclophosphamide	Tumor cells	↑ CALR translocation	[181,182]
			↑ release of HMGB1	[181]
		Tregs	Depletion of this population	[182,183]
		T cells	Promotes Th1 polarization	[183]
		NK cells	↑ activation	[182]
		Myeloid cells	Modulation of this population	[182]
		MM cells	↑ secretory response, ↓ CD47	[184]
		Macrophages	↑ CD64 expression, ↑ ADCP	[184]
Arginase inhibitors	nor-NOHA	High-density neutrophils	Reversion of the immune-suppressive properties of this population	[103]
	CB-1158	High-density neutrophils	Reversion of the immune-suppressive properties of this population	[103]
	BEC	M2-type macrophages	Reversion of the anti-myeloma effect of M2-type macrophages in the context of Th2 adoptive cell therapy	[185]
		MDSCs	Reversion of the inhibitory effect of conditioned media from MDSCs on the anti-myeloma efficacy of bortezomib	[102]

Table 1. *Cont.*

Drug Group	Drug Name	Target Cell Population	Immune Effects/Molecular Mechanisms	Reference
IDO inhibitors	INCB014943	DCs	Reversion of effector T cell suppression induced by DCs	[74]
	1-methyl-DL-Trp	IDO$^+$ MM cells	Reversion of Treg expansion induced by IDO	[186]
		Osteoclasts	Reversion of the suppression of T-cell proliferation and CTLs activity induced by osteoclasts	[187]
		Macrophages	Reversion of the inhibition of CD4$^+$ T cell proliferation and cytokine production induced by macrophages	[188]

4.1. Immunomodulatory Drugs (IMiDs)

IMiDs are a class of immunomodulatory drugs with pleiotropic effects on myeloma cells and other immune cells, for which antiangiogenic, cytotoxic and immunomodulatory activities have been reported [4]. Currently, there are three types of IMiDs approved for the treatment of MM namely, thalidomide and its analogues, lenalidomide and pomalidomide and their immunomodulatory properties have been widely described.

Different authors have reported that T cells increase their cytokine production after IMiD exposure. Indeed, Haslett et al. observed the enhancement of cytotoxic and proliferative responses by T cells, mainly by the CD8$^+$ subset, and the increase in IL-2 and IFN-γ production promoted by thalidomide [155]. Conversely, Schafer et al. showed that lenalidomide and to a greater extent pomalidomide, increased IL-2 production by both CD4$^+$ and CD8$^+$ T cells, with a slightly more potent effect on the CD4$^+$ subpopulation [157]. In the same work, pomalidomide and lenalidomide enhanced AP-1 transcriptional activity in stimulated T cells, finding that the proximal AP-1 binding site of the IL-2 promoter is involved in the IMiD response [157]. Besides this, pomalidomide promoted the nuclear translocation of NFAT2 via PI3K and its subsequent binding to the IL-2 promoter, further enhancing IL-2 transcription [158]. Franssen et al. provided additional mechanisms for their activity on immune populations, specifically the decrease in the cereblon substrate proteins Ikaros and Aiolos in CD4$^+$ T cells, CD8$^+$ T cells, NK cells and B-cells. This explained the increase in activated T cells in lenalidomide-refractory patients after treatment with lenalidomide combined with low-dose cyclophosphamide and prednisone [159]. Moreover, these authors also found that pretreatment of PBMCs with lenalidomide enhanced PBMC-mediated killing of both lenalidomide-sensitive and lenalidomide-resistant myeloma cell lines [159].

IMiDs have also been shown to increase NK cell cytotoxic activity [156,158,160,167]. Firstly, this effect was suggested to occur indirectly via induction of IL-2 production in T cells [156,158,160]. However, Hideshima et al. have recently described the phosphorylation and activation of Zap-70 and the involvement of cereblon as two direct and independent mechanisms of pomalidomide-mediated upregulation of granzyme-B expression and NK cell activity [167]. Additionally, other authors observed that IMiDs may enhance the susceptibility of myeloma cells to NK cell-mediated recognition and killing by increasing the expression of MICA and PVR (the ligand for DNAM-1 receptor) in myeloma cells [161]. Specifically, these authors observed that IMiDs increase mRNA expression and promoter activity of MICA and PVR, and suggested that IMiDs may shift both Ikaros and Aiolos depletion and IRF4 downregulation into increased MICA expression, indicating that all these transcription factors repress MICA gene expression [161].

Activation of T and NK cells by IMiDs has also been explained by indirect mechanisms. In this sense, lenalidomide has been reported to reduce PD-L1 expression on RPMI-8226 and primary MM cells, and the combination of lenalidomide and pidilizumab (anti-PD-1) significantly enhanced NK-cell cytotoxicity against myeloma cells [35]. Fujiwara et al.

found that pomalidomide treatment promoted tumor killing by CTLs through inhibition of IFN-γ-induced PD-L1 expression in different tumor cells, including myeloma cells [162]. Contrary to these findings, other authors did not observe any reduction of PD-L1 expression after lenalidomide treatment in different MM cell lines [163].

IMiDs have also been proposed to increase anti-tumor immunity by enhancing DC function [164]. Both pomalidomide and lenalidomide increase the endocytic activity of DCs and the expression of MHC Class I and CD86 in these cells [164]. Moreover, when DCs were treated with pomalidomide and lenalidomide prior to antigen presentation assay, both IMiDs effectively increased $CD8^+$ T-cell cross-priming, but only pomalidomide was effective in increasing $CD4^+$ T-cell priming [164].

Furthermore, lenalidomide and pomalidomide strongly inhibited Treg proliferation and had an inhibitory effect on the suppressor function of these cells, which was associated with decreased FOXP3 expression [165]. Other authors observed that lenalidomide reduced Treg differentiation by myeloma cells due to ICOSL downregulation, whereas dexamethasone induced apoptosis of Tregs, therefore identifying for the first time an immune synergism that explained the observed immune-modulation associated with the lenalidomide + dexamethasone combination therapy [166].

Finally, the IFN-regulated gene *CD38* is repressed by Ikaros and Aiolos, and treatment with lenalidomide increased surface expression of CD38 in several MM cell lines leading to higher efficacy of ADCC mediated by daratumumab [168].

4.2. Proteasome Inhibitors (PIs)

The use of PIs, such as bortezomib, carfilzomib and ixazomib, has been incorporated into several regimens for the treatment of MM [189]. In addition to directly induced tumor cell death [190], PIs can exert ICD. In this sense, Chang et al. examined the generation of immune-mediated antitumor effects in response to bortezomib in a murine ovarian tumor model [169]. Treatment with bortezomib resulted in a higher recruitment of $CD8^+$ T lymphocytes into the tumor and higher amounts of tumor-infiltrating IFN-γ^+ T lymphocytes. Moreover, in vitro treatment of ovarian tumor cells with bortezomib led to the surface upregulation of Hsp60 and Hsp90, two ICD markers, which promoted the phagocytosis of tumor cells by DCs [169]. Regarding MM, the delivery of an activating signal from bortezomib-killed myeloma cells to DCs is mediated by the exposure of Hsp90 on the surface of apoptotic cells [170]. Indeed, DCs pulsed with bortezomib-killed myeloma cells are potent inducers of tumor-specific IFN γ–producing T cells [170]. Both bortezomib and carfilzomib promoted in myeloma cell lines the exposure of CALR, another protein marker of ICD [171]. Finally, combined treatment of carfilzomib and chloroquine (which blocks autophagy) increased both apoptosis and cell surface exposure of CALR, therefore increasing the immunogenic ability of carfilzomib [171].

4.3. Histone Deacetylase Inhibitors (HDACi)

HDACi exert antimyeloma effects through multiple mechanisms of action including epigenetic, protein stabilizing and immunogenic effects [191], although data regarding the latter are still contradictory and controversial as exposed below.

Moreno-Bost et al. observed that the sequential treatment of MM cells with 5-azacitidine followed by the HDACi MGCD0103 (mocetinostat) increased their susceptibility to the specific lysis mediated by MAGE-A3-specific CTLs and the secretion of IFN-γ by the latter [172]. In other study, valproic acid (VPA) induced the upregulation of MICA/B and ULBP2 in MM cell lines and patients' myeloma cells, and, consequently, degranulation and cytotoxic activity of NK cells were enhanced in presence of VPA-pretreated myeloma cells [173]. Additionally, sodium butyrate, another HDACi, also upregulates MICA in MM cell lines when combined with a matrix metalloproteinase inhibitor III and phenylarsine oxide, a drug that hinders surface ligand internalization [174]. Moreover, the cytotoxic efficacy of cytokine-induced killer (CIK) cells in targeting myeloma is higher when MM cells are pretreated with a combination of these three drugs [174]. Panobinostat, a pan-HDACi

approved for the treatment of relapsed MM, also upregulates ULPBP2/5/6 and MICA/B in MM cells [163].

Regarding effects on the PD-1/PD-L1 axis, the HDAC6 selective inhibitor ACY-241 significantly decreases PD-L1 expression on pDCs, which in turn attenuates PD-L1/PD-1-mediated NK suppression and enhances NK cell-mediated MM cell cytotoxicity [177]. Furthermore, combined treatment of ACY-241 and anti-PD-L1 triggered a more robust cytolytic activity and degranulation against MM cells than each agent alone [177]. With respect to PD-L1 expression in MM cells, panobinostat, entinostat (class I HDAC-specific inhibitor) and ricolinostat (HDAC6 inhibitor) upregulated PD-L1 in these cells probably by histone acetylation of the *PDL1* gene promoter [163]. In line with these results, reanalyzing gene expression microarray data generated in our lab [192], we have found an increase in PD-L1 mRNA expression after treatment of MM.1S cells with panobinostat. On the contrary, Bae et al. found that ACY-241 decreased PD-L1 expression on $CD138^+$ tumor cells [178]. ACY-241 also reduced PD-L1 expression on Tregs and PD-1 expression on $CD3^+$ T cells, upregulated CD80/86 and MHC molecules (class I and II) on both tumor and DCs and upregulated costimulatory and activation molecules on antigen-specific CTLs [178]. Panobinostat, however, has been shown to impair DC function to stimulate antigen-specific immune responses [176].

Interestingly, several HDACi upregulate the expression of CD38 [175,179,180]. In ex vivo cultures, panobinostat and, to a greater extent, ricolinostat upregulated CD38 expression in myeloma cells from both newly diagnosed and relapsed/refractory patients, which improved the cytotoxic effects of daratumumab [175,179]. Specifically, the inhibition of HDAC6 by ricolinostat prevents the deacetylation of H3K27 in the *CD38* promoter [179]. Moreover, second-generation HDAC6 inhibitors such as ACY-241 and WT-161 also increase CD38 expression in MM cells [179]. Importantly, the class I HDAC-specific inhibitor entinostat enhances CD38 expression alone and in combination with IFN-α, ATRA or both. However HDAC6 inhibition impairs the upregulation of CD38 in MM cells by IFN-α and ATRA, which constitutes an aspect to take into account when considering the possibility of adding a HDACi to the combined treatment of daratumumab, IFN-α and ATRA [180].

4.4. Cyclophosphamide

The alkylating agent cyclophosphamide has been used in the treatment of MM for over 60 years and, at low doses, it also presents significant immunomodulatory activity [193]. In this sense, treatment of different tumor models with mafosfamide (a chemical compound related to cyclophosphamide) or cyclophosphamide induces CALR translocation [181,182] and the release of HMGB1 [181], both of them being surrogate ICD markers. Accordingly, cyclophosphamide-treated mice showed an increase in tumor infiltrating DCs with an activated phenotype [181]. Moreover, type I IFN, a cytokine known to stimulate DC activation and T-cell priming, has a synergistic antitumor effect with cyclophosphamide [181]. In addition to ICD induction, cyclophosphamide also depleted Tregs [182,183], promoted Th1 polarization [183], increased activated NK cells [182] and modulated the myeloid population [182] in different tumor models.

In MM, there is still no clear evidence of the involvement of ICD mechanism in the in vivo responses to cyclophosphamide, although other immune-modulating effects have been observed. Thus, in vitro exposure of MM.1S cells to low doses of cyclophosphamide leads to a secretory response, which, along with downregulation of the "don't eat me" antigen CD47, greatly augments macrophage induced ADCP of daratumumab-coated MM cells [184]. Additionally, after cyclophosphamide treatment, macrophages presented increased levels of the CD64 Fcγ receptor, required for ADCP, possibly further enhancing phagocytosis [184]. These results have been confirmed in the clinical setting in the CyBorD-DARA trial [194].

4.5. Arginase Inhibitors

As mentioned previously, Arg-1 exerts immunosuppressive effects and, accordingly, several arginase inhibitors have demonstrated beneficial effects, especially in solid tumors [195]. In MM, however, there are still few studies that have explored the effect of arginase inhibitors. It has been described that the efficacy of bortezomib against MM cells was reduced in presence of either serum obtained from MM patients or conditioned media from MDSCs from MM patients, being both effects reverted by treatment with arginase inhibitors [102]. In other study, two Arg-1 inhibitors, nor-NOHA and CB-1158, reverted the immune-suppressive properties of both MGUS and MM-high-density neutrophils (HDNs) [103]. Contrary to the detrimental effects of arginase, it has also been published that arginase produced by activated macrophages may inhibit the growth of tumor cells [196]. In line with this, Th2 adoptive cell therapy eradicated myeloma cells in a murine model, which was associated with massive infiltration of M2-type macrophages producing arginase and was strongly inhibited by treatment with the arginase inhibitor BEC [185].

4.6. IDO Inhibitors

The effects of IDO inhibitors against MM are still controversial. The induction of high IDO production by DCs suppresses effector T cell activation, whereas treatment with the highly selective IDO inhibitor INCB014943 significantly reverses these effects [74]. Furthermore, it has also been observed that IDO is functionally expressed in MM cells [186]. In this sense, IDO^+ myeloma cells induce an expansion of the overall Treg population and reduce the percentage of IL-2 and IFN-γ-expressing T cells, being both effects partially reverted by D,L-1-methyl-tryptophan (1-methyl-DL-Trp), a chemical inhibitor of IDO [186]. Conversely, Pfeifer et al. found that IDO is expressed in myeloma cells in a low degree and is not upregulated after treatment with the cytokines IFN-γ, HGF and TNF-α [197]. However, IFN-γ stimulation of mesenchymal stromal cells (MSCs) specifically induced IDO in these cells, which may directly induce the apoptosis of myeloma cells by tryptophan depletion or the accumulation of tryptophan metabolites, being these effects abrogated by specific IDO inhibitors like 1-methyl-DL-Trp [197].

Interestingly, IDO is significantly upregulated during osteoclastogenesis [187]. Moreover, expression of IDO in osteoclasts is further enhanced following INF-γ stimulation [187]. Accordingly, both T-cell proliferation and cytotoxic capacity of CTLs are significantly inhibited in presence of osteoclasts and these effects are partly overcome by treatment with the IDO inhibitor 1-methyl-DL-Trp [187].

IL-32γ derived from MM cells significantly induced the production of IDO in macro phages, a phenomenon predominantly dependent on the STAT3 and NF-κB pathways [188]. IDO produced by IL-32γ-educated macrophages inhibits proliferation and effector function of $CD4^+$ T cells, being T cell proliferation almost completely restored by adding 1-methyl-DL-Trp [188].

5. Concluding Remarks

Due to the wide range of all the immune alterations described so far in monoclonal gammopathies, and also considering the contradictory results in some cases, it is still difficult to accurately predict which patients with MGUS or SMM will progress to active MM based on such alterations. Therefore, further deepening on cellular and molecular impairment of patients' immune system and its interplay with myeloma cells will help to elucidate its implication in disease progression. It would be important to ascertain whether alterations in the immune system are responsible for the progression of MGUS to MM or if in contrast, abnormalities in the tumor plasma cells induce an immunosuppressive microenvironment, favoring the transition to the malignant stages of the disease. In addition, it would be of interest to investigate whether such immune impairment is the result of a sequential additive process or otherwise consequence of a specific global alteration already

present at the MGUS phase. Moreover, the precise understanding of the nature of immune alterations could contribute to the discovery of new targets for therapeutic approaches.

Additionally, it is of utmost importance to discern the immune-stimulating mechanisms of the drugs described above in the context of MM, principally of those for which controversial data have been reported as HDACi, and of the most novel ones (i.e., arginase inhibitors, IDO inhibitors, etc.). In line with this, new preclinical studies and clinical trials currently ongoing with some of them will help to elucidate their real perspectives as antimyeloma agents, and especially their capacity to potentiate the efficacy of immunotherapeutic mAbs.

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