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Role of Bone Marrow Stromal Cells in the Regulation of NKG2D and DNAM-1 Ligand Expression on Multiple Myeloma Cells

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INTRODUCTION

1. Multiple Myeloma (MM)

1.1 General Features

Multiple Myeloma (MM) is a hematologic disease characterized by the accumulation of malignant plasma cells (PCs) in the bone marrow (BM) microenvironment, the presence of clonal immunoglobulins in the blood and/or urine and organ dysfunction. MM represents about 13% of hematologic neoplasia and it is more common in men than in women with a median age at diagnosis of 70 years. Clinical features of MM are referred to as the CRAB criteria: hyper-Calcemia, Renal failure, Anemia and Bone lesions; moreover, immunodeficiency plays a pivotal role in the progression of this malignancy (Palumbo & Anderson, 2011; Röllig et al., 2015). Although the ever increasing number and complexity of drug classes [*e.g.* genotoxic drugs, the proteasome inhibitors, the immunomodulatory drugs (IMiDs), the monoclonal antibodies (mAbs) and autologous hematopoietic stem cell transplantation (HSCT)] to treat this hematologic malignancy significantly improved patient survival and quality of life, MM remains an incurable disease, with a survival of 4-5 years from the diagnosis (Mateos et al., 2010; Laubach et al., 2016; Ravi & Gonsalves, 2021; Wallington-Beddoe & Mynott, 2021).

Multiple Myeloma is a multi-stage disease, starting from the pre-malignant phase *"Monoclonal Gammopathy of Undetermined Significance (MGUS)",* which can evolve into active intramedullary and extramedullary MM passing through the pre-symptomatic stage *"Smoldering MM (SMM)"*.

In the MGUS phase, the level of serum monoclonal immunoglobulins is < 3g/dL, the content of malignant PCs in the BM is less than 10% and organs are not damaged. MGUS is present in 3 to 4% of the people older than 50 years and progresses to malignant MM at the rate of 1% per year. Unfortunately, there are no unequivocal genetic or phenotypic markers that can distinguish MGUS from MM tumor cells. Moreover, it is not known to what extent, intrinsic genetic or epigenetic changes in the tumor cell, can affect the progression from MGUS to MM. Thus, it is not yet possible to predict if and when this progression will occur (Kyle et al., 2010; Agarwal & Ghobrial, 2013).

Smoldering MM is an intermediate stage between MGUS and active MM and is characterized by high concentration of serum monoclonal immunoglobulins (> 3g/dL), more than 10% of

malignant PCs in the BM and by the absence of the typical MM-related end-organ damage. Approximately 10% of SMM per year progresses to MM in the first five years from diagnosis (Kyle et al., 2007; Rajkumar et al., 2015).

Signs and symptoms of active intramedullary MM include anemia, lytic bone lesions, hypercalcemia, renal dysfunction and increased risk of infection. Extramedullary MM is characterized by a high percentage (20%) of malignant PCs in the BM, also detectable in the blood, the involvement of several organs including skin, liver, lymphatic system, pleura, and central nervous system and a low rate of survival (Mitsiades et al., 2004).

The origin of the malignant PCs remains largely unknown. The normal PC is transformed to the premalignant MGUS/SMM state and eventually to symptomatic MM through the accumulation of a number of sequential genetic and epigenetic events, several changes in the BM microenvironment which support the MM cells and probably a failure of the immune system to eliminate the malignant clone. A variety of genetics hits (gene translocation, suppression, duplication or mutation), and secondary events (cell cycle dysregulation, epigenetic modifications) are involved in tumor cell transformation, rendering MM a highly heterogeneous disease. About 50% to 60% of MM cases are hyperdiploid with trisomies in odd chromosomes (chromosomes 3, 5, 7, 9, 11, 15, 19, and 21) (Neri and Bahlis, 2013) and translocations involving the immunoglobulin heavy chain. Beyond these initial recurrent abnormalities, secondary genetic events highly contribute to MM progression. In particular, RB (Retinoblastoma) protein seems to be constitutively phosphorylated in MM cells and IL (Interleukin)-6 further shifts RB to its phosphorylated form. Cyclin D1 deregulation is a rather initial incident in MM pathogenesis. Cyclin D1 is overexpressed in a high proportion of MM cells with translocation t(11;14)(q13;q32). The RAS/RAF/MEK/ERK (Rat Sarcoma/Rapidly Accelerated Fibrosarcoma/Mitogen-activated protein Kinase Kinase/Extracellular signal-Regulated Kinases) pathway has a key role in multiple functions of PCs and angiogenesis. Mutations of KRAS/NRAS/BRAF (Kirsten Rat Sarcoma virus/ Neuroblastoma RAS viral oncogene homolog/v-Raf murine sarcoma viral oncogene homolog B) are found in almost half of MM patients resulting in more aggressive clinical behavior and poorer prognosis. The translocation t (4;14) leads to amplified expression of FGFR3 (Fibroblast Growth Factor Receptor 3), which also activates the RAS/MAPK (Mitogen-Activated Protein Kinase) pathway. Bcl-2 (B-cell lymphoma-2) is probably a critical survival factor for MM cells in the hypo-proliferative early stages of the disease since it seems to lose its importance in the aggressive proliferative phase of the disease and its overexpression makes these cancer cells resistant to apoptosis. High expression of cyclin D1 and Bcl-2 together with low levels of Bcl-

xl (Bcl-2-associated X protein) and MCL-1 (Induced myeloid leukemia cell differentiation protein) are detected in MM cell lines and primary patient samples with translocation t(11;14). Mutations of p53 are uncommon in MM (10%) and are mainly observed in the terminal stage of the disease. IL-6, which has a crucial role in MM pathogenesis, can directly trigger PI3K (Phosphoinositide 3-kinase) and activate AKT (PKB, Protein Kinase B) and the RAS/MAPK pathway (Carrasco et al., 2007; Shaffer et al., 2008; Lohr et al., 2014;).

Sequencing and gene expression profiling studies have also identified numerous epigenetic defects in MM, including locus-specific DNA hypermethylation of cancer-related and B cell specific genes, genome-wide DNA hypomethylation and genetic defects, copy number variations and/or abnormal expression patterns of various chromatin modifying enzymes. Recent studies have demonstrated that generalized gene hypomethylation is associated with the transition from MGUS to MM, whereas hypermethylation of specific target genes correlates with progression of intramedullary into extramedullary MM (Morgan et al., 2014; De Smedt et al., 2018).

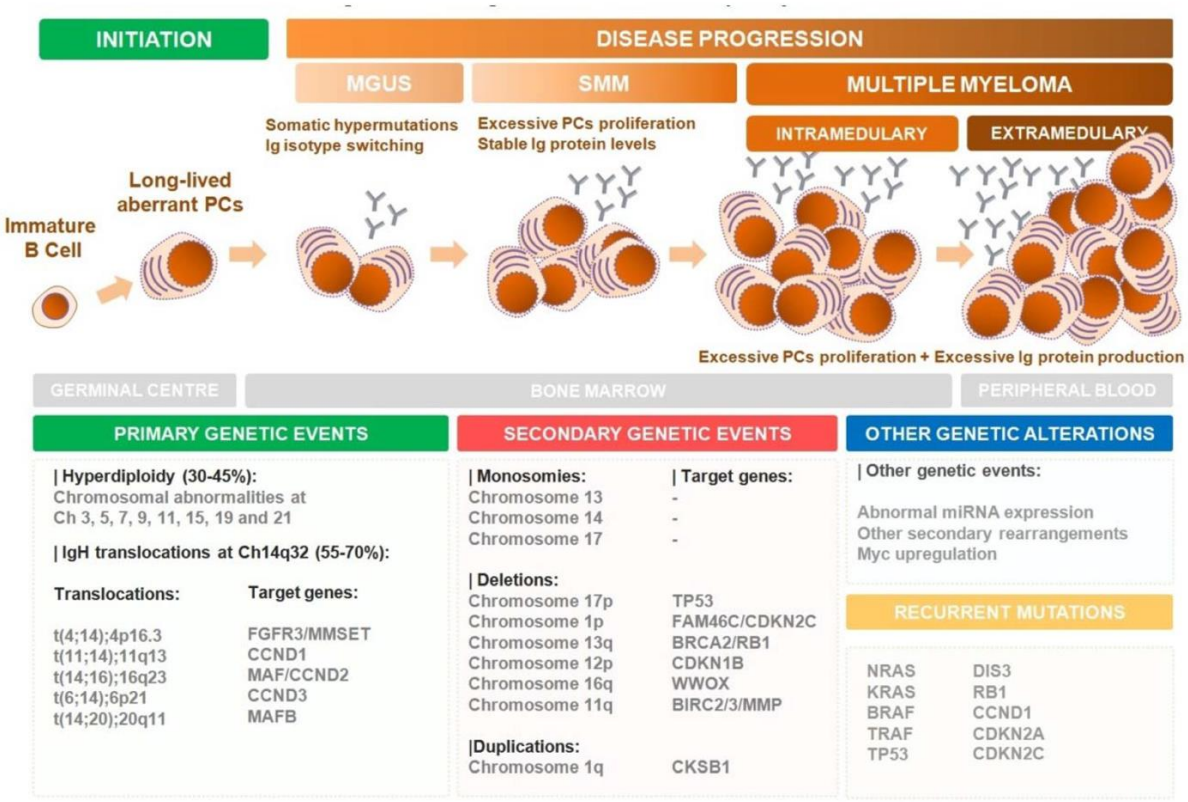

Fig.1. Genetic alterations during MM progression. (Pinto et al., 2020)

2. BM Microenvironment: Role of Bone Marrow Stromal Cells (BMSCs) on MM Progression

MM is considered a prototype of malignancy characterized by complex bi-directional interactions between tumor cells and BM microenvironment, which strongly contribute to sustain survival, proliferation, drug resistance and progression of the disease (Maiso et al., 2021). Within BM microenvironment, MM cells contact with different cellular components, including bone marrow stromal cells (BMSCs), fibroblasts, adipocytes, endothelial cells (ECs), osteoclasts (OCs), osteoblasts (OBs), immune cells and hematopoietic cells and non-cellular compartment of the extracellular matrix and the liquid milieu of cytokines, chemokines, growth factors and extracellular vesicles (EVs). Among cellular components, a pivotal role in MM pathogenesis is played by BMSCs. These cells are fibroblast-like cells, with notable secretory, immunomodulatory and homing properties. Phenotypically, BMSCs can be characterized according three criteria established from the International Society for Cellular Therapy (ISCT) (Dominici et al., 2006; Krampera et al., 2013; Viswanathan et al., 2019): 1) the adhesion to plastic surface in normal culture condition; 2) the capability to differentiate into adipocytes, osteocytes and chondrocytes *in vitro* under appropriate stimuli; 3) the expression for different surface markers, such as CD73, CD90, CD105 and CD106 in the absence of hematopoietic marker CD45.

2.1 Comparison of BMSCs from Healthy Donors (HD-BMSCs) and MM Patients (MM-BMSCs)

Several studies highlight that BMSCs derived from MM patients (MM-BMSCs) are functionally and genetically different from BMSCs derived from healthy donors (HD-BMSCs) (Xu et al., 2018). Most studies have been conducted after isolation of BMSCs by adherence to plastic and subsequent *in vitro* expansion or by exposing BMSCs to MM cells. In addition, a 3D tissue-engineered bone (TE-bone) model based on silk scaffolds with a mineralized bone matrix was established. This model was used to recapitulate the *in vivo* interactions between MM, BMSCs and ECs (Reagan et al., 2014). By using these different approaches, it was demonstrated that regardless of the disease stage, the surface immunophenotype of MM-BMSCs is similar to that of HD-BMSCs. However, MM-BMSCs show reduced osteogenic potential, lower proliferation rate and reduced efficiency to suppress T cell proliferation compared to HD-BMSCs (Li et al., 2010; Xu et al., 2012; André et al., 2013). MM-BMSCs

differ in their production of many soluble factors, such as cytokines, chemokines, growth factors and EVs, which contribute to MM cell growth and chemoresistance leading to disease progression. For instance, MM-BMSCs express higher amount of IL-6, which is the most potent growth factor involved in, IL-1β, IL-3, GM-CSF (Granulocyte Monocyte-Colony Stimulating Factor), TNF (Tumor Necrosis Factor)-α, HGF (Hepatocyte Growth Factor) and GDF15 (Growth Differentiation Factor 15) (Corre et al., 2007, 2012; Zdzisińska et al., 2008).

Important differences between HD- and MM-BMSCs were also found in the content of secreted EVs, such as exosomes and microvesicles (MVs). Compared to HD-BMSC-derived EVs, those produced by MM-BMSCs contain lower level of miR15a, which is identified as an oncosuppressor; on the other hand, they contain high level of IL-6, chemokine CCL2 (CC motif Ligand 2), IL-1ra, MCP-1 (Monocyte Chemoattractant Protein-1), MIP (Macrophage Inflammatory Protein)-1α, MIP-1β, SDF (Stromal cell-Derived Factor)-1 and γ-catenin, which promote the progression of this neoplasia (Roccaro et al., 2013).

Substantial differences were found in the expression of specific miRNAs when HD-BMSCs and MM-BMSCs were subjected to osteogenic differentiation conditions: miR-135b was upregulated in MM-BMSCs and impaired their osteogenic differentiation by targeting SMAD5 (Xu et al., 2013). Similarly, miR-138 was significatively increased in MM-BMSCs compared to HD-BMSCs, and inhibition of miR-138 resulted in enhanced osteogenic differentiation of MM-BMSCs (Tsukamoto et al., 2018). The expression of other miRNAs has been found upregulated in MM-MSCs (miR-221, miR203a-3p.1, miR-223) or decreased (miR-342, miR-363, or miR-29b) respect to that of HD-BMSCs, modulating the expression of genes involved in osteoblastogenesis (Raimondo et al., 2020).

Other studies showed that frequency of BMSCs was significantly higher in patients with active MM, suggesting a differentiation blockade responsible for accumulating BMSCs to support a protective microenvironment for MM cells. Additionally, MM-BMSCs transcriptional signature was found enriched in genes with a critical role in functions related to MM pathogenesis, such as IL-17 pathway and TNF signaling via NF-kB, osteoblastogenesis inhibition, BMSC proliferation, immune-suppressive potential, and a reinforced pro-adipogenous phenotype (Alameda et al., 2020). After exposure to MM cells, MM-BMSCs acquire a pro-senescence profile and transdifferentiate into tumor-associated fibroblasts, which promoted MM progression and tumor angiogenesis in *in vivo* models (Kanehira et al., 2017). Furthermore, a reduced expression of Dicer1 and miR-93/miR-20a was associated with elevated expression of the cell cycle inhibitor p21, resulting in senescence of BMSCs, reduced

osteogenic, increased adipogenic differentiation and promotion of MM cell growth (J. Guo et al., 2018).

Molecular mechanisms underlying these phenotypic and genetic differences remain largely unknown. However, a number of *in vitro* experiments demonstrated that BMSCs undergo chromosomal aberrations following the exposure to MM cells. The interactions of HD-BMSCs with MM cells mediated by cell adhesion, or through soluble factors and EVs are key and initiating elements of the transition from HD-BMSCs to MM-BMSCs. These interactions would progressively mediate dysregulation of gene expression, changes in the miRNA and epigenetic profiles, and genomic abnormalities which would shape functional and phenotypic changes from HD-BMSCs to MM-BMSCs. Transversal factors affecting these changes in BMSCs would be hypoxic conditions and the featured immunosuppressed microenvironment of MM and patient aging (Arnulf et al., 2007; Corre et al., 2007; Garayoa et al., 2009).

2.2 Contribution of MM-BMSCs to Myeloma Bone Disease (MBD)

Multiple myeloma is the disease with the highest incidence of bone involvement among all the malignant diseases. Abnormalities in conventional radiography can be found in approximately 80% of patients with newly diagnosed MM. Bone disease in MM is characterized by lytic bone lesions, which can cause severe bone pain, pathologic fractures and hypercalcemia. Bone resorption is due principally to an increased osteoclast activity and decreased osteoblast function. This imbalance is principally favored by the interaction between MM cells and BMSCs: 1) MM cells upregulate the expression of RANKL (Receptor Activator of Nuclear Factor K B Ligand) in BMSCs, whereas the expression of OPG (Osteoprotegerin) is reduced, thus favoring osteoclastogenesis and OC activation through RANKL/RANK signaling; 2) interaction with MM cells leads to increased secretion of Activin A in BMSCs via adhesion-mediated JNK (c-Jun N-terminal Kinase) activation. Besides inhibiting OB differentiation, increased activin A levels would stimulate OC formation and activity; 3) MM cells reduce the expression of both EphB2 and EphB4 in BMSCs thereby impairing OB differentiation and no longer preventing OC formation, allowing increased osteoclastogenesis; 4) MM cell interacting-BMSCs would promote the production of Wnt5a which in turn would increase OC formation and activity (Silvestris et al., 2007; Yaccoby, 2010; Colucci et al., 2011; Lin & Hankenson, 2011).

2.3 Contribution of MM-BMSCs to MM Growth and Survival

BMSC-MM cell communication involves direct adhesive interactions as well as soluble factors able to engage autocrine and paracrine loops, which generate a tumor-promoting microenvironment. VLA-4 (Very Late Antigen-4), LFA-1 (Lymphocyte function-associated antigen-1), MUC-1 (Mucin-1) or CD40 present on MM cells bind VCAM1 (Vascular Cell Adhesion Molecule 1), ICAM1 (Intercellular Adhesion Molecule 1) or CD40L on BMSCs, leading to the activation of many pathways which promote an induction of cell cycle progression and anti-apoptotic proteins and inhibition of pro-apoptotic signals on MM cells (Hideshima & Anderson, 2021). Specifically, VLA-4-VCAM1 and Notch pathways trigger the activation of NF-kB in both cell types, leading to the production of IL-6, VEGF and IGF1 (Insuline Growth Factor 1) by BMSCs, which are associated with MM cell proliferation and survival (Hideshima et al., 2007). The myeloma-BMSC interactions further enhance the production of growth factors, including IGF-1, HGF, VEGF (Vascular Endothelial Growth Factor) and Gas6 (Growth arrest specific gene 6), or cytokines, such as IL-6 and TNF-a, and the chemokines SDF-1 and IL-8, which sustain MM cell growth and chemoresistance and positively correlates with disease progression. Most of these soluble factors concur to further induce the activity of NF-kB family transcription factors, which are strongly implicated in the pathogenesis of MM cells, as described above (Mitsiades et al., 2004; Furukawa et al., 2017; Roy et al., 2018).

Among these factors, an important role is played by Gas6 and IL-8. Gas6 and Pros1 (Protein S 1) are members of the family of vitamin K-dependent protein and are recognized by TAM (Tyro3, Axl, MerTK) receptors. Pros1 acts as ligand for Tyro3 and MerTK, whereas Gas6 is ligand for all three TAMs. Optimal signaling through the receptor is induced only when Pros1 and Gas6 act as bridging ligands for phosphatidyl serine (PtdSer), typically expressed on the outer membrane of apoptotic cells. This leads to homodimerization and autophosphorylation of the TAM and triggering of downstream signaling pathways such as MEK/ERK, PI3K/AKT and JAK/STAT (Lew et al., 2014; Rothlin et al., 2015). MerTK and Axl are highly expressed in both solid and hematologic tumors. Several studies demonstrated the importance of TAM-Gas6/Pros1 axis also in MM. In detail, it was observed an increased amount of Gas6 in the serum of MM patients and its implication in proliferation and chemoresistance of malignant PCs (Furukawa et al., 2017; Z. Zhang et al., 2020). Furthermore, an overexpression of TAMs, especially Axl, was also observed in MM; in this regard, a novel human monoclonal antibody,

named DAXL-88, was generated that recognizes both human and murine AXL and inhibits tumor cell migration and invasion induced by Gas6 (Y. Duan et al., 2019).

IL-8, alternatively known as CXCL8, is a member of the CXC chemokine family and was originally described as a neutrophil chemoattractant (Yoshimura et al., 1987). This cytokine is normally produced by a wide range of cells, including lymphocytes, monocytes, endothelial cells, fibroblasts, hepatocytes, and keratinocytes. The biological effects of IL-8 are mediated through the binding of IL-8 to two cell-surface G protein–coupled receptors, termed CXCR1 and CXCR2. After binding with these receptors, IL-8 signaling promotes activation of the primary effectors PI3K or PLC (Phospholipase C), promoting the activation of Akt, PKC, calcium mobilization and/or MAPK (Mitogen-Activated Protein Kinase) signaling cascades. The overexpression of IL-8 in many tumors and cancer cell lines has led to investigation of its role in tumor progression. IL-8 promotes tumor growth, angiogenesis, and metastasis in a variety of human cancers (Waugh & Wilson, 2008). In MM, IL-8 is mostly produced by BMSCs within BM microenvironment. Regarding the role of IL-8 in MM cell proliferation, some reports have been published with contradictory results. In one study, increased MM cell proliferation mediated by the presence of the chemokine was reported, whereas no differences in proliferation rate were observed in the others (Pellegrino et al., 2005; Kline et al., 2007). However, other studies demonstrated that the expression of IL-8 is increased in both MM cells and BMSCs after treatment with anti-myeloma drugs, thus promoting survival and resistance to bortezomib of malignant PCs via NF-kB transcription factor activity and plays a major role in osteoclastogenesis (Markovina et al., 2010; Herrero et al., 2016).

As previously commented, BMSCs can also impact MM progression through the production of exosomes and MVs. Exosomes are small EVs produced in the endosomal compartment with a diameter of 50/100 nm expressing the specific surface markers CD63 and CD81 and containing proteins and micro-miRNAs. EVs from HD- and MM-BMSCs were found to be functional distinct, due to their different content of miRNAs, oncogenic proteins, cytokines and adhesion molecules. Whereas MM-BMSCs exosomes can increase myeloma proliferation, HD-BMSCs exosomes were able to significantly reduce MM growth in in vitro and in vivo assays (Roccaro et al., 2013). Moreover, BMSC-derived exosomes could be taken up not only by MM cells but also by MDSCs in the BM and activate these MDSCs enhancing their immunosuppressive functions, thus favoring MM progression (J. Wang et al., 2015). Exosomes have been shown to mediate the transfer of the lncRNA LINC00461 to MM cells, where it sponged miR15a/16 and enhanced MM cell proliferation and survival (Deng et al., 2019). Instead, MM-derived exosomes were found enriched in miR-146a and miR-21, which once transferred to BMSCs

induced the secretion of cytokines and chemokines (including CXCL1, IL6, IL8, IP-10, MCP-1, and CCL5) and increased BMSC proliferation and transformation to cancer-associated fibroblasts, finally resulting in increased MM cell viability and migration (De Veirman et al., 2016; Cheng et al., 2017). On the other hand, MVs are larger EVs generated directly by plasma membrane budding with a diameter greater than 1 μm and express on the surface phosphatidylserine for internalization by malignant PCs. Of interest, mass spectrometry analysis revealed that MM-BMSCs MVs were enriched in VLA-4, which facilitated its uptake and transfer to MM cells (Dabbah et al., 2020). MM-BMSCs-derived MVs are shown to be incorporated by MM cells increasing MM viability, proliferation and translation initiation through the activation of various biochemical pathways in MM cells, including MAPK and NF-kB signaling; conversely, HD-BMSC-derived MVs decrease these effects (Dabbah et al., 2017). MM-BMSC-derived MVs are also enriched of MiR-10a which was found to promote MM proliferation (Umezu et al., 2019).

2.4 Contribution of MM-BMSCs to a Pro-Inflammatory and Immunosuppressive Microenvironment

BMSCs are known to display potent immunosuppressive and anti-inflammatory activities, modulating the innate and adaptative immune systems through a plethora of contact-dependent and independent mechanisms.

As compared to HD-BMSCs, co-culture of T cells with MM-BMSCs results in increased Th17/Treg ratio. This altered immunomodulatory capacity was dependent on different factors secreted by MM-BMSCs, such as IL-6, VCAM1 and CD40, able to promote Th17 differentiation. Consistently, increased numbers of Th17 cells in MM have been related to MM growth and progression (André et al., 2015). Other studies have shown a pro-inflammatory profile of MM-BMSCs. Single-cell transcriptomic analysis of the MM BM has identified an activated inflammatory stromal cell population associated with TNF signaling. Several groups also reported that MM-BMSCs produce a large amount of pro-inflammatory cytokines after interaction with MM cells, resulting in MM growth and progression. In fact, many of these inflammatory cytokines also function as growth factors for MM cells or may induce the secretion of MM growth factors by other cells in the microenvironment (Zdzisińska et al., 2008). On the other hand, immunosuppression is a common feature of MM patients associated with the evolution of the disease. This immunosuppression is mediated by high concentrations of immunosuppressive soluble factors, loss of effective antigen presentation, effector cell

dysfunction, and by the recruitment of immunosuppressive populations, such as myeloid-derived suppressor cells (MDSCs). In the BM microenvironment, MM-BMSCs contribute to the expansion, activation and survival of MDSCs through the secretion of HGF and activation of the STAT3 and STAT1 pathways leading to increased BCL-XL and MCL-1 levels (Yen et al., 2013). These MDSCs produced upregulated immunosuppressive factors (*e.g.* Arginase1 and TNF-α) and have an increased ability to digest bone matrix and they have been reported to support MM progression by suppressing T cell responses (Díaz-Tejedor et al., 2021)., inducing Treg differentiation, inhibiting the maturation of dendritic cells (DCs) and their capacity to process and present antigens. In addition, BMSCs also reduce neutrophil activation and proliferation of natural killer (NK) cells, thus regulating innate immune system responses (Shi et al., 2011).

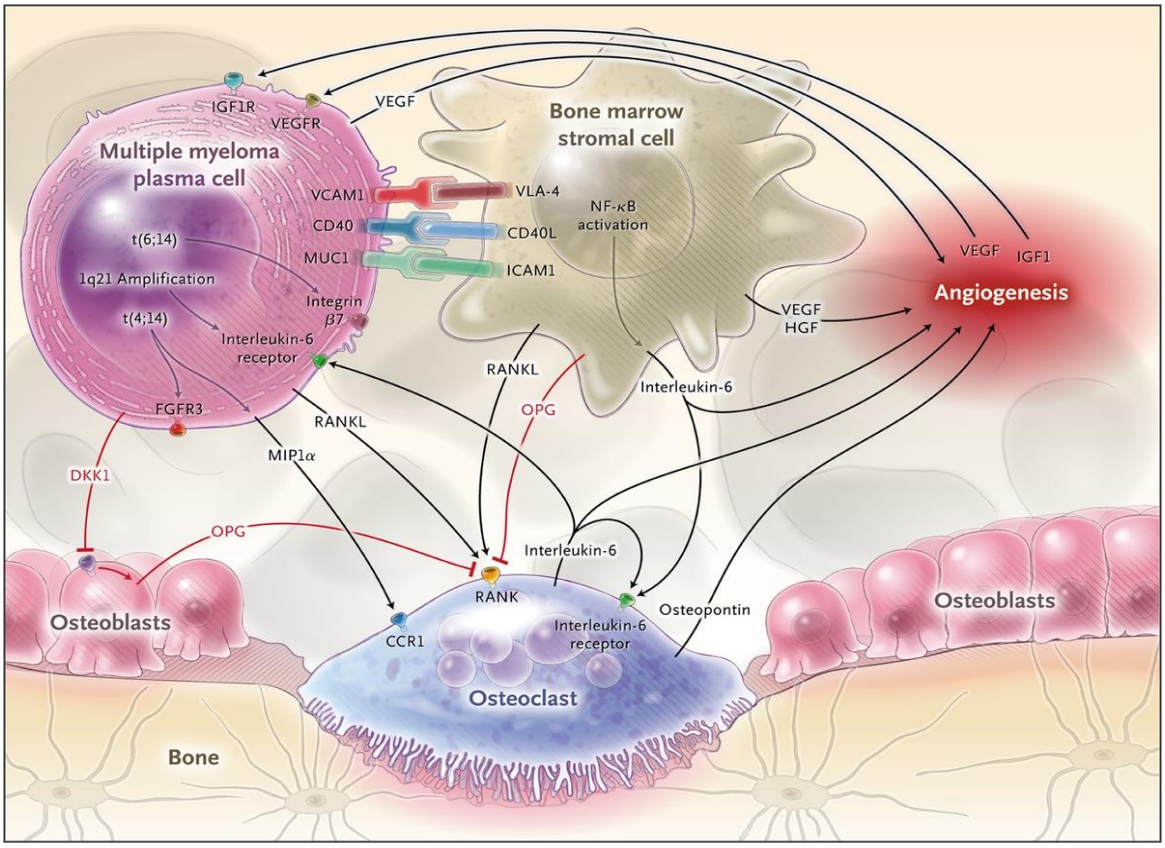

Fig. 2. Interaction between PCs and BM in MM. (Palumbo & Anderson, 2011)

3. NF-kB Pathway in MM

Progression of MM is promoted by an accumulation of genetic and cytogenetic aberrations. These abnormalities promote proliferation, survival and resistance to chemotherapy of malignant PCs. A peculiar feature of MM is the constitutive activation of NF-kB (Nuclear Factor kappa-light-chain-enhancer of activated B cells) pathway (Gilmore, 2007; Matthews et al., 2016). NF-kB is a family of transcription factors including p50, p52, p65, RelB and c-Rel, important for the normal development of B cells and PCs (Ghosh et al., 2002). These proteins are constitutively expressed in physiologic conditions by cells but retained in the cytoplasm in an inactive form by inhibitors. There are two general activation pathways, classical and alternative. Different stimuli activate NF-kB through the classical pathway: following stimulation, the inhibitory subunits IkBα, IkBβ and IkBε are phosphorylated by the complex IKKα, IKKβ and IKKγ and degraded by proteasoma; as a result, the heterodimers p65/p50 and c-Rel/p50 can translocate into the nucleus and activate the transcription of target genes. On the other hand, the alternative pathway involves the activation of the kinase NIK (NF-kB-Inducing Kinase), which allows the release of the heterodimer p52/RelB and its translocation into the nucleus.

Mutations of these pathways have been identified in 17% of MM tumors (Annunziata et al., 2007; Keats et al., 2007). Translocations or amplification were found to increase transcription of five positive regulators, which include NIK, NF-kB1 plus three TNFR (CD40, TACI, LTBR). In addition, there was one example of a translocation that deleted a negative regulatory region from NIK so that it was less susceptible to proteasomal degradation. Deletions and mutations were shown to inactivate different negative regulators of the classical (CYLD) or alternative (cIAP1&2, TRAF2, TRAF3, and NF-kB2) (Demchenko et al., 2010).

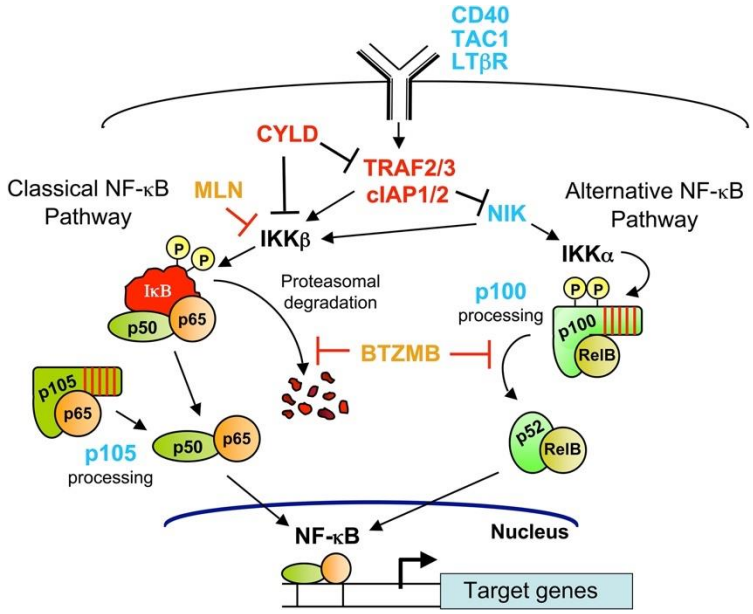

Fig. 3. NF-kB signaling pathway in MM cells. (Gilmore, 2007)

In addition to genetic mutations, NF-kB activation is promoted by the interactions between MM cells and BM microenvironment, mainly with BMSCs. These cells produce a large amount of different growth factors, cytokines, chemokines (*e.g.* VEGF, HGF, IGF, BAFF, APRIL, Gas6, IL-6, IL-8) and EVs able to activate NF-kB pathways in MM cells, promoting their proliferation and chemoresistance (Markovina et al., 2010; Yang et al., 2017; Huynh et al., 2018).

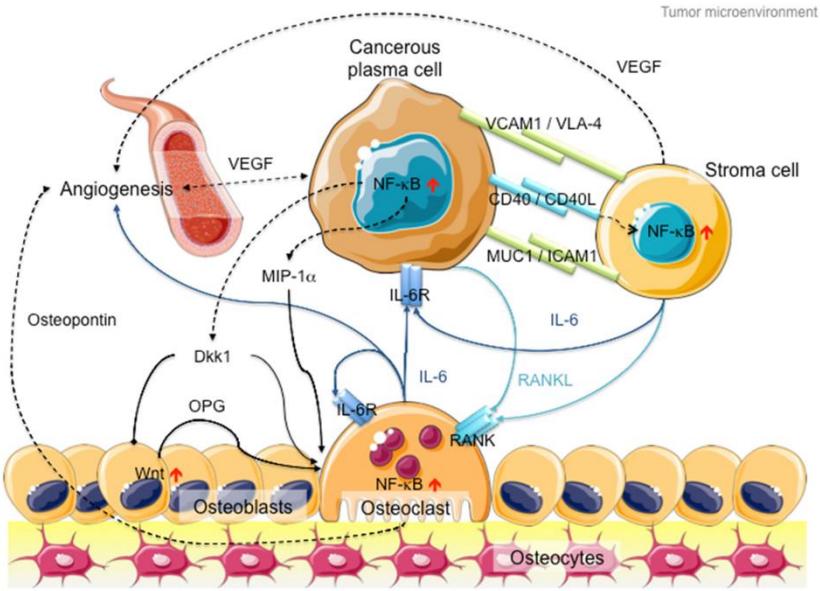

Fig. 4. Contribution of BM microenvironment in NF-kB signaling pathway in MM cells. (Wong et al., 2020)

Indeed, inhibition of NF-kB pathways represents an attractive therapeutic approach. For instance, the proteasome inhibitor Bortezomib able to block the two major pathways leading to NF-kB activation has excellent clinical activity in MM patients (Mitsiades et al., 2002; Hideshima et al., 2003); moreover, dexamethasone induces an upregulation of IkBα, which inhibits the classical pathway (Keats et al., 2007). On the other hand, NF-kB pathways could be targeted in MM by blocking its extrinsic activation by factors produced in the BM microenvironment, or by directly targeting intrinsic components of this pathway. It has been demonstrated that the sequestration of BAFF and APRIL by Atacicept or the use of the IKKβ kinase inhibitors efficiently prevent MM growth and induce apoptosis (Rossi et al., 2009; Hideshima et al., 2009).

4. Natural Killer (NK) cells

4.1 General Features

Natural Killer (NK) cells belong to the type I innate lymphoid cells and represent 5-20% of total lymphocytes in the blood. NK cells are cytotoxic and immunoregulatory lymphocytes acting against virus-infected cells and tumor cells without a prior exposure to the antigens (Vivier et al., 2008; Gross et al., 2013). They originate in the BM from a common lymphoid progenitor through the expression of two specific transcription factors, Eomesdermin and T-bet and begin to circulate in the blood, colonizing various organs, including BM, blood, spleen, liver, skin, lung, secondary lymphoid organs and pregnant uterus (Caligiuri, 2008; Yu et al., 2013).

Morphologically, NK cells appear as large lymphocytes with a big nucleus and a cytoplasm containing granules rich in lytic enzymes. Phenotipically, they are positive for the expression of CD56 and CD16, but negative for the expression of CD3; therefore, they are defined as $CD56^{+}CD3^{-}$ cells (Robertson et al., 1990). The molecule CD56 is an isoform of the adhesion molecule for neuronal cells NCAM (Neural Cell Adhesion Molecule) and acts as a regulator of the interactions between NK and target cells. The molecule CD16 is the low affinity receptor for the constant fragment (Fc) of immunoglobulins IgG (FcγRIII) and activates the antibody-dependent cell-mediated cytotoxicity (ADCC) (Leibson, 1997).

Two major subsets of NK cells are found in humans that can be distinguished by their levels of CD56 expression, namely $CD56^{dim}$ and $CD56^{bright}$. $CD56^{dim}$ NK cells are fully mature, make up about 90% of the NK cells in peripheral blood, and predominantly mediate cytotoxic responses. In contrast, $CD56^{bright}$ cells are more immature, make up about 5-15% of total NK cells, and are primarily cytokine producers [*e.g.* IFN (Interferon)-γ, TNF-α], while playing a limited role in cytolytic responses. On the other hand, $CD56^{bright}$ cells are better able to leave the vasculature and constitute the majority of NK cells found in either lymph nodes or the decidual tissues of pregnant women. In the first trimester of human pregnancy, $CD56^{bright}$ NK cells can surprisingly constitute about 70% of the lymphocytes in the decidual tissue, and recent evidence suggests that they play important roles in promoting angiogenesis during pregnancy (Bryceson & Long, 2008; Fu et al., 2014). More recently, another NK cell subset (named $CD56^{dim}CD16^{dim}$) characterized by low expression of either CD56 and CD16 and equipped with both high degranulation capacity and IFN-γ production has been discovered (Stabile et al., 2015).

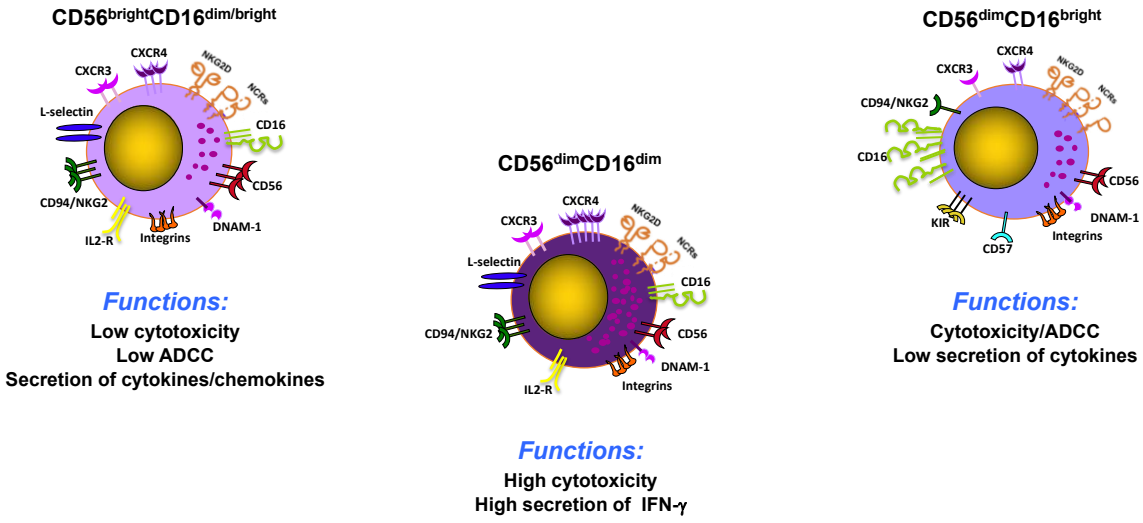

Fig. 5. NK cell subsets. (Gismondi et al., 2015)

As described above, NK cells play major role in tumor immunosurveillance, controlling cancer initiation, even if their activity is often inefficacious in advanced stages. Distinct NK cell subsets can exert different roles and specific functions in solid and hematological cancers (Stabile et al., 2017). This is due to the impact of the tumor microenvironment on NK cell phenotypic and functional features. Regarding solid tumors, it is well known that a prevalence of non-cytotoxic $CD56^{bright}$ NK cells with low expression of perforin ($CD56^{bright}perforin^{low}$) was observed in lung and breast cancers, where an upregulation of the chemokines CXCL9 and CXCL10 which favors $CD56^{bright}$ recruitment was observed (Carrega et al., 2008, 2014). In prostate cancer, $CD56^{bright}$ NK cells with low or null degranulation potential, lower expression of activating receptors and higher expression of the inhibitory receptor ILT2 were found (Pasero et al., 2015). On the other hand, $CD56^{bright}$ NK cells result to have a pro-angiogenic phenotype in different solid tumors, such as breast, melanoma and colorectal cancers (Levi et al., 2015). Several evidence support the concept that NK cells play a preferential role in the control of the onset of hematological cancer. In acute myeloid leukemia (AML), $CD56^{dim}CD16^{+}$ NK cells with low expression of activating NCRs (Natural Cytotoxicity Receptors) and high expression of inhibitory receptors KIRs (Killer cell Immunoglobulin Receptors) ($CD16^{+}KIR^{high}NCR^{low}$) that fail to recognize and kill autologous and allogenic blasts were described (Costello et al., 2002). An impairment of NK cell functions was also observed in B-acute lymphoid leukemia (B-ALL), myelodysplastic syndromes (MDS) and MM (Epling-Burnette et al., 2007; Konjević et al., 2016). About $CD56^{dim}CD16^{dim}$ NK cell subpopulation, it was recently demonstrated that

their frequency is increased in breast cancer and in both BM and PB of pediatric T-ALL and B-ALL, but they result poorly functional (Mamessier et al., 2013; Stabile et al., 2015).
Overall, these findings suggest that NK cells undergo modulation of receptor repertoire and blockade of their maturation in solid and hematological tumor microenvironment, which results in development of non-cytotoxic NK cells with impaired of effector functions, leading to tumor escape from NK cell recognition and killing.

4.2 NK Cell Activation and Cytotoxicity

Natural Killer cells were initially defined by their ability to kill tumor cells without prior sensitization. The role of NK cells has been expanded to include the elimination of virally infected cells and secretion of cytokines that mediate crosstalk and regulation of other immune cells. The capability of NK cells to lyse a target cell is mediated by two major mechanisms: ADCC and natural cytotoxicity (Gross et al., 2013; Leibson, 1997).
In ADCC, the Fcγ receptor (FcγRIIIa or CD16) expressed by NK cells binds to the Fc portion of the subclasses IgG1 and IgG3 on opsonized target cells, activating NK cell cytotoxicity and production of cytokines. In this context, ADCC is thought to play an important role in mediating the antitumor effects of many monoclonal antibodies (mAbs) used in the new immunotherapeutic approaches for the treatment of solid and hematologic tumors (Seidel et al., 2013; W. Wang, 2015).
Natural cytotoxicity is a direct recognition mechanism and is mediated by a balance of signals derived from a repertoire of activating and inhibitory receptors. Two main hypotheses explain this mechanism of NK cell activation: *"missing-self"* and *"induced-self"*. According to *"missing-self"* hypothesis, virus-infected or tumor cells reduce or lose the expression of MHC (Major Histocompatibility Complex) class I molecule, recognized by NK cell inhibitory receptors and normally expressed by all nucleated cells of the organism. The expression of these proteins prevents NK cells from activating and killing normal cells; therefore, their reduction or loss increase the susceptibility of a cell to lysis by NK cells (Ljunggren & Kärre, 1990). On the other hand, according to *"induced-self"* hypothesis virus-infected or tumor cells increase the expression of proteins which are generally poorly expressed by normal cells; these proteins act as ligands of NK cell activating receptors and induce the activation of these effector lymphocytes (Raulet, 2004).

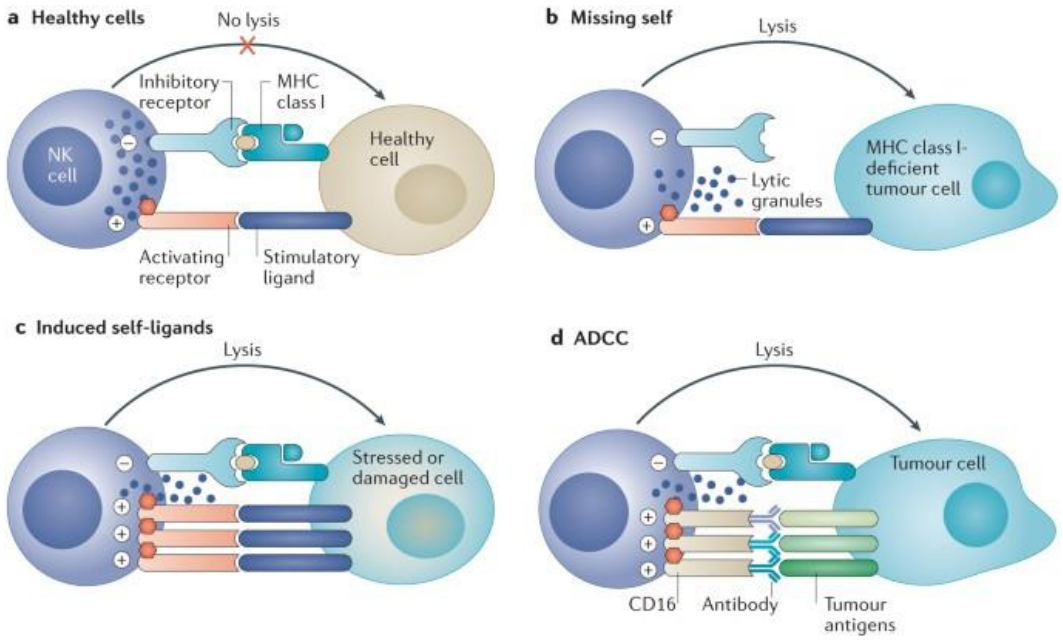

Fig. 5. Regulatory mechanisms of NK cell activation. (Morvan & Lanier, 2016)

In addition to successful target recognition, activation of NK cell cytotoxicity can also be triggered or enhanced by other factors, either physiological or exogenous. Interleukins (mostly IL-2, IL-12, IL-15, IL-18, and IL-21) play the main role in NK cell activity modulation (Y. Wu et al., 2017). The application of PMA-ION (Phorbol 12-Myristate 13-Acetate Ionophore Ionomycin), which leads to increased NK cell degranulation, is another classical approach used to induce NK cell activity (Romera-Cárdenas et al., 2016). Many compounds have also been identified as activators of PKC (Protein Kinase C), which plays an important role in the lytic signaling pathway in NK cells; hence, its activation is crucial to maintain NK cell cytotoxicity (Rana & Whalen, 2015). Stimulation of NK cells generally leads to increased killing of target cells and enhanced IFN-γ and TNF-α production, or higher level of degranulation.

NK cell cytotoxicity is a multi-step starting with receptor-ligand interactions and formation of an immunological synapse (IS). This is followed by recruitment of filamentous actin (F-actin) to the IS and polarization of the lytic granules and the microtubule-organizing center (MTOC) toward the IS. Then, the granules dock at the synapse and are ready for the final step: granule-membrane fusion and release of the cytotoxic contents at the center of the IS (Chen et al., 2007; Mace et al., 2012; Mizesko et al., 2013).

Cytotoxic granules contain perforin, pore-forming protein, and granzymes-serine proteases. Perforin generates pores in the target cell membrane allowing granzymes to enter the cell and initiate the apoptosis by caspase-dependent and independent pathways. In death receptor-mediated cytotoxicity, death ligands [FasL, TNF, TRAIL (TNF-related apoptosis-inducing

ligand) and LTα] expressed by activated NK cells engage the death receptors on the target cell surface, thereby activating the caspase cascade leading to the death of this cell (Screpanti et al., 2001; Smyth et al., 2005).

4.3 NK Cell Receptor Repertoire

As described above, NK cells are able to detect and kill target cells via a complex mechanism based on a dynamic integration between activating and inhibitory signals generated by the binding of different surface receptors on NK cells that recognize their ligands expressed by target cells (Vivier et al., 2004; Raulet & Vance, 2006).

4.3.1 NK Cell Activating Receptors

Activating receptors are necessary to activate NK cell effector functions. It was demonstrated that by blocking one or more activating receptors with antibodies, NK cells lost the capability to detect and lyse tumor cells (Long et al., 2013).
There are multiple MHC-dependent or MHC-independent activating receptors on NK cells such as NKG2D (Natural Killer Group 2 D), DNAM-1 (DNAX Accessory Molecule-1, CD226), NCRs (Natural Citotoxicity Receptors) and 2B4, in addition to the mentioned CD16 (Chester et al., 2015).
Here, some of these most important activating receptors are illustrated, focusing on the receptors NKG2D and DNAM-1, that have a prominent role in tumor immunosurveillance.

4.3.1.1 NKG2D

NKG2D belongs to NKG2 family of C-type lectin-like receptors. It is expressed by NK cells, $CD4^+$ and $CD8^+$ T cells and NKT cells and recognize MIC (MHC class I chain-related molecule)A, MICB and ULBP (ULl6-Binding Proteins)1-6 proteins (Lanier, 2015). NKG2D expression is regulated at transcriptional and post-translational levels by different cytokines: IL-2, IL-7, IL-12 and IL-15 upregulate its expression, while TGF-β, IFN-β1 e IL-21 inhibit it (Castriconi et al., 2003; Burgess et al., 2006; Zhang et al., 2008). NKG2D activates NK cells by cooperating with other activating receptors, such as CD16, NKp46, and 2B4. There are two different adaptor proteins, DAP10 and DAP12, both of which can associate with NKG2D and mediate activation via two different signaling pathways. Studies have shown that there are two

NKG2D splicing variants in mouse NK cells. Resting murine NK cells express the longer protein (NKG2DL), which can associate with DAP10 only. In contrast, activated murine NK cells express the shorter protein (NKG2DS) which can associate with either DAP10 or DAP12. Human NK cells express only NKG2DL, which associates with DAP10 exclusively (Gilfillan et al., 2002; Rabinovich et al., 2006; Nabekura et al., 2017). The intracellular segment of DAP10 contains a YxxM motif which recruits PI3K and the Grb2 (Growth factor Receptor-Bound protein 2)-Vav1 (vav guanine nucleotide exchange factor 1) complex, promoting Ca^{2+} influx, cellular degranulation, and secretion of cytokines (Upshaw et al., 2006).

4.3.1.2 DNAM-1

DNAM-1 belongs to the Ig-SF and is expressed by NK cells, T cells, and monocytes. This receptor recognizes PVR (Poliovirus receptor, CD155) and Nectin-2 (CD112), physiologically expressed on the surface of neural, epithelial, endothelial cells, but overexpressed by some epithelial and hematologic tumors (Bottino et al., 2003; Fuchs & Colonna, 2006; Martinet & Smyth, 2015). DNAM-1 expression is stimulated by IL-2 and IL-15, but it is inhibited by TGF-β (Wilson et al., 2011; Hromadnikova et al., 2016). This receptor can transmit activating signals through synergetic effects with the integrin LFA-1 and the activating receptor 2B4. The intracellular region of DNAM-1 contains a special signaling structure, characterized by four tyrosine residues Y293, Y300, Y322, and Y325 and one serine residue, S329. A first phosphorylation occurs in the S329 by PKC (Protein Kinase C), allowing the association with LFA-1, which can bind to ICAM (Intercellular Adhesion Molecule)-1 on the target cells and promote the formation of IS. At the same time, interaction of DNAM-1 with its ligand triggers phosphorylation of the tyrosine residue Y322 in the intracellular domain by tyrosine kinase Fyn recruiting the complex Grb2-Vav1 and inducing Ca^{2+} mobilization and NK cell degranulation (Shibuya et al., 1999; Z. Zhang et al., 2015). In addition, the intracellular region of the DNAM-1 molecule also contains motifs for binding to the isoforms of actin-binding protein 4.1G. 4.1G is an important molecule in the membrane cytoskeleton structure, which can interact with the membrane-associated guanylate kinase (MAGUK) homologue and plays a part in the formation and anchoring of membrane protein complexes. The binding of these two protein families to the DNAM-1 molecule may be related to the formation of the cytoskeleton, the clustering of CD226 molecule, the narrowing of CD226 and LAF-1 molecules, and the entry of CD226 into lipid rafts (Shirakawa et al., 2006).

4.3.1.3 NCRs

The NCR family includes three members: NKp46 (NCR1, CD335), NKp44 (NCR2, CD336), and NKp30 (NCR3, CD337). These receptors lack ITAM sequences in the intracellular domain and need the association with different adaptors which can generate activating signals (Bottino et al., 2000).

NKp46 transmits activating signals in association with ITAM-related adaptors FcεRIγ and CD3ζ (Westgaard et al., 2004). NKp46 can recognize different ligands, including proteins overexpressed by melanoma and myeloma tumors, viral ligands, such as HA (Hemagglutinin), bacterial ligands, such as vimentin on the mycobacterium tuberculosis-infected cell surfaces and parasitic ligands, such as PfEMP1 (Plasmodium falciparum Erythrocyte Membrane Protein 1) (Barrow et al., 2019). NKp46 triggers NK cell cytotoxicity by synergistic effects with other activating receptors, such as 2B4, CD2, NKG2D, and DNAM-1, leading to enhanced Ca^{2+} flux (Bryceson et al., 2006; Zamai et al., 2020).

The ligands of NKp30 include the tumor cell ligands, such as B7-H6, BAG6/-BAT3 (BCL2-associated athanogene 6/nuclear HLA-B associated transcript-3 protein), and galectin-3, the viral ligands, such as HA of vaccinia virus and poxvirus and pp65 of human cytomegalovirus and the parasitic ligands PfEMP1 (Barrow et al., 2019). NKp30 acts synergistically with NKp46 and NKp44 in triggering the cytotoxicity of NK cells, thus playing an important role in anti-infection immunity and antitumor immunity. However, high levels of soluble BAG6 could be detected in the plasma of CLL, and its concentration increases in the advanced disease stages. This BAG6 isoform released by CLL cells was found to inhibit NK cell cytotoxicity. Differently, exosomal BAG6 could enhance NK cell killing (Reiners et al., 2013).

NKp44 is only expressed on activated NK cells. NKp44 ligands include the tumor cell ligands PCNA (Proliferating Cell Nuclear Antigen), PDGF-DD (Platelet-Derived Growth Factor DD), nidogen-1, and NKp44L [an isomer of MLL5 (Mixed-Lineage Leukemia-5 protein)], the viral ligand HA and bacterial ligands, such as Mycobacterium tuberculosis cell wall components. Furthermore, some subtypes of HLA-DP are also ligands of NKp44 (Barrow et al., 2019). NKp44 can transmit activating signal through the ITAM sequences of KAPAP/DAP12 (Cantoni et al., 1999). However, the intracellular domain of this receptor also contains ITIM sequences which could be efficiently phosphorylated, thus suggesting a possible role of inhibitory receptor for NKp44 (Campbell et al., 2004). Consistently it was demonstrated that tumor cells could overexpress PCNA, which can associate with HLA-I molecules forming an

inhibitory ligand complex recognized byNKp44 which suppresses the NK cell killing activity through its ITIM sequences (Horton et al., 2013).

4.3.1.4 2B4

The activating receptor 2B4 belongs to SLAM (Signaling Lymphocytic Activation Molecule) family. 2B4 is expressed on NK cells, $CD8^+$ T cells, monocytes, and other immune cells and binds to CD48, a molecule which is generally expressed on hematopoietic-origin cells and EBV-infected B cells (Brown *et al.,* 1998; Pahima *et al.,* 2019). The intracellular region of 2B4 contains ITSM (Immunoreceptor Tyrosine-based Switch Motifs) sequences, which are composed of TxYxxV/1 and are phosphorylated after the binding of 2B4 with its ligand, inducing an activating signaling cascade responsible for NK cell activation (Agresta *et al.,* 2018). Similar to NKG2D, 2B4 acts as an NK cell co-receptor and its function of transmitting signals in most cases relies on the participation of other activating receptors, such as NCRs, NKG2D, and DNAM-1. Such effects activate NK cell cytotoxicity and IFN-γ production and play an important role in antiviral and antitumor immunity (Sivori et al., 2000; Kim et al., 2010).

4.3.2 NK Cell Inhibitory Receptors

Most of inhibitory receptors recognize MHC class I molecules and generate inhibitory signals to suppress NK cell function and participate in autoimmune tolerance under physiological conditions to avoid the killing of normal cells. However, some non-MHC-restricted inhibitory receptors are also known. Inhibitory receptors are characterized by long intracellular tails with ITIM (Immunoreceptor Tyrosine-based Inhibitory Motifs) sequences, which are phosphorylated when receptors recognize their specific ligands. After phosphorylation, ITIMs activate PT-Pases (Protein Tyrosine-Phosphatases), mainly including SHP (Src Homology region 2-containing protein tyrosine phosphatase)-1 and SHP-2, downregulating multiple activating signal molecules by dephosphorylation (Yokoyama & Kim, 2006).
Inhibitory receptors mainly include KIR (Killer cell Immunoglobulin Receptors)-2DL, KIR-3DL, LIRs (Leucocyte Immunoglobulin-like Receptor), the heterodimer CD94/NKG2A (Natural Killer Group 2) and TIGIT.

4.3.2.1 KIRs and LIRs

KIR-2DL and -3DL belong to the immunoglobulin-superfamily (Ig-SF) and they differ for the number of Ig-like domains: KIR-2DL has two Ig-like domains and KIR-3DL has three Ig-like domains. The majorities of KIRs are highly specific for classic MHC-I molecules (HLA-A, HLA-B, and HLA-C). For instance, KIR2DL1, KIR2DL2, and KIR2DL3 are specific receptors of HLA-C molecules, and KIR3DL1 and KIR3DL2 can combine with HLA-A or HLA-B. Unlike other KIRs, KIR2DL4 recognizes both soluble and membrane HLA-G (Thielens et al., 2012).

LIR family also belongs to Ig-SF and includes LIR1 and LIR2, but only LIR1 is expressed by NK cells. This inhibitory receptor can recognize multiple MHC class I molecules, such as HLA-A, HLA-B, HLA-C and HLA-G. by specific interactions between the first and second domain of LRI1 and α3-domain and β2-microglobulin of the MHC-I molecules. In addition, it has been demonstrated that LIR1 binds UL18, a human cytomegalovirus (HCMV) encoded protein, with a greater affinity than for MHC-I molecules. This may represent an escape mechanism for CMV virus to the NK cell-mediated killing (Li et al., 2011).

4.3.2.2 CD94/NKG2A

The heterodimer CD94/NKG2A is a C-type lectin receptor with a carbohydrate-binding protein domain. The molecule CD94 is able to couple with different members of NKG2 family (A/C/E), but among these, only NKG2A contains ITIM sequences in the intracellular tail, transducing an inhibitory signal after the recognition of its specific ligand, the molecule HLA-E, a non-classical HLA class I molecule, which have a very limited polymorphism, unlike HLA proteins that are recognized by KIR and LIR receptors. Instead, CD94-NKG2C and CD94-NKG2E lack ITIM motifs and interact with the adaptor molecule DAP12 that contains an immuno-tyrosine activating motif (ITAM). It is interesting to note that, despite CD94-NKG2C and CD94-NKG2A are one activating and one inhibitory receptor, they are specific for the same molecule, HLA-E, but the reason and the implications of this remain unclear (Borrego et al., 2005).

4.3.2.3 TIGIT

TIGIT (T cell Immunoreceptor with Ig and ITIM domains) also belongs to Ig-SF and plays a critical role in limiting both innate and adaptive immunity. Indeed, it is expressed not only by

NK cells, but also by $CD8^+$ and $CD4^+$ T cells, Tregs and follicular T helper cells in humans. TIGIT is composed of an extracellular Ig variable domain, a type 1 transmembrane domain, and a cytoplasmic tail with two inhibitory motifs conserved in mouse and human: an ITIM and an ITT (Ig tail-tyrosine)-like motif. TIGIT binds the same ligands for DNAM-1, PVR and Nectin-2. It was also demonstrated that TIGIT binds PVR with higher affinity than competing receptor DNAM-1. After binding with PVR, the ITT-like motif is phosphorylated at Y225 and binds to cytosolic adapter Grb230 and β-arrestin to recruit SHIP-1. SHIP-1 impedes PI3K and MAPK signaling. SHIP-1 also impairs TRAF6 and NF-kB activation, leading to inhibition of IFN-γ production by NK cells (Li et al., 2014; Yin et al., 2018).

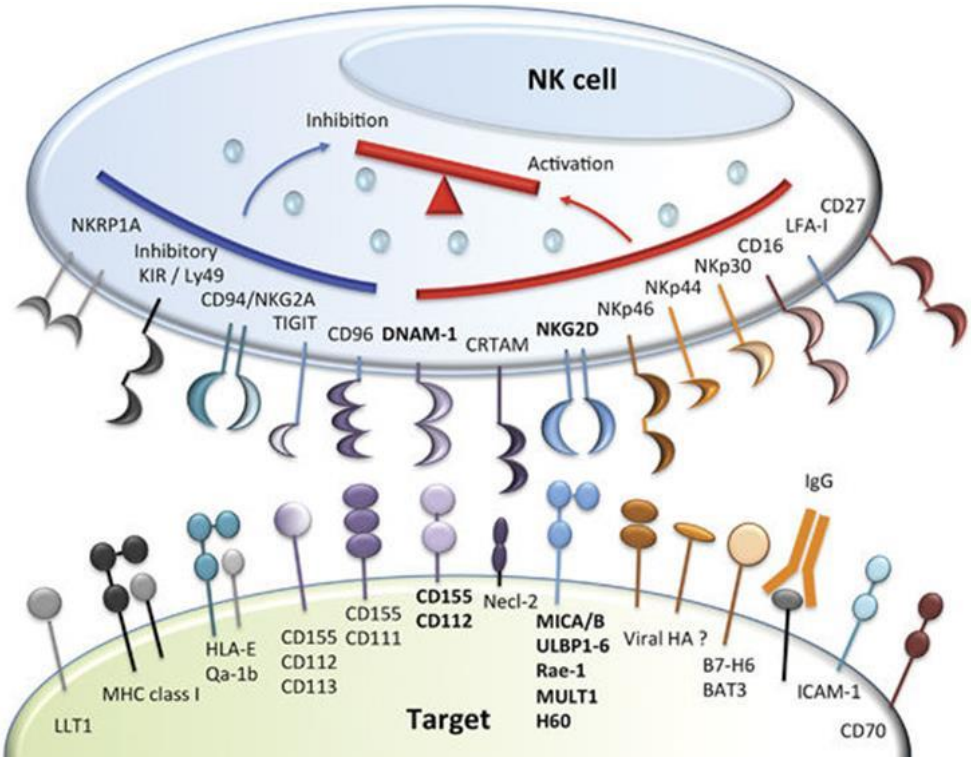

Fig. 6. NK cell receptor repertoire. (Chan et al., 2014)

5. Anti-MM Activity of NK Cells

It is well known that NK cells play an important role in the control of MM progression. *In vitro* studies demonstrated that NK cells purified from the blood of healthy subjects can kill primary malignant PCs and MM cell lines (Frohn et al., 2002; Katodritou et al., 2011); this occurs because MM cells express high levels of activating receptor NCRs, NKG2D and DNAM-1 ligands on their surface and can be recognized by NK cells (Carbone et al., 2005; El-Sherbiny et al., 2007). Moreover, many evidence supporting the capability of NK cells to kill MM cells have been obtained also through *in vivo* experiments in murine models where depletion of NK cells promoted MM growth and progression (Ponzetta et al., 2015).

However, MM have developed numerous strategies to evade immunosurveillance and detection by NK cells. First, in the BM microenvironment of MM patients, chemokines necessary for NK cell recruitment (*e.g.* SDF-1) are strongly reduced (Ponzetta et al., 2015). At the same time, there is an accumulation of immune cells with immunosuppressive properties, such as Treg and MDSCs. These cells and malignant PCs secrete high levels of immunosuppressive cytokines, such as TGF (Transforming Growth Factor)-β, IL-6 and IL-10. TGF-β induces the downregulation of NK cell activating receptors and impairs NK cell cytotoxicity; IL-6 has been shown to impair NK cell activity in experimental models, human disease, and when administered to patients with advanced cancer; IL-10 inhibits production of pro-inflammatory IFN-γ and TNF-α and promotes development of NK-resistant tumor phenotypes. Besides cytokines, other soluble factors are known to suppress NK-mediated antitumor capabilities: for instance, PGE2 (Prostaglandin E2) inhibits NK cell activating signals transduced by NCR, NKG2D, and CD16 (Urashima et al., 1996; Tsuruma et al., 1999; Martinet et al., 2010; Van Valckenborgh et al., 2012).

During MM progression, NK cells and malignant PCs undergo major phenotypic changes. Recently, a comprehensive immune phenotyping analysis by flow cytometry of freshly obtained NK cells from the peripheral blood and BM of MM patients and healthy donors was performed (Pazina et al., 2021). It was found that patients with active MM tend to have lower frequencies of NK cells as a percentage of total lymphocytes in PB, while relapsed and post-autologous stem cell transplanted patients have significantly higher NK cell frequencies, but lower percentage of $CD56^{dim}$ NK cell subset, which may be a consequence of the repopulation of NK cells after depletion by high-dose chemotherapy and SCT. Furthermore, downregulation of the expression of the activating receptors NKG2D, DNAM1, 2B4, NKp30 and CD16 and increase of the levels of inhibitory receptors KIRs, TIM-3 and PD-1 were also observed in late

stage of disease (Martínez-Sánchez et al., 2016; Pazina et al., 2021). Several differences were also found regarding NK cell phenotype in BM and PB of MM patients. The percentage of total and terminally mature $CD57^{+}CD56^{dim}$ NK cells was significantly lower in BM compared to PB. The $CD56^{bright}$ NK cell subset is significantly more activated in the BM, as assessed by a greater fraction of cells expressing the activation marker CD69 (Pazina et al., 2021).

On the other hand, compared to MGUS, malignant PCs in an active MM stage have lower levels of NK cell activating receptor ligands MICA and MICB, but higher expression levels of NK cell inhibitory ligands MHC-I, HLA-E and PD-L1 (Carbone et al., 2005). Reduction of the expression level of MICA on MM cell surface is associated with an increase of the soluble form (sMICA) in the serum of MM patients (Rebmann et al., 2007; Von Lilienfeld-Toal et al., 2010). sMICA induces internalization of surface NKG2D, NCRs and chemokine receptors and promotes an impairment of NK effector functions. In addition, sMICA has been shown to promote the accumulation of MDSCs and macrophages with an immunosuppressive phenotype (Xiao et al., 2015).

Taken together, these data demonstrate that immunoescape of malignant PCs and NK cell immunity alterations already detectable in early myeloma, progress in a clinical stage-dependent manner and that immunotherapy modalities based on efficient NK cell effector function are likely to exert a more effective anti-myeloma effect when used in early-stage disease (Jurisic et al., 2007).

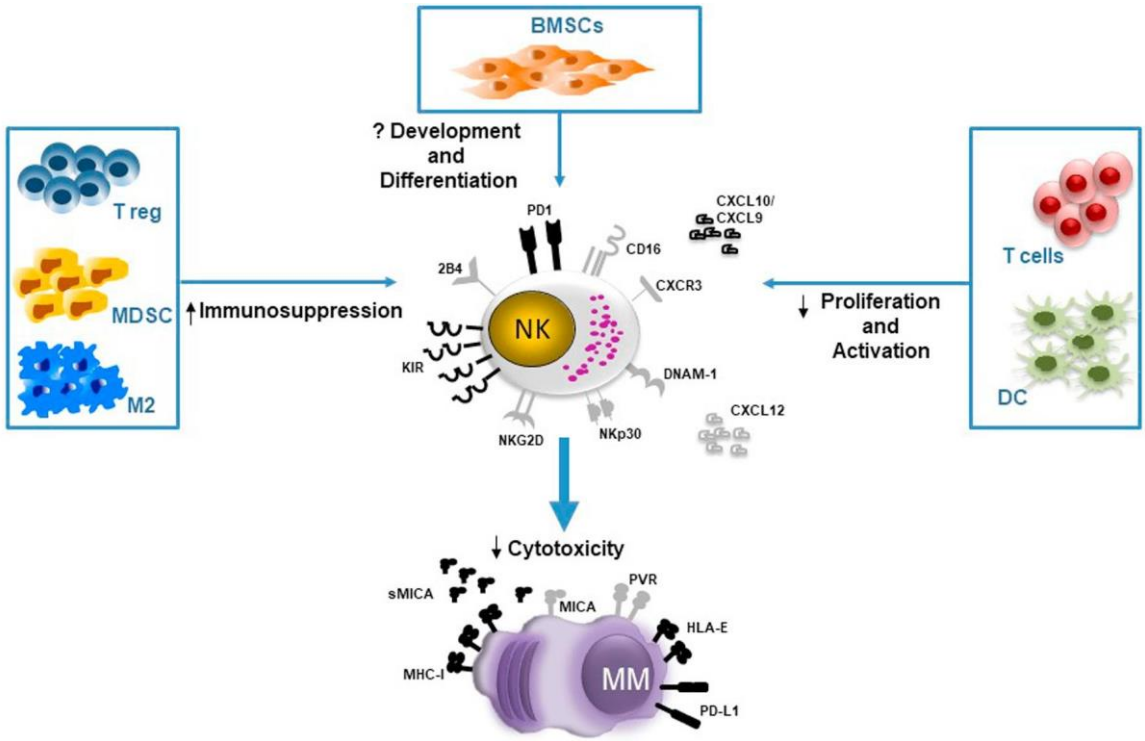

Fig. 7. Molecular mechanisms preventing NK cell activity in MM. (Fionda et al., 2018)

Several strategies to revert anti-MM activity of NK cells have been developed in the last years, such as the use of IMiDs, the synthesis of monoclonal antibodies (mAbs) and cell therapy.

IMiDs [Thalidomide, Lenalidomide (CC-5013) and Pomalidomide (CC4047)], have the ability to modulate humoral as well as cellular components of the innate and adaptive immune responses, including NK cell activity against cancer cells. In particular, an increased number of NK cells expressing higher levels of the activating receptors NKG2D, NKp30 and NKp44 has been observed in MM patients during IMiDs therapy (Hayashi et al., 2005; Quach et al., 2010); moreover, IMiDs enhance NK cell ADCC activity toward MM cells and can sensitize myeloma cells to NK cell cytotoxicity by inhibiting the expression of PD-L1 (Benson et al., 2010). The main molecular target of IMiDs is cereblon (CRBN) (Ito et al., 2010), a protein functioning as the receptor substrate of the cullin-4- RING ubiquitin ligase ($CLR4^{CRBN}$) complex, containing also cullin-4 (CUL4), the DNA damage binding protein-1 (DDB1), and the RING-finger protein (ROC1) (Fischer et al., 2014). Moreover, CRBN can function in a ubiquitin-independent manner by binding and modulating the membrane expression and/or the function of different proteins. IMiDs can differently regulate the ubiquitination and degradation of CRBN substrates. Indeed, through their glutarimide ring, IMiDs can interact with CRBN, thus altering the substrate specificity of $CLR4^{CRBN}$ complex or disrupting CRBN binding to other proteins (Eichner et al., 2016). Recent studies demonstrated CRBN-dependent and independent effects of IMiDs in NK cells. IMiDs treated-human NK cells have increased granzyme B (GZM-B) expression and cytotoxicity via Zap-70 activation and CRBN-induced degradation of IKZF3 (Hideshima et al., 2021). More recently, it was demonstrated that CRBN is implicated in the regulation of key effector functions of NK cells, such as migration and cytotoxicity, via Rac1 activation. Indeed, Lenalidomide is able to increase the ability of NK cells to migrate in response to chemotactic stimuli and to form conjugates with target cells, but these effects are blocked by silencing CRBN expression or by using a chemical inhibitor able to induce CRBN degradation (Fionda et al., 2021).

Monoclonal antibodies (mAbs) are mostly used for the treatment of hematologic cancers. In MM context, several mAbs have been synthetized, such as CT-011 e IPH2102, which are anti-PD-1 and anti-KIRs mAbs respectively and neutralize competitive negative signals derived from these inhibitory receptors resulting in enhanced trafficking, immune complex formation, and cytotoxicity of NK cells (Romagné et al., 2009; Benson et al., 2010). The mAb Elotuzumab promotes NK cell ADCC and prevents the adhesion to BM of MM cells, reducing their drug resistance by targeting SLAMF7 molecule, which is expressed by malignant PCs and at low

levels by NK and T cells (Collins et al., 2013); on the other hand, Daratumumab targets CD38, a molecule expressed by MM cells, enhancing their apoptosis (Overdijk et al., 2016).

Regarding cell therapy, there are several strategies that improve the quality of life and prolong the survival of patients with MM. In addition to autologous or allogeneic transplantation of stem cells from peripheral blood, a new technique has been developed in recent years: the in vitro activation and expansion of autologous or allogeneic NK cells and their re-infusion in patients with MM (Alici et al., 2008; Blade et al., 2010). Furthermore, genetically modified NK cells expressing specific chimeric receptors for certain antigens present on malignant PCs (CARs: "Chimeric Antigen Receptors") also represent a promising strategy (CARs: "Chimeric Antigen Receptors") (Rezvani et al., 2017).

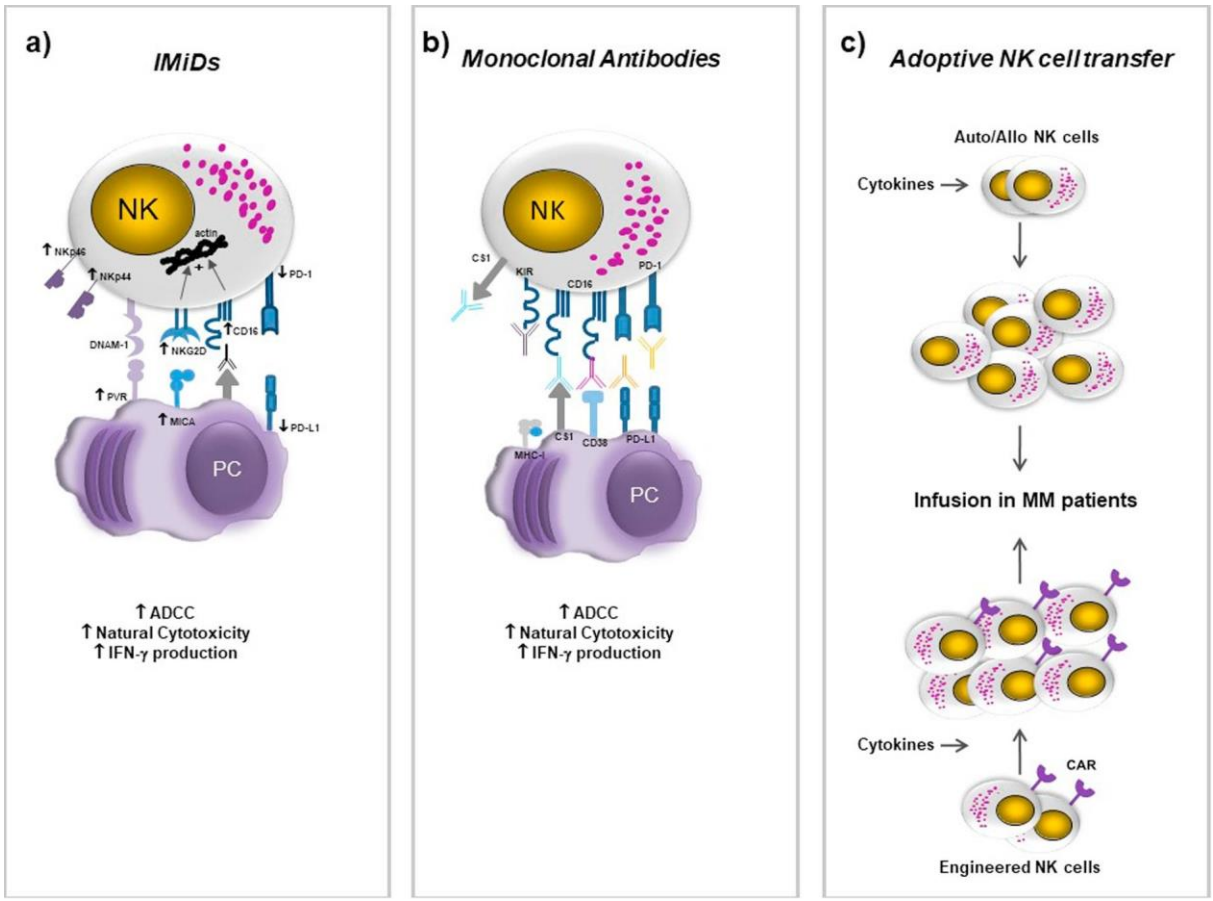

Fig. 8. NK cell-based therapies in MM. (Fionda et al., 2018)

6. Regulation of the Expression of NKG2D and DNAM-1 Ligands on MM cells

Multiple Myeloma cells express high levels of NKG2D and DNAM-1 ligands, which allow recognition and killing of malignant PCs by these effector lymphocytes (Carbone et al., 2005; El-Sherbiny et al., 2007). Therefore, understanding how the expression of these molecules is regulated could help to implement therapeutic strategies aimed to increase the recognition of MM cells by immune system cells.

Several studies from our laboratory have allowed the identification of transcriptional and post-translational regulatory mechanisms of NKG2D and DNAM-1 ligands on MM cells. It was demonstrated that the treatment with the genotoxic drugs Melphalan and Doxorubicin and the proteasome inhibitor Bortezomib. increase surface expression of MICA, MICB and PVR on MM cells. This mainly occurs through the activation of the kinases ATM (Ataxia Telangiectasia Mutated) and ATR (ATM and Rad 3 Related) as a result of the DNA damage response induced by the production of ROS (Reactive Oxygen Species). These two kinases promote the phosphorylation and the nuclear accumulation of the transcription factor E2F, which binds and activates *mica*, *micb* and *pvr* promoter (Soriani et al., 2009, 2014). To evade immune response, malignant PCs can inhibit the expression of NKG2D and DNAM-1 ligands by several mechanisms. For instance, it was shown that the transcription factor HSF1 (Heat Shock Transcription Factor 1) acts as an activator of *mica* and *micb* genes (B. Zhang et al., 2009; Schilling et al., 2015), but its action is inhibited by the binding with the molecular chaperones HSP-90 (Heat Shock Protein-90), which is overexpressed by MM cells (Fionda et al., 2009). Furthermore, MM cells constitutively express the kinase GSK3 (Glycogen Synthase Kinase 3), which activates a repressor of *mica* gene, the transcription factor STAT3 (Bedel et al., 2011), by phosphorylating its Y705. Indeed, treatment of MM cells with specific inhibitors of these pathways results in the increased expression of these ligands (Fionda et al., 2013). Moreover, it was demonstrated that MM cells treated with IMiDs upregulate the expression of MICA and PVR. This occurs because IMiDs inhibit the action of Ikaros and Aiolos, two repressors of *mica* and *pvr* promoters, and IRF-4, a transcription repressor of mica (Fionda et al., 2015). Pharmacological inhibition of members of BET (Bromodomain and Extra-Terminal) family represses the action of IRF-4 and c-MYC with consequent induction of MICA expression (Abruzzese et al., 2016). As described previously, either NKG2D or DNAM-1 ligands are regulated at post-translational level. In particular, MICB can undergo a proteolytic cleavage by metalloproteinase ADAM10, promoting its release in a soluble form. The same effect was detected for the long form of MICA in MM cells and it was demonstrated that the treatment

with Melphalan and Doxorubicin can enhance the action of ADAM10 (Zingoni et al., 2015); on the other hand, a post-translational modification named SUMOylation induces PVR intracellular retention and inhibits its translocation to the cell surface (Zitti et al., 2017). Instead, Nectin-2 is subject to the action of ubiquitination which promotes its degradation(Molfetta et al., 2019). It was shown that the inhibition of these post-translational modifications can enhance the recognition and killing of MM cells by NK cells.

MATERIALS AND METHODS

1. Reagents and Antibodies

The selective CXCR2 antagonist SB225002 and 7-AAD were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Anti-CD138/FITC (MI15) anti-CD138/Percp-cy5.5, anti-CD38/APC (HIT2), anti-CD107a/APC (H4A3), anti-CD3/FITC (SK7), anti-CD56/PE (NCAM16.2), anti-CD45/APC-H7 (2D1), anti-CD90/PeCy5 (5E10), anti-CD105/APC (266), anti-CD106/PE (51-10C9), anti-CD73/Percp-cy5.5 (AD2), anti-CD146/BUV395 (P1H12) anti-Mertk/BB700 (125518) and anti-Axl/BV711 (108724) monoclonal antibodies (mAbs) were purchased from BD Biosciences (Franklin Lakes, New Jersey, USA). Anti-CXCR2/PE (REA208) mAb was purchased from Miltenyi Biotec (Auburn, CA, USA). Anti-DNAM1 (DX11) mAb was purchased from Biorad (Hercules, CA, USA). Anti-MICA (MAB159227), anti-MICB (MAB236511), anti-ULBP1 (MAB170818), anti-ULBP2/5/6 (MAB165903), anti-ULBP3 (MAB166510), anti-Nect2 (R2.525), anti-NKG2D (MAB149810), anti-CXCR1 (MAB330) unconjugated mAbs and anti-PVR/PeCy7 (SKII.4), anti-MICA/AF488 and anti-Tyro3/AF488 (FAB859G) conjugated mAbs were purchased from R&D System (Minneapolis, USA). Anti-PVR mAb was kindly provided by Prof. M. Colonna (Washington University, St Louis, MO). Anti-MHC class I (W6/32) mAb was purchased from ATCC (Manassas, Virginia, USA). Allophycocyanin conjugated with goat anti mouse (GAM-APC) (Poly4053) mAb was purchased from Jackson Immuno-Research Laboratories (Cambridgeshire, UK).
Anti-p65 (C-20) and anti-Oct-1 (C-21X) mAbs were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). Anti-Gas6 (D3A3G) and anti-phosphop65 (93H1) mAbs were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-Actin (AC-15) mAb was purchased from Sigma-Aldrich. Donkey anti-rabbit (NA934V) or sheep anti-mouse (NA931V) secondary Hrp-linked mAbs were purchased from GE Healthcare (Wisconsin, MA, USA).

2. Cell lines and Clinical Samples

Human MM cell line SKO007(J3) was kindly provided by Prof. P. Trivedi (Sapienza, University of Rome, Italy). The human MM cell lines JJN3, KMS27, MM1S, and LP1 were kindly provided by Prof. Nicola Giuliani (University of Parma, Parma, Italy). MM cell lines were maintained at 37 °C and 5% CO2 in RPMI1640 (Euroclone, Milan, IT) supplemented

with 10% FCS, 2 mM L-glutamine, 100 U/mL penicillin and 100 U/mL streptomycin (complete RPMI1640). The human 293T embryonic kidney cells were purchased from ATCC (Manassas, VA, USA) and were maintained at 37°C and 5% CO_2 in DMEM medium (Euroclone) supplemented with 10% FCS, 2 mM L-glutamine, 100 U/mL penicillin and 100 U/mL streptomycin (complete DMEM). All cell lines were mycoplasma-free (EZ-PCR Mycoplasma Test Kit, Biological Industries, Kibbutz Beit-Haemek, Israel).

Bone marrow samples from MM patients were managed at the Division of Hematology, Department of Translational Medicine and Precision, Sapienza University of Rome. Informed consent in accordance with the Declaration of Helsinki was obtained from all patients, and approval was obtained from the Ethics Committee of the Sapienza University of Rome (RIF.CE: 5191). BM samples were centrifuged at 1400 rpm for 10 min to collect BM plasma which were used for ELISA assay. Then, BM samples were resuspended with a red blood lysis buffer 1X [1.5 M NH_4Cl, 100 mM $NaHCO_3$ and 10 mM EDTA in ddH_2O] and wash twice with physiologic solution. The percentage of primary malignant PCs was calculated by incubating BM cells with mAbs anti-CD138/FITC and anti-CD38/APC for 20 min in ice. After washing with PBS, cells were acquired with a FACSCanto II (BD Biosciences); MM cells are $CD138^+CD38^+$. BM cells were separated in two different fractions, $CD138^+$ and $CD138^-$, by using a commercial kit purchased from Miltenyi Biotech. Following the protocol, BM cellular pellet was resuspended with MACS Buffer (80 µl for 2 x 10^7 cells) and incubated for 15 min at 4 ° C with magnetic beads conjugated with an anti-CD138 mAb. After washing in MACS Buffer, the cell pellet was resuspended in 500 µl of the same solution and loaded on a LS column. More than 95% of the purified cells expressed CD138 and CD38.

$CD138^-$ fraction was harvested to obtain BMSCs. Cells were plated (1×10^6 per cm^2) in MEMα medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin and 100 U/mL streptomycin (complete MEMα) at 37 °C and 5% CO_2 . After 24 h, non-adherent cells were removed to select only adherent cells. In keeping with the ISCT recommendations (Domini *et al.,* 2006; Krampera *et al.,* 2013; Vishwanathan *et al.,* 2019), BMSCs were incubated for 20 min in ice with a combination of mAbs, including anti-CD45/APC-H7, anti-CD90/PeCy5, anti-CD105/APC, anti-CD73/Percp-cy5.5, anti-CD106/PE and anti-CD146/BUV395 and analyzed by a FACS LRSFORTESSA flow cytometer (BD Biosciences) and FlowJoV10 (Treestar Inc, Ashland, OR, USA) software. BMSCs were used only until fifth passage.

Patient	Sex/Age	Clinical stage	Monoclonal Ig	% of PCs
1	M/74	MM PD	IgGλ	8
2	F/63	Relapse	IgGλ	90
3	M/65	Relapse	IgGλ	70
4	M/61	Relapse	IgGk	55
5	M/67	Relapse	IgGk	15
6	M/73	Onset	IgGλ	61
7	F/74	Onset	IgGλ	23
8	M/65	Relapse	IgGλ	29
9	F/74	Smoldering	IgGκ	64
10	F/70	Onset	IgGκ	48
11	M/79	Relapse	IgGκ	34
12	M/59	Smoldering	IgGκ	40
13	M/55	Onset	IgGκ	38
14	M/61	Relapse	IgGκ	57
15	F/76	Onset	IgGκ	55
16	F/57	Smoldering	IgGλ	10
17	F/75	MGUS	IgGλ	7
18	M/80	Onset	IgGκ	25
19	M/88	Onset	IgGκ	15
20	F/60	Onset	IgGλ	25
21	F/71	Smoldering	IgGκ	4

Table 1. Clinical parameters of MM patients used for the analysis of MICA and PVR classified according to Durie and Salmon's Staging System.

Patient	Sex/Age	Clinical stage	Monoclonal Ig	% of PCs
1	M/74	MM PD	IgGλ	8
2	F/63	Relapse	IgGλ	90
3	M/65	Relapse	IgGλ	70
4	F/76	Onset	IgGκ	55
5	M/67	Relapse	IgGκ	15
6	M/61	Onset	IgGλ	61
7	F/74	Onset	IgGλ	23
8	M/73	Relapse	IgGκ	29
9	F/74	Smoldering	IgGλ	64
10	F/70	Onset	IgGκ	48
11	F/62	Onset	IgGκ	48
12	M/59	Smoldering	IgGκ	69
13	M/68	Relapse	IgGκ	9
14	F/65	Onset	IgGλ	73
15	F/67	Onset	IgGλ	22
16	M/79	Relapse	IgGκ	34
17	M/55	Onset	IgGκ	38
18	M/61	Relapse	IgGκ	57
19	F/71	Relapse	IgGλ	39
20	M/71	MGUS	IgGκ	10
21	F/65	MGUS	IgGκ	8
22	M/63	Relapse	IgGλ	1
24	F/71	MGUS	IgGκ	15
25	M/53	Smoldering	IgGλ	31

Table 2. Clinical parameters of MM patients used for the analysis of IL-8 and Gas6 by ELISA classified according to Durie and Salmon's Staging System.

3. Adipogenic and Osteogenic Differentiation

For adipogenic differentiation, 1×10^5 BMSCs were seeded on 6-well plates and cultured for 14 days with adipogenic induction medium containing 1 µmol/L dexamethasone, 0.5 mmol/L isobutyl methylxanthine and 100 µmol/L indomethacin. Cells cultured in complete MEMα served as a negative control. Adipogenic differentiation was assessed by Oil-Red-O staining (Sigma-Aldrich) according to the manufacturer instructions. Briefly, BMSCs were washed twice with PBS and fixed with Formalin 10% for 1 h. After two washes with water, BMSCs were incubated with isopropanol 60% for 5 min and then covered with Oil Red O Working Solution for 20 min. Cells were washed 5 times with water, incubated with hematoxylin for 1 min and then visualized under an optical microscope: lipid droplets appeared red and nuclei appeared blue. Adipogenic differentiation was also validated by Real-Time PCR analysis of *ppar-γ1* and *ppar-γ2* gene expression.

For osteogenic differentiation, BMSCs were seeded at 1×10^5 on 6-well plates and cultured for 21 days in osteogenic induction medium containing 0.1 µmol/L dexamethasone, 10 mmol/L β-glycerol phosphate, and 200 µmol/L ascorbate-2-phosphate. Cells cultured in complete MEMα were used as a negative control. Osteogenic differentiation was detected by von Kossa staining (Sigma-Aldrich). Following the protocol, BMSCs were washed twice with PBS and incubated with Formalin 10% for 15 min at 4°C. After washing with water, BMSCs were incubated with $AgNO_3$ for 30 min and then washed twice with water. After UV exposure for 1 h, cells were visualized under an optical microscope. Osteogenic differentiation was also validated by Real-Time PCR analysis of *osteopontin* and *runx2* gene expression.

4. Preparation of BMSC-CM and MVs

BMSC-conditioned medium (BMSC-CM) was collected from 72 h-culture of confluent BMSCs and was used to stimulate MM cells. BMSC-CM and complete RPMI1640 medium were separated in different fractions by Amicon Ultra-15 centrifugal filter devices (Merck Millipore, St.Louis, MO, USA) permeable to a range of molecular-weight according to the manufacturer's instructions. In detail, collection tubes were pre-activated with free serum-medium by centrifugation at 1000 x g for 10 min. The medium was carried in upper chamber of collection tubes and centrifuged at 5000 x g to allow to the medium to flow through the filters and to be fractioned. The upper fractions were recovered, by inverting the support and centrifugation at 1000 x g for 2 min. A cascade fractioning with a descending molecular weight

permeability were required to obtain BMSC-CM containing exclusively proteins with molecular weight higher than 100 KDa, 50 KDa, 30 KDa or 10 KDa.

In some experiments, BMSC-CM was pre-treated with proteinase K (250 μg/ml) for 1 h at 65°C and then inactivated by adding 0,1 mM phenylmethylsulfonyl fluoride (PMSF) and incubated at 95°C for 10 min.

For MV isolation, MVs-free medium was obtained by centrifugation of FBS at 100,000 x g for 3 h in a Beckman ultracentrifuge (Beckman Coulter, Brea, CA, USA). BMSCs cells were cultured for 72 h in MEM-α medium supplemented with 10% of FBS-MV free. Conditioned media was first centrifuged at 800 x g for 5 min to remove cells and then centrifuged at 4500 x g for 5 min to discard large debris. After ultracentrifugation at 20,000 x g for 60 min at 4°C, both supernatant and pelleted MVs were recovered. The supernatant was 0.22 μm filtered and used as conditioned medium lacking MVs (BMSC-CM DEPL.). The MVs were washed and re-suspended in PBS and MV total protein concentration was measured at 280 nm using a NanoDrop spectrophotometer (Thermo Fisher Scientific).

Transmission electron microscopy (TEM) of BMSC-isolated MVs was performed as following described. Briefly, MVs were fixed in 2% PFA and adsorbed on formvar-carbon-coated copper grids. The grids were then incubated in 1% glutaraldehyde for 5 min, washed with deionized water eight times, and then negatively stained with 2% uranyl oxalate (pH 7) for 5 min and methyl cellulose/uranyl for 10 min at 4°C. Excess methyl cellulose/uranyl was blotted off, and the grids were air-dried and observed with a TEM (Morgagni 268D, Philips Electronics, Eindhoven, The Netherlands) at an accelerating voltage of 80 kV. Digital images were taken with Mega View imaging software. DLS experiments were performed to measure MVs size and concentration. All the measurements were made at 25°C on a Zetasizer Nano ZS90 spectrometer (Malvern, UK) equipped with a 5 mW HeNe laser (wavelength $\lambda = 632.8$ nm) and a non-invasive back-scattering optical setup (NIBS). For each sample, the detected intensity was processed by a digital logarithmic correlator, which computes a normalized intensity autocorrelation function. Then, the distribution of the diffusion coefficient D was obtained by using the CONTIN method (Provencher SW. CONTIN: a general purpose constrained regularization program for inverting noisy linear algebraic and integral equations. Computer Physics Communications 1982; 27:229-42). D was converted into an effective hydrodynamic diameter DH through the Stokes–Einstein equation: $DH = KBT/(3\pi\eta D)$, where KBT is the system's thermal energy and η represents the solvent viscosity. Solvent-resistant micro cuvettes (ZEN0040, Malvern, Herrenberg, Germany) have been used for experiments with a sample volume of 40 μL. The count rates obtained were then corrected for the attenuator used. Results

are given as mean ± standard deviation of five replicates. MV size distribution and concentration were calculated by a recently proposed DLS-based non-invasive tool (Palmieri et al., 2014; Papi et al., 2005). For flow cytometry analysis, about 5 μg of MVs were labeled with mAbs CD45, CD90, CD73, CD105 or specific isotypes for 1 h at RT with low rotation (300 rpm). Then, MVs were washed with PBS and centrifuged at 13200 rpm for 1 h at 4°C and acquired using a FACSCanto II and data analysis was performed using the FlowJoV10 software. The size of MVs was estimated by comparing the forward scatter signals with those of reference microspheres obtained from flow cytometry sub-micron particle size reference kit (Thermo Fisher Scientific).

5. Immunofluorescence and Cytometric Analysis

SKO007(J3) MM cell line was cultured in 24-well tissue culture plates for 72 h at a concentration of 1.5 x 10^5 cells/mL in direct contact with 0.2 x 10^5 BMSCs, in presence of a transwell system (3-μm porous transwell support, Corning, New York, USA), in complete, fractioned, Gas6/IL-8-depleted or pre-treated with proteinase K BMSC-CM or RPMI1640. For direct contact experiments, SKO007(J3) cells were stained with 1 μM of Carboxy Fluorescein Succinimidyl Ester (CFSE; Thermo Fisher Scientific) for 30 min at 37°C and analyzed by using FACSCanto II. After 72 h, SKO007(J3) cells were stained with 0.5 μg of anti-MICA, anti-MICB, anti-ULBP1, anti-ULBP2/5/6, anti-ULBP3, anti-PVR/CD155 or anti-Nect2 primary mAbs for 30 min, washed with PBS, and stained GAM-APC secondary conjugated mAb. Nonspecific fluorescence was assessed by using 0.5 μg of an isotype-matched irrelevant mAb (R&D Systems) followed by the same secondary antibody. In all experiments, cells were stained with Propidium Iodide (PI, 1 μg/mL) in order to assess cell viability (always higher than 90% after the different treatments). Fluorescence was analyzed using a FACSCanto II flow cytometer and data were analyzed using FlowJoV10 software. In some experiments, SKO007(J3) cells were pretreated for 1 h at 4°C with anti-CXCR1 or IgG control (1 μg/10^6 cells) or were pre-treated with for 1 h at 37°C with a CRCX2 antagonist, SB225002 (12.5 nM) or vehicle control (DMSO). 10 μg of MVs were used to stimulate SKO007(J3) cells seeded in complete RPMI1640 or in BMSC-CM or in BMSC-CM Depleted. After 72 h, cells were stained with anti-PVR/Pecy7 conjugated mAb and analyzed as described above. Nonspecific fluorescence was assessed by using the respective isotype-matched irrelevant anti-IgG/Pecy7 mAb. All samples were also stained with Fixable Viability Stain APCH7 to discriminate cell viability.

U266, ARP1, MM1S, LP1 and KMS27 MM cell lines were seeded in BMSC-CM or complete RPMI1640 and after 72 h stained with anti-MICA or anti-PVR mAbs and analyzed as described above.

The membrane expression of NKG2D and DNAM-1 ligands was analyzed by direct staining immunofluorescence on patient-derived PCs (2 x 10^6 cells/mL) cultured for 48 h in BMSC-CM or complete RPMI1640 supplemented with IL-3 (20 ng/mL) and IL-6 (2 ng/mL). Primary MM cells were stained with anti-MICA/AF488 (5 μL/10^6 cells) and anti-PVR/PeCy7 (10 μL/10^6 cells) conjugated mAbs. Nonspecific fluorescence was assessed by using the respective isotype-matched irrelevant mAbs: anti-IgG/AF488 (5 μL/10^6 cells) and anti-IgG/PeCy7 (10 μL/10^6 cells). All samples were also stained with Fixable Viability Stain APC-H7 to discriminate cell viability and analyzed as described above.

6. Annexin V

Apoptotic cell death was evaluated using APC Annexin-V Apoptosis Detection Kit with PI (Thermo Fisher Scientific) according to the manufacturer's instruction. Briefly, 1.5 x 10^5 SKO007(J3) were cultured in 24-well plates in complete RPMI1640 medium or BMSC-CM. After 72 h, cells were washed once with PBS and resuspended with Binding Buffer 1X to 10^6/mL. Then, cells were incubated with 5 μL of Annexin-V/APC for 15 min at RT. After wash with Binding Buffer 1X, cells were resuspended in 200 μL of Binding Buffer 1X and 5 μL of PI Staining Solution were added. Cell populations were acquired using FACSCanto II flow cytometer and analyzed by using FlowJoV10 software.

7. Enzyme-Linked Immunosorbent Assay (ELISA)

BMSC-CM and whole MVs derived from BMSCs or BM plasma were analyzed for CXCL8/IL-8 or Gas6 ELISA following instructions provided by the manufacturer (R&D Systems). Briefly, a 96-well microplate was coated with Capture Antibody overnight, washed and incubated with Block Buffer for 1 h. After three washes, 100 μL of Standard or samples were added for 2 h and then incubated with Detection Antibody for 2 h. Then, microplate was washed three times and incubated for 20 min first with Streptavidin-HRP and then with Substrate Solution. After blocking reaction with Stop Solution, absorbance was measured at 450/540 nm with Victor2 Microplate Reader (Perkin Elmer–GMI Waltham, MA, USA); IL-8 and Gas6 concentrations

were calculated in correlation to a standard curve of control samples. Each incubation was performed at RT.

BMSC-CM was collected from 72 h culture of 2 x 10^4 BMSCs in 1 mL of serum-free medium in 24-well plates. Analysis of MVs was performed in the absence of lysis buffer and PBS was used as a control.

8. *In Vitro* NK Cell Expansion

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood of healthy donors by Lymphoprep (Nycomed, Oslo, Norway) gradient centrifugation. In detail, human peripheral blood was diluted in PBS and gently added to Lymphoprep with a ratio 2:1. Samples were centrifuged at 1600 rpm at room temperature (RT) for 20 min without brake. PBMCs layer was collected, washed twice with PBS, counted, and then co-cultured for 10 days with irradiated (30Gy) Epstein-Barr virus (EBV)-transformed B-cell line RPMI8266 at 37°C in a humidified 5% CO_2 atmosphere. On day 10, NK cell growth was assessed by staining with anti-CD56/PE and anti-CD3/FITC mAbs. Fluorescence was evaluated by FACSCanto II flow cytometer and FlowJoV10 software. The cell population was routinely more than 90% $CD56^+CD3^-$ cells.

9. Degranulation Assay and Cytotoxicity Assay

To assess the functional effect on NK cells of NKG2D and DNAM1 ligand upregulation on MM cells stimulated by BMSCs-CM, degranulation assays were performed with *in vitro* expanded primary NK cells as source of effector cells. NK cell degranulation was evaluated with the lysosomal marker CD107a. BMSCs-CM treated SKO007(J3) cells were incubated with NK cells at E:T ratio of 2.5:1 in a U-bottom 96-well tissue culture plate in complete RPMI 1640 medium at 37°C and 5% CO_2 for 3 h. 50 µM Monensin (Golgi-stop; Sigma-Aldrich) was added after 1 h.

Thereafter, cells were washed with PBS and stained with anti-CD3/FITC, anti-CD56/BV421 and anti-CD107a/APC mAbs (5 µL/10^6 cells) for 30 min at 4°C to gate the $CD3^-CD56^+$ NK cell population. All samples were also stained with Fixable Viability Stain APC-H7 (BD Biosciences) to discriminate cell viability. In some experiments, 10^6 cells were pretreated for 20 min at RT with 1 µg of anti-NKG2D and anti-DNAM-1 blocking mAbs. CD107a expression was evaluated on $CD3^-CD56^+$ NK cell population. Fluorescence was analyzed using a FACSCanto II flow cytometer and a FlowJo Flow V10 Cytometric Analysis Software.

$CD138^-$ BM sample fraction was used to perform autologous and heterologous degranulation assays. Briefly, $CD138^-$ cells were seeded at 10^6/mL in complete RPMI1640 supplemented with IL-2 (200 U/mL) for 48 h. Then, they were incubated at E:T ratio of 2.5:1 for 3 h at 37°C and 5% CO_2 with SKO007(J3) or autologous $CD138^+$ cells pre-treated with BMSC-CM or complete RPMI1640. CD107a expression was evaluated on $CD138^-CD3^-CD45^+CD56^+$ NK cells and analyzed as described above.

In some degranulation assays, healthy donor-derived PBMCs were used as effector cells. They were incubated at E:T ratio of 1:1 for 3 h at 37°C and 5% CO_2 with SKO007(J3) pLKO.1-Control/Gas6 shRNA. When a blocking Ab was used, one million of cells were pretreated for 20 min at RT with 1 μg of anti-NKG2D. CD107a expression was evaluated on $CD3^-CD14^-CD19^-CD45^+CD56^+$ NK cells and analyzed as described above.

NK cell cytotoxicity was evaluated by 7-AAD assay. SKO007(J3) target cells were seeded in complete RPMI1640 or BMSC-CM for 72 h. Then, they were labeled with 1 μM CFSE for 30 min at 37°C and incubated with NK cells at different E:T ratios at 37°C and 5% CO_2. CFSE-labeled target cells alone were used as negative control in each test. After coculture for 4 h, the cell mixture was stained with 7-AAD (5 μg/mL) for 20 min, washed with PBS and fixed by PFA 1%. Fluorescence was analyzed using FACSCanto II flow cytometer and FlowJoV10 software. NK cell cytotoxicity was calculated as cells positive for both CFSE and 7-AAD, after substracting the spontaneous lysis in negative control.

10. Western Blotting

SKO007(J3) cells (5 x 10^6) were stimulated by BMSC-CM or complete RPMI1640 for 24 h at 37°C. For the analysis of Gas6 expression, SKO007(J3) pLKO.1-Control and pLKO.1-Gas6 shRNA were pretreated overnight with Brefeldin A (BFA, 5 μg/mL). Then, cells were pelleted, washed with PBS, resuspended in lysis buffer [1% NP-40, 10% glycerol, 0.1% SDS, 0.5% Sodium Deoxycholate, 1 mM PMSF, 10 mM NaF, 1 mM Na_3VO_4, complete protease inhibitor mixture (Roche, Bale Switzerland) in PBS] and subsequently incubated 15 min on ice. The lysate was centrifuged at 13200 rpm for 15 min at 4°C and the supernatant was collected as whole cell extract. Protein concentration was determined by the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). In detail, a solution containing reagent A and reagent B at ratio 1:50 was plated in 96 flat microplate wells (200 μL/well). Two wells of protein free-Pierce BCA solution were necessary to measure the basal absorbance, while 3 μL of protein suspension were added in duplicate for each condition. The microplate was incubated at 37°C for 30 min,

and absorbance was measured at 595 nm in a Victor 2 Microplate Reader (Perkin Elmer). For nuclear protein extraction, SKO007(J3) (10 x 10^6) cells were first resuspended in a lysis buffer [50 mM KCl, 0.5% NP40, 25 mM Hepes, 1 mM PMSF, 20 µg of apoprotein, 10 µg of leupeptin, 1 mM Na_3VO_4, 100 µM DTT] for 4 min at 4°C and centrifuged at 5000 rpm for 5 min. Then the supernatants were transferred in a new eppendorf and pellets were resuspended in a wash buffer [50 mM KCl, 25 Mm Hepes, 1 mM PMSF, 20 µg of apoprotein, 10 µg of leupeptin, 1 mM Na_3VO_4, 100 µM DTT] and centrifuged at 5000 rpm for 5 min. Pellets were then resuspended in a nuclear extraction buffer [500 mM KCl, 25 mM Hepes, 1 mM PMSF, 20 µg of apoprotein, 10 µg of leupeptin, 1 mM Na_3VO_4, 100 µM DTT, 10% glycerol], by gentle rotation for 180 min at 4°C. Protein concentration was determined by the Pierce BCA Protein Assay Kit as previously described. Nuclear extracts or whole extracts (65µg) were run on 10% denaturing SDS-polyacrylamide gels. Proteins were then electroblotted on nitrocellulose membranes (GE Healthcare), stained with Ponceau to verify that similar amounts of proteins had been loaded in each lane and incubated for at least 1 h in 3% bovine serum albumin (BSA) diluted in TBST buffer. Nitrocellulose membranes were incubated for 1 h or overnight with anti-p65, anti-phospho65, anti-Gas6, anti-Oct-1 or anti-Actin primary mAbs diluted in TBST-3%BSA. Nitrocellulose membranes were washed in TBST buffer and incubated with donkey anti-rabbit or sheep anti-mouse (1:10000) secondary mAbs diluted in TBST-3%BSA for 1 h at RT. After three washes with TBST, immunoreactive bands were visualized by incubating nitrocellulose membranes with ECL substrate WESTAR ηC ULTRA 2.0 (Cyanagen, Bologna, Italy) for 2 min at RT and acquired by using ChemiDoc Imagers (Biorad).

11. Preparation of Plasmid DNA

Plasmid DNA (100 ng) was added to bacteria cell suspension, and incubated in ice for 5 min, at 42°C for 1 min and in ice again for 5 min. After addition of 100 µL of S.O.C medium (Thermo Fisher Scientific), the reaction was seeded on LB agar plate containing ampicillin (AMP) (100 µg/mL) to select the growth of plasmid transformed-bacteria. After overnight incubation at 37°C, a single resistant bacteria colony was seeded in 120 mL of Terrific Broth (Sigma-Aldrich) containing AMP (100 µg/mL) and growth at 37°C for 18 h. Bacteria pellet was obtained by centrifugation at 3000 rpm for 20 min at 4°C and plasmid DNA was prepared using "Qiagen plasmid maxi kit" (Qiagen, Hilden, Germany). Bacteria pellet was resuspended with 10 mL of buffer P1 and lysed with 10 mL of buffer P2. After incubation at RT for 5 min, 10 mL of pre-chilled buffer P3 were added into bacteria suspension, vigorously inverted 4-6 times every 5

min, incubated on ice for 20 min and centrifuged for 30 min at 4000 rpm at 4°C. Supernatant was applied in a pre-equilibrated Qiagen-tip column via 10 mL of QBT buffer. After washing of column with 30 mL of QC wash buffer, plasmid DNA was eluted with 15 mL of QF buffer, and precipitated by adding of 10,5 mL of Isopropanol, followed by a centrifugation at 4000 rpm for 45 min. DNA pellet was washed with 2 mL of pre-chilled 70% ethanol, centrifuged at 13200 rpm for 15 min at 4°C and dried. Plasmid DNA was dissolved in a suitable volume of TE buffer and concentration was determined by measuring light absorbance at 260nm (A260) and the ratio of A260/A280 by Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific).

12. Plasmids

To generate the MICA-270 promoter plasmid, a fragment of 270 bp was obtained by cutting the 3.2 k wild type (WT) GFP reporter (Dr. Skov, University of Copenhagen, Frederiksberg, Denmark) with the enzymes KpnI and BglII, and cloned in pGL3-basic luciferase vector (Promega, Madison, Wisconsin, USA). The fragment (B) of the human PVR/CD155 promoter cloned in pGL2-basic luciferase vector (Promega) [pGL2-basic-PVR (B) promoter plasmid] was kindly provided by Dr. Bernhardt G. (Hannover Medical School, Hannover, Germany) (Solecki *et al.,* 1997).

The lentiviral vectors pLKO.1sh-Gas6 and pLKO.1sh-Axl, expressing a short hairpin RNA for Gas6 and Axl respectively and the puromycin gene resistance, were generated by inserting shRNA sequences in pLKO.1 lentiviral vectors. In detail, pLKO.1 lentiviral vector was cut with restriction enzymes Age1 and EcoRI and annealed reverse and forward shRNA sequences for Gas6 and Axl were cloned in open pLKO.1. Forward and reverse shRNA sequences were as follows:

human Gas6 shRNA forward: 5'-
CCGGGCAGACAATCTCTGTTGAGGACTCGAGTCCTCAACAGAGATTGTCTGCTTT
TTG-3';

human Gas6 shRNA reverse: 5'-
AATTCAAAAAGCAGACAATCTCTGTTGAGGACTCGAGTCCTCAACAGAGATTGTC
TGC-3';

human Axl shRNA forward: 5'-
CCGGCGAAATCCTCTATGTCAACATCTCGAGATGTTGACATAGAGGATTTCGTTT
TTG-3';

human Axl shRNA reverse: 5'
AATTCAAAAACGAAATCCTCTATGTCAACATCTCGAGATGTTGACATAGAGGATT
TCG-3'.

For knocking down IL-8 and Mertk, we used pLKO.1-shIL-8 (TRCN0000369178) and pLKO.1-shMertk (TRCN0000442967) lentiviral vectors and the control vector pLKO non-targeting shRNA (MISSION™ Sigma-Aldrich).

The retroviral vector pMSCV-Neo-IKBα-DN encoding for the neomycin gene resistance and the IKBα dominant negative (IKBα-DN), a mutated form of the human IKBα with the substitution of serine 32 and 36 by alanine residues insert, and the empty retroviral vector pMSCV-Neo (Murine Stem Cell Virus-Neomycin) were kindly provided by Prof. Hischott (McGill University, Montreal, Quebec, Canada). The lentiviral vector pHAGE-3xNF-kB-LUC-GFP expressing the green fluorescence gene insert and containing the luciferase gene driven by NF-kB-responsive consensus sequences was purchased from Addgene (Cambridge, Massachusetts, USA).

Envelope plasmid pVSG-5, lentiviral packaging plasmid pPAX2, retroviral packaging plasmid pGAG-Pol-Env, and pTK-Green Renilla (TK Renyl) control reporter vector for luciferase assay were purchased from Addgene (Cambridge).

13. Nucleofection

2 x 10^6 SKO007(J3) cells were mixed to plasmid DNA (2 µg of pGL3 basic-empty vector or pGL3 basic-270bp MICA promoter, pGL2 basic empty vector or pGL2 basic-PVR-B promoter fragment plus 1 µg of TK-Renyl) resuspended in 100 µL of SF cell line solution and transferred in a cuvette for nucleofection in a 4D-Nucleofector System (Lonza Walkersville, Maryland, USA). To decrease variations in the experiments due to different transfection efficiency, cells were transfected in single batches that were then separated into different treatment groups. Transfected cells were resuspended in 5 mL of complete RPMI1640 in T25 flask. After 24 h, cells were divided in three equal fractions and stimulated by 500 µL of BMSCs-CM in 24 well plate for 48 h or as control by 500 µL of complete RPMI1640. After additional 48 h, cells were harvested, and protein extracts were prepared for the renilla and luciferase assays.

14. Electroporation by Gene Pulser

TK renyl (2 µg), and pGL3 basic-empty vector, or pGL3 basic-270bp MICA promoter (5 µg) combined with pRC-CMV-empty vector or pRC-CMV-IKBαDN (5 µg) were added to SKO007(J3) cells (5 x 10^6/500 µL) of serum-free RPMI1640). For PVR promoter experiments, SKO007(J3) cells were co-transfected to express TK renyl (2 µg) and pGL2 basic-empty vector or pGL2 basic-PVR-B promoter fragment (5 µg) in combination with pRC-CMV-empty vector, or pRC-CMV-IKBαDN (5 µg). To decrease variations in the experiments due to different transfection efficiency, cells were transfected in single batches that were then separated into different treatment groups. All cells were electroporated in micro pulser cuvettes (Biorad). The gene pulser was set to deliver 210 mV/950 µF. Transfected cells were resuspended in 5 mL of complete medium RPMI in T25 flask. 24 h later, the cells were divided in three fractions and stimulated by 3 mL of BMSC-CM or as control 500 µL of complete RPMI1640 medium in 12 well plate for 48 h.

15. Luciferase Assay

Luciferase assays were performed using Promega Nano-Glo® Dual-Luciferase® Reporter Assay (Promega). Transfected SKO007(J3) cells were washed in PBS and resuspended 55 µL of lysis buffer 1X at RT for 15 min. Then samples were centrifuged at 13200 rpm for 15 min at 4°C and supernatant containing proteins was collected and transferred in a new eppendorf. Protein concentration was determined by the Pierce BCA Protein Assay Kit. The LAR II luciferase substrate (100 µL) were added to 65 µg of cell extract in a 96 well plate and immediately read in a GloMax®-Multi Detection System (Promega). Soon after, the renyl substrate Stop &Glo reagent (100 µL) was added in untreated sample to normalize DNA uptake.

16. *In Vitro* Virus Production and Cell Infection

HEK 293T cells (1.5 x 10^6) were seeded in a T25 flask. After 24 h, semiconfluent HEK 293T cells were washed with PBS and pre-treated for 30 min with 5 mL of serum-free Optimem medium (Thermo Fisher Scientific). For the generation of lentivirus 10 µg of plasmid DNA [1.25 µg of VSG-5, 3.75 µg of PAX2 (for lentivirus) or of GAG-Pol-Env (for retrovirus) and 5 µg of viral vector] was incubated for 5 min in 250 µL of serum-free Optimem. Then plasmid

DNA was mixed to 20 µL of Lipofectamine 2000 (Thermo Fisher Scientific) resuspended in the same volume of Optimem. After 20 min incubation, the solution was added to HEK293T cells for 5 h, then removed and replaced by complete DMEM medium. After a further 48 h culture, virus-containing supernatants were harvested, filtered in 0.45 µm filter and used to infect SKO007(J3) cells (0.5 x 10^6) or BMSCs (10^5), pre-treated for 1 h with Polybrene 4 µg/mL (Sigma-Aldrich). Cells were infected twice for 2 h with viral supernatant in the presence of Polybrene (8 µg/mL). Post-infection, cells were allowed to expand for 24 h and were then selected for neomycin (1 mg/mL) resistance for pMSCV retroviral vector or puromycin (1 µg/mL) resistance for pRETRO-SUPER and pLKO.1 vectors. After 4 days of selection, SKO007(J3) pLKO.1-Control or pLKO.1-Gas6/Mertk/Axl shRNA were seeded at 1,5 x 10^5/mL for 72 h without puromycin and then used for FACS analysis, Real-Time PCR and Western Blotting assay. For GFP-expressing viruses (pHAGE-3xNF-kB-LUC-GFP), the infection efficiency was measured by using FACSCanto II for GFP expression at day 3 after infection.

17. Real-Time Polymerase Chain Reaction

Total RNA was extracted using the total RNA mini Kit (Blood/Cultured cells) following instruction provided by the manufacturer Geneaid Biotech Ltd (Taipei, Taiwan). In detail, MM cells were lysed for 5 min in 400 µL of RB buffer. RNA was precipitated by adding of 70% ethanol (500 µL) to sample lysate and transferred in RB Column for a centrifugation at 13200 rpm for 1 min. To eliminate DNA, the RB Column was washed with W1 Buffer (400 µL) and Washing buffer (600 µL). The column matrix was then dried by centrifugation at 13200 rpm for 5 min, and the RNA was eluted in 50 µL of RNAse free water by centrifugation. The concentration and quality of the extracted total RNA were determined by measuring light absorbance at 260 nm (A260) and the ratio of A260/A280 by Nanodrop 2000 spectrophotometer. Reverse transcription was carried out in a 25 µL reaction volume with 1.5 µg total RNA according to the manufacturer's protocol for Moloney murine leukemia virus reverse transcriptase (Promega). In detail, each RNA sample was supplemented by Oligo(dT) primers (0.5 µg) and denatured at 70°C for 5 min. cDNA was then generated at 42°C for 1h in the presence of the enzyme reverse transcriptase (10 U), supplemented with RNAsin (2.5 units) and dNTP (12.5 nM). Real-Time PCR was performed using TaqMan assays and the ABI Prism 7900 Sequence Detection system (Applied Biosystems, Waltham, MA, USA). cDNAs were

amplified in triplicate with primers for *mica* (Hs00792195_m1), *pvr* (Hs00197846_m1), *cxcl8/il-8* (Hs00174103_m1), *gas6* (Hs1090305_m1) and *gapdh* (Hs03929097_g1) conjugated with fluorochrome FAM.

cDNAs obtained from RNA derived by differentiated BMSCs were amplified in triplicate with primers for human *osteopontin*, *runx2*, *ppar-γ1*, *ppar-γ2* and for *gapdh* by using the Power-SYBR green mix with ROX (Applied Biosystems). Primer sequences were as follows:

human *osteopontin* forward: 5'-TTGCAGCCTTCTCAGCCAA-3';

osteopontin reverse: 5'-GGAGGCAAAAGCAAATCACTG-3';

human *runx2* forward: 5'-ATGTGTGTTTGTTTCAGCAGCA-3';

runx2 reverse: 5'-TCCCTAAAGTCACTCGGTATGTGTA-3';

human *ppar-γ1* forward: 5'-CTATGGAGTTCATGCTTGTG-3';

ppar-γ1 reverse: 5'-GTACTGAGTACTGACATTTATTT-3';

human *ppar-γ2* forward: 5'-CGAGGACACCGGAGAGGG-3';

ppar-γ2 reverse: 5'-TGTGGTTTAGTGTTGGCTTCTT-3';

human *gapdh* forward: 5'-TCGACAGTCAGCCGCATCT-3';

gapdh reverse: 5'-CCGTTGACTCCGACCTTCA-3';

The level of ligand expression was measured using threshold cycle (Ct). The Ct was obtained by subtracting the Ct value of the gene of interest from the housekeeping gene (*gapdh*) Ct value. In the current study, we used Ct of the untreated sample as the calibrator. The fold change was calculated according to the formula $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct$ was the difference between Ct of the sample and the Ct of the calibrator (according to the formula, the value of the calibrator in each run is 1). The analysis was performed using the SDS version 2.4 software (Applied Biosystems).

18. Statistical Analysis

Statistical significance between two groups was determined by performing two-tailed, paired Student's t-test. Differences between multiple groups were analyzed with two-way analysis of variance (ANOVA). GraphPad Prism 6 (GraphPad, San Diego, CA, USA) software was used. Graphs show mean values, and error bars represent the SEM.

AIM OF THE STUDY

MM is a neoplasia caused by the uncontrolled proliferation of malignant PCs in the BM. A crucial role in the progression of MM is played by BMSCs, which, through direct interactions and/or production of numerous soluble factors, promote proliferation, survival and accumulation of malignant PCs in the BM.

Natural killer cells are key effectors in the immune response against MM. In particular, the engagement of the NKG2D, DNAM-1 and NCRs activating receptors plays a prominent role in the recognition and killing of MM cells by NK cells. However, malignant PCs have developed strategies to evade immunosurveillance and detection by NK cells. Therefore, understanding the mechanisms regulating the expression of these molecules on MM cells is essential for implementing new therapeutic strategies aimed to increase their susceptibility to immune system.

In this study, we evaluated the possible impact of BMSCs in the regulation of the expression of NK cell activating ligands on human MM cells.

RESULTS

1. BMSC-derived Soluble Factor(s) Enhance MICA and PVR Expression on Human MM Cells

Several studies from our laboratory elucidated the molecular mechanisms underlying the regulation of NKG2D and DNAM-1 ligands on human MM cells (Fionda et al., 2009, 2013, 2015; Soriani et al., 2009, 2014; Abruzzese et al., 2016; Zitti et al., 2017; Molfetta et al., 2019; Petillo et al., 2021). Here, we evaluated the possible impact of BMSCs on NK cell-mediated recognition and attack of MM cells.

To this aim, we isolated BMSCs from BM aspirates samples of newly diagnosed MGUS and ONSET-MM patients. BMSCs were phenotypically characterized by multiparametric flow cytometry analysis. In accordance with the criteria established from ISCT (Dominici et al., 2006; Krampera et al., 2013; Viswanathan et al., 2019), BMSCs express the combination of surface markers CD73, CD90, CD105, CD106 and CD146, but are negative for the hematopoietic marker CD45 (Fig. 1A). As another important criterion for defining BMSCs is their multipotency, we also evaluated their capability to differentiate into adipocytes and osteocytes if cultured in appropriate medium. By von Kossa staining we proved that BMSC-derived osteocytes showed calcium deposits, while via Oil Red O staining we revealed the presence of lipid droplets in BMSC-derived adipocytes (Fig. 1B, D). In addition, we confirmed an increased expression of osteocyte (*osteopontin* and *runx2*) or adipocyte (*ppar-γ1* and *ppar-γ2*) specific genes in these differentiated cells (Fig. 1C, E).

To evaluate whether BMSCs could affect NKG2D and DNAM-1 ligand expression on MM cells, CFSE-stained SKO007(J3) cells were seeded on a confluent monolayer of MGUS or ONSET BMSCs. After 72 h, NKG2D and DNAM-1 ligand expression on SKO007(J3) cell line was analyzed by flow cytometry. As shown in Fig. 1F, G, co-culture with BMSCs induced an upregulation of NKG2DL MICA and DNAM-1L PVR, whereas no effect was found for surface expression of MICB, ULBPs and Nectin-2. Moreover, a similar upregulation was observed for both MGUS and ONSET BMSCs, thus indicating that the capability of these cells to increase MICA and PVR expression on human MM cells was independent on the disease stage.

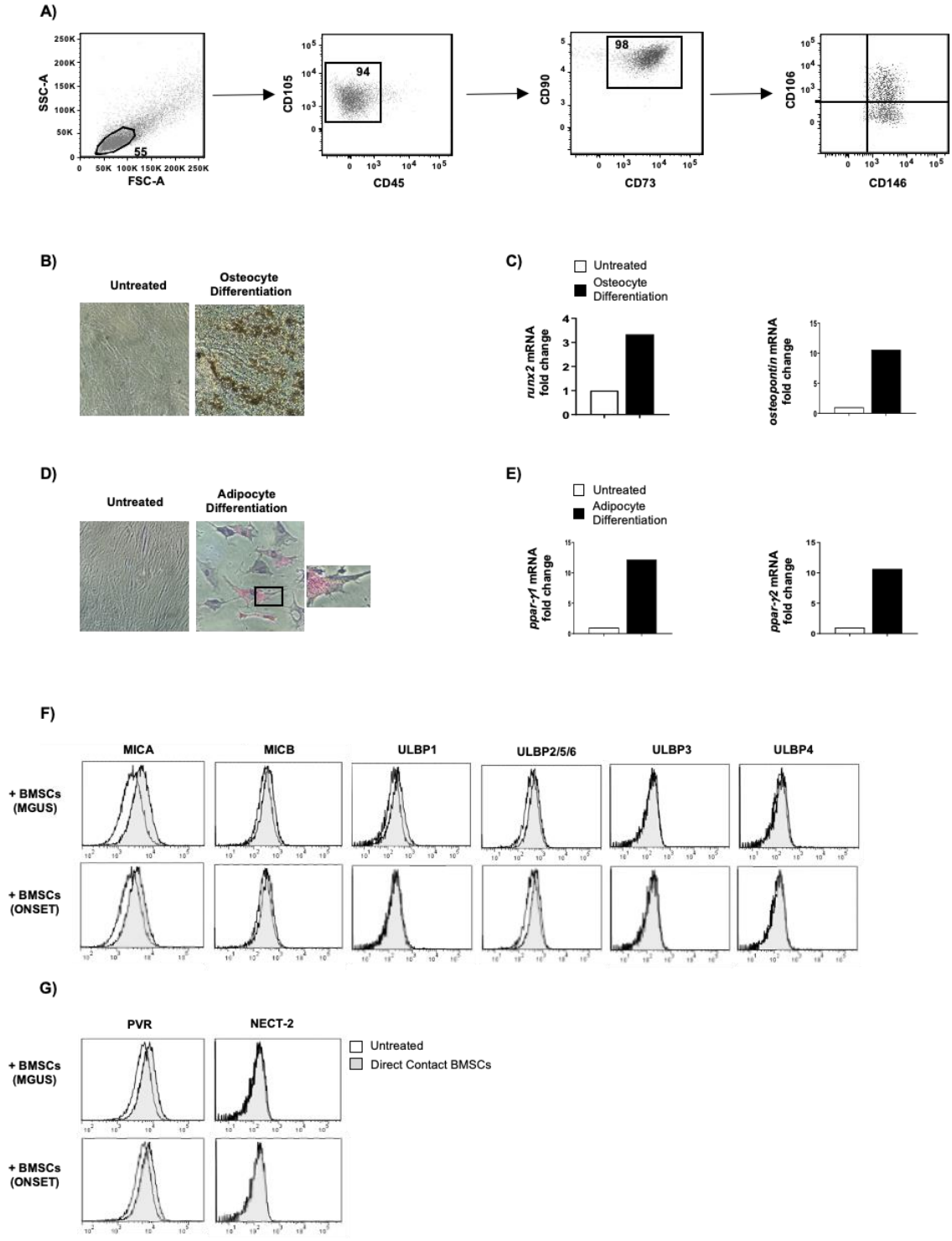

Fig. 1. Direct contact co-culture with BMSCs increases NK cell activating ligand expression on human MM cells. Primary BMSCs isolated from CD138$^-$ fraction of BM mononuclear cells from ONSET MM patients were subjected to multiparameter flow cytometry analysis. Cells were stained with anti-CD45/APC-H7, anti-CD90/PeCy5, anti-CD105/APC, anti-CD73/Percp-cy5.5, anti-CD106/PE and anti-CD146/BUV395 mAbs. Dot plots represent an example of gating strategy used (**A**). BMSCs were growth in osteogenic (**B**) or adipogenic (**D**) medium and assayed by von Kossa or Red Oil staining to evaluate their capacity to generate osteocytes or adipocytes. Total RNA was isolated from differentiated cells and assayed for the expression of osteogenic (**C**) or adipogenic genes (**E**) by Real-Time PCR analysis. Data, expressed as fold change units, were normalized with GAPDH and referred to the untreated cells, considered as calibrator. One out of three independent experiments is shown. The expression of MICA, MICB, ULBP1, ULBP2/5/6, ULBP3 and ULBP4 (**F**) or PVR and Nect-2 (**G**) was analyzed by flow cytometry analysis on CFSE-labeled SKO007(J3) MM cell line cultured in complete RPMI1640 for 72 h in the presence (Direct contact BMSCs) or absence (Untreated) of a confluent monolayer of BMSCs obtained from MGUS or ONSET MM patients. White histograms represent the basal expression of the indicated ligand, while gray histograms represent the expression following the co-culture in direct contact with the BMSCs. Shown results are representative of one of four independent experiments.

To discriminate the possible contribution of direct interactions and/or soluble factor(s) by BMSCs in NK cell ligand upregulation on MM cells, we performed co-culture experiments in the presence of a transwell with 3 μm pores which allows only diffusion of soluble factors. SKO007(J3) cells were placed in the upper chamber and BMSCs or complete RPMI1640 medium in the lower chamber. We observed an increase of MICA and PVR surface expression on SKO007(J3) cell when co-cultured with BMSCs even in the presence of this insert allowing only passage of soluble molecules (Fig. 2A). Accordingly, the same results were obtained by culturing SKO007(J3) cells in conditioned medium collected from 72 h culture of confluent BMSCs (BMSC-CM) (Fig. 2B), suggesting that BMSC-derived soluble factor(s) enhance the expression of MICA and PVR on MM cells. The possibility that these changes could be due to toxic and no specific effects of BMSC-CM on MM cells was excluded as these treatments did not affect cell viability over the time chosen for these experiments (as assessed by annexin V and propidium iodide staining) on SKO007(J3) cells (Fig. 2C). Based on these observations, we extended our analysis to additional MM cell lines and primary $CD138^+$ MM cells. As shown in Fig. 2D, we found that treatment with BMSC-CM was able to increase the basal expression of MICA on ARP-1 and U266 cell lines and of PVR on U266, MM1S, LP1 and KMS27 cell lines. We also observed that MM patient-derived PCs cultured in heterologous BMSC-CM for 48h expressed higher levels of MICA and PVR than primary MM cells cultured in RPMI1640 medium alone (Fig. 2E and Table 1).

All together, these results indicate that BMSCs are able to upregulate MICA and PVR expression on human MM cells and soluble factors play an important role in these mechanisms.

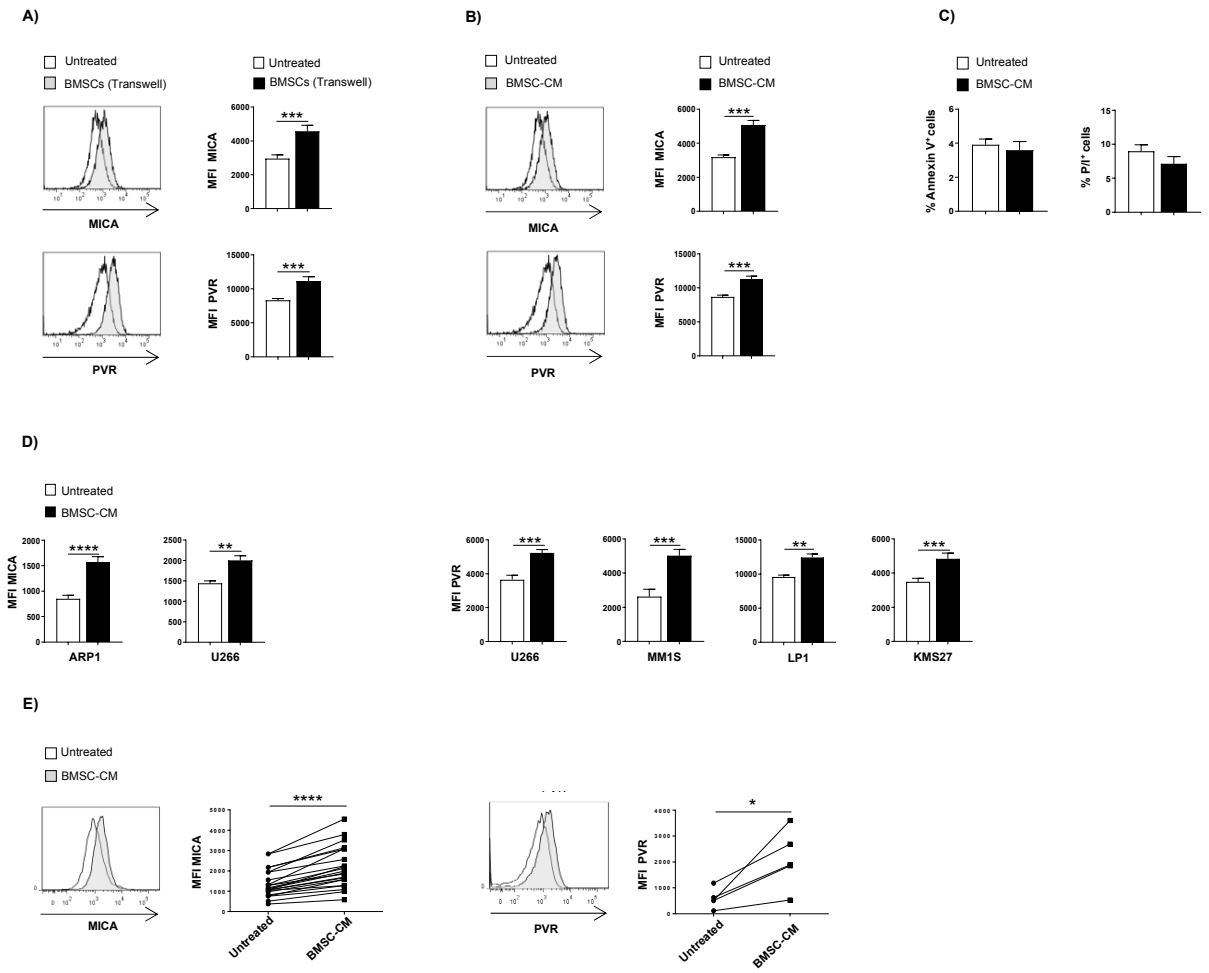

Fig. 2. BMSC-derived soluble factor(s) increase the expression of MICA and PVR on human MM cells. MICA and PVR expression was analyzed by flow cytometry on SKO007(J3) MM cell line cultured for 72 h with MM-BMSCs using a transwell support **(A)** or with BMSC-CM (**B**). A representative experiment is shown (left panels). Histograms represent the MFI of specific mAb - MFI of isotype control (right panels). Data show mean ± SEM calculated based on at least three independent experiments. *(****$p < 0.002$; paired Student t-test). (**C**) Staining of BMSC-CM treated SKO007(J3) cell line using Annexin-V/APC and Propidium Iodide. Histograms indicate the percentage of Annexin V or Propidium positive cells and were calculated based on at least 3 independent experiments. MICA and PVR expression was analyzed by FACS on the indicated MM cell lines (**D**) or on primary malignant PCs (**E**) untreated or treated with BMSC-CM for 72 h or 48 h, respectively. Histograms represent the MFI of specific mAb - MFI of isotype control. Data show mean ± SEM calculated based on at least three independent experiments. For patient-derived PCs cells, a representative experiment is shown on the left, data are shown in right panel where each dot represents a single patient and indicates the MFI of specific mAb-MFI of isotype *(***$p < 0.005$; ***$p < 0.002$; ****$p < 0.001$; paired Student t-test).

2. BMSCs Render MM Cells More Susceptible to NK Cell Recognition and Lysis

Because changes in the expression of MICA and PVR on tumor cells can modify their recognition by NK cells via NKG2D and DNAM-1 activating receptors, we tested whether co-culture of MM cells with BMSCs could also lead to increased NK cell activation. To this aim, we analyzed the degranulation of NK cells isolated from healthy donors against SKO007(J3) cells by evaluating the expression of the CD107a, a surrogate marker for cytotoxic granule exocytosis, by FACS analysis. Surface CD107a expression was determined by gating on NK cells upon their interaction with SKO007(J3) cells untreated or treated with BMSC-CM, used as targets (E:T ratio of 2.5:1). The upregulation of MICA and PVR on MM cells was verified before the degranulation assay. As shown in Fig. 3A-C, basal expression of CD107a on NK cells was enhanced following treatment of SKO007(J3) cells with BMSC-CM. Based on these findings, we evaluated the role of NKG2D and DNAM-1 receptors in MM cell recognition by performing the degranulation assay in the presence of specific blocking mAbs. Treatment of NK cells with NKG2D and DNAM-1 blocking mAbs decreased CD107a expression, whereas no change of expression was observed upon treatment with a control mAb, leading to the conclusion that NK cell degranulation involves these activating receptors.

To investigate whether BMSCs affect MM susceptibility to NK cell-mediated killing, we performed a CFSE-7AAD cytotoxicity assay. We incubated NK cells isolated from healthy donors with SKO007(J3) after culture in complete RPMI1640 medium or BMSC-CM. We found that killing of MM cells significantly increased following the treatment with BMSC-CM at all E:T ratios analyzed (Fig. 3D-F).

Importantly, a higher extent of degranulation was also observed in patient-derived NK cells against SKO007(J3) cells (Fig. 3G-I) or autologous primary $CD138^+$ PCs treated with BMSC-CM (Fig. 3L, M).

Overall, our results demonstrate that BMSC-mediated MICA and PVR upregulation on MM cells enhances NK cell degranulation and killing by promoting their NKG2D and DNAM-1-mediated recognition.

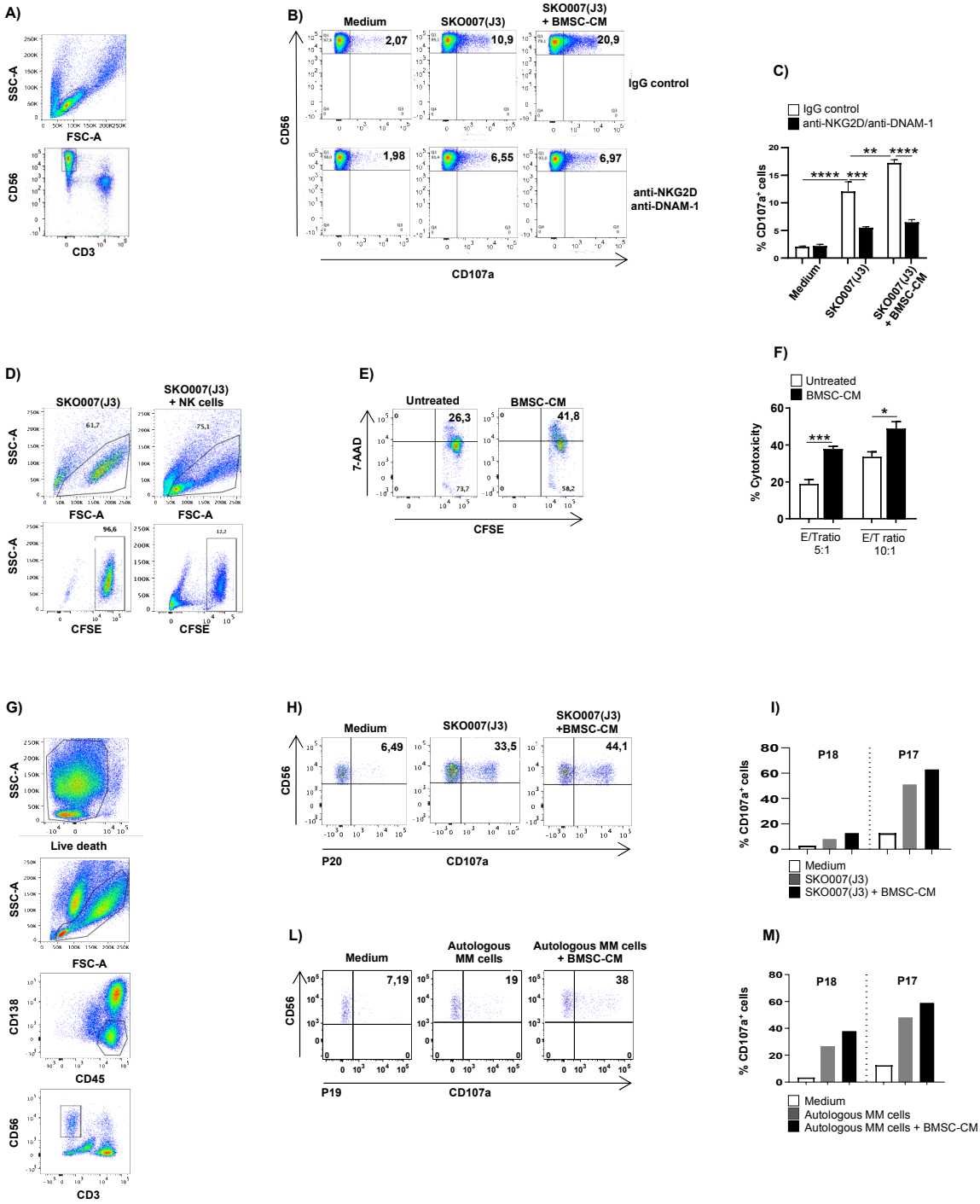

Fig. 3. BMSC-CM enhances the ability of human MM cells to stimulate NK cell degranulation and cytotoxicity by promoting NKG2D/DNAM-1 recognition. Cultured NK cells were incubated with SKO007(J3) cells, untreated or cultured for 72 h with BMSC-CM and used as target cells in a degranulation assay at E/T ratio of 2.5:1. Cell surface expression of CD107a was analyzed on $CD3^-$ $CD56^+$ NK cells [the gating strategy is shown (**A**)]. To evaluate the role of NKG2D and DNAM-1, the assay was performed in parallel treating NK cells with blocking anti-NKG2D and anti–DNAM-1 or anti-IgG mAbs used as control. Results are expressed as the percentage of $CD107a^+$ cells. A representative experiment is shown in (**B**). Histogram represents the mean ± SEM from three independent experiments (**C**) (*$p < 0.05$, ANOVA). CFSE-labeled SKO007(J3) untreated or treated with BMSC-CM for 72 h were co-cultured with NK cells isolated from healthy donors (E:T ratio 5:1). After staining of cell mixture with 7-AAD, dead target cells ($CFSE^+$ $7\text{-}AAD^+$) were analyzed by FACS analysis [(the gating strategy is shown in (**D**)]. A representative experiment is shown in (**E**). Results are expressed as the percentage of $CFSE^+$ $7\text{-}AAD^+$ cells. Histogram represents the mean ± SEM from three independent experiments (**F**) (*$p < 0.05$; ***$p < 0.002$; ANOVA). $CD138^-$ BM cells were incubated with SKO007(J3) (**H**) or autologous primary malignant PCs (**L**) untreated or treated with BMSC-CM for 72 h and used as target cells in a degranulation assay. The assay was performed at E: T ratio of 2.5:1. Cell surface expression of CD107a was analyzed on $CD3^-CD138^-CD45^+CD56^+$ NK cells [the gating strategy is shown in (**G**)]. Results obtained from 3 patients for each condition [P17, P18 and P20 in (**H, I**); P17, P18 and P19 in (**L**, **M**)] are shown.

3. MICA and PVR Upregulation on Human MM Cells by BMSCs Occurs at Transcriptional Level and Involves NF-kB Transcription Factor

To explore the possibility that BMSCs could upregulate MICA and PVR expression of MM cells at transcriptional level, total RNA was isolated from SKO007(J3) cells cultured in complete RPMI1640 medium or BMSC-CM for 48 h and analyzed by real-time quantitative RT-PCR. We observed an upregulation of *mica* and *pvr* mRNAs in SKO007(J3) cells treated with BMSC-CM (Fig. 4A, D); moreover, similar results were obtained in BMSC-CM-treated malignant PCs isolated from different MM patients (Fig. 4B, E). Then, we analyzed the effects of BMSC-CM on SKO007(J3) cells transiently transfected with *mica* and *pvr* gene promoters. As shown in Figure 4C, F, BMSC-CM enhanced the activity of the reporter gene driven by a 270 bp fragment of the *mica* or a 343 bp fragment (B) of the *pvr* promoter.

Collectively, these data indicate that MICA and PVR mRNA expression and promoter activity are enhanced by BMSC-CM in MM cells.

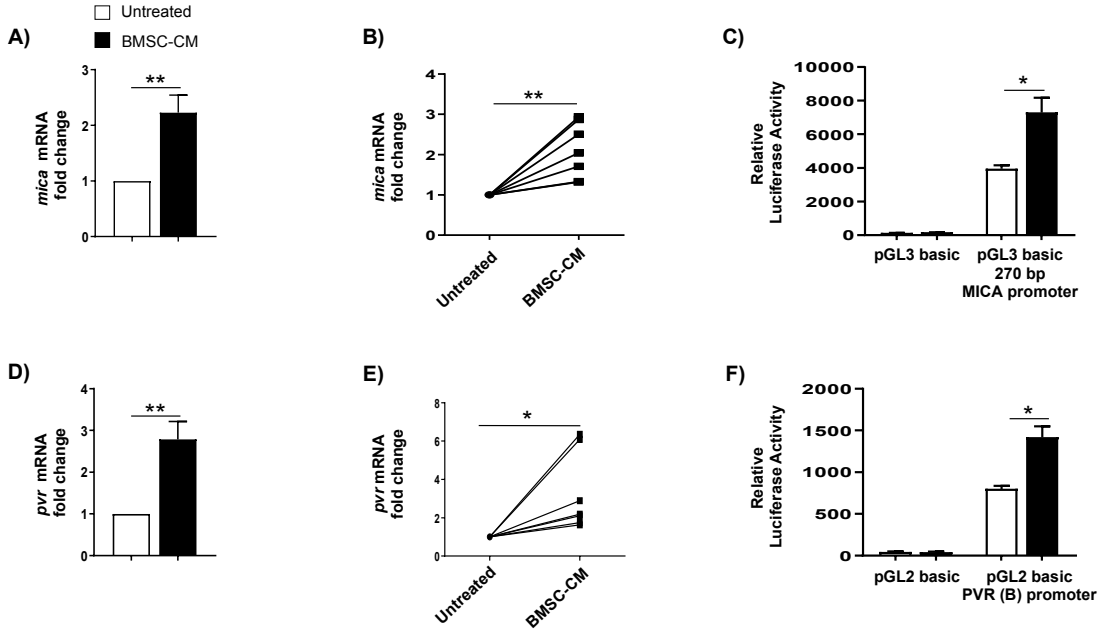

Fig. 4. BMSC-CM increases MICA and PVR mRNA expression and promoter activity in human MM cells. Real-Time PCR analysis for the expression of *mica* (**A, B**) or *pvr* (**D, E**) genes on total mRNA obtained from SKO007(J3) cells (**A, D**) or patient-derived PCs (**B, E**) after 48 h stimulation with BMSC-CM or complete RPMI1640 medium. Data, expressed as fold change units, were normalized with GAPDH and referred to the untreated cells, considered as calibrator. For SKO007(J3) cells, histograms represent the mean ± SEM from three independent experiments. For primary myeloma cells, data are shown where each dot represents a single patient. (*$p < 0.05$; **$p < 0.005$; paired Student t-test). SKO007(J3) cells were transiently transfected with pGL3 basic empty vector/MICA 270 bp promoter plasmid (**C**) or pGL2 basic empty vector/PVR (B) promoter plasmid (**F**) as described in materials and methods. After 48 h of treatment with BMSC-CM, SKO007(J3) cells were harvested, and protein extracts were prepared for the luciferase assay. Data, expressed as relative luciferase activity, were normalized to protein concentration and renilla activity and represent the mean ± SEM from three independent experiments (*$p < 0.05$; paired Student t-test).

To identify possible molecule(s) involved in the upregulation of MICA and PVR by BMSCs, we focused our attention on NF-kB family transcription factors. These proteins are constitutively active in MM cells and a number of studies demonstrated increased NF-kB activity in MM cells in response to soluble factors produced by BMSCs (Markovina et al., 2010; Huynh et al., 2018; Roy et al., 2018). We first confirmed the capability of BMSC-CM to induce NF-kB activation in MM cells in our experimental setting. As nuclear translocation is an important step leading to NF-kB activation, total and nuclear extracts were prepared from SKO007(J3) cells cultured for 24 h in BMSC-CM or complete RPMI1640 medium and were analyzed by western blotting assay. We observed that treatment with BMSC-CM does not affect total amount of NF-kB subunit p65 (Fig. 5A), but can induce nuclear translocation of this protein (Fig. 5B). Then, we evaluated the possible effects of BMSC-CM on the activity of this transcription factor. To this purpose, we infected SKO007(J3) cells with a lentivirus construct expressing luciferase directed by three NF-kB responsive sites (pHAGE-3xNF-kB-LUC-GFP). We found that culture of MM cells with BMSC-CM for 72 h significantly enhances luciferase expression thus indicating an increased NF-kB transcriptional activity (Fig. 5C).

Overall, these findings indicate that BMSC-CM can induce NF-kB pathway activation in MM cells.

To examine the role of NF-kB in MICA and PVR regulation by BMSC-derived soluble factor(s), we transduced a dominant negative form of IKBα (pMSCV-Neo-IKBα-DN), the cytoplasmic repressor of NF-kB, by retroviral infection in SKO007(J3) cells. IKBα-DN, mutated in the amino acid residues Serine 32 (Ser^{32}) and Serine 36 (Ser^{36}), cannot be phosphorylated and degraded by proteasome, blocking p65 nuclear translocation. After selection in G418, the IKBα-DN overexpression was demonstrated in western blotting assays. As shown in Fig. 5D, treatment with phorbol myristate acetate (PMA), a known NF-kB inducer, was able to induce p65 nuclear translocation in SKO007(J3) cells infected with an empty control vector (pMSCV-Neo) but not in pMSCV-Neo-IKBα-DN overexpressing cells.

Flow cytometry analysis was performed on SKO007(J3) cells overexpressing IKBα-DN or empty control vector cultured in BMSC-CM or complete RPMI1640 medium. We observed that inhibition of NF-kB transcription activity induced an increase in the expression of the basal levels of MICA, but not of PVR and blocks the induction of both ligands by the BMSC-CM (Fig. 5E, F, H, I). Quantitative real-time RT-PCR also confirmed these data at mRNA level (Fig. 10B, E). Accordingly, the activity of *mica* and *pvr* promoters was also blocked when BMSC-CM-treated cells were co-transfected with IKBα-DN expression vector (Fig. 5G, L).

These data show that BMSC-derived soluble factor(s) enhance MICA and PVR expression on human MM cells *via* NF-kB transcription factor activity.

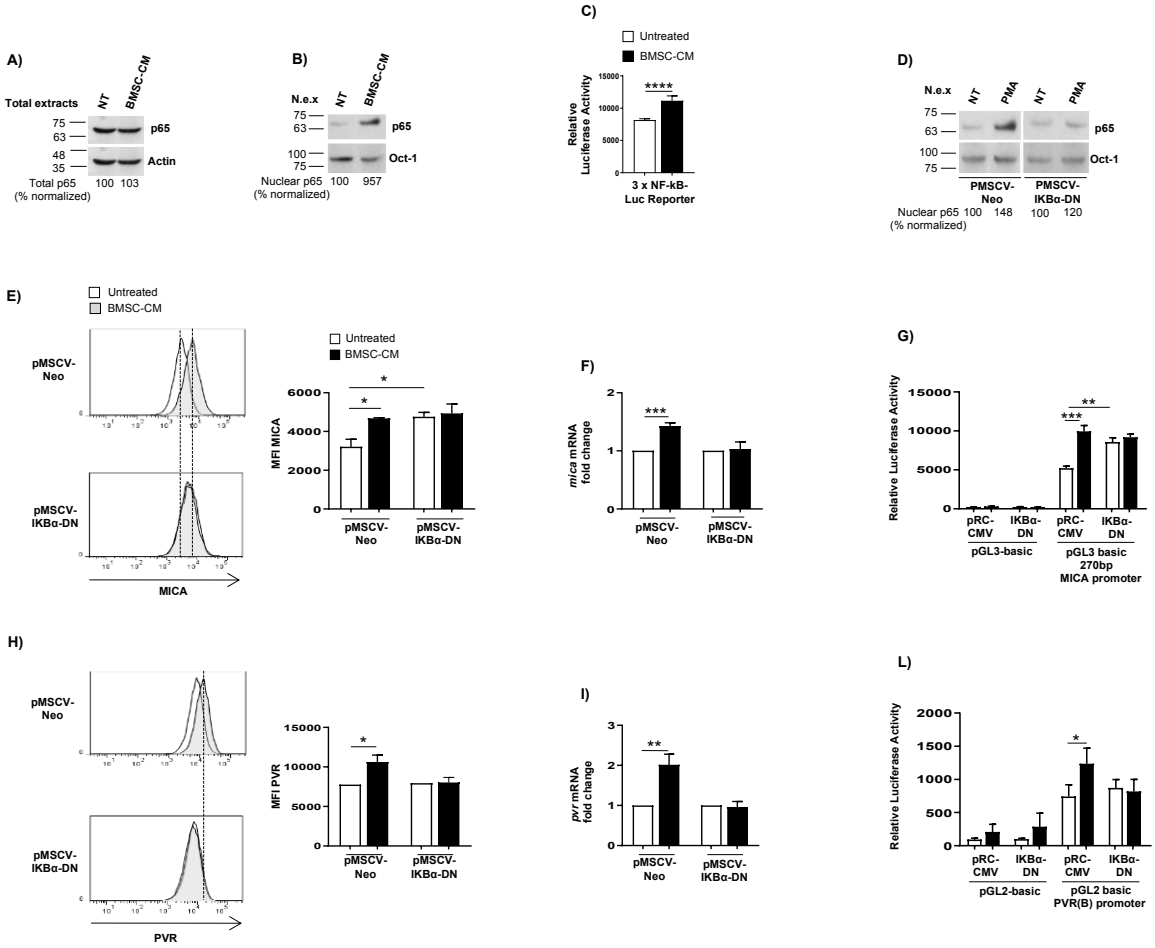

Fig. 5. IKBα-DN blocks BMSC-CM-mediated induction of MICA and PVR expression on human MM cells. Western Blot analysis of total (**A**) or nuclear extracts (N.e.x.) (**B**) obtained from SKO007(J3) MM cells or from SKO-007(J3) cells infected with pMSCV-Neo or pMSCV-IKBα-DN untreated or treated for 30 min with PMA cultured for 24 h in BMSC-CM or complete RPMI1640 (NT) (**D**). The proteins transferred to nitrocellulose membranes were immunoblotted for p65 and Actin or Oct-1. Numbers represent densitometric analysis of p65 normalized to Actin or Oct-1 relative to untreated cells. SKO007(J3) cells were infected with lentivirus pHAGE-3xNF-kB-LUC-GFP obtained as described in Materials and Methods. After 72 h culture in BMSC-CM, SKO007(J3) cells were harvested and analyzed for luciferase activity (**C**). Results are expressed as relative luciferase activity and represent the mean ± SEM from 4 independent experiments (****$p < 0.001$; paired Student t-test). FACS and Real-Time PCR analysis of MICA (**E, F**) and PVR expression (**H, I**) in SKO007(J3) cells transduced with pMSCV-Neo or pMSCV-IKBα-DN, untreated or treated with BMSC-CM. For MICA and PVR surface expression, a representative experiment is shown (left panel). Histograms represent the MFI of specific mAb - MFI of isotype control (right panel). The MFI of MICA and PVR was calculated based on at least 3 independent experiments ± SEM (*$p < 0.05$; ANOVA). For MICA and PVR mRNAs, data expressed as fold change units, were normalized with GAPDH and referred to the untreated cells, considered as calibrator and represent the mean of 3 experiments (**$p < 0.005$; ***$p < 0.001$; ANOVA). SKO007(J3) cells were transiently co-transfected with pGL3 basic empty/MICA 270 bp promoter plasmid (**G**) or pGL2 basic empty/PVR (B) promoter plasmid (**L**) plus pRC-CMV empty vector or IKBα-DN and analyzed for luciferase activity as described above. Results are expressed as relative luciferase activity and represent the mean ± SEM from four independent experiments (*$p <$ 0.05; **$p < 0.005$; ***$p < 0.002$; ANOVA).

4. MICA and PVR Induction on Human MM cells Is Mediated by Different BMSC-derived Proteins

To investigate about the chemical nature of BMSC-derived soluble factor(s) responsible for MICA and PVR upregulation on MM cells, we cultured SKO007(J3) MM cells in BMSC-CM pre-treated with proteinase K, a broad-spectrum serine protease. By flow cytometry analysis, we observed that protein degradation of BMSC-CM by proteinase K blocked its capability to increase MICA and PVR expression on SKO007(J3) MM cells (Fig. 6A, B), suggesting that the soluble factor(s) produced by BMSCs involved in these mechanisms may be protein(s). Then, using centrifugal filtration with a semi permeable membrane of different molecular weight cutoff (Centricon), we separated BMSC-CM and complete RPMI1640 medium, used as a control, into fractions ranging from 100 to 10 KDa. Fractioned BMSC-CM or complete RPMI1640 medium containing exclusively proteins with molecular weight higher than 100 KDa, 50 KDa, 30 KDa or 10 KDa were used to stimulate SKO007(J3) cells for 72h. We observed that PVR expression resulted significantly enhanced only on MM cells treated with fraction >100 KDa, whereas MICA levels augmented on SKO007(J3) cells exposed to the smaller fraction >50 KDa (Fig. 6C, D).

These data demonstrate that protein(s) of different molecular sizes secreted by BMSCs mediate the induction of MICA and PVR expression on MM cells.

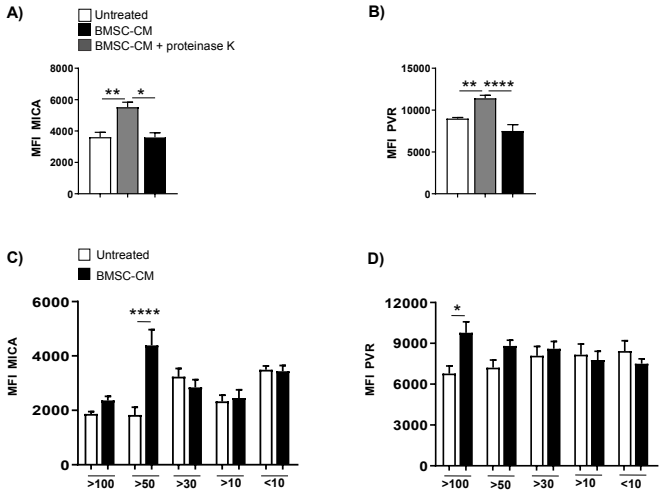

Fig. 6. BMSC-derived proteins responsible for MICA and PVR upregulation of human MM cells. MICA (**A**) and PVR (**B**) surface expression was analyzed on SKO007(J3) MM cell line by FACS analysis cultured in BMSC-CM pre-treated or not with proteinase K or complete RPMI1640 (Untreated) for 72 h. Histograms represent the MFI of specific mAb - MFI of isotype control. The MFI of MICA and PVR was calculated based on at least 3 independent experiments ± SEM (*$p < 0.05$; **$p < 0.005$; ****$p < 0.001$; ANOVA). MICA (**C**) and PVR (**D**) surface expression was analyzed on SKO007(J3) cell line by FACS analysis cultured in fractions of different molecular weight obtained from BMSC-CM or complete RPMI1640 (Untreated) for 72 h. Histograms represent the MFI of specific mAb - MFI of isotype control. The MFI of MICA and PVR was calculated based on at least 3 independent experiments ± SEM (*$p < 0.05$; ****$p < 0.001$; paired Student t-test).

5. Increased PVR Expression on Human MM Cells by BMSCs Requires IL-8-Bearing Microvesicles

To identify possible BMSC-derived proteins which upregulate PVR expression on human MM cells, we based on the observations that: 1) PVR upregulation on human MM cells by BMSCs is mediated by proteins with a molecular weight higher than 100 KDa; 2) microvesicles (MVs) are large extracellular vesicles (EVs), represent crucial mediators of intercellular communication between MM and BMSCs and activate different signaling pathways in MM cells (*e.g.* NF-kB) (Dabbah et al., 2017; Marcus et al., 2016). For these reasons, we examined the possible role of BMSC-derived MVs in the regulation of PVR expression on human MM cells. To this aim, we isolated EVs by BMSC-CM *via* ultracentrifugation and validated their identity by transmission electron microscopy and DLS experiments that demonstrated membrane vesicles of size 200–1000 nm resembling MVs (Fig. 7A, B). Since MVs generate directly from cell membrane budding, we performed flow cytometry analysis for the same molecules expressed by BMSCs; accordingly, BMSC-derived MVs are negative for the hematopoietic marker CD45, but they bear CD90, CD105 and CD73 (Fig. 7C).

To evaluate the possible contribution of these EVs on PVR upregulation on human MM cells, we cultured SKO007(J3) MM cells for 72 h with MVs or BMSC-CM depleted of MVs (BMSC-CM DEPL.) or their combination. We found that only the combined treatment of MVs with BMSC-CM DEPL. enhanced PVR expression as well as complete BMSC-CM (Fig. 7D). When used alone neither BMSC-derived MVs or BMSC-CM DEPL. were not able to increase PVR surface expression, thus indicating a cooperative activity of MVs with other(s) soluble factor(s) produced by BMSCs.

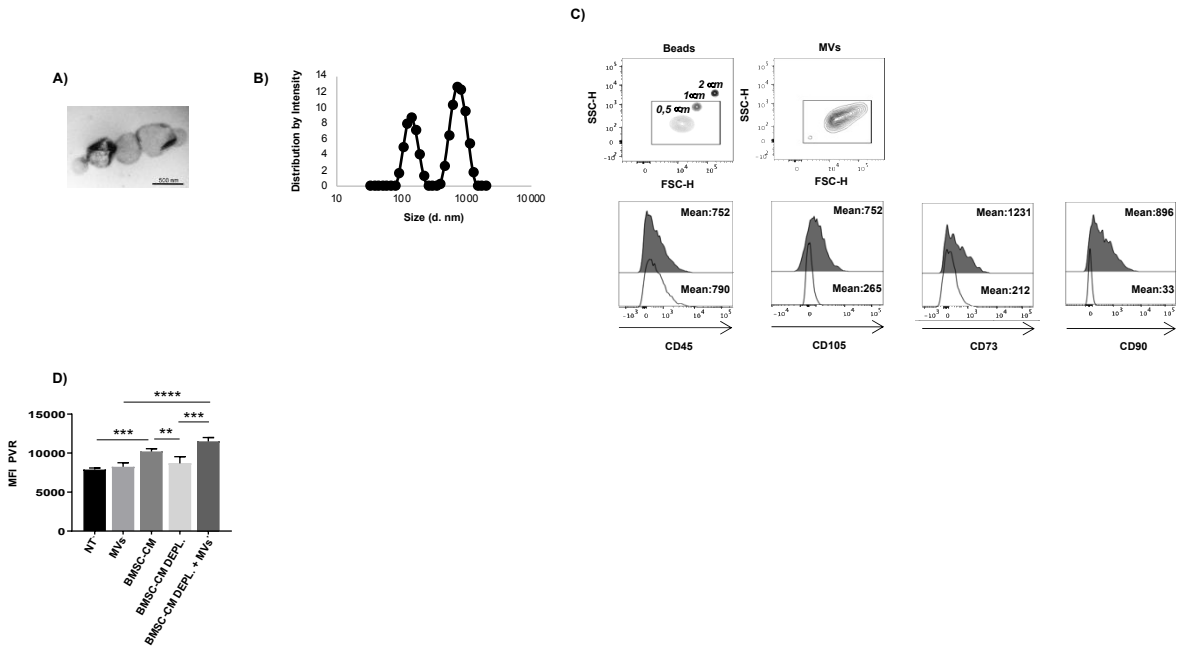

Fig. 7. BMSCs regulate PVR expression on human MM cells via MVs. Electron microscope analysis of MV morphology and size. A representative image of MVs prepared from BMSCs is shown in (**A**). Size distribution of BMSC-derived MVs was analyzed through DLS. A representative experiment out of three is shown in (**B**). Flow cytometry characterization of MVs released by BMSCs (**C**). Reference size beads were used to gate MVs, defined as elements of smaller size than 1μm (upper panel). The grey colored histograms represent MVs stained with negative (CD45) and positive (CD105, CD73, and CD90) BMSC markers, while white histograms represent isotype control (lower panel). PVR surface expression was analyzed on SKO007(J3) cell line untreated or treated for 72 h with complete or MV-depleted BMSC-CM (BMSC-CM DEPL.) alone or in combination with BMSC-derived MVs (**D**). Histograms represent the MFI of specific mAb - MFI of isotype control. The MFI of PVR was calculated based on at least 3 independent experiments ± SEM (***p* < 0.005; ****p* < 0.002; *****p* < 0.001; ANOVA).

MVs are also known to encapsulate or bind on their surface different cytokines (Fitzgerald *et al.,* 2018). Thus, we tried to identify possible factor-bearing MVs responsible for PVR upregulation on human MM cells.

Analysis of microarray public data of MM patients (datasets GSE2658, GSE6477 available at http://www.ncbi.nlm.nih.gov/geo/) indicates that a significant positive correlation exists between PVR and chemokine receptor expression, CXCR1 (R = 0.335 in Hanamura MM Dataset of R2 platform for genomic analysis) and CXCR2 (R = 0.239 in Chng MM Dataset of R2 platform for genomic analysis) (Fig. 8A, B). Based on these observations, to gain further insight into the nature of the BMSC-secreted factors capable of inducing PVR expression on human MM cells, we focused our attention on CXCR1/2 ligand CXCL8/IL-8, a chemokine strongly produced by BMSCs and able to trigger NF-kB activation in MM cells (Markovina *et al.,* 2010). By flow cytometry analysis we first revealed that CXCR1 and CXCR2 are expressed on SKO007(J3) MM cell line and treatment with BMSC-CM does not change the levels of these proteins (Fig. 8C). Then, we collected cell-free supernatants from BMSCs after 72 h culture and tested them for the presence of IL-8 by ELISA assay. We observed that IL-8 is

expressed by BMSCs with no significant difference between MGUS and ONSET BMSCs (Fig. 8D and Table 2).

To evaluate the possible contribution of IL-8 in PVR upregulation, we used two different approaches to block CXCR1 and CXCR2 activity. SKO007(J3) cells were treated with an anti-CXCR1 blocking mAb or a selective CXCR2 antagonist, SB225002, before stimulation with BMSC-CM. We observed that both treatments significantly block PVR upregulation by BMSC-CM at protein (Fig. 8E, G) and mRNA level (Fig. 8F, H). Consistently, a significant reduction of PVR upregulation was observed in SKO007(J3) cells treated with conditioned medium obtained from IL-8 silenced-BMSCs (Fig. 8I, L).

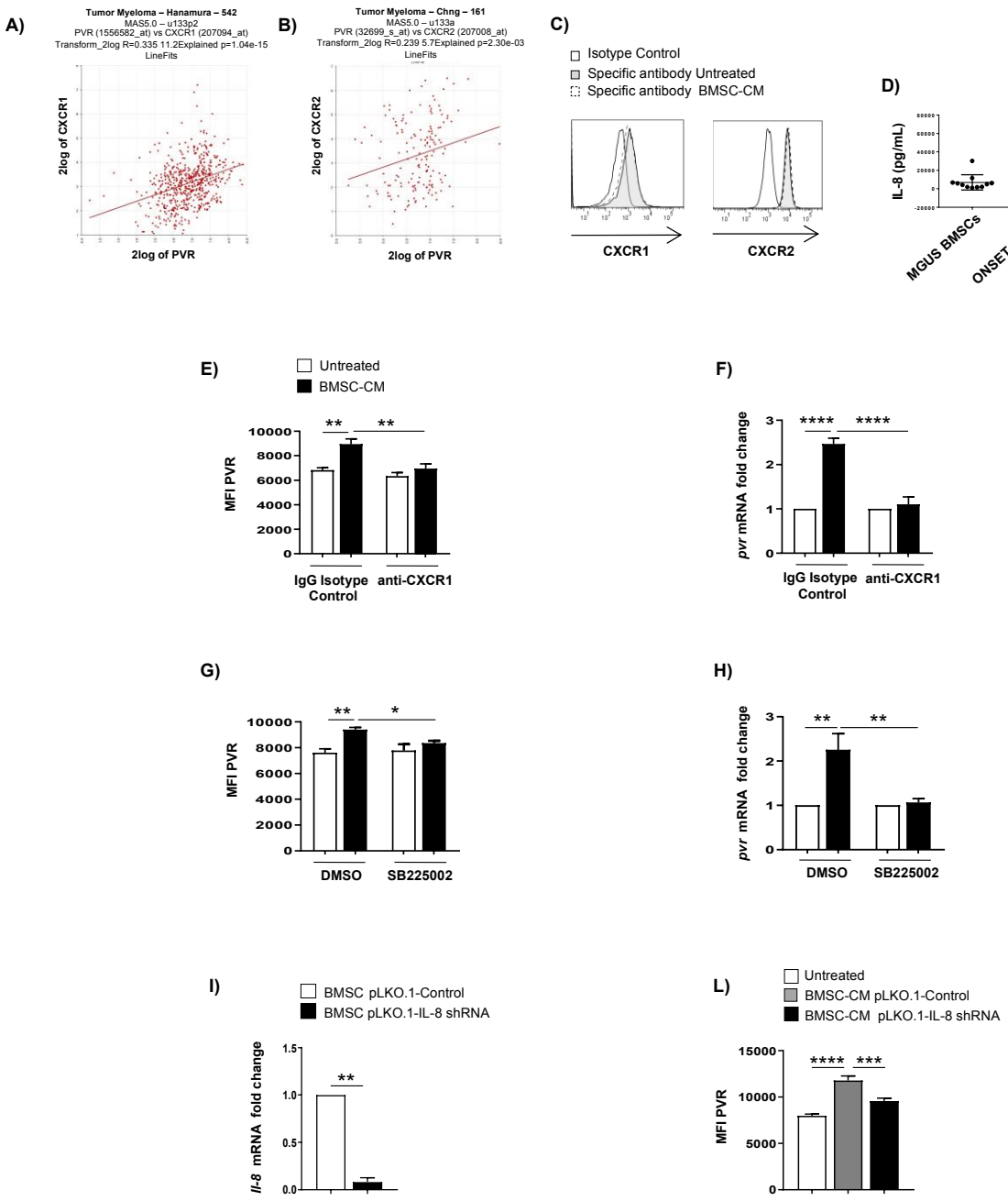

Fig. 8. Blockade of CXCR1/2 receptors prevents PVR upregulation by BMSC-CM. Correlation analysis of expression values from MM patients between PVR and CXCR1 (probe set 1556582_at for PVR vs. probe set 207094_at for CXCR1) (**A**). R-value: 0.335, p-value = 1.04e−15, (Hanamura MM Dataset of R2), and between PVR and CXCR2 (probe set 32699_at for PVR vs. probe set 207008_at for CXCR2) (**B**). R-value: 0.239, p-value = 2.30e−03, (Chng-161 MM Dataset of R2). CXCR1 and CXCR2 expression was analyzed by flow cytometry on SKO007(J3) cells untreated or treated with BMSC-CM for 72 h (**C**). A representative experiment out of three is shown. MGUS or Active MM-BMSCs were cultured in serum-free MEMα medium for 72 h and then BMSC-CM was assayed for IL-8 by ELISA (**D**). PVR surface expression was analyzed by flow cytometry on SKO007(J3) cells were pre-treated with anti-CXCR1 or IgG control (**E**) or with SB225002 or vehicle control (DMSO) (**G**) and stimulated or not (Untreated) by BMSC-CM. Real Time PCR analysis of total PVR mRNA obtained from SKO007(J3) cells after 48 h stimulation with BMSC-CM and pre-treatment with anti-CXCR1 blocking mAb (**F**) or SB225002 (**H**). Real-Time PCR analysis for *il-8* gene expression on total mRNA obtained from BMSCs infected with lentiviral vector expressing IL-8 shRNA (pLKO.1-IL-8 shRNA) or scramble control (pLKO.1-Control) (**I**). Data, expressed as fold change units, were normalized with GAPDH and referred to the untreated cells, considered as calibrator. Data were calculated based on at least 3 independent experiments ± SEM (**$p < 0.005$; ****$p < 0.001$; paired Student t-test; ANOVA). Cytofluorimetric analysis of PVR surface expression on SKO007(J3) cells treated or untreated for 72 h with CM derived from BMSCs transduced with pLKO.1-IL-8 shRNA or pLKO.1-Control (**L**). Histograms represent the MFI of specific mAb - MFI of isotype control. Data were calculated based on at least three independent experiments ± SEM (*$p < 0.05$; **$p < 0.005$; ***$p < 0.002$; ****$p < 0.001$; ANOVA).

Then, we evaluated the presence of IL-8 in two different fractions of BMSC-CM, containing molecules with molecular weight higher or lower than 100 KDa, we found that the fraction >100 KDa contained the majority of IL-8 (Fig. 9A). These findings indicate that despite its very low molecular weight (8 KDa), IL-8 is an important component of the fraction >100 KDa of BMSC-CM and this could indicate a possible association with MVs. To confirm this hypothesis, we performed ELISA for IL-8 by using whole MVs obtained from BMSCs. We revealed the presence of IL-8 on MVs surface (Figure 9B); accordingly, IL-8 content was almost lost in BMSC-CM DEPL. (Fig. 9C). Moreover, as shown in Fig. 9D, E, pre-treatment of SKO007(J3) cells with anti-CXCR1 blocking mAb and SB225002 abolished PVR upregulation by the combination of BMSC-CM DEPL with BMSC-derived MVs.

Taken together, these results demonstrate that IL-8 is associated with BMSC-derived MVs which are also required for PVR upregulation via CXCR1/CXCR2 signaling activation; moreover, they indicate that MVs are necessary but not sufficient per sé to enhance PVR expression on human MM cells.

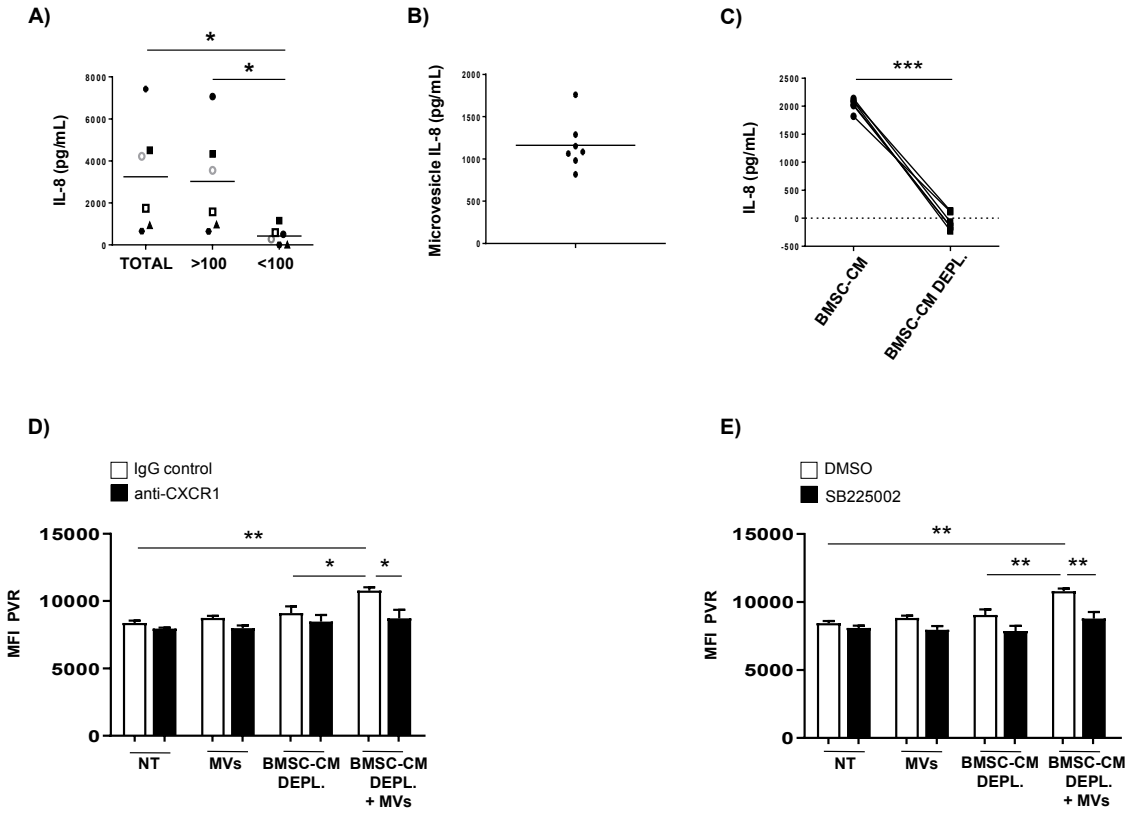

Fig. 9. BMSC-derived MVs bear IL-8. Total and fractioned (<100 or >100 KDa) BMSC-CM (**A**), whole MVs (**B**) and BMSC-CM or BMSC-CM DEPL. (**C**) were analyzed for IL-8 by ELISA. Data were calculated based on at least 3 independent experiments ± SEM (*$p < 0.05$; ANOVA). PVR surface expression was analyzed on SKO007(J3) pre-treated with anti-CXCR1 or IgG control (**D**) or with SB225002 or vehicle control (DMSO) (**E**) cultured in complete RPMI1640 (NT), complete BMSC-CM or BMSC-CM DEPL. alone or in combination with BMSC-derived MVs. Histograms represent the MFI of specific mAb - MFI of isotype control. Data were calculated based on at least three independent experiments ± SEM (*$p < 0.05$; **$p < 0.005$; ANOVA).

6. Gas6 Protein Is a Regulator of MICA Expression on Human MM Cells

To identify a possible BMSC-derived protein responsible for MICA upregulation on human MM cells, we focused our attention on Gas6, a protein of 75 KDa able to enhance the activity of several different signaling pathways in human MM cells, including NF-kB (Furukawa et al., 2017).

We first tested the presence of Gas6 receptors (TAM receptors) on MM cells. By flow cytometry analysis, we observed the expression of Mertk and Axl, but not of Tyro3, on the surface of SKO007(J3) cell line (Fig. 10A). Then, we performed ELISA assays to verify the presence of this growth factor in BM microenvironment. As previously reported (Furukawa et al., 2017), we found a very high production of Gas6 in plasma derived from BM aspirates of MM patients (Fig. 10B and Table 2). We also confirmed that an important source of this factor is represented by BMSCs in our experimental settings, as suggested by the presence of Gas6 in BMSC-CM (Fig. 10C and Table 2).

Based on these findings, we infected BMSCs with a lentiviral vector expressing Gas6 shRNA (pLKO.1-Gas6 shRNA) or the vector control pLKO.1. After puromycin selection, we obtained Gas6-silenced BMSCs as demonstrated by ELISA assay (Fig. 10D). Finally, by FACS analysis we demonstrated that CM derived from BMSCs lacking Gas6 was not able to augment MICA on SKO007(J3) MM cells (Fig. 10E), thus revealing a role for this factor in the regulation of this NKG2D ligand. Accordingly, the ability of BMSC-CM to enhance NF-kB transcription factor activity was also abolished by the absence of Gas6 (Fig. 10F).

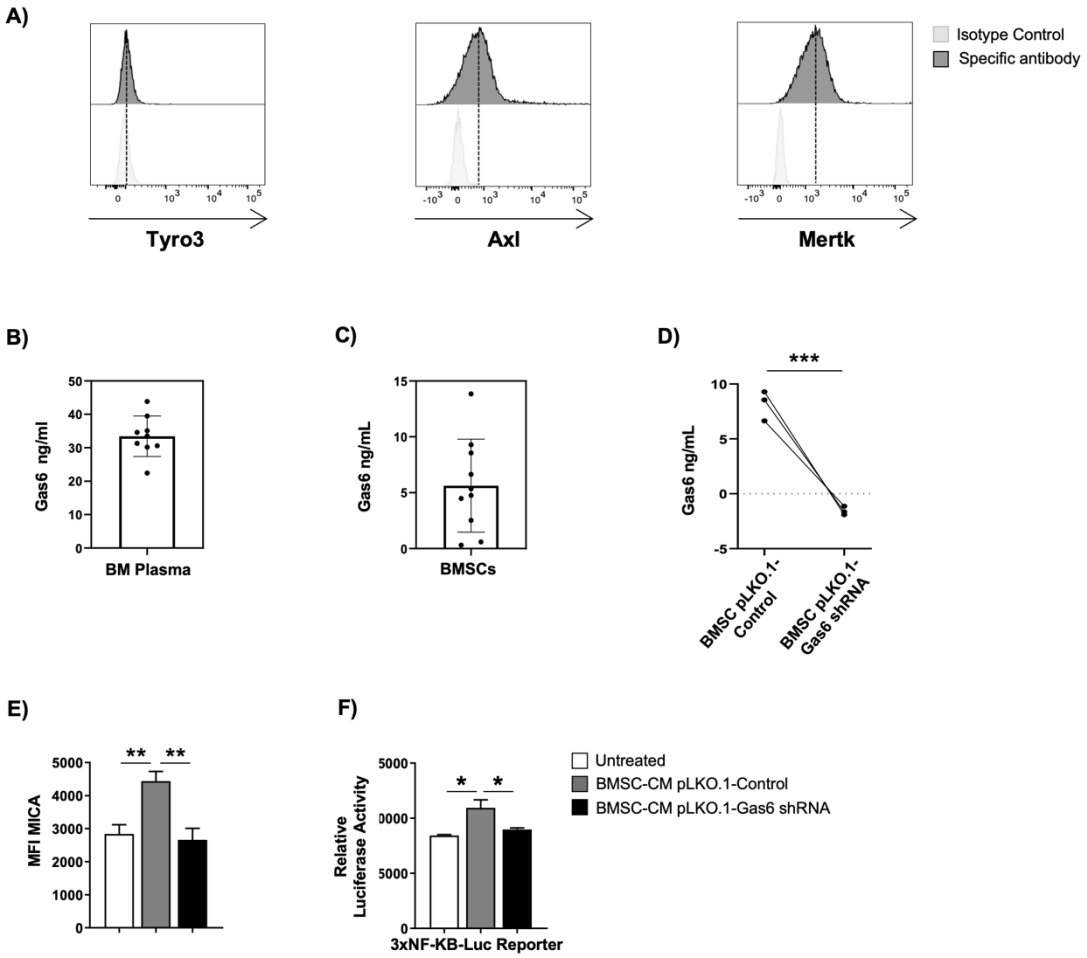

Fig. 10. Gas6-depleted BMSCs are not able to induce MICA expression on human MM cells. TAM (Tyro3, Axl, Mertk) receptor expression was analyzed on SKO007(J3) by FACS analysis. A representative experiment is shown in (**A**). Plasma from BM aspirates of MM patients (**B**) and CM derived from 72 h culture in serum-free medium of BMSCs untreated (**C**) or infected with lentiviral vector expressing Gas6 shRNA (pLKO.1-Gas6 shRNA) or scramble control (pLKO.1-Control) (**D**) were assayed for Gas6 by ELISA. (**E**). MICA surface expression was analyzed by FACS on SKO007(J3) untreated or treated for 72 h with CM derived from BMSCs pLKO.1-Gas6 shRNA or pLKO.1-Control. Histograms represent the MFI of specific mAb - MFI of isotype control. Data were calculated based on at least three independent experiments ± SEM (**$p < 0.005$; ANOVA). (**F**) SKO007(J3) cells were infected with lentivirus pHAGE-3xNF-kB-LUC-GFP cultured for 72 h in CM derived from BMSCs pLKO.1-Gas6 shRNA or pLKO.1-Control. Cells were harvested and analyzed for luciferase activity. Results are expressed as relative luciferase activity and represent the mean ± SEM from 4 independent experiments (*$p < 0.05$; ANOVA).

Since Gas6 is an important autocrine factor for MM cell proliferation and survival (Furukawa et al., 2017), we wanted to investigate the possible impact of the endogenous production of this protein on basal expression of MICA on human MM cells. To this aim, we silenced Gas6 expression in SKO007(J3) cells by shRNA approach (Fig. 11A) and analyzed MICA expression. We found that the reduction of Gas6 causes a significant downregulation of basal expression of this NKG2DL at both protein and mRNA level in MM cells (Fig. 11B, C). Importantly, by annexin V and propidium iodide staining, we could exclude toxic and no specific effects of *gas6* gene interference on SKO007(J3) cell line (Fig. 11D). Accordingly, we also observed that Gas6 depleted-SKO007(J3) cells have reduced levels of basal NF-kB activity

as indicated by a lower phosphorylation of p65 subunit (Fig. 11E). These observations suggest the involvement of this pathway also in Gas6-mediated regulation of basal expression of MICA. Thus, we tested the possible role of TAM receptors in these regulatory circuits. We transduced SKO007(J3) cell line with lentiviral vectors expressing Mertk (pLKO.1-Mertk shRNA) or Axl shRNA (pLKO.1-Axl shRNA) or control vector pLKO.1. As shown in Fig. 11F-M, we found that inhibition of either Mertk or Axl expression strongly reduced MICA expression at both protein and mRNA levels in MM cells.

These data indicate that Gas6/TAM receptors signaling sustains MICA expression on MM cells.

Finally, to analyze the functional consequence of changes in MICA expression in Gas6 depleted-MM cells, we performed a degranulation assay. To this aim, we cultured healthy donors PBMCs without (medium) or with target cells (SKO007(J3) pLKO.1-Control or SKO007(J3) pLKO.1-Gas6 shRNA). We observed that CD107a expression was significantly reduced on gated NK cells contacting SKO007(J3) pLKO.1-Gas6 shRNA (displaying low levels of MICA), when compared to SKO007(J3) pLKO.1-control. Accordingly, a blocking anti-NKG2D mAb impaired NK cell degranulation against SKO007(J3) pLKO.1-control, but it failed to reduce NK cell degranulation contacting SKO007(J3) pLKO.1-Gas6 shRNA (Fig. 11N-P).

Overall, our results identify Gas6 protein as a novel regulator of MICA expression in human MM cells; both autocrine and paracrine production of this factor significantly contributes to sustain the expression of the activating ligand on these tumors cells rendering them more susceptible to recognition by NK cells.

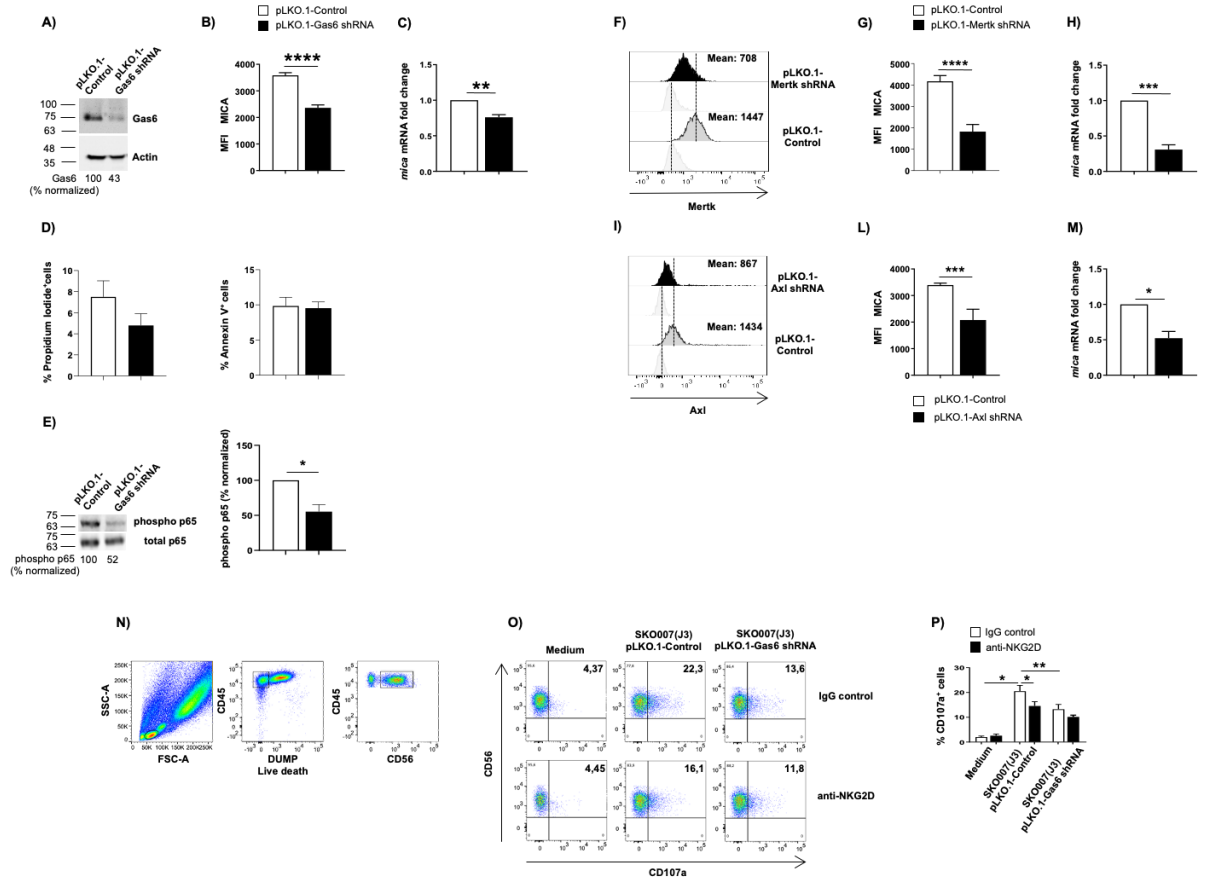

Fig. 11. Genetic ablation of Gas6 or TAM receptors reduces MICA expression on human MM cells and susceptibility to recognition by NK cells. Western Blot analysis on total extracts obtained from SKO007(J3) pLKO.1-Gas6 shRNA or pLKO.1-Control. The proteins transferred to nitrocellulose membranes were immunoblotted for Gas6 and Actin or phospho p65 and total p65. Numbers represent densitometric analysis of Gas6 normalized to Actin (**A**) or phospho p65 normalized to total p65 (**E**) relative to untreated cells. Histogram represents the mean ± SEM from three independent experiments (*$p < 0.05$; paired Student t-test). MICA surface expression was analyzed by flow cytometry on SKO007(J3) pLKO.1-Gas6 shRNA (**B**), pLKO.1-Mertk shRNA (**F**) and pLKO.1-Axl shRNA (**I**) or pLKO.1-Control. Histograms represent the MFI of specific mAb - MFI of isotype control. Data were calculated based on at least three independent experiments ± SEM (***$p < 0.002$; ****$p < 0.001$; paired Student t-test). SKO007(J3) pLKO.1-Gas6 shRNA or pLKO.1-Control were stained using Annexin-V/APC and Propidium Iodide (**D**). Histograms indicate the percentage of Annexin V or propidium positive cells and were calculated based on at least 3 independent experiments. Mertk and Axl surface expression was analyzed by flow cytometry on SKO007(J3) cells pLKO.1-Mertk, pLKO.1-Axl shRNA or pLKO.1-Control. A representative experiment is shown. Real-Time PCR analysis for *mica* gene expression on total mRNA obtained from SKO007(J3) pLKO.1-Gas6 shRNA (**C**), pLKO.1-Mertk shRNA (**H**) and pLKO.1-Axl shRNA (**M**) or pLKO.1-Control. Data, expressed as fold change units, were normalized with GAPDH and referred to pLKO.1-Control, considered as calibrator. Data were calculated based on at least 3 independent experiments ± SEM (*$p < 0.05$; **$p < 0.005$; paired Student t-test). Healthy donor-derived PBMCs were incubated with SKO007(J3) pLKO.1-Gas6 shRNA or pLKO.1-Control at E/T ratio of 1:1. CD107a expression was analyzed on $CD3^-CD11^-CD14^-CD45^+CD56^+$ NK cells as shown in (**N**). To evaluate the role of NKG2D, the assay was performed in parallel treating NK cells with blocking anti-NKG2D or anti-IgG mAbs used as control. Results are expressed as the percentage of $CD107a^+$ cells. A representative experiment is shown in (**O**). Histogram represents the mean ± SEM from three independent experiments (**P**) (*$p < 0.05$; **$p < 0.005$ ANOVA).

DISCUSSION

NK cells are important innate effector lymphocytes in the response against tumor and virus-infected cells. Several *in vitro* and *in vivo* studies demonstrated that these lymphocytes play an important role in control of MM progression (Frohn et al., 2002; Katodritou et al., 2011; Ponzetta et al., 2015). The recognition and killing of MM cells by NK cells are mediated by activating receptor ligands expressed on these cancer cells (Carbone et al., 2005; El-Sherbiny et al., 2007). However, during MM progression the expression of these molecules results strongly reduced, leading to an impairment of anti-MM activity of NK cells (Carbone et al., 2005; El-Sherbiny et al., 2007).

The comprehension of molecular mechanisms underlying the expression of NK cell activating ligands on human MM cells is crucial for the development of therapeutic approaches aimed to render these cancer cells more susceptible to NK cell-mediated attack. Our group identified different transcriptional (Fionda et al., 2009, 2013, 2015; Soriani et al., 2009, 2014; Abruzzese et al., 2016; Petillo et al., 2021) and post-translational mechanisms (Molfetta et al., 2019; Zingoni et al., 2015; Zitti et al., 2017) implicated in the regulation of NKG2D and DNAM-1 ligand expression on human MM cells.

Here, we evaluated the possible impact of the BM microenvironment on these mechanisms, focusing on BMSCs.

By direct contact and the secretion of numerous soluble factors, BMSCs favor proliferation and survival of malignant PCs thus promoting disease progression (Mitsiades et al., 2006; Xu et al., 2018); however, the possible impact of BMSCs on the mechanisms regulating the anti-MM immune response remains unexplored.

We demonstrated that BMSCs induce a significant upregulation of MICA and PVR mRNA and protein in MM cell lines and primary malignant PCs, thus enhancing the recognition and NK cell degranulation via engagement of NKG2D and DNAM-1 receptors.

Although BMSCs can be genetically and functionally altered during MM progression (Xu et al., 2018), we did not find any difference between MGUS or ONSET MM-BMSCs, suggesting that the capability of BMSCs to induce the expression of MICA and PVR on human MM cells is not dependent on the stage of the disease.

We demonstrated that BMSC-derived soluble factor(s) are key mediators of increased MICA and PVR levels on human MM cells; indeed, we found the upregulation of these ligands by performing direct contact co-culture as well as through transwell support or culturing MM cells in BMSC-CM. Moreover, the observation that BMSC-CM is sufficient per sè to fully induce

MICA and PVR expression indicates that soluble factor(s) mediating these effects are constitutively produced by BMSCs and that interaction with MM cells does not affect the expression and/or function of these molecules.

The contribution of soluble factors in the regulation of these ligands has been previously described. Different cytokines, such as TGF-β, IL-10, IFN-γ, and TNF-α can control MICA expression in different types of cancer cells (Duan et al., 2019). For instance, MICA expression is blocked by IFN-γ in melanoma cells, while TGF-β was found to inhibit the expression of this NKG2DL in malignant glioma (Eisele, 2006; Zhang et al., 2008).

Our findings also demonstrate that activation of NF-kB pathway is required for NK cell ligand regulation by BMSCs.

NF-kB was found to be a positive regulator of *mica* gene in different cellular contexts. In activated T lymphocytes and epithelial cancer cell lines, NF-kB acts by binding to a specific sequence in the long intron 1 of the *mica* gene (Molinero et al., 2004). Furthermore, in human endothelial cells TNF-α-induced NF-kB binds a regulatory control site at -130 bp upstream of the *mica* transcription start site (D. Lin et al., 2012). On the other hand, the involvement of NF-kB transcription factor in the regulation of *pvr* gene has only recently been demonstrated in neuroblastoma cells (Brandetti et al., 2021).

We showed that IKBα-DN overexpression induces a specific increase in the basal expression of MICA but abrogates the stimulating effect of BMSC-CM on the expression of this ligand. These findings suggest a double role of NF-kB in MICA regulation as a negative as well as a positive transcriptional regulator of this gene. Indeed, it is likely that NF-kB controls the expression of one or more repressor(s) of *mica* gene. A possible candidate could be IRF-4, a known NF-kB gene target (Boddicker et al., 2015), recently emerged as a negative regulator of *mica* transcription in MM cells (Fionda et al., 2015; Abruzzese et al., 2016; Petillo et al., 2021).

NF-kB also mediates *mica* promoter activation by BMSC-CM. Future studies will clarify if an NF-kB responsive element located at -130 bp from transcriptional start site (D. Lin et al., 2012), comprised in the region of *mica* promoter (270 bp from transcriptional start site) is involved in the induction of *mica* promoter by BMSC-CM; interestingly, this NF-kB responsive element overlaps a region of *mica* promoter previously identified as a heat shock response element (HSE) (Fionda et al., 2009; B. Zhang et al., 2009; Schilling et al., 2015) and a Ikaros consensus site (Fionda et al., 2015) critical for the induction of this promoter.

Differently, IKBα-DN overexpression inhibits only PVR upregulation by BMSC-CM; these findings indicate that DNAM-1L regulation may require the cooperation between NF-kB and other transcription factors which remain to be defined. A search for sequence homology

revealed the presence of putative NF-kB binding sites (-254-GGGGAGGGCCAG-243; -162-GTGGGTATTCCC-150) in the region of *pvr*(B) promoter spanning -343 bp from the transcription start site with increased activity in BMSC-CM treated cells. The possible contribution of these regulatory elements in BMSC-mediated PVR up-regulation will be examined in future studies.

Of note, we also addressed the involvement in MICA and PVR regulation of proteins of different molecular weight produced by BMSCs. Protein(s) larger than 100 KDa can augment only PVR expression, while proteins with a molecular weight ranging from 50 to 100 KDa can specifically increase MICA expression.

Interestingly, we described a novel role for IL8-bearing MVs in the regulation of PVR expression.

MVs are large EVs are key mediators in BMSC-MM cell communication, and activate different pathways in cancer cells, including NF-kB (Marcus et al., 2016; Dabbah et al., 2017, 2020).

We found that MVs released by BMSCs are able to induce PVR upregulation on MM cells only in combination with other(s) soluble factor(s); however, their depletion abolishes the effect of BMSC-CM, suggesting that MVs are necessary, but insufficient per sè to mediate these mechanisms.

In parallel, we identified IL-8 as a critical mediator of PVR upregulation on human MM cells by BMSCs. We observed that PVR expression was selectively reduced both at level of mRNA and protein upon blockade of CXCR1/2 receptors on MM cells as well as by depletion of IL-8 via shRNA in BMSCs.

Based on the knowledge that many cytokines can be released by cells in encapsulated or associated form with EVs (Fitzgerald et al., 2018), we demonstrated that the majority of IL-8 produced by BMSCs is associated with MV surface. Further studies are needed for the identification of the other(s) BMSC-derived factor(s) which synergistically cooperate with IL-8-bearing MVs in these mechanisms. In this regard, there is evidence of a crosstalk between receptors of IL-8 and growth factors in endothelial and cancer cells. IL-8 activation of CXCR1/2 can transactivate growth factor receptors, such as EGFR or VEGFR, via direct interaction or receptor phosphorylation by prolonging and reinforcing their signaling and cellular responses (Schraufstatter et al., 2003; Petreaca et al., 2007; Kyriakakis et al., 2011). Alternatively, CXCR1/2-IL-8 interaction may be promoted by other(s) soluble factor(s) released by BMSCs. Consistently, other ligands and chemokine receptors can affect the functions of CXCR1 and CXCR2. For instance, the atypical receptor CCRL2 is required for CXCR2-dependent neutrophil recruitment; in this context, CCRL2 does not bind IL-8 but forms

heterodimers with CXCR2 and regulates IL-8 induced-CXCR2 signaling (Del Prete et al., 2017).

We also described new regulatory mechanisms for MICA. Indeed, we identified Gas6 as a key regulator of basal and BMSC-mediated expression of MICA.

Overexpression of Gas6 and TAM receptors always predicts a poor clinical prognosis and outcome in many cancer types, since they favor multiple aspects of tumor progression and metastasis, including tumor cell proliferation, migration, invasion, survival, angiogenesis, therapeutic resistance (Ammoun et al., 2014; Chiu et al., 2015; G. Wu et al., 2018). Importantly, Gas6/TAM signaling pathway also contributes to create an immunosuppressive tumor microenvironment, leading cancer cells to evade immune system (Tanaka & Siemann, 2020).

In the context of MM, it is well known that autocrine and paracrine production of Gas6 in the BM significantly highly sustains cancer cell proliferation and survival via NF-kB (Furukawa et al., 2017).

Our findings demonstrate that Gas6 is required for both basal and stromal-induced MICA expression on MM cells. Indeed, genetic depletion of Gas6 as well as Mertk or Axl receptors lead to a significant downmodulation of basal MICA; in addition, BMSCs lacking Gas6 loose the capability to increase the expression of this NKG2DL on MM cells. Consistently, these different conditions are also associated with a reduced activation of NF-kB pathway in MM cells.

Ligands of NK cell inhibitory receptors can be regulated by TAM pathway on cancer cells. Mertk activation led to PD-L1 upregulation in breast and lung cancer cells (Du et al., 2021). Moreover, pharmacologic inhibition and genetic knockout of Axl decrease surface level expression of MHC-I on myeloid and cancer cells (Aguilera et al., 2016; Z. Guo et al., 2017). These findings are consistent with a role for this pathway in mediating tumor cell escape from immune surveillance, also as a negative regulator of anti-tumor NK cell activity (Paolino et al., 2014).

Our data show that Gas6 sustains the expression of an activating ligand, however MM progression is associated with reduced surface levels of MICA due to proteolytic cleavage (Rebmann et al., 2007; Von Lilienfeld-Toal et al., 2010; Zingoni et al., 2015). sMICA induces internalization of surface NKG2D, NCRs and chemokine receptors and promotes an impairment of NK cell effector functions. In addition, sMICA has been shown to promote the accumulation of MDSCs and macrophages with an immunosuppressive phenotype (Xiao et al., 2015).

Although MICA and PVR upregulation results in the promotion of NK cell effector activity against MM cells, both BMSC-derived IL-8 and Gas6 can also increase the expression of different adhesion molecules, such as VCAM-1 and ICAM-1 (Furukawa et al., 2017; Markovina et al., 2010); PVR itself is also an adhesion molecule which may contribute to support MM cell survival and proliferation by enhancing the adhesion to BMSCs. Furthermore, PVR molecule is not only a DNAM-1 activating receptor ligand but can also bind CD96/TIGIT inhibitory receptors. During MM progression NK cells undergo significant phenotypic changes due to decreased activating receptors and increased inhibitory receptors, leading to functional impairment (Martínez-Sánchez et al., 2016; Pazina et al., 2021). In particular, DNAM-1 and NKG2D expression is downregulated on NK cells from MM patients with active disease compared to patients in remission. Consistently, PVR overexpression was correlated with tumor progression and unfavorable prognosis in different cancer cell types (Molfetta et al., 2020).

Taking in consideration these findings, we could hypothesize that MICA and PVR overexpression render MM cells more susceptible to NK cell mediated attack at early stage of diseases, while leading to a defective NK cell activity during disease progression: in MM late stage, receptor repertoire of these cytotoxic lymphocytes results altered and the interaction PVR/TIGIT or PVR/CD96 may play a dominant role in the MM microenvironment; moreover, accumulation of sMICA also contributes to create an immunosuppressive microenvironment.

In conclusion, this study allowed us to identify a new mechanism for regulating the expression of activator ligands of NK cells on MM cells. The results obtained indicate that BMSCs influence the expression of the *mica* and *pvr* genes through the production of Gas6 and IL-8, respectively; these mechanisms occur at transcriptional level and involve NF-kB transcription factor activity.

BIBLIOGRAPHY

1. Abruzzese, M. P., Bilotta, M. T., Fionda, C., Zingoni, A., Soriani, A., Vulpis, E., Borrelli, C., Zitti, B., Petrucci, M. T., Ricciardi, M. R., Molfetta, R., Paolini, R., Santoni, A., & Cippitelli, M. (2016). Inhibition of bromodomain and extra-terminal (BET) proteins increases NKG2D ligand MICA expression and sensitivity to NK cell-mediated cytotoxicity in multiple myeloma cells: Role of cMYC-IRF4-miR-125b interplay. *Journal of Hematology and Oncology*, *9*(1). https://doi.org/10.1186/s13045-016-0362-2
2. Agarwal, A., & Ghobrial, I. M. (2013). Monoclonal Gammopathy of Undetermined Significance and Smoldering Multiple Myeloma: A Review of the Current Understanding of Epidemiology, Biology, Risk Stratification, and Management of Myeloma Precursor Disease. *Clinical Cancer Research*, *19*(5), 985–994. https://doi.org/10.1158/1078-0432.CCR-12-2922
3. Agresta, L., Hoebe, K. H. N., & Janssen, E. M. (2018). The Emerging Role of CD244 Signaling in Immune Cells of the Tumor Microenvironment. *Frontiers in Immunology*, *9*. https://doi.org/10.3389/fimmu.2018.02809
4. Aguilera, T. A., Rafat, M., Castellini, L., Shehade, H., Kariolis, M. S., Hui, A. B.-Y., Stehr, H., von Eyben, R., Jiang, D., Ellies, L. G., Koong, A. C., Diehn, M., Rankin, E. B., Graves, E. E., & Giaccia, A. J. (2016). Reprogramming the immunological microenvironment through radiation and targeting Axl. *Nature Communications*, *7*(1), 13898. https://doi.org/10.1038/ncomms13898
5. Alici, E., Sutlu, T., Björkstrand, B., Gilljam, M., Stellan, B., Nahi, H., Quezada, H. C., Gahrton, G., Ljunggren, H. G., & Dilber, M. S. (2008). Autologous antitumor activity by NK cells expanded from myeloma patients using GMP-compliant components. *Blood*, *111*(6), 3155–3162. https://doi.org/10.1182/blood-2007-09-110312
6. Ammoun, S., Provenzano, L., Zhou, L., Barczyk, M., Evans, K., Hilton, D. A., Hafizi, S., & Hanemann, C. O. (2014). Axl/Gas6/NFκB signalling in schwannoma pathological proliferation, adhesion and survival. *Oncogene*, *33*(3), 336–346. https://doi.org/10.1038/onc.2012.587
7. André, T., Meuleman, N., Stamatopoulos, B., de Bruyn, C., Pieters, K., Bron, D., & Lagneaux, L. (2013). Evidences of Early Senescence in Multiple Myeloma Bone Marrow Mesenchymal Stromal Cells. *PLoS ONE*, *8*(3), e59756. https://doi.org/10.1371/journal.pone.0059756
8. André, T., Najar, M., Stamatopoulos, B., Pieters, K., Pradier, O., Bron, D., Meuleman, N., & Lagneaux, L. (2015). Immune impairments in multiple myeloma bone marrow mesenchymal stromal cells. *Cancer Immunology, Immunotherapy*, *64*(2), 213–224. https://doi.org/10.1007/s00262-014-1623-y
9. Annunziata, C. M., Davis, R. E., Demchenko, Y., Bellamy, W., Gabrea, A., Zhan, F., Lenz, G., Hanamura, I., Wright, G., Xiao, W., Dave, S., Hurt, E. M., Tan, B., Zhao, H., Stephens, O., Santra, M., Williams, D. R., Dang, L., Barlogie, B., … Staudt, L. M. (2007). Frequent Engagement of the Classical and Alternative NF-κB Pathways by Diverse Genetic Abnormalities in Multiple Myeloma. *Cancer Cell*, *12*(2), 115–130. https://doi.org/10.1016/j.ccr.2007.07.004
10. Arnulf, B., Lecourt, S., Soulier, J., Ternaux, B., Lacassagne, M. N., Crinquette, A., Dessoly, J., Sciaini, A. K., Benbunan, M., Chomienne, C., Fermand, J. P., Marolleau, J. P., & Larghero, J. (2007). Phenotypic and functional characterization of bone marrow mesenchymal stem cells derived from patients with multiple myeloma. *Leukemia*, *21*(1), 158–163. https://doi.org/10.1038/sj.leu.2404466
11. Barrow, A. D., Martin, C. J., & Colonna, M. (2019). The Natural Cytotoxicity Receptors in Health and Disease. *Frontiers in Immunology*, *10*. https://doi.org/10.3389/fimmu.2019.00909
12. Bedel, R., Thiery-Vuillemin, A., Grandclement, C., Balland, J., Remy-Martin, J.-P., Kantelip, B., Pallandre, J.-R., Pivot, X., Ferrand, C., Tiberghien, P., & Borg, C. (2011). Novel Role for

STAT3 in Transcriptional Regulation of NK Immune Cell Targeting Receptor MICA on Cancer Cells. *Cancer Research*, *71*(5), 1615–1626. https://doi.org/10.1158/0008-5472.CAN-09-4540
13. Benson, D. M., Bakan, C. E., Mishra, A., Hofmeister, C. C., Efebera, Y., Becknell, B., Baiocchi, R. A., Zhang, J., Yu, J., Smith, M. K., Greenfield, C. N., Porcu, P., Devine, S. M., Rotem-Yehudar, R., Lozanski, G., Byrd, J. C., & Caligiuri, M. A. (2010). The PD-1/PD-L1 axis modulates the natural killer cell versus multiple myeloma effect: A therapeutic target for CT-011, a novel monoclonal anti-PD-1 antibody. *Blood*, *116*(13), 2286–2294. https://doi.org/10.1182/blood-2010-02-271874
14. Blade, J., Rosinol, L., Cibeira, M. T., Rovira, M., & Carreras, E. (2010). Hematopoietic stem cell transplantation for multiple myeloma beyond 2010. *Blood*, *115*(18), 3655–3663. https://doi.org/10.1182/blood-2009-08-238196
15. Boddicker, R. L., Kip, N. S., Xing, X., Zeng, Y., Yang, Z.-Z., Lee, J.-H., Almada, L. L., Elsawa, S. F., Knudson, R. A., Law, M. E., Ketterling, R. P., Cunningham, J. M., Wu, Y., Maurer, M. J., O'Byrne, M. M., Cerhan, J. R., Slager, S. L., Link, B. K., Porcher, J. C., … Feldman, A. L. (2015). The oncogenic transcription factor IRF4 is regulated by a novel CD30/NF-κB positive feedback loop in peripheral T-cell lymphoma. *Blood*, *125*(20), 3118–3127. https://doi.org/10.1182/blood-2014-05-578575
16. Borrego, F., Masilamani, M., Kabat, J., Sanni, T. B., & Coligan, J. E. (2005). The cell biology of the human natural killer cell CD94/NKG2A inhibitory receptor. *Molecular Immunology*, *42*(4), 485–488. https://doi.org/10.1016/j.molimm.2004.07.031
17. Bottino, C., Biassoni, R., Millo, R., Moretta, L., & Moretta, A. (2000). The human natural cytotoxicity receptors (NCR) that induce HLA class I-independent NK cell triggering. *Human Immunology*, *61*(1), 1–6. https://doi.org/10.1016/S0198-8859(99)00162-7
18. Bottino, C., Castriconi, R., Pende, D., Rivera, P., Nanni, M., Carnemolla, B., Cantoni, C., Grassi, J., Marcenaro, S., Reymond, N., Vitale, M., Moretta, L., Lopez, M., & Moretta, A. (2003). Identification of PVR (CD155) and Nectin-2 (CD112) as Cell Surface Ligands for the Human DNAM-1 (CD226) Activating Molecule. *The Journal of Experimental Medicine*, *198*(4), 557–567. https://doi.org/10.1084/jem.20030788
19. Brandetti, E., Focaccetti, C., Pezzolo, A., Ognibene, M., Folgiero, V., Cotugno, N., Benvenuto, M., Palma, P., Manzari, V., Rossi, P., Fruci, D., Bei, R., & Cifaldi, L. (2021). Enhancement of Neuroblastoma NK-Cell-Mediated Lysis through NF-kB p65 Subunit-Induced Expression of FAS and PVR, the Loss of Which Is Associated with Poor Patient Outcome. *Cancers*, *13*(17), 4368. https://doi.org/10.3390/cancers13174368
20. Brown, M. H., Boles, K., Anton van der Merwe, P., Kumar, V., Mathew, P. A., & Neil Barclay, A. (1998). 2B4, the Natural Killer and T Cell Immunoglobulin Superfamily Surface Protein, Is a Ligand for CD48. *Journal of Experimental Medicine*, *188*(11), 2083–2090. https://doi.org/10.1084/jem.188.11.2083
21. Bryceson, Y. T., & Long, E. O. (2008). Line of attack: NK cell specificity and integration of signals. *Current Opinion in Immunology*, *20*(3), 344–352. https://doi.org/10.1016/j.coi.2008.03.005
22. Bryceson, Y. T., March, M. E., Ljunggren, H.-G., & Long, E. O. (2006). Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. *Blood*, *107*(1), 159–166. https://doi.org/10.1182/blood-2005-04-1351
23. Burgess, S. J., Marusina, A. I., Pathmanathan, I., Borrego, F., & Coligan, J. E. (2006). IL-21 down-regulates NKG2D/DAP10 expression on human NK and CD8+ T cells. *Journal of Immunology (Baltimore, Md. : 1950)*, *176*(3), 1490–1497. https://doi.org/10.4049/jimmunol.176.3.1490
24. Caligiuri, M. A. (2008). Human natural killer cells. *Blood*, *112*(3), 461–469. https://doi.org/10.1182/blood-2007-09-077438
25. Campbell, K. S., Yusa, S., Kikuchi-Maki, A., & Catina, T. L. (2004). NKp44 Triggers NK Cell Activation through DAP12 Association That Is Not Influenced by a Putative Cytoplasmic

Inhibitory Sequence. *The Journal of Immunology*, *172*(2), 899–906. https://doi.org/10.4049/jimmunol.172.2.899
26. Cantoni, C., Bottino, C., Vitale, M., Pessino, A., Augugliaro, R., Malaspina, A., Parolini, S., Moretta, L., Moretta, A., & Biassoni, R. (1999). NKp44, A Triggering Receptor Involved in Tumor Cell Lysis by Activated Human Natural Killer Cells, Is a Novel Member of the Immunoglobulin Superfamily. *Journal of Experimental Medicine*, *189*(5), 787–796. https://doi.org/10.1084/jem.189.5.787
27. Carbone, E., Neri, P., Mesuraca, M., Fulciniti, M. T., Otsuki, T., Pende, D., Groh, V., Spies, T., Pollio, G., Cosman, D., Catalano, L., Tassone, P., Rotoli, B., & Venuta, S. (2005). HLA class I, NKG2D, and natural cytotoxicity receptors regulate multiple myeloma cell recognition by natural killer cells. *Blood*, *105*(1), 251–258. https://doi.org/10.1182/blood-2004-04-1422
28. Carrasco, D. R., Sukhdeo, K., Protopopova, M., Sinha, R., Enos, M., Carrasco, D. E., Zheng, M., Mani, M., Henderson, J., Pinkus, G. S., Munshi, N., Horner, J., Ivanova, E. v., Protopopov, A., Anderson, K. C., Tonon, G., & DePinho, R. A. (2007). The Differentiation and Stress Response Factor XBP-1 Drives Multiple Myeloma Pathogenesis. *Cancer Cell*, *11*(4), 349–360. https://doi.org/10.1016/j.ccr.2007.02.015
29. Carrega, P., Bonaccorsi, I., di Carlo, E., Morandi, B., Paul, P., Rizzello, V., Cipollone, G., Navarra, G., Mingari, M. C., Moretta, L., & Ferlazzo, G. (2014). CD56 ^{bright} Perforin ^{low} Noncytotoxic Human NK Cells Are Abundant in Both Healthy and Neoplastic Solid Tissues and Recirculate to Secondary Lymphoid Organs via Afferent Lymph. *The Journal of Immunology*, *192*(8), 3805–3815. https://doi.org/10.4049/jimmunol.1301889
30. Carrega, P., Morandi, B., Costa, R., Frumento, G., Forte, G., Altavilla, G., Ratto, G. B., Mingari, M. C., Moretta, L., & Ferlazzo, G. (2008). Natural killer cells infiltrating human nonsmall-cell lung cancer are enriched in CD56brightCD16− cells and display an impaired capability to kill tumor cells. *Cancer*, *112*(4), 863–875. https://doi.org/10.1002/cncr.23239
31. Castriconi, R., Cantoni, C., della Chiesa, M., Vitale, M., Marcenaro, E., Conte, R., Biassoni, R., Bottino, C., Moretta, L., & Moretta, A. (2003). Transforming growth factor 1 inhibits expression of NKp30 and NKG2D receptors: Consequences for the NK-mediated killing of dendritic cells. *Proceedings of the National Academy of Sciences*, *100*(7), 4120–4125. https://doi.org/10.1073/pnas.0730640100
32. Chan, C. J., Smyth, M. J., & Martinet, L. (2014). Molecular mechanisms of natural killer cell activation in response to cellular stress. *Cell Death & Differentiation*, *21*(1), 5–14. https://doi.org/10.1038/cdd.2013.26
33. Chen, X., Trivedi, P. P., Ge, B., Krzewski, K., & Strominger, J. L. (2007). Many NK cell receptors activate ERK2 and JNK1 to trigger microtubule organizing center and granule polarization and cytotoxicity. *Proceedings of the National Academy of Sciences*, *104*(15), 6329–6334. https://doi.org/10.1073/pnas.0611655104
34. Cheng, Q., Li, X., Liu, J., Ye, Q., Chen, Y., Tan, S., & Liu, J. (2017). Multiple Myeloma-Derived Exosomes Regulate the Functions of Mesenchymal Stem Cells Partially via Modulating miR-21 and miR-146a. *Stem Cells International*, *2017*, 1–9. https://doi.org/10.1155/2017/9012152
35. Chester, C., Fritsch, K., & Kohrt, H. E. (2015). Natural Killer Cell Immunomodulation: Targeting Activating, Inhibitory, and Co-stimulatory Receptor Signaling for Cancer Immunotherapy. *Frontiers in Immunology*, *6*. https://doi.org/10.3389/fimmu.2015.00601
36. Chiu, K.-C., Lee, C.-H., Liu, S.-Y., Chou, Y.-T., Huang, R.-Y., Huang, S.-M., & Shieh, Y.-S. (2015). Polarization of tumor-associated macrophages and Gas6/Axl signaling in oral squamous cell carcinoma. *Oral Oncology*, *51*(7), 683–689. https://doi.org/10.1016/j.oraloncology.2015.04.004
37. Collins, S. M., Bakan, C. E., Swartzel, G. D., Hofmeister, C. C., Efebera, Y. A., Kwon, H., Starling, G. C., Ciarlariello, D., Bhaskar, S., Briercheck, E. L., Hughes, T., Yu, J., Rice, A., & Benson, D. M. (2013). Elotuzumab directly enhances NK cell cytotoxicity against myeloma via

CS1 ligation: Evidence for augmented NK cell function complementing ADCC. *Cancer Immunology, Immunotherapy*, *62*(12), 1841–1849. https://doi.org/10.1007/s00262-013-1493-8
38. Colucci, S., Brunetti, G., Oranger, A., Mori, G., Sardone, F., Specchia, G., Rinaldi, E., Curci, P., Liso, V., Passeri, G., Zallone, A., Rizzi, R., & Grano, M. (2011). Myeloma cells suppress osteoblasts through sclerostin secretion. *Blood Cancer Journal*, *1*(6), e27–e27. https://doi.org/10.1038/bcj.2011.22
39. Corre, J., Labat, E., Espagnolle, N., Hébraud, B., Avet-Loiseau, H., Roussel, M., Huynh, A., Gadelorge, M., Cordelier, P., Klein, B., Moreau, P., Facon, T., Fournié, J.-J., Attal, M., & Bourin, P. (2012). Bioactivity and Prognostic Significance of Growth Differentiation Factor GDF15 Secreted by Bone Marrow Mesenchymal Stem Cells in Multiple Myeloma. *Cancer Research*, *72*(6), 1395–1406. https://doi.org/10.1158/0008-5472.CAN-11-0188
40. Corre, J., Mahtouk, K., Attal, M., Gadelorge, M., Huynh, A., Fleury-Cappellesso, S., Danho, C., Laharrague, P., Klein, B., Rème, T., & Bourin, P. (2007). Bone marrow mesenchymal stem cells are abnormal in multiple myeloma. *Leukemia*, *21*(5), 1079–1088. https://doi.org/10.1038/sj.leu.2404621
41. Costello, R. T., Sivori, S., Marcenaro, E., Lafage-Pochitaloff, M., Mozziconacci, M.-J., Reviron, D., Gastaut, J.-A., Pende, D., Olive, D., & Moretta, A. (2002). Defective expression and function of natural killer cell–triggering receptors in patients with acute myeloid leukemia. *Blood*, *99*(10), 3661–3667. https://doi.org/10.1182/blood.V99.10.3661
42. Dabbah, M., Attar-Schneider, O., Tartakover Matalon, S., Shefler, I., Jarchwsky Dolberg, O., Lishner, M., & Drucker, L. (2017). Microvesicles derived from normal and multiple myeloma bone marrow mesenchymal stem cells differentially modulate myeloma cells' phenotype and translation initiation. *Carcinogenesis*, *38*(7), 708–716. https://doi.org/10.1093/carcin/bgx045
43. Dabbah, M., Jarchowsky-Dolberg, O., Attar-Schneider, O., Tartakover Matalon, S., Pasmanik-Chor, M., Drucker, L., & Lishner, M. (2020). Multiple myeloma BM-MSCs increase the tumorigenicity of MM cells via transfer of VLA4-enriched microvesicles. *Carcinogenesis*, *41*(1), 100–110. https://doi.org/10.1093/carcin/bgz169
44. de Smedt, E., Lui, H., Maes, K., de Veirman, K., Menu, E., Vanderkerken, K., & de Bruyne, E. (2018). The Epigenome in Multiple Myeloma: Impact on Tumor Cell Plasticity and Drug Response. *Frontiers in Oncology*, *8*. https://doi.org/10.3389/fonc.2018.00566
45. de Veirman, K., Wang, J., Xu, S., Leleu, X., Himpe, E., Maes, K., de Bruyne, E., van Valckenborgh, E., Vanderkerken, K., Menu, E., & van Riet, I. (2016). Induction of miR-146a by multiple myeloma cells in mesenchymal stromal cells stimulates their pro-tumoral activity. *Cancer Letters*, *377*(1), 17–24. https://doi.org/10.1016/j.canlet.2016.04.024
46. Dejardin, E. (2006). The alternative NF-κB pathway from biochemistry to biology: Pitfalls and promises for future drug development. *Biochemical Pharmacology*, *72*(9), 1161–1179. https://doi.org/10.1016/j.bcp.2006.08.007
47. del Prete, A., Martínez-Muñoz, L., Mazzon, C., Toffali, L., Sozio, F., Za, L., Bosisio, D., Gazzurelli, L., Salvi, V., Tiberio, L., Liberati, C., Scanziani, E., Vecchi, A., Laudanna, C., Mellado, M., Mantovani, A., & Sozzani, S. (2017). The atypical receptor CCRL2 is required for CXCR2-dependent neutrophil recruitment and tissue damage. *Blood*, *130*(10), 1223–1234. https://doi.org/10.1182/blood-2017-04-777680
48. Demchenko, Y. N., Glebov, O. K., Zingone, A., Keats, J. J., Bergsagel, P. L., & Kuehl, W. M. (2010). Classical and/or alternative NF-kappaB pathway activation in multiple myeloma. *Blood*, *115*(17), 3541–3552. https://doi.org/10.1182/blood-2009-09-243535
49. Deng, M., Yuan, H., Liu, S., Hu, Z., & Xiao, H. (2019). Exosome-transmitted LINC00461 promotes multiple myeloma cell proliferation and suppresses apoptosis by modulating microRNA/BCL-2 expression. *Cytotherapy*, *21*(1), 96–106. https://doi.org/10.1016/j.jcyt.2018.10.006
50. Díaz-Tejedor, A., Lorenzo-Mohamed, M., Puig, N., García-Sanz, R., Mateos, M.-V., Garayoa, M., & Paíno, T. (2021). Immune System Alterations in Multiple Myeloma: Molecular

Mechanisms and Therapeutic Strategies to Reverse Immunosuppression. *Cancers*, *13*(6), 1353. https://doi.org/10.3390/cancers13061353
51. Dominici, M., le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F. C., Krause, D. S., Deans, R. J., Keating, A., Prockop, D. J., & Horwitz, E. M. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy*, *8*(4), 315–317. https://doi.org/10.1080/14653240600855905
52. Du, W., Zhu, J., Zeng, Y., Liu, T., Zhang, Y., Cai, T., Fu, Y., Zhang, W., Zhang, R., Liu, Z., & Huang, J. (2021). KPNB1-mediated nuclear translocation of PD-L1 promotes non-small cell lung cancer cell proliferation via the Gas6/MerTK signaling pathway. *Cell Death & Differentiation*, *28*(4), 1284–1300. https://doi.org/10.1038/s41418-020-00651-5
53. Duan, S., Guo, W., Xu, Z., He, Y., Liang, C., Mo, Y., Wang, Y., Xiong, F., Guo, C., Li, Y., Li, X., Li, G., Zeng, Z., Xiong, W., & Wang, F. (2019). Natural killer group 2D receptor and its ligands in cancer immune escape. *Molecular Cancer*, *18*(1). https://doi.org/10.1186/s12943-019-0956-8
54. Duan, Y., Luo, L., Qiao, C., Li, X., Wang, J., Liu, H., Zhou, T., Shen, B., Lv, M., & Feng, J. (2019). A novel human anti-AXL monoclonal antibody attenuates tumour cell migration. *Scandinavian Journal of Immunology*, *90*(2). https://doi.org/10.1111/sji.12777
55. Eichner, R., Heider, M., Fernández-Sáiz, V., van Bebber, F., Garz, A.-K., Lemeer, S., Rudelius, M., Targosz, B.-S., Jacobs, L., Knorn, A.-M., Slawska, J., Platzbecker, U., Germing, U., Langer, C., Knop, S., Einsele, H., Peschel, C., Haass, C., Keller, U., … Bassermann, F. (2016). Immunomodulatory drugs disrupt the cereblon–CD147–MCT1 axis to exert antitumor activity and teratogenicity. *Nature Medicine*, *22*(7), 735–743. https://doi.org/10.1038/nm.4128
56. Eisele, G. (2006). TGF- and metalloproteinases differentially suppress NKG2D ligand surface expression on malignant glioma cells. *Brain*, *129*(9). https://doi.org/10.1093/brain/awl205
57. El-Sherbiny, Y. M., Meade, J. L., Holmes, T. D., McGonagle, D., Mackie, S. L., Morgan, A. W., Cook, G., Feyler, S., Richards, S. J., Davies, F. E., Morgan, G. J., & Cook, G. P. (2007). The requirement for DNAM-1, NKG2D, and NKp46 in the natural killer cell-mediated killing of myeloma cells. *Cancer Research*, *67*(18), 8444–8449. https://doi.org/10.1158/0008-5472.CAN-06-4230
58. Epling-Burnette, P. K., Bai, F., Painter, J. S., Rollison, D. E., Salih, H. R., Krusch, M., Zou, J., Ku, E., Zhong, B., Boulware, D., Moscinski, L., Wei, S., Djeu, J. Y., & List, A. F. (2007). Reduced natural killer (NK) function associated with high-risk myelodysplastic syndrome (MDS) and reduced expression of activating NK receptors. *Blood*, *109*(11), 4816–4824. https://doi.org/10.1182/blood-2006-07-035519
59. Fionda, C., Abruzzese, M. P., Zingoni, A., Cecere, F., Vulpis, E., Peruzzi, G., Soriani, A., Molfetta, R., Paolini, R., Ricciardi, M. R., Petrucci, M. T., Santoni, A., & Cippitelli, M. (2015). The IMiDs targets IKZF-1/3 and IRF4 as novel negative regulators of NK cell-activating ligands expression in multiple myeloma. *Oncotarget*, *6*(27), 23609–23630. https://doi.org/10.18632/oncotarget.4603
60. Fionda, C., Malgarini, G., Soriani, A., Zingoni, A., Cecere, F., Iannitto, M. L., Ricciardi, M. R., Federico, V., Petrucci, M. T., Santoni, A., & Cippitelli, M. (2013). Inhibition of Glycogen Synthase Kinase-3 Increases NKG2D Ligand MICA Expression and Sensitivity to NK Cell-Mediated Cytotoxicity in Multiple Myeloma Cells: Role of STAT3. *The Journal of Immunology*, *190*(12), 6662–6672. https://doi.org/10.4049/jimmunol.1201426
61. Fionda, C., Soriani, A., Malgarini, G., Iannitto, M. L., Santoni, A., & Cippitelli, M. (2009). Heat Shock Protein-90 Inhibitors Increase MHC Class I-Related Chain A and B Ligand Expression on Multiple Myeloma Cells and Their Ability to Trigger NK Cell Degranulation. *The Journal of Immunology*, *183*(7), 4385–4394. https://doi.org/10.4049/jimmunol.0901797
62. Fionda, C., Stabile, H., Molfetta, R., Kosta, A., Peruzzi, G., Ruggeri, S., Zingoni, A., Capuano, C., Soriani, A., Paolini, R., Gismondi, A., Cippitelli, M., & Santoni, A. (2021). Cereblon

regulates NK cell cytotoxicity and migration via Rac1 activation. *European Journal of Immunology*, *51*(11), 2607–2617. https://doi.org/10.1002/eji.202149269
63. Fionda, C., Stabile, H., Molfetta, R., Soriani, A., Bernardini, G., Zingoni, A., Gismondi, A., Paolini, R., Cippitelli, M., & Santoni, A. (2018). Translating the anti-myeloma activity of Natural Killer cells into clinical application. *Cancer Treatment Reviews*, *70*, 255–264. https://doi.org/10.1016/j.ctrv.2018.10.005
64. Fischer, E. S., Böhm, K., Lydeard, J. R., Yang, H., Stadler, M. B., Cavadini, S., Nagel, J., Serluca, F., Acker, V., Lingaraju, G. M., Tichkule, R. B., Schebesta, M., Forrester, W. C., Schirle, M., Hassiepen, U., Ottl, J., Hild, M., Beckwith, R. E. J., Harper, J. W., … Thomä, N. H. (2014). Structure of the DDB1–CRBN E3 ubiquitin ligase in complex with thalidomide. *Nature*, *512*(7512), 49–53. https://doi.org/10.1038/nature13527
65. Fitzgerald, W., Freeman, M. L., Lederman, M. M., Vasilieva, E., Romero, R., & Margolis, L. (2018). A System of Cytokines Encapsulated in ExtraCellular Vesicles. *Scientific Reports*, *8*(1), 8973. https://doi.org/10.1038/s41598-018-27190-x
66. Frohn, C., Höppner, M., Schlenke, P., Kirchner, H., Koritke, P., & Luhm, J. (2002). Anti-myeloma activity of natural killer lymphocytes. *British Journal of Haematology*, *119*(3), 660–664. https://doi.org/10.1046/j.1365-2141.2002.03879.x
67. Fu, B., Tian, Z., & Wei, H. (2014). Subsets of human natural killer cells and their regulatory effects. In *Immunology* (Vol. 141, Issue 4, pp. 483–489). https://doi.org/10.1111/imm.12224
68. Fuchs, A., & Colonna, M. (2006). The role of NK cell recognition of nectin and nectin-like proteins in tumor immunosurveillance. In *Seminars in Cancer Biology* (Vol. 16, Issue 5, pp. 359–366). https://doi.org/10.1016/j.semcancer.2006.07.002
69. Furukawa, M., Ohkawara, H., Ogawa, K., Ikeda, K., Ueda, K., Shichishima-Nakamura, A., Ito, E., Imai, J., Yanagisawa, Y., Honma, R., Watanabe, S., Waguri, S., Ikezoe, T., & Takeishi, Y. (2017). Autocrine and Paracrine Interactions between Multiple Myeloma Cells and Bone Marrow Stromal Cells by Growth Arrest-specific Gene 6 Cross-talk with Interleukin-6. *Journal of Biological Chemistry*, *292*(10), 4280–4292. https://doi.org/10.1074/jbc.M116.733030
70. Garayoa, M., Garcia, J. L., Santamaria, C., Garcia-Gomez, A., Blanco, J. F., Pandiella, A., Hernández, J. M., Sanchez-Guijo, F. M., del Cañizo, M. C., Gutiérrez, N. C., & San Miguel, J. F. (2009). Mesenchymal stem cells from multiple myeloma patients display distinct genomic profile as compared with those from normal donors. *Leukemia*, *23*(8), 1515–1527. https://doi.org/10.1038/leu.2009.65
71. Ghosh, S., & Karin, M. (2002). Missing Pieces in the NF-κB Puzzle. *Cell*, *109*(2), S81–S96. https://doi.org/10.1016/S0092-8674(02)00703-1
72. Gilfillan, S., Ho, E. L., Cella, M., Yokoyama, W. M., & Colonna, M. (2002). NKG2D recruits two distinct adapters to trigger NK cell activation and costimulation. *Nature Immunology*, *3*(12), 1150–1155. https://doi.org/10.1038/ni857
73. Gilmore, T. D. (2007). Multiple myeloma: lusting for NF-kappaB. *Cancer Cell*, *12*(2), 95–97. https://doi.org/10.1016/j.ccr.2007.07.010
74. Gismondi, A., Stabile, H., Nisti, P., & Santoni, A. (2015). Effector Functions of Natural Killer Cell Subsets in the Control of Hematological Malignancies. *Frontiers in Immunology*, *6*. https://doi.org/10.3389/fimmu.2015.00567
75. Gross, E., Sunwoo, J. B., & Bui, J. D. (2013). Cancer Immunosurveillance and Immunoediting by Natural Killer Cells. *The Cancer Journal*, *19*(6), 483–489. https://doi.org/10.1097/PPO.0000000000000005
76. Guo, J., Zhao, Y., Fei, C., Zhao, S., Zheng, Q., Su, J., Wu, D., Li, X., & Chang, C. (2018). Dicer1 downregulation by multiple myeloma cells promotes the senescence and tumor-supporting capacity and decreases the differentiation potential of mesenchymal stem cells. *Cell Death & Disease*, *9*(5), 512. https://doi.org/10.1038/s41419-018-0545-6

77. Guo, Z., Li, Y., Zhang, D., & Ma, J. (2017). Axl inhibition induces the antitumor immune response which can be further potentiated by PD-1 blockade in the mouse cancer models. *Oncotarget*, *8*(52), 89761–89774. https://doi.org/10.18632/oncotarget.21125
78. Hayashi, T., Hideshima, T., Akiyama, M., Podar, K., Yasui, H., Raje, N., Kumar, S., Chauhan, D., Treon, S. P., Richardson, P., & Anderson, K. C. (2005). Molecular mechanisms whereby immunomodulatory drugs activate natural killer cells: Clinical application. *British Journal of Haematology*, *128*(2), 192–203. https://doi.org/10.1111/j.1365-2141.2004.05286.x
79. Hayden, M. S., & Ghosh, S. (2008). Shared Principles in NF-κB Signaling. *Cell*, *132*(3), 344–362. https://doi.org/10.1016/j.cell.2008.01.020
80. Herrero, A. B., García-Gómez, A., Garayoa, M., Corchete, L. A., Hernández, J. M., San Miguel, J., & Gutierrez, N. C. (2016). Effects of IL-8 Up-Regulation on Cell Survival and Osteoclastogenesis in Multiple Myeloma. *The American Journal of Pathology*, *186*(8), 2171–2182. https://doi.org/10.1016/j.ajpath.2016.04.003
81. Hideshima, T., & Anderson, K. C. (2021). Signaling Pathway Mediating Myeloma Cell Growth and Survival. *Cancers*, *13*(2), 216. https://doi.org/10.3390/cancers13020216
82. Hideshima, T., Ikeda, H., Chauhan, D., Okawa, Y., Raje, N., Podar, K., Mitsiades, C., Munshi, N. C., Richardson, P. G., Carrasco, R. D., & Anderson, K. C. (2009). Bortezomib induces canonical nuclear factor-κB activation in multiple myeloma cells. *Blood*, *114*(5), 1046–1052. https://doi.org/10.1182/blood-2009-01-199604
83. Hideshima, T., Mitsiades, C., Tonon, G., Richardson, P. G., & Anderson, K. C. (2007). Understanding multiple myeloma pathogenesis in the bone marrow to identify new therapeutic targets. *Nature Reviews Cancer*, *7*(8), 585–598. https://doi.org/10.1038/nrc2189
84. Hideshima, T., Ogiya, D., Liu, J., Harada, T., Kurata, K., Bae, J., Massefski, W., & Anderson, K. C. (2021). Immunomodulatory drugs activate NK cells via both Zap-70 and cereblon-dependent pathways. *Leukemia*, *35*(1), 177–188. https://doi.org/10.1038/s41375-020-0809-x
85. Hideshima, T., Richardson, P., & Anderson, K. C. (2003). Novel therapeutic approaches for multiple myeloma. *Immunological Reviews*, *194*(1), 164–176. https://doi.org/10.1034/j.1600-065X.2003.00053.x
86. Horton, N. C., Mathew, S. O., & Mathew, P. A. (2013). Novel Interaction between Proliferating Cell Nuclear Antigen and HLA I on the Surface of Tumor Cells Inhibits NK Cell Function through NKp44. *PLoS ONE*, *8*(3), e59552. https://doi.org/10.1371/journal.pone.0059552
87. Hromadnikova, I., Li, S., Kotlabova, K., & Dickinson, A. M. (2016). Influence of in vitro IL-2 or IL-15 alone or in combination with Hsp 70 derived 14-mer peptide (TKD) on the expression of NK cell activatory and inhibitory receptors on peripheral blood T cells, B cells and NKT cells. *PLoS ONE*, *11*(3). https://doi.org/10.1371/journal.pone.0151535
88. Huynh, M., Pak, C., Markovina, S., Callander, N. S., Chng, K. S., Wuerzberger-Davis, S. M., Bakshi, D. D., Kink, J. A., Hematti, P., Hope, C., Asimakopoulos, F., Rui, L., & Miyamoto, S. (2018). Hyaluronan and proteoglycan link protein 1 (HAPLN1) activates bortezomib-resistant NF-B activity and increases drug resistance in multiple myeloma. *Journal of Biological Chemistry*, *293*(7), 2452–2465. https://doi.org/10.1074/jbc.RA117.000667
89. Ito, T., Ando, H., Suzuki, T., Ogura, T., Hotta, K., Imamura, Y., Yamaguchi, Y., & Handa, H. (2010). Identification of a Primary Target of Thalidomide Teratogenicity. *Science*, *327*(5971), 1345–1350. https://doi.org/10.1126/science.1177319
90. Jurisic, V., Srdic, T., Konjevic, G., Markovic, O., & Colovic, M. (2007). Clinical stage-depending decrease of NK cell activity in multiple myeloma patients. *Medical Oncology*, *24*(3), 312–317. https://doi.org/10.1007/s12032-007-0007-y
91. Kanehira, M., Fujiwara, T., Nakajima, S., Okitsu, Y., Onishi, Y., Fukuhara, N., Ichinohasama, R., Okada, Y., & Harigae, H. (2017). A Lysophosphatidic Acid Receptors 1 and 3 Axis Governs Cellular Senescence of Mesenchymal Stromal Cells and Promotes Growth and Vascularization of Multiple Myeloma. *Stem Cells*, *35*(3), 739–753. https://doi.org/10.1002/stem.2499

92. Katodritou, E., Terpos, E., North, J., Kottaridis, P., Verrou, E., Gastari, V., Chadjiaggelidou, C., Sivakumaran, S., Jide-Banwo, S., Tsirogianni, M., Kapetanos, D., Zervas, K., & Lowdell, M. W. (2011). Tumor-primed natural killer cells from patients with multiple myeloma lyse autologous, NK-resistant, bone marrow-derived malignant plasma cells. *American Journal of Hematology*, *86*(12), 967–973. https://doi.org/10.1002/ajh.22163
93. Keats, J. J., Fonseca, R., Chesi, M., Schop, R., Baker, A., Chng, W. J., van Wier, S., Tiedemann, R., Shi, C. X., Sebag, M., Braggio, E., Henry, T., Zhu, Y. X., Fogle, H., Price-Troska, T., Ahmann, G., Mancini, C., Brents, L. A., Kumar, S., … Bergsagel, P. L. (2007). Promiscuous Mutations Activate the Noncanonical NF-κB Pathway in Multiple Myeloma. *Cancer Cell*, *12*(2), 131–144. https://doi.org/10.1016/j.ccr.2007.07.003
94. Kim, E.-O., Kim, N., Kim, T.-J., Kim, K., Kim, T. W., Kumar, V., & Lee, K.-M. (2010). Unidirectional signaling triggered through 2B4 (CD244), not CD48, in murine NK cells. *Journal of Leukocyte Biology*, *88*(4), 707–714. https://doi.org/10.1189/jlb.0410198
95. Kline, M., Donovan, K., Wellik, L., Lust, C., Jin, W., Moon-Tasson, L., Xiong, Y., Witzig, T. E., Kumar, S., Rajkumar, S. V., & Lust, J. A. (2007). Cytokine and chemokine profiles in multiple myeloma; significance of stromal interaction and correlation of IL-8 production with disease progression. *Leukemia Research*, *31*(5), 591–598. https://doi.org/10.1016/j.leukres.2006.06.012
96. Konjević, G., Vuletić, A., & Mirjačić Martinović, K. (2016). Natural killer cell receptors: alterations and therapeutic targeting in malignancies. *Immunologic Research*, *64*(1), 25–35. https://doi.org/10.1007/s12026-015-8695-4
97. Krampera, M., Galipeau, J., Shi, Y., Tarte, K., & Sensebe, L. (2013). Immunological characterization of multipotent mesenchymal stromal cells—The International Society for Cellular Therapy (ISCT) working proposal. *Cytotherapy*, *15*(9), 1054–1061. https://doi.org/10.1016/j.jcyt.2013.02.010
98. Kyle, R. A., Durie, B. G. M., Rajkumar, S. v, Landgren, O., Blade, J., Merlini, G., Kröger, N., Einsele, H., Vesole, D. H., Dimopoulos, M., San Miguel, J., Avet-Loiseau, H., Hajek, R., Chen, W. M., Anderson, K. C., Ludwig, H., Sonneveld, P., Pavlovsky, S., Palumbo, A., … Boccadoro, M. (2010). Monoclonal gammopathy of undetermined significance (MGUS) and smoldering (asymptomatic) multiple myeloma: IMWG consensus perspectives risk factors for progression and guidelines for monitoring and management. *Leukemia*, *24*(6), 1121–1127. https://doi.org/10.1038/leu.2010.60
99. Kyle, R. A., Remstein, E. D., Therneau, T. M., Dispenzieri, A., Kurtin, P. J., Hodnefield, J. M., Larson, D. R., Plevak, M. F., Jelinek, D. F., Fonseca, R., Melton, L. J., & Rajkumar, S. V. (2007). Clinical Course and Prognosis of Smoldering (Asymptomatic) Multiple Myeloma. *New England Journal of Medicine*, *356*(25), 2582–2590. https://doi.org/10.1056/NEJMoa070389
100. Kyriakakis, E., Cavallari, M., Pfaff, D., Fabbro, D., Mestan, J., Philippova, M., de Libero, G., Erne, P., & Resink, T. J. (2011). IL-8-mediated angiogenic responses of endothelial cells to lipid antigen activation of iNKT cells depend on EGFR transactivation. *Journal of Leukocyte Biology*, *90*(5), 929–939. https://doi.org/10.1189/jlb.0211097
101. Lanier, L. L. (2015). NKG2D Receptor and Its Ligands in Host Defense. *Cancer Immunology Research*, *3*(6), 575–582. https://doi.org/10.1158/2326-6066.CIR-15-0098
102. Laubach, J., Garderet, L., Mahindra, A., Gahrton, G., Caers, J., Sezer, O., Voorhees, P., Leleu, X., Johnsen, H. E., Streetly, M., Jurczyszyn, A., Ludwig, H., Mellqvist, U. H., Chng, W. J., Pilarski, L., Einsele, H., Hou, J., Turesson, I., Zamagni, E., … Richardson, P. G. (2016). Management of relapsed multiple myeloma: Recommendations of the International Myeloma Working Group. In *Leukemia* (Vol. 30, Issue 5, pp. 1005–1017). https://doi.org/10.1038/leu.2015.356
103. Leibson, P. J. (1997). Signal transduction during natural killer cell activation: Inside the mind of a killer. In *Immunity* (Vol. 6, Issue 6, pp. 655–661). https://doi.org/10.1016/S1074-7613(00)80441-0

104. Levi, I., Amsalem, H., Nissan, A., Darash-Yahana, M., Peretz, T., Mandelboim, O., & Rachmilewitz, J. (2015). Characterization of tumor infiltrating Natural Killer cell subset. *Oncotarget*, *6*(15), 13835–13843. https://doi.org/10.18632/oncotarget.3453
105. Lew, E. D., Oh, J., Burrola, P. G., Lax, I., Zagórska, A., Través, P. G., Schlessinger, J., & Lemke, G. (2014). Differential TAM receptor–ligand–phospholipid interactions delimit differential TAM bioactivities. *ELife*, *3*. https://doi.org/10.7554/eLife.03385
106. Li, B., Fu, J., Chen, P., & Zhuang, W. (2010). Impairment in Immunomodulatory Function of Mesenchymal Stem Cells from Multiple Myeloma Patients. *Archives of Medical Research*, *41*(8), 623–633. https://doi.org/10.1016/j.arcmed.2010.11.008
107. Li, J.-M., Southerland, L., Hossain, M. S., Giver, C. R., Wang, Y., Darlak, K., Harris, W., Waschek, J., & Waller, E. K. (2011). Absence of Vasoactive Intestinal Peptide Expression in Hematopoietic Cells Enhances Th1 Polarization and Antiviral Immunity in Mice. *The Journal of Immunology*, *187*(2), 1057–1065. https://doi.org/10.4049/jimmunol.1100686
108. Li, M., Xia, P., Du, Y., Liu, S., Huang, G., Chen, J., Zhang, H., Hou, N., Cheng, X., Zhou, L., Li, P., Yang, X., & Fan, Z. (2014). T-cell Immunoglobulin and ITIM Domain (TIGIT) Receptor/Poliovirus Receptor (PVR) Ligand Engagement Suppresses Interferon-γ Production of Natural Killer Cells via β-Arrestin 2-mediated Negative Signaling. *Journal of Biological Chemistry*, *289*(25), 17647–17657. https://doi.org/10.1074/jbc.M114.572420
109. Lin, D., Lavender, H., Soilleux, E. J., & O'Callaghan, C. A. (2012). NF-κB regulates MICA gene transcription in endothelial cell through a genetically inhibitable control site. *Journal of Biological Chemistry*, *287*(6), 4299–4310. https://doi.org/10.1074/jbc.M111.282152
110. Lin, G. L., & Hankenson, K. D. (2011). Integration of BMP, Wnt, and notch signaling pathways in osteoblast differentiation. *Journal of Cellular Biochemistry*, *112*(12), 3491–3501. https://doi.org/10.1002/jcb.23287
111. Ljunggren, H. G., & Kärre, K. (1990). In search of the "missing self": MHC molecules and NK cell recognition. *Immunology Today*, *11*(C), 237–244. https://doi.org/10.1016/0167-5699(90)90097-S
112. Lohr, J. G., Stojanov, P., Carter, S. L., Cruz-Gordillo, P., Lawrence, M. S., Auclair, D., Sougnez, C., Knoechel, B., Gould, J., Saksena, G., Cibulskis, K., McKenna, A., Chapman, M. A., Straussman, R., Levy, J., Perkins, L. M., Keats, J. J., Schumacher, S. E., Rosenberg, M., … Golub, T. R. (2014). Widespread genetic heterogeneity in multiple myeloma: Implications for targeted therapy. *Cancer Cell*, *25*(1), 91–101. https://doi.org/10.1016/j.ccr.2013.12.015
113. Long, E. O., Sik Kim, H., Liu, D., Peterson, M. E., & Rajagopalan, S. (2013). Controlling Natural Killer Cell Responses: Integration of Signals for Activation and Inhibition. *Annual Review of Immunology*, *31*(1), 227–258. https://doi.org/10.1146/annurev-immunol-020711-075005
114. Mace, E. M., Wu, W. W., Ho, T., Mann, S. S., Hsu, H.-T., & Orange, J. S. (2012). NK Cell Lytic Granules Are Highly Motile at the Immunological Synapse and Require F-Actin for Post-Degranulation Persistence. *The Journal of Immunology*, *189*(10), 4870–4880. https://doi.org/10.4049/jimmunol.1201296
115. Maiso, P., Mogollón, P., Ocio, E. M., & Garayoa, M. (2021). Bone Marrow Mesenchymal Stromal Cells in Multiple Myeloma: Their Role as Active Contributors to Myeloma Progression. *Cancers*, *13*(11), 2542. https://doi.org/10.3390/cancers13112542
116. Mamessier, E., Pradel, L. C., Thibult, M.-L., Drevet, C., Zouine, A., Jacquemier, J., Houvenaeghel, G., Bertucci, F., Birnbaum, D., & Olive, D. (2013). Peripheral Blood NK Cells from Breast Cancer Patients Are Tumor-Induced Composite Subsets. *The Journal of Immunology*, *190*(5), 2424–2436. https://doi.org/10.4049/jimmunol.1200140
117. Marcus, H., Attar-Schneider, O., Dabbah, M., Zismanov, V., Tartakover-Matalon, S., Lishner, M., & Drucker, L. (2016). Mesenchymal stem cells secretomes' affect multiple myeloma translation initiation. *Cellular Signalling*, *28*(6), 620–630. https://doi.org/10.1016/j.cellsig.2016.03.003

118. Markovina, S., Callander, N. S., O'Connor, S. L., Xu, G., Shi, Y., Leith, C. P., Kim, K., Trivedi, P., Kim, J., Hematti, P., & Miyamoto, S. (2010). Bone marrow stromal cells from multiple myeloma patients uniquely induce bortezomib resistant NF-κB activity in myeloma cells. *Molecular Cancer*, *9*(1), 176. https://doi.org/10.1186/1476-4598-9-176
119. Martinet, L., Jean, C., Dietrich, G., Fournié, J. J., & Poupot, R. (2010). PGE2inhibits natural killer and γδ T cell cytotoxicity triggered by NKR and TCR through a cAMP-mediated PKA type I-dependent signaling. *Biochemical Pharmacology*, *80*(6), 838–845. https://doi.org/10.1016/j.bcp.2010.05.002
120. Martinet, L., & Smyth, M. J. (2015). Balancing natural killer cell activation through paired receptors. In *Nature Reviews Immunology* (Vol. 15, Issue 4, pp. 243–254). https://doi.org/10.1038/nri3799
121. Martínez-Sánchez, M. v., Periago, A., Legaz, I., Gimeno, L., Mrowiec, A., Montes-Barqueros, N. R., Campillo, J. A., Bolarin, J. M., Bernardo, M. v., López-Álvarez, M. R., González, C., García-Garay, M. C., Muro, M., Cabañas-Perianes, V., Fuster, J. L., García-Alonso, A. M., Moraleda, J. M., Álvarez-Lopez, M. R., & Minguela, A. (2016). Overexpression of KIR inhibitory ligands (HLA-I) determines that immunosurveillance of myeloma depends on diverse and strong NK cell licensing. *OncoImmunology*, *5*(4). https://doi.org/10.1080/2162402X.2015.1093721
122. Mateos, M. V., Oriol, A., Martínez-López, J., Gutiérrez, N., Teruel, A. I., de Paz, R., García-Laraña, J., Bengoechea, E., Martín, A., Mediavilla, J. D., Palomera, L., de Arriba, F., González, Y., Hernández, J. M., Sureda, A., Bello, J. L., Bargay, J., Peñalver, F. J., Ribera, J. M., … Miguel, J. F. S. (2010). Bortezomib, melphalan, and prednisone versus bortezomib, thalidomide, and prednisone as induction therapy followed by maintenance treatment with bortezomib and thalidomide versus bortezomib and prednisone in elderly patients with untreated multiple myelom. *The Lancet Oncology*, *11*(10), 934–941. https://doi.org/10.1016/S1470-2045(10)70187-X
123. Matthews, G. M., de Matos Simoes, R., Dhimolea, E., Sheffer, M., Gandolfi, S., Dashevsky, O., Sorrell, J. D., & Mitsiades, C. S. (2016). NF-κB dysregulation in multiple myeloma. In *Seminars in Cancer Biology* (Vol. 39, pp. 68–76). https://doi.org/10.1016/j.semcancer.2016.08.005
124. Mitsiades, C. S., Mitsiades, N., Munshi, N. C., & Anderson, K. C. (2004). Focus on multiple myeloma. *Cancer Cell*, *6*(5), 439–444. https://doi.org/10.1016/j.ccr.2004.10.020
125. Mitsiades, C. S., Mitsiades, N., Poulaki, V., Schlossman, R., Akiyama, M., Chauhan, D., Hideshima, T., Treon, S. P., Munshi, N. C., Richardson, P. G., & Anderson, K. C. (2002). Activation of NF-KB and upregulation of intracellular anti-apoptotic proteins via the IGF-1/Akt signaling in human multiple myeloma cells: therapeutic implications. *Oncogene*, *21*(37), 5673. http://search.ebscohost.com/login.aspx?direct=true&db=a9h&AN=8638755&site=ehost-live&scope=site
126. Mitsiades, C. S., Mitsiades, N. S., Munshi, N. C., Richardson, P. G., & Anderson, K. C. (2006). The role of the bone microenvironment in the pathophysiology and therapeutic management of multiple myeloma: Interplay of growth factors, their receptors and stromal interactions. *European Journal of Cancer*, *42*(11), 1564–1573. https://doi.org/10.1016/j.ejca.2005.12.025
127. Mizesko, M. C., Banerjee, P. P., Monaco-Shawver, L., Mace, E. M., Bernal, W. E., Sawalle-Belohradsky, J., Belohradsky, B. H., Heinz, V., Freeman, A. F., Sullivan, K. E., Holland, S. M., Torgerson, T. R., Al-Herz, W., Chou, J., Hanson, I. C., Albert, M. H., Geha, R. S., Renner, E. D., & Orange, J. S. (2013). Defective actin accumulation impairs human natural killer cell function in patients with dedicator of cytokinesis 8 deficiency. *Journal of Allergy and Clinical Immunology*, *131*(3), 840–848. https://doi.org/10.1016/j.jaci.2012.12.1568
128. Molfetta, R., Milito, N. D., Zitti, B., Lecce, M., Fionda, C., Cippitelli, M., Santoni, A., & Paolini, R. (2019). The Ubiquitin-proteasome pathway regulates Nectin2/CD112 expression and

impairs NK cell recognition and killing. *European Journal of Immunology*, *49*(6), 873–883. https://doi.org/10.1002/eji.201847848
129. Molfetta, R., Zitti, B., Lecce, M., Milito, N. D., Stabile, H., Fionda, C., Cippitelli, M., Gismondi, A., Santoni, A., & Paolini, R. (2020). CD155: A Multi-Functional Molecule in Tumor Progression. *International Journal of Molecular Sciences*, *21*(3), 922. https://doi.org/10.3390/ijms21030922
130. Molinero, L. L., Fuertes, M. B., Girart, M. V., Fainboim, L., Rabinovich, G. a, Costas, M. a, & Zwirner, N. W. (2004). NF-kappa B regulates expression of the MHC class I-related chain A gene in activated T lymphocytes. *Journal of Immunology (Baltimore, Md. : 1950)*, *173*(9), 5583–5590. https://doi.org/10.4049/jimmunol.173.9.5583
131. Morgan, G. J., Johnson, D. C., Weinhold, N., Goldschmidt, H., Landgren, O., Lynch, H. T., Hemminki, K., & Houlston, R. S. (2014). Inherited genetic susceptibility to multiple myeloma. *Leukemia*, *28*(3), 518–524. https://doi.org/10.1038/leu.2013.344
132. Morvan, M. G., & Lanier, L. L. (2016). NK cells and cancer: you can teach innate cells new tricks. *Nature Reviews Cancer*, *16*(1), 7–19. https://doi.org/10.1038/nrc.2015.5
133. Nabekura, T., Gotthardt, D., Niizuma, K., Trsan, T., Jenus, T., Jonjic, S., & Lanier, L. L. (2017). Cutting Edge: NKG2D Signaling Enhances NK Cell Responses but Alone Is Insufficient To Drive Expansion during Mouse Cytomegalovirus Infection. *The Journal of Immunology*, *199*(5), 1567–1571. https://doi.org/10.4049/jimmunol.1700799
134. Overdijk, M. B., Jansen, J. H. M., Nederend, M., Lammerts van Bueren, J. J., Groen, R. W. J., Parren, P. W. H. I., Leusen, J. H. W., & Boross, P. (2016). The Therapeutic CD38 Monoclonal Antibody Daratumumab Induces Programmed Cell Death via Fcγ Receptor–Mediated Cross-Linking. *The Journal of Immunology*, *197*(3), 807–813. https://doi.org/10.4049/jimmunol.1501351
135. Pahima, H., Puzzovio, P. G., & Levi-Schaffer, F. (2019). 2B4 and CD48: A powerful couple of the immune system. *Clinical Immunology*, *204*, 64–68. https://doi.org/10.1016/j.clim.2018.10.014
136. Palmieri, V., Lucchetti, D., Gatto, I., Maiorana, A., Marcantoni, M., Maulucci, G., Papi, M., Pola, R., de Spirito, M., & Sgambato, A. (2014). Dynamic light scattering for the characterization and counting of extracellular vesicles: a powerful noninvasive tool. *Journal of Nanoparticle Research*, *16*(9), 2583. https://doi.org/10.1007/s11051-014-2583-z
137. Palumbo, A., & Anderson, K. (2011). Multiple Myeloma. *The New England Journal of Medicine*, *364*(11), 1046–1060. https://doi.org/10.1007/978-1-4614-8520-9
138. Paolino, M., Choidas, A., Wallner, S., Pranjic, B., Uribesalgo, I., Loeser, S., Jamieson, A. M., Langdon, W. Y., Ikeda, F., Fededa, J. P., Cronin, S. J., Nitsch, R., Schultz-Fademrecht, C., Eickhoff, J., Menninger, S., Unger, A., Torka, R., Gruber, T., Hinterleitner, R., … Penninger, J. M. (2014). The E3 ligase Cbl-b and TAM receptors regulate cancer metastasis via natural killer cells. *Nature*, *507*(7493), 508–512. https://doi.org/10.1038/nature12998
139. Papi, M., Arcovito, G., de Spirito, M., Amiconi, G., Bellelli, A., & Boumis, G. (2005). Simultaneous static and dynamic light scattering approach to the characterization of the different fibrin gel structures occurring by changing chloride concentration. *Applied Physics Letters*, *86*(18), 183901. https://doi.org/10.1063/1.1915526
140. Pasero, C., Gravis, G., Granjeaud, S., Guerin, M., Thomassin-Piana, J., Rocchi, P., Salem, N., Walz, J., Moretta, A., & Olive, D. (2015). Highly effective NK cells are associated with good prognosis in patients with metastatic prostate cancer. *Oncotarget*, *6*(16), 14360–14373. https://doi.org/10.18632/oncotarget.3965
141. Pazina, T., MacFarlane, A. W., Bernabei, L., Dulaimi, E., Kotcher, R., Yam, C., Bezman, N. A., Robbins, M. D., Ross, E. A., Campbell, K. S., & Cohen, A. D. (2021). Alterations of NK Cell Phenotype in the Disease Course of Multiple Myeloma. *Cancers*, *13*(2), 226. https://doi.org/10.3390/cancers13020226

142. Pellegrino, A., Ria, R., Pietro, G. di, Cirulli, T., Surico, G., Pennisi, A., Morabito, F., Ribatti, D., & Vacca, A. (2005). Bone marrow endothelial cells in multiple myeloma secrete CXC-chemokines that mediate interactions with plasma cells. *British Journal of Haematology*, *129*(2), 248–256. https://doi.org/10.1111/j.1365-2141.2005.05443.x
143. Petillo, S., Capuano, C., Molfetta, R., Fionda, C., Mekhloufi, A., Pighi, C., Antonangeli, F., Zingoni, A., Soriani, A., Petrucci, M. T., Galandrini, R., Paolini, R., Santoni, A., & Cippitelli, M. (2021). Immunomodulatory effect of NEDD8-activating enzyme inhibition in Multiple Myeloma: upregulation of NKG2D ligands and sensitization to Natural Killer cell recognition. *Cell Death & Disease*, *12*(9), 836. https://doi.org/10.1038/s41419-021-04104-w
144. Petreaca, M. L., Yao, M., Liu, Y., DeFea, K., & Martins-Green, M. (2007). Transactivation of Vascular Endothelial Growth Factor Receptor-2 by Interleukin-8 (IL-8/CXCL8) Is Required for IL-8/CXCL8-induced Endothelial Permeability. *Molecular Biology of the Cell*, *18*(12), 5014–5023. https://doi.org/10.1091/mbc.e07-01-0004
145. Pinto, V., Bergantim, R., Caires, H. R., Seca, H., Guimarães, J. E., & Vasconcelos, M. H. (2020). Multiple Myeloma: Available Therapies and Causes of Drug Resistance. *Cancers*, *12*(2), 407. https://doi.org/10.3390/cancers12020407
146. Ponzetta, A., Benigni, G., Antonangeli, F., Sciumè, G., Sanseviero, E., Zingoni, A., Ricciardi, M. R., Petrucci, M. T., Santoni, A., & Bernardini, G. (2015). Multiple myeloma impairs bone marrow localization of effector natural killer cells by altering the chemokine microenvironment. *Cancer Research*, *75*(22), 4766–4777. https://doi.org/10.1158/0008-5472.CAN-15-1320
147. Quach, H., Ritchie, D., Stewart, A. K., Neeson, P., Harrison, S., Smyth, M. J., & Prince, H. M. (2010). Mechanism of action of immunomodulatory drugs (IMiDS) in multiple myeloma. In *Leukemia* (Vol. 24, Issue 1, pp. 22–32). https://doi.org/10.1038/leu.2009.236
148. Rabinovich, B., Li, J., Wolfson, M., Lawrence, W., Beers, C., Chalupny, J., Hurren, R., Greenfield, B., Miller, R., & Cosman, D. (2006). NKG2D splice variants: a reexamination of adaptor molecule associations. *Immunogenetics*, *58*(2–3), 81–88. https://doi.org/10.1007/s00251-005-0078-x
149. Raimondo, S., Urzì, O., Conigliaro, A., lo Bosco, G., Parisi, S., Carlisi, M., Siragusa, S., Raimondi, L., de Luca, A., Giavaresi, G., & Alessandro, R. (2020). Extracellular Vesicle microRNAs Contribute to the Osteogenic Inhibition of Mesenchymal Stem Cells in Multiple Myeloma. *Cancers*, *12*(2), 449. https://doi.org/10.3390/cancers12020449
150. Rajkumar, S. V., Landgren, O., & Mateos, M.-V. (2015). Smoldering multiple myeloma. *Blood*, *125*(20), 3069–3075. https://doi.org/10.1182/blood-2014-09-568899
151. Rana, K., & Whalen, M. (2015). Activation of protein kinase C and protein kinase D in human natural killer cells: effects of tributyltin, dibutyltin, and tetrabromobisphenol A. *Toxicology Mechanisms and Methods*, *25*(9), 680–688. https://doi.org/10.3109/15376516.2015.1070226
152. Raulet, D. H. (2004). Interplay of natural killer cells and their receptors with the adaptive immune response. In *Nature Immunology* (Vol. 5, Issue 10, pp. 996–1002). https://doi.org/10.1038/ni1114
153. Raulet, D. H., & Vance, R. E. (2006). Self-tolerance of natural killer cells. *Nature Reviews Immunology*, *6*(7), 520–531. https://doi.org/10.1038/nri1863
154. Ravi, G., & Gonsalves, W. I. (2021). Current diagnosis, risk stratification and treatment paradigms in newly diagnosed multiple myeloma. *Cancer Treatment and Research Communications*, *29*, 100444. https://doi.org/10.1016/j.ctarc.2021.100444
155. Reagan, M. R., Mishima, Y., Glavey, S. v., Zhang, Y., Manier, S., Lu, Z. N., Memarzadeh, M., Zhang, Y., Sacco, A., Aljawai, Y., Shi, J., Tai, Y.-T., Ready, J. E., Kaplan, D. L., Roccaro, A. M., & Ghobrial, I. M. (2014). Investigating osteogenic differentiation in multiple myeloma using a novel 3D bone marrow niche model. *Blood*, *124*(22), 3250–3259. https://doi.org/10.1182/blood-2014-02-558007
156. Rebmann, V., Schütt, P., Brandhorst, D., Opalka, B., Moritz, T., Nowrousian, M. R., & Grosse-Wilde, H. (2007). Soluble MICA as an independent prognostic factor for the overall

survival and progression-free survival of multiple myeloma patients. *Clinical Immunology (Orlando, Fla.)*, *123*(1), 114–120. https://doi.org/10.1016/j.clim.2006.11.007
157. Reiners, K. S., Topolar, D., Henke, A., Simhadri, V. R., Kessler, J., Sauer, M., Bessler, M., Hansen, H. P., Tawadros, S., Herling, M., Krönke, M., Hallek, M., & Pogge von Strandmann, E. (2013). Soluble ligands for NK cell receptors promote evasion of chronic lymphocytic leukemia cells from NK cell anti-tumor activity. *Blood*, *121*(18), 3658–3665. https://doi.org/10.1182/blood-2013-01-476606
158. Rezvani, K., Rouce, R., Liu, E., & Shpall, E. (2017). Engineering Natural Killer Cells for Cancer Immunotherapy. In *Molecular Therapy* (Vol. 25, Issue 8, pp. 1769–1781). https://doi.org/10.1016/j.ymthe.2017.06.012
159. Robertson, M. J., Caligiuri, M. A., Manley, T. J., Levine, H., & Ritz, J. (1990). Human natural killer cell adhesion molecules. Differential expression after activation and participation in cytolysis. *Journal of Immunology (Baltimore, Md. : 1950)*, *145*(10), 3194–3201.
160. Roccaro, A. M., Sacco, A., Maiso, P., Azab, A. K., Tai, Y. T., Reagan, M., Azab, F., Flores, L. M., Campigotto, F., Weller, E., Anderson, K. C., Scadden, D. T., & Ghobrial, I. M. (2013). BM mesenchymal stromal cell-derived exosomes facilitate multiple myeloma progression. *Journal of Clinical Investigation*, *123*(4), 1542–1555. https://doi.org/10.1172/JCI66517
161. Röllig, C., Knop, S., & Bornhäuser, M. (2015). Multiple myeloma. *The Lancet*, *385*(9983), 2197–2208. https://doi.org/10.1016/S0140-6736(14)60493-1
162. Romagné, F., André, P., Spee, P., Zahn, S., Anfossi, N., Gauthier, L., Capanni, M., Ruggeri, L., Benson, D. M., Blaser, B. W., della Chiesa, M., Moretta, A., Vivier, E., Caligiuri, M. A., Velardi, A., & Wagtmann, N. (2009). Preclinical characterization of 1-7F9, a novel human anti-KIR receptor therapeutic antibody that augments natural killer-mediated killing of tumor cells. *Blood*, *114*(13), 2667–2677. https://doi.org/10.1182/blood-2009-02-206532
163. Romera-Cárdenas, G., Thomas, L. M., Lopez-Cobo, S., García-Cuesta, E. M., Long, E. O., & Reyburn, H. T. (2016). Ionomycin Treatment Renders NK Cells Hyporesponsive. *PLOS ONE*, *11*(3), e0150998. https://doi.org/10.1371/journal.pone.0150998
164. Rossi, J.-F., Moreaux, J., Hose, D., Requirand, G., Rose, M., Rouillé, V., Nestorov, I., Mordenti, G., Goldschmidt, H., Ythier, A., & Klein, B. (2009). Atacicept in relapsed/refractory multiple myeloma or active Waldenström's macroglobulinemia: a phase I study. *British Journal of Cancer*, *101*(7), 1051–1058. https://doi.org/10.1038/sj.bjc.6605241
165. Rothlin, C. v., Carrera-Silva, E. A., Bosurgi, L., & Ghosh, S. (2015). TAM Receptor Signaling in Immune Homeostasis. *Annual Review of Immunology*, *33*(1), 355–391. https://doi.org/10.1146/annurev-immunol-032414-112103
166. Roy, P., Sarkar, U., & Basak, S. (2018). The NF-κB Activating Pathways in Multiple Myeloma. *Biomedicines*, *6*(2), 59. https://doi.org/10.3390/biomedicines6020059
167. Schilling, D., Kühnel, A., Tetzlaff, F., Konrad, S., & Multhoff, G. (2015). NZ28-induced inhibition of HSF1, SP1 and NF-κB triggers the loss of the natural killer cell-activating ligands MICA/B on human tumor cells. *Cancer Immunology, Immunotherapy*, *64*(5), 599–608. https://doi.org/10.1007/s00262-015-1665-9
168. Schraufstatter, I. U., Trieu, K., Zhao, M., Rose, D. M., Terkeltaub, R. A., & Burger, M. (2003). IL-8-Mediated Cell Migration in Endothelial Cells Depends on Cathepsin B Activity and Transactivation of the Epidermal Growth Factor Receptor. *The Journal of Immunology*, *171*(12), 6714–6722. https://doi.org/10.4049/jimmunol.171.12.6714
169. Screpanti, V., Wallin, R. P. A., Ljunggren, H.-G., & Grandien, A. (2001). A Central Role for Death Receptor-Mediated Apoptosis in the Rejection of Tumors by NK Cells. *The Journal of Immunology*, *167*(4), 2068–2073. https://doi.org/10.4049/jimmunol.167.4.2068
170. Seidel, U. J. E., Schlegel, P., & Lang, P. (2013). Natural Killer Cell Mediated Antibody-Dependent Cellular Cytotoxicity in Tumor Immunotherapy with Therapeutic Antibodies. *Frontiers in Immunology*, *4*. https://doi.org/10.3389/fimmu.2013.00076

171. Shaffer, A. L., Emre, N. C. T., Lamy, L., Ngo, V. N., Wright, G., Xiao, W., Powell, J., Dave, S., Yu, X., Zhao, H., Zeng, Y., Chen, B., Epstein, J., & Staudt, L. M. (2008). IRF4 addiction in multiple myeloma. *Nature*, *454*(7201), 226–231. https://doi.org/10.1038/nature07064
172. Shi, M., Liu, Z. W., & Wang, F. S. (2011). Immunomodulatory properties and therapeutic application of mesenchymal stem cells. In *Clinical and Experimental Immunology* (Vol. 164, Issue 1, pp. 1–8). https://doi.org/10.1111/j.1365-2249.2011.04327.x
173. Shibuya, K., Lanier, L. L., Phillips, J. H., Ochs, H. D., Shimizu, K., Nakayama, E., Nakauchi, H., & Shibuya, A. (1999). Physical and Functional Association of LFA-1 with DNAM-1 Adhesion Molecule. *Immunity*, *11*(5), 615–623. https://doi.org/10.1016/S1074-7613(00)80136-3
174. Shirakawa, J., Wang, Y., Tahara-Hanaoka, S., Honda, S., Shibuya, K., & Shibuya, A. (2006). LFA-1-dependent lipid raft recruitment of DNAM-1 (CD226) in CD4+ T cell. *International Immunology*, *18*(6), 951–957. https://doi.org/10.1093/intimm/dxl031
175. Silvestris, F., Lombardi, L., de Matteo, M., Bruno, A., & Dammacco, F. (2007). Myeloma bone disease: Pathogenetic mechanisms and clinical assessment. *Leukemia Research*, *31*(2), 129–138. https://doi.org/10.1016/j.leukres.2006.04.014
176. Sivori, S., Parolini, S., Falco, M., Marcenaro, E., Biassoni, R., Bottino, C., Moretta, L., & Moretta, A. (2000). 2B4 functions as a co-receptor in human NK cell activation. *European Journal of Immunology*, *30*(3), 787–793. https://doi.org/10.1002/1521-4141(200003)30:3<787::AID-IMMU787>3.0.CO;2-I
177. Smyth, M. J., Cretney, E., Kelly, J. M., Westwood, J. A., Street, S. E. A., Yagita, H., Takeda, K., Dommelen, S. L. H. van, Degli-Esposti, M. A., & Hayakawa, Y. (2005). Activation of NK cell cytotoxicity. *Molecular Immunology*, *42*(4), 501–510. https://doi.org/10.1016/j.molimm.2004.07.034
178. Soriani, A., Iannitto, M. L., Ricci, B., Fionda, C., Malgarini, G., Morrone, S., Peruzzi, G., Ricciardi, M. R., Petrucci, M. T., Cippitelli, M., & Santoni, A. (2014). Reactive Oxygen Species– and DNA Damage Response–Dependent NK Cell Activating Ligand Upregulation Occurs at Transcriptional Levels and Requires the Transcriptional Factor E2F1. *The Journal of Immunology*, *193*(2), 950–960. https://doi.org/10.4049/jimmunol.1400271
179. Soriani, A., Zingoni, A., Cerboni, C., Lannitto, M. L., Ricciardi, M. R., Gialleonardo, V. di, Cippitelli, M., Fionda, C., Petrucci, M. T., Guarini, A., Foà, R., & Santoni, A. (2009). ATM-ATR-dependent up-regulation of DNAM-1 and NKG2D ligands on multiple myeloma cells by therapeutic agents results in enhanced NK-cell susceptibility and is associated with a senescent phenotype. *Blood*, *113*(15), 3503–3511. https://doi.org/10.1182/blood-2008-08-173914
180. Stabile, H., Fionda, C., Gismondi, A., & Santoni, A. (2017). Role of Distinct Natural Killer Cell Subsets in Anticancer Response. *Frontiers in Immunology*, *8*. https://doi.org/10.3389/fimmu.2017.00293
181. Stabile, H., Nisti, P., Morrone, S., Pagliara, D., Bertaina, A., Locatelli, F., Santoni, A., & Gismondi, A. (2015). Multifunctional human CD56low CD16low natural killer cells are the prominent subset in bone marrow of both healthy pediatric donors and leukemic patients. *Haematologica*, *100*(4), 489–498. https://doi.org/10.3324/haematol.2014.116053
182. Tanaka, M., & Siemann, D. W. (2020). Gas6/Axl Signaling Pathway in the Tumor Immune Microenvironment. *Cancers*, *12*(7), 1850. https://doi.org/10.3390/cancers12071850
183. Thielens, A., Vivier, E., & Romagné, F. (2012). NK cell MHC class I specific receptors (KIR): From biology to clinical intervention. In *Current Opinion in Immunology* (Vol. 24, Issue 2, pp. 239–245). https://doi.org/10.1016/j.coi.2012.01.001
184. Tsukamoto, S., Løvendorf, M. B., Park, J., Salem, K. Z., Reagan, M. R., Manier, S., Zavidij, O., Rahmat, M., Huynh, D., Takagi, S., Kawano, Y., Kokubun, K., Thrue, C. A., Nagano, K., Petri, A., Roccaro, A. M., Capelletti, M., Baron, R., Kauppinen, S., & Ghobrial, I. M. (2018). Inhibition of microRNA-138 enhances bone formation in multiple myeloma bone marrow niche. *Leukemia*, *32*(8), 1739–1750. https://doi.org/10.1038/s41375-018-0161-6

185. Tsuruma, T., Yagihashi, A., Hirata, K., Torigoe, T., Araya, J., Watanabe, N., & Sato, N. (1999). Interleukin-10 reduces natural killer (NK) sensitivity of tumor cells by downregulating NK target structure expression. *Cellular Immunology*, *198*(2), 103–110. https://doi.org/10.1006/cimm.1999.1586
186. Umezu, T., Imanishi, S., Yoshizawa, S., Kawana, C., Ohyashiki, J. H., & Ohyashiki, K. (2019). Induction of multiple myeloma bone marrow stromal cell apoptosis by inhibiting extracellular vesicle miR-10a secretion. *Blood Advances*, *3*(21), 3228–3240. https://doi.org/10.1182/bloodadvances.2019000403
187. Upshaw, J. L., Arneson, L. N., Schoon, R. A., Dick, C. J., Billadeau, D. D., & Leibson, P. J. (2006). NKG2D-mediated signaling requires a DAP10-bound Grb2-Vav1 intermediate and phosphatidylinositol-3-kinase in human natural killer cells. *Nature Immunology*, *7*(5), 524–532. https://doi.org/10.1038/ni1325
188. Urashima, M., Ogata, A., Chauhan, D., Hatziyanni, M., Vidriales, M. B., Dedera, D. A., Schlossman, R. L., & Anderson, K. C. (1996). Transforming growth factor-beta1: differential effects on multiple myeloma versus normal B cells. *Blood*, *87*(5), 1928–1938. http://www.bloodjournal.org/content/87/5/1928.abstract
189. van Valckenborgh, E., Schouppe, E., Movahedi, K., de Bruyne, E., Menu, E., de Baetselier, P., VanDerkerken, K., & van GinDerachter, J. A. (2012). Multiple myeloma induces the immunosuppressive capacity of distinct myeloid-Derived suppressor cell subpopulations in the bone marrow. In *Leukemia* (Vol. 26, Issue 11, pp. 2424–2428). https://doi.org/10.1038/leu.2012.113
190. Viswanathan, S., Shi, Y., Galipeau, J., Krampera, M., Leblanc, K., Martin, I., Nolta, J., Phinney, D. G., & Sensebe, L. (2019). Mesenchymal stem versus stromal cells: International Society for Cell & Gene Therapy (ISCT®) Mesenchymal Stromal Cell committee position statement on nomenclature. *Cytotherapy*, *21*(10), 1019–1024. https://doi.org/10.1016/j.jcyt.2019.08.002
191. Vivier, E., Nunès, J. A., & Vély, F. (2004). Natural Killer Cell Signaling Pathways. *Science*, *306*(5701), 1517–1519. https://doi.org/10.1126/science.1103478
192. Vivier, E., Tomasello, E., Baratin, M., Walzer, T., & Ugolini, S. (2008). Functions of natural killer cells. *Nature Immunology*, *9*(5), 503–510. https://doi.org/10.1038/ni1582
193. von Lilienfeld-Toal, M., Frank, S., Leyendecker, C., Feyler, S., Jarmin, S., Morgan, R., Glasmacher, A., Märten, A., Schmidt-Wolf, I. G. H., Brossart, P., & Cook, G. (2010). Reduced immune effector cell NKG2D expression and increased levels of soluble NKG2D ligands in multiple myeloma may not be causally linked. *Cancer Immunology, Immunotherapy*, *59*(6), 829–839. https://doi.org/10.1007/s00262-009-0807-3
194. Wallington-Beddoe, C. T., & Mynott, R. L. (2021). Prognostic and predictive biomarker developments in multiple myeloma. *Journal of Hematology & Oncology*, *14*(1), 151. https://doi.org/10.1186/s13045-021-01162-7
195. Wang, J., de Veirman, K., de Beule, N., Maes, K., de Bruyne, E., van Valckenborgh, E., Vanderkerken, K., & Menu, E. (2015). The bone marrow microenvironment enhances multiple myeloma progression by exosome-mediated activation of myeloid-derived suppressor cells. *Oncotarget*, *6*(41), 43992–44004. https://doi.org/10.18632/oncotarget.6083
196. Wang, W. (2015). NK cell-mediated antibody-dependent cellular cytotoxicity in cancer immunotherapy. *Frontiers in Immunology*, *6*. https://doi.org/10.3389/fimmu.2015.00368
197. Waugh, D. J. J., & Wilson, C. (2008). The Interleukin-8 Pathway in Cancer. *Clinical Cancer Research*, *14*(21), 6735–6741. https://doi.org/10.1158/1078-0432.CCR-07-4843
198. Westgaard, I. H., Berg, S. F., Vaage, J. T., Wang, L. L., Yokoyama, W. M., Dissen, E., & Fossum, S. (2004). Rat NKp46 activates natural killer cell cytotoxicity and is associated with FcεRIγ and CD3ζ. *Journal of Leukocyte Biology*, *76*(6), 1200–1206. https://doi.org/10.1189/jlb.0903428

199. Wilson, E. B., El-Jawhari, J. J., Neilson, A. L., Hall, G. D., Melcher, A. A., Meade, J. L., & Cook, G. P. (2011). Human tumour immune evasion via TGF-β blocks NK cell activation but not survival allowing therapeutic restoration of anti-tumour activity. *PLoS ONE*, *6*(9). https://doi.org/10.1371/journal.pone.0022842
200. Wong, A. H.-H., Shin, E. M., Tergaonkar, V., & Chng, W.-J. (2020). Targeting NF-κB Signaling for Multiple Myeloma. *Cancers*, *12*(8), 2203. https://doi.org/10.3390/cancers12082203
201. Wu, G., Ma, Z., Cheng, Y., Hu, W., Deng, C., Jiang, S., Li, T., Chen, F., & Yang, Y. (2018). Targeting Gas6/TAM in cancer cells and tumor microenvironment. *Molecular Cancer*, *17*(1), 20. https://doi.org/10.1186/s12943-018-0769-1
202. Wu, Y., Tian, Z., & Wei, H. (2017). Developmental and Functional Control of Natural Killer Cells by Cytokines. *Frontiers in Immunology*, *8*. https://doi.org/10.3389/fimmu.2017.00930
203. Xiao, G., Wang, X., Sheng, J., Lu, S., Yu, X., & Wu, J. D. (2015). Soluble NKG2D ligand promotes MDSC expansion and skews macrophage to the alternatively activated phenotype. *Journal of Hematology & Oncology*, *8*(1), 13. https://doi.org/10.1186/s13045-015-0110-z
204. Xu, S., Cecilia Santini, G., de Veirman, K., vande Broek, I., Leleu, X., de Becker, A., van Camp, B., Vanderkerken, K., & van Riet, I. (2013). Upregulation of miR-135b Is Involved in the Impaired Osteogenic Differentiation of Mesenchymal Stem Cells Derived from Multiple Myeloma Patients. *PLoS ONE*, *8*(11), e79752. https://doi.org/10.1371/journal.pone.0079752
205. Xu, S., de Veirman, K., de Becker, A., Vanderkerken, K., & van Riet, I. (2018). Mesenchymal stem cells in multiple myeloma: a therapeutical tool or target? *Leukemia*, *32*(7). https://doi.org/10.1038/s41375-018-0061-9
206. Xu, S., Evans, H., Buckle, C., de Veirman, K., Hu, J., Xu, D., Menu, E., de Becker, A., vande Broek, I., Leleu, X., Camp, B. v, Croucher, P., Vanderkerken, K., & van Riet, I. (2012). Impaired osteogenic differentiation of mesenchymal stem cells derived from multiple myeloma patients is associated with a blockade in the deactivation of the Notch signaling pathway. *Leukemia*, *26*(12), 2546–2549. https://doi.org/10.1038/leu.2012.126
207. Yaccoby, S. (2010). Osteoblastogenesis and tumor growth in myeloma. *Leukemia & Lymphoma*, *51*(2), 213–220. https://doi.org/10.3109/10428190903503438
208. Yang, H., Zheng, Y., Zhang, Y., Cao, Z., & Jiang, Y. (2017). Mesenchymal stem cells derived from multiple myeloma patients protect against chemotherapy through autophagy-dependent activation of NF-κB signaling. *Leukemia Research*, *60*(July), 82–88. https://doi.org/10.1016/J.LEUKRES.2017.07.002
209. Yen, B. L., Yen, M.-L., Hsu, P.-J., Liu, K.-J., Wang, C.-J., Bai, C.-H., & Sytwu, H.-K. (2013). Multipotent Human Mesenchymal Stromal Cells Mediate Expansion of Myeloid-Derived Suppressor Cells via Hepatocyte Growth Factor/c-Met and STAT3. *Stem Cell Reports*, *1*(2), 139–151. https://doi.org/10.1016/j.stemcr.2013.06.006
210. Yin, X., Liu, T., Wang, Z., Ma, M., Lei, J., Zhang, Z., Fu, S., Fu, Y., Hu, Q., Ding, H., Han, X., Xu, J., Shang, H., & Jiang, Y. (2018). Expression of the Inhibitory Receptor TIGIT Is Up-Regulated Specifically on NK Cells With CD226 Activating Receptor From HIV-Infected Individuals. *Frontiers in Immunology*, *9*. https://doi.org/10.3389/fimmu.2018.02341
211. Yokoyama, W. M., & Kim, S. (2006). Licensing of natural killer cells by self-major histocompatibility complex class I. *Immunological Reviews*, *214*(1), 143–154. https://doi.org/10.1111/j.1600-065X.2006.00458.x
212. Yoshimura, T., Matsushima, K., Oppenheim, J. J., & Leonard, E. J. (1987). Neutrophil chemotactic factor produced by lipopolysaccharide (LPS)-stimulated human blood mononuclear leukocytes: partial characterization and separation from interleukin 1 (IL 1). *Journal of Immunology (Baltimore, Md. : 1950)*, *139*(3), 788–793.
213. Yu, J., Freud, A. G., & Caligiuri, M. A. (2013). Location and cellular stages of natural killer cell development. *Trends in Immunology*, *34*(12), 573–582. https://doi.org/10.1016/j.it.2013.07.005

214. Zamai, L., del Zotto, G., Buccella, F., Gabrielli, S., Canonico, B., Artico, M., Ortolani, C., & Papa, S. (2020). Understanding the Synergy of NKp46 and Co-Activating Signals in Various NK Cell Subpopulations: Paving the Way for More Successful NK-Cell-Based Immunotherapy. *Cells*, *9*(3), 753. https://doi.org/10.3390/cells9030753
215. Zdzisińska, B., Bojarska-Junak, A., Dmoszyńska, A., & Kandefer-Szerszeń, M. (2008). Abnormal cytokine production by bone marrow stromal cells of multiple myeloma patients in response to RPMI8226 myeloma cells. *Archivum Immunologiae et Therapiae Experimentalis*, *56*(3), 207. https://doi.org/10.1007/s00005-008-0022-5
216. Zhang, B., Au, Q., Yoon, I. S., Tremblay, M.-H., Yip, G., Zhou, Y., Barber, J. R., & Ng, S. C. (2009). Identification of small-molecule HSF1 amplifiers by high content screening in protection of cells from stress induced injury. *Biochemical and Biophysical Research Communications*, *390*(3), 925–930. https://doi.org/10.1016/j.bbrc.2009.10.079
217. Zhang, C., Zhang, J., Niu, J., Zhou, Z., Zhang, J., & Tian, Z. (2008). Interleukin-12 improves cytotoxicity of natural killer cells via upregulated expression of NKG2D. *Human Immunology*, *69*(8), 490–500. https://doi.org/10.1016/j.humimm.2008.06.004
218. Zhang, Z., Wang, W., Ma, D., Xiong, J., Kuang, X., Zhang, S., Fang, Q., & Wang, J. (2020). Heme oxygenase-1 inhibition mediates Gas6 to enhance bortezomib-sensitivity in multiple myeloma via ERK/STAT3 axis. *Aging*, *12*(8), 6611–6629. https://doi.org/10.18632/aging.102996
219. Zhang, Z., Wu, N., Lu, Y., Davidson, D., Colonna, M., & Veillette, A. (2015). DNAM-1 controls NK cell activation via an ITT-like motif. *The Journal of Experimental Medicine*, *212*(12), 2165–2182. https://doi.org/10.1084/jem.20150792
220. Zingoni, A., Cecere, F., Vulpis, E., Fionda, C., Molfetta, R., Soriani, A., Petrucci, M. T., Ricciardi, M. R., Fuerst, D., Amendola, M. G., Mytilineos, J., Cerboni, C., Paolini, R., Cippitelli, M., & Santoni, A. (2015). Genotoxic Stress Induces Senescence-Associated ADAM10-Dependent Release of NKG2D MIC Ligands in Multiple Myeloma Cells. *The Journal of Immunology*, *195*(2), 736–748. https://doi.org/10.4049/jimmunol.1402643
221. Zitti, B., Molfetta, R., Fionda, C., Quatrini, L., Stabile, H., Lecce, M., de Turris, V., Ricciardi, M. R., Petrucci, M. T., Cippitelli, M., Gismondi, A., Santoni, A., & Paolini, R. (2017). Innate immune activating ligand SUMOylation affects tumor cell recognition by NK cells. *Scientific Reports*, *7*(1). https://doi.org/10.1038/s41598-017-10403-0