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Targeting the bone marrow microenvironment in multiple myeloma

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Summary: Multiple myeloma (MM) is characterized by clonal expansion of malignant plasma cells in the bone marrow (BM). Despite the significant advances in treatment, MM is still a fatal malignancy. This is mainly due to the supportive role of the BM microenvironment in differentiation, migration, proliferation, survival, and drug resistance of the malignant plasma cells. The BM microenvironment is composed of a cellular compartment (stromal cells, osteoblasts, osteoclasts, endothelial cells, and immune cells) and a non-cellular compartment. In this review, we discuss the interaction between the malignant plasma cell and the BM microenvironment and the strategy to target them.

Keywords: *myeloma, bone marrow, immune cells, BMSCs, vessel formation*

Introduction

Multiple myeloma (MM) is characterized by clonal expansion of malignant plasma cells in the bone marrow (BM) leading to multiple bone lytic lesions, anemia, and immunodeficiency at the time of diagnosis (1). It is the second most common hematologic malignancy in the United States (2). MM is almost always preceded by a premalignant disease well known as monoclonal gammopathy of undetermined significance (MGUS). MGUS is found in approximately 2% of the general population aged 50 years and in 5% of those older than 70, and it progresses to MM at a rate of 1% per year (3). The response rate and overall survival (OS) of MM have significantly improved due to the introduction of novel agents such as thalidomide, lenalidomide, and bortezomib, and autologous stem cell transplantation (4). However MM continues to be a mostly incurable disease. Therefore, there is an urgent need for the development of other therapeutic agents that not only target the tumor clone but its permissive microenvironment.

MM cells grow and expand almost exclusively within the BM, thus suggesting the importance of the BM microenvironment in supporting MM cell growth and survival. The clonal plasma cell trafficking in an out of the BM allows the progression or 'metastasis' of the disease to new BM sites (5). The BM microenvironment consists of a cellular compartment and a non-cellular compartment. The cellular compartment can be subdivided into hematopoietic cell types including myeloid cells, T lymphocytes, B lymphocytes, NK cells, and osteoclasts, while non-hematopoietic cells include bone marrow stromal cells (BMSCs), fibroblasts, osteoblasts, endothelial cells, and blood vessels. The non-cellular compartment includes the extracellular matrix (ECM), oxygen concentration, and the liquid milieu (cytokines, growth factors, and chemokines), which are produced and/or affected by the cellular compartment within the bone marrow microenvironment. These microenvironment compartments exert differential effects on MM cell progression and may also work synergistically. In this review, we discuss how each BM microenvironment compartment supports MM cell growth and disease progression, as well as how to target the environment to prevent MM progression (*Fig. 1*).

The immune microenvironment in MM

An important step in the progression of tumors is evasion and suppression of the host immune system (6, 7). The role for the immune system in suppressing tumor growth was demonstrated *in vivo* using immunodeficient mouse models. For instance, $Rag2^{-/-}$ mice and SCID (severe combined immunodeficiency) mice, which lack both B and T cells, develop spontaneous adenocarcinomas (8) or T-cell lymphomas (9), respectively. In the normal microenvironment the effector cells, mainly the natural killer (NK) cells and cytotoxic T lymphocytes (CTLs), are capable of driving potent anti-tumor responses. However, tumor cells often induce an immunosuppressive microenvironment, which favors the expansion of immunosuppressive cell populations,

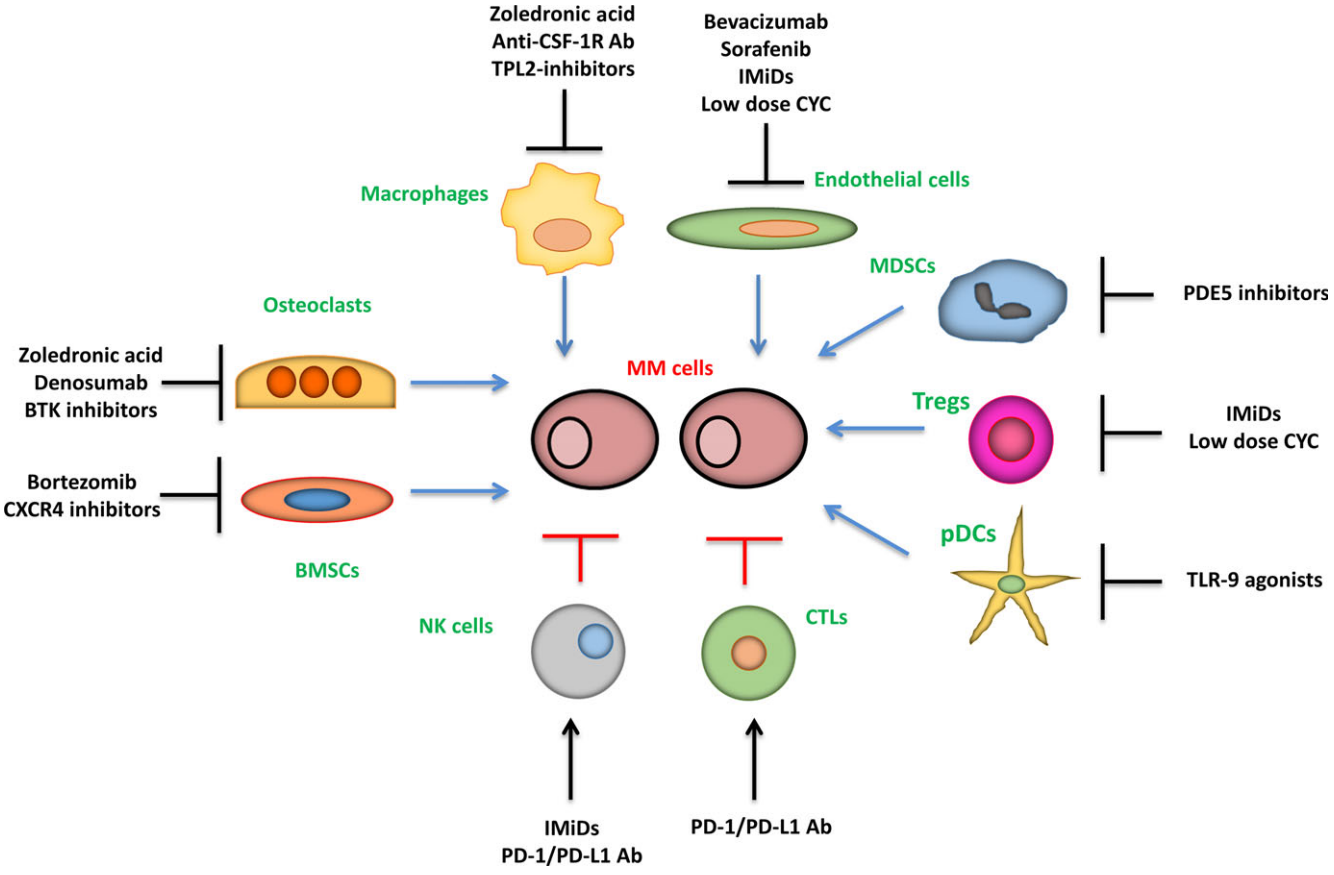

***Fig. 1.* Targeting the cellular compartment of the bone marrow (BM) microenvironment in multiple myeloma (MM).** Treatments suppressing the functions of myeloid derived suppressor cells (MDSCs), Tregs, plasmacytoid dendritic cells (pDCs), macrophages, bone marrow stromal cells (BMSCs), osteoclasts, and endothelial cells in MM microenvironment are under development, while IMiDs and PD-1 and PD-L1 antibody (PD-1/PD-L1 Ab) induce NK cell and cytotoxic T-lymphocyte (CTL) functions.

such as myeloid derived suppressor cells (MDSCs) and regulatory T cells (Tregs). MM is associated with both cellular and humoral immune deficiencies (10–12), indicating that the evolution of disease in MM is associated with an immunosuppressive milieu that fosters immune escape and tumor growth.

Myeloid derived suppressor cells (MDSCs)

MDSCs are a heterogeneous population of immature myeloid cells that differentiate into macrophages, granulocytes, or dendritic cells under normal conditions. However, under pathological conditions such as cancer, differentiation of immature myeloid cells is inhibited, resulting in accumulation of MDSCs (13). In cancer patients and tumor models, MDSCs accumulate in the tumor microenvironment due to release of soluble factors by tumor cells or cells in the microenvironment (14, 15). Previous reports showed that cancer patients with higher MDSC levels have shorter survival compared to patients with lower MDSC levels (16, 17). Depletion of MDSCs in tumor-bearing mice using Gr-1 antibody inhibited tumor outgrowth (18, 19), suggesting that MDSCs may be a target of anti-tumor treatment. There are two main subsets of MDSCs, granulocytic MDSC (G-MDSC) and monocytic MDSC (Mo-MDSC). In mice, G-MDSCs are identified as $CD11b^{+}$ $Ly6G^{hi}$ $Ly6C^{low}$ and Mo-MDSCs as $CD11b^{+}$ $Ly6G^{-}$ $Ly6C^{high}$, while in humans G-MDSCs are $CD11b^{+}$ $CD33^{+}$ HLA-$DR^{-/low}$ $CD14b^{-}$ and Mo-MDSCs are $CD11b^{+}$ $CD33^{+}$ HLA-$DR^{-/low}$ $CD14^{+}$. In tumor-bearing mice, G-MDSCs are the main MDSC subset to be expanded in the peripheral lymphoid organs (20). MDSCs can suppress T-cell proliferation through expression of immune suppressive factors, such as arginase, reactive oxygen species (ROS), and nitric oxide (NO). G-MDSCs have increased levels of ROS and low levels of NO, whereas Mo-MDSCs have increased level of NO but low levels of ROS (20, 21). It is also reported that MDSCs can induce the development of Tregs *in vivo*, which are anergic and suppressive (22).

Previous reports have shown an increase in the number of MDSCs in the peripheral blood (PB) (23, 24) and BM (25) of MM patients compared to healthy donors. MDSCs induce MM growth by suppressing T-cell-mediated immune responses, while MM cells induce the development of MDSCs from healthy donor peripheral blood mononuclear cells, confirming a bidirectional interaction between MDSCs and MM cells and immune effector cells (24). A recent report showed that purified MDSCs from patients with MM was able to induce more Treg development than MDSCs from age-matched controls (26). Interestingly, MDSCs from 5TGM1 murine MM mice displayed a significantly high potential to differentiate into mature and functional osteoclasts than those from non-tumor controls, which indicates that tumor-induced MDSCs exacerbate cancer-associated bone destruction by directly serving as osteoclast precursors (27).

Given that novel agents such as the immunomodulatory drug lenalidomide and the proteasome inhibitor bortezomib target both MM cells and the BM microenvironment (28), the effect of these drugs against MDSCs was studied. However, neither bortezomib nor lenalidomide were able to alter the suppressive activity of MDSCs (24). This indicates that additional strategies are needed to target MDSCs in the MM microenvironment. Phosphodiesterase-5 (PDE5) inhibitors reduced the suppressive machinery of tumor recruited MDSCs through downregulation of arginase 1 and nitric oxide synthase-2 expression in murine tumor models (18, 29, 30). Noonan *et al.* (31) recently reported that PDE5 inhibitor, tadalafil, reduced MDSC function in a relapsed/refractory MM patient. The strategy targeting MDSCs in MM with PDE5 inhibitors may represent a novel approach that can augment the efficacy of tumor-directed therapies.

Regulatory T cells (Tregs)

Among the T cells recruited to the tumor sites that have immunosuppressive functions are Tregs. T-cell infiltration has been shown to occur within the tumor microenvironment due to microenvironment-secreted chemokines/cytokines (32). Tregs are a subset of $CD4^{+}$ T lymphocytes that suppress functions of antigen-presenting cells (APCs) and effector T cells by direct contact or by release of anti-inflammatory cytokines (IL-10 and TGF-β); they are characterized by the expression of transcription factor FOXP3 (33). These cells accumulate in the tumor microenvironment and the peripheral blood of patients with cancer (34, 35). The increased frequency of Tregs has been generally considered as a marker of poor prognosis due to Treg-mediated suppression of anti-tumor immunity (36, 37). It has been shown by using the diphtheria toxin inducible 'depletion of regulatory T cell' (DEREG) mice (38) that Treg depletion induces regression of solid tumors, which was associated with an increased intratumoral accumulation of activated $CD8^{+}$ cytotoxic T cells (39, 40). These data indicate that targeting Tregs in cancer can be a potential anti-tumor strategy.

Many groups have reported an increase of functional Tregs in MM patient's PB compared to healthy donors (23, 41–44). A positive association of Treg frequency with international staging system (ISS) and paraprotein level was observed (42). The correlation between increased numbers of functional Tregs and disease progression was also shown in a MM mouse model (45). MM patients with higher percentage of Tregs in the peripheral blood are shown to have shorter time to progression (44) and shorter overall survival (43). Beyer *et al.* (41) showed that Tregs from MM patients express increased levels of IL-10 and TGF-β compared to healthy controls, indicating a more suppressive function of MM patients derived Tregs. However, there are some conflicting results (46, 47). The mechanism of how MM cells induce Tregs is not well understood. Feyler *et al.* (48) showed in an *in vitro* experimental model that MM cells can directly induce Tregs in an APC-independent manner mediated, at least in part, through MM expression of ICOS-L. These data showing the association of Tregs with MM progression suggest that Tregs could be targeted along with the tumor cells in MM.

Low-dose cyclophosphamide (CYC) has been shown to reduce the numbers and function of Tregs, and to induce anti-tumor, immune-mediated effects (49, 50). In a MM mouse model, low-dose CYC showed a transient depletion of Tregs resulting in reduced occurrence of MM and improved survival rate (51). Lenalidomide and pomalidomide, which are immunomodulatory drugs (IMiDS) used as anti-MM treatment, are reported to inhibit expansion and function of Tregs (52). However, for more specific and effective targeting of Tregs, it is vital to understand how Treg development, homeostasis, and effector function are controlled at the molecular level.

Dendritic cells (DCs)

Dendritic cells (DCs) are BM-derived professional APCs that present self and non-self antigens to T cells and promote immunity or tolerance (53). Antigen presentation by DCs induces naive T cells to differentiate into effector and memory T cells, but it can also lead to different forms of T-cell tolerance, depending on the functional status of the DCs. Myeloid DCs (mDCs) and Plasmacytoid DCs (pDCs) are the two major DC subsets that have been identified based on their origin, phenotype, and function (54). Several studies have documented an increase of DCs in human tumor sites, which often correlated with adverse prognosis (55–57). Indeed, loss of immune function of tumor-infiltrating DCs has been linked to the suppressive effects of the tumor microenvironment mediated by various cytokines (58). Recent findings have demonstrated that tumor-infiltrating pDCs from solid tumors express high levels of ICOS-L, which explains their ability to induce Tregs (59, 60). It was also shown that TGF-β secreted by DCs from breast cancer patients was partially associated with induction of Tregs (61). Similar findings of induction of Tregs by DCs were observed in MM patients (62).

DCs play an important role in normal plasma cell differentiation and survival (63, 64). However, the frequency and function of DCs in MM patients compared to healthy individuals is still controversial (65, 66). Chauhan *et al.* (67) showed that pDCs are increased in MM patient's BM compared to healthy controls and pDCs confer growth, survival, chemotaxis, and drug resistance against MM cells. Targeting Toll-like receptors with CpG oligodeoxynucleotides both restores pDC immune function and abrogates pDC-induced MM cell growth. Toll-like receptor 9 (TLR-9) agonist inhibited pDC-induced MM cell growth through Interferon secretion and activation of TLR9/MyD88 signaling axis (68). Kukreja *et al.* (69) reported that DCs enhanced clonogenic growth of MM cell lines and primary tumor cells from MM patients. This effect was inhibited by blockade of the RANK–RANK ligand and BAFF–APRIL-mediated interactions. These data suggest that MM–DC interactions may directly impact the biology of MM and may be a target for therapeutic intervention.

Natural killer cells (NK cells)

NK cells represent a heterogeneous lymphocyte population with cytotoxic anti-tumor capacity and multiple immunoregulatory properties. One of the NK cell activating receptors is Natural Killer group 2D (NKG2D) which recognizes various proteins expressed on the surface of target cells in response to several forms of cellular stress. MHC class I polypeptide-related sequence A (MICA) is one of the ligands for NKG2D. Target tumor cells ectopically expressing MICA are efficiently killed via NKG2D despite the expression of MHC class I molecules (70).

NK cells in MM patients are increased in the PB (71, 72) and BM (73, 74) compared to healthy individuals. However, the expansion of NK cells in MM patients is not associated with their activation. It is reported that the NKG2D expression on the surface of NK cells from MM patients is decreased (75, 76), which may lead to the escape of MM from immunosurveillance. One of the mechanisms proposed

is the increased levels of soluble MICA in the circulation of MM patients, which triggers the downregulation of NKG2D and impaired lymphocyte cytotoxicity (75). The functional defect of NK cells in MM patients can also be explained by the expression of programmed cell death 1 (PD-1) on NK cells of MM patients (77). Engagement of PD-1 with their ligand PD-L1, which is expressed on MM cells, can down-modulate the NK cell versus MM effect.

In MM, the therapeutic efficacy of IMiDs is known to originate, at least in part, from the activation of NK cells. IMiDs are able to stimulate T cells to produce IFN-γ and IL-2 leading to NK cell activation (78, 79). Lenalidomide upregulates CD16, CD40L, and LFA1 on NK cells, thereby facilitating ADCC against MM cells (80). Salvage therapy with lenalidomide after allogenic stem cell transplantation for MM leads to an increase of activated $NKp44^+$ NK cells (81). Also the proteasome inhibitor bortezomib has been shown to promote NK cell activation by increasing the levels of MICA on the surface of MM cells (75). These results show that, at least in part, the efficacy of novel anti-MM agents is associated with NK cell activation.

Macrophages

Cells of the monocyte–macrophage lineage are one of the major components of the leukocyte infiltration in tumors. There is strong evidence that these cells promote inflammatory circuits that ultimately lead to tumor progression, tumor-cell invasion, and metastasis (82).

Zheng *et al.* (83) first showed that physical interaction between macrophages and MM cells activates signaling pathways that protect MM cells from apoptosis induced by drug treatment, thereby contributing to MM development of drug resistance. A subsequent study (84) found that the interactions between P-selectin glycoprotein ligand 1 (PSGL-1) and intercellular adhesion molecule-1 (ICAM-1) on myeloma cells and E/P selectins and CD18 on macrophages, respectively, allowed macrophages to protect myeloma cells from drug-induced apoptosis through stimulation of SRC, ERK1/2 kinases, and c-MYC and suppression of drug-induced caspase activation. However, macrophages are also able to protect myeloma cells from apoptosis through non-contact-mediated mechanisms (85), suggesting that macrophages promote myeloma cell survival through both a contact-mediated and non-contact-mediated mechanisms.

In a recent study (86), the role of macrophages in MM has been further investigated, and interesting findings have clarified molecular mechanisms of activation of MM-associated macrophages. Human myeloma–associated monocytes/macrophages (MAM), but not MM cells, were found to be the predominant source of interleukin-1b (IL-1b), IL-10, and tumor necrosis factor-α, whereas IL-6 originates from both stromal cells and macrophages consistent with previous results. TLR2 and TLR6 expression on human myeloma BM $CD14^+$ monocytic cells correlated with local processing of versican, a proteoglycan TLR2/6 agonist, suggesting that the versican-TLR2/6-mitogen-activated protein 3 (MAP3) kinase, TPL2 (Cot/MAP3K8) pathway may ultimately activate MM-associated macrophages. Indeed, ablation of TPL2 in the genetically engineered *in vivo* myeloma model, Vk*MYC, led to prolonged disease latency associated with plasma cell growth defect through the abrogation of the 'inflammatory switch' of MM macrophages associated with development of MM. Interestingly, pharmacologic TPL2 inhibition in human monocytes led to dose-dependent attenuation of IL-1b induction/secretion in response to TLR2 stimulation, suggesting that this may represent a new therapeutic target in MM, acting on the microenvironment and not on MM cells (86).

Tumor-associated macrophages are also a rich source of potent proangiogenic cytokines and growth factors, such as vascular endothelial growth factor, IL-8, and fibroblast growth factor-2, and express a broad array of angiogenesis modulating enzymes including matrix metalloproteinases, cycloxygenase-2, and colony-stimulating factor-1 (87). Neovessel formation and angiogenesis are important pathogenic mechanisms associated with MM progression (discussed later); these observations support the hypothesis that macrophages may also support MM growth indirectly through the paracrine stimulation of MM-associated neoangiogenesis.

Interestingly, MM-associated macrophages are also able to participate directly, through vessel incorporation (vasculogenic mimicry), to the formation of MM-associated neovessels (88). Thus, macrophages seem to greatly contribute to MM-associated neovascularization through both the paracrine secretion of angiogenic factors (angiogenic pathway) and a vasculogenic pathway, and may therefore represent an important target for designing novel anti-neovessel drugs in MM.

PD-1/PD-L1 pathway as a target of modifying the MM immune microenvironment

PD-1 is a type I transmembrane protein which belongs to the CD28 family (89). PD-1 is expressed on activated and exhausted T and B cells and has two ligands, PD-L1 and

PD-L2. PD-L1 is not expressed on normal epithelial tissues but it is aberrantly expressed on a variety of solid tumors (90). Binding of PD-L1 to PD-1 reduces cytokine production and activation of the target T cells, leading to an immunosuppressive microenvironment.

It is reported that PD-L1 is not expressed on normal plasma cells, while it is expressed on primary MM cells (91). *In vitro* analysis has shown that cytokines (91) and BMSCs (92) increase PD-L1 expression on MM cells, indicating that the BM microenvironment plays a role in the activation of the PD-1/PD-L1 pathway. It has been demonstrated that PD-1 expression is upregulated on T cells (93) and NK cells (77) isolated from patients with MM compared to healthy donors, likely leading to an inhibition of anti-tumor immunity through the expression of PD-L1.

Clinical trials targeting PD-1/PD-L1 pathway to overcome tumor-associated immune suppression have shown promising results for a variety of solid tumors. Some groups have reported the efficacy of inhibiting the PD-1/PD-L1 pathway in preclinical studies of MM. Rosenblatt *et al.* (93) showed that CT-011, an anti-PD1 antibody, enhanced activated T-cell responses after DC/tumor fusion stimulation in MM. Hallet *et al.* (94) showed that PD-L1 blockade combined with stem cell transplant and whole-cell vaccination increased the survival of myeloma-bearing mice. Kearl *et al.* (95) showed that PD-L1 antibody improves survival of murine MM when combined with whole body irradiation. Considering the elevation of PD-1 and its ligand in the MM microenvironment, inhibition of the PD-1/PD-L1 pathway has the potential to change the strategy of the microenvironment targeted therapy in MM.

Other cellular compartments

Bone marrow stromal cells (BMSCs)

MM cells adhere to BMSCs and ECM into the BM. Adhesion of tumor cells to BMSCs activates many pathways resulting in the upregulation of cell cycle regulating proteins and anti-apoptotic proteins (96). Differences in MM- versus normal BMSCs adhesion molecule expression may facilitate MM cell entrapment in the BM. MM BMSCs express adhesion molecules that bind MM cells inter-cellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) at higher levels than normal BMSCs (97, 98). Beta-1 and beta-2 integrin mediated MM cell adhesion may also be stronger to MM BMSCs than to normal BMSCs (99).

The interaction between MM cells and BMSCs triggers NF-κB signaling pathway and IL-6 secretion in BMSCs. In turn, IL-6 enhances the production and secretion of vascular endothelial growth factor (VEGF) by MM cells. The existence of this paracrine loop optimizes the BM milieu for MM tumor-cell growth (100). BMSC-MM cell interaction is also mediated through Notch. The interaction of Notch–Notch ligand leads to activate Notch-signaling pathways both in MM cells as well as in BMSCs, with induction of IL-6, VEGF, and insulin-like growth factor (IGF-1) secretion, and is associated with MM cell proliferation and survival (101, 102). Moreover, BMSCs from MM patients expresses several pro-angiogenic molecules such as VEGF, basic-fibroblast growth factor (bFGF), angiopoietin 1 (Ang-1), TGF-β, platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), and IL-1 (103). Gene-expression profiling data showed that growth differentiation factor 15 (GDF15) is aberrantly secreted by BMSCs in MM (104). GDF15 not only increases survival of stroma-dependent MM cells (105), but also enhances the tumor-initiating potential and self-renewal of MM cells (106). BMSCs in MM seem to support proliferation of the stem-like population of MM cells to a greater extent than normal BMSCs, suggesting that MM BMSCs are more specifically selective for the growth of tumor-initiating cells than normal BMSCs (107).

BMSCs from MM patients have also been shown to release exosomes, which are transferred to MM cells, thereby resulting in modulation of tumor growth *in vivo*. This finding suggests that exosomes might constitute a novel mechanism for intercellular transfer of genetic information in clonal plasma cell disorders (108).

Multiple drugs can also target the BMSC–MM interactions. Bortezomib inhibits MM cell growth triggered by BMSC adhesion, as well as production and secretion of cytokines that mediate MM cell growth and survival (109). In addition, bortezomib directly induces osteoblastic differentiation in BMSCs to combat osteolysis through RUNX-2 (110). The CXCR4 inhibitor AMD3100 enhances sensitivity of MM cell to multiple therapeutic agents by disrupting adhesion of MM cells to BMSCs (111). Novel drug screens using BMSC–MM cell co-cultures may allow the development of therapeutic agents that are clinically relevant to target the BMSCs in MM (112).

Osteoblasts

It has been reported that osteoblasts may contribute to MM pathogenesis by supporting MM cell growth and survival (113). This could potentially result from the ability of osteoblasts to secrete IL-6 in co-culture system with MM

cells. Other mechanisms include the possible role of osteoblasts in stimulating MM cell survival by blocking TRAIL-mediated programmed MM cell death by secreting osteoprotegerin (OPG) (114).

In addition, it is clear that suppression of osteoblast activity is responsible for both osteolytic process and progression of MM tumor burden (115). One of the factors responsible for suppression of osteoblast activity in MM is Dickkopf-1 (DKK1) (116). DKK1 is a Wnt signaling pathway inhibitor, which acts by binding to LRP5/6 preventing Wnt signaling and leading to translocation of β-catenin to the nucleus (117). Elevated DKK1 levels in BM and PB from patients with MM correlated with the gene-expression patterns of DKK1 and were associated with the presence of focal bone lesions (116). Recombinant human DKK1 or BM serum containing an elevated level of DKK1 inhibited the differentiation of osteoblast precursor cells *in vitro* (116). Studies have shown that blocking DKK1 and activating Wnt signaling prevents bone disease in MM, but is also associated with a reduction in tumor burden (118–120). Fulciniti *et al.* (120) showed that the anti-Dkk1 antibody (BHQ880) increases osteoblast differentiation *in vitro* and increases osteoblast number and trabecular bone *in vivo*. Blockade of DKK1 can restore osteoblast activity in MM, which may lead to improvement of MM bone disease.

Proteasome inhibitors have direct effects on osteoblasts to promote osteoblast differentiation and bone formation. Garett *et al.* showed that proteasome inhibitors regulate bone morphogenetic protein-2 (BMP-2) gene expression of osteoblastic cells in part through inhibiting the proteolytic processing of Gli3 protein (121). Bortezomib increases the number of osteoblasts in a 5T2MM mouse model (122). It was also reported that the serum concentrations of bone-specific alkaline phosphatase (BAP) and osteocalcin, which indicates osteoblast activity, increased significantly in MM patients treated with bortezomib (123). Bone-specific nanoparticles loaded with bortezomib showed increased osteoblastic activity contributing to increased bone volume as well as inhibition in MM growth *in vivo* (124). These results indicate the potential for proteasome inhibitors to increase osteoblastogenesis in the BM microenvironment in MM.

Osteoclasts

The balance between bone resorption and bone formation is lost in the majority of cases in MM, resulting in bone destruction and development of osteolytic lesions (125). Several factors are implicated in osteoclast activation, including RANKL, macrophage inflammatory protein-1a (MIP-1a), IL-3, and IL-6, which are produced by MM cells and play a role to increase osteoclast activity (126).

RANKL is a member of the tumor necrosis factor (TNF) family and plays a major role in the increased osteoclastogenesis implicated in MM bone disease. RANK is a transmembrane signaling receptor expressed by osteoclast cells. MM cell binding to neighboring BMSC within the BM results in increased RANKL expression. This leads to an increase in osteoclast activity through the binding of RANKL to its receptor, on osteoclast precursor cells, which further promotes their differentiation through NF-κB and JunN-terminal kinase pathway (127). RANKL is also involved in inhibition of osteoclast apoptosis. Blocking RANKL with soluble form of RANK has been shown to modulate not only bone loss, but also tumor burden, in MM *in vivo* models (128). Denosumab is a fully human monoclonal antibody that binds RANKL with high affinity and specificity and inhibits RANKL-RANK interaction, resulting in rapid and sustained suppression of the markers of osteoclastic bone resorption (129).

Bruton's tyrosine kinase (BTK) plays a significant role in B-cell development (130). BTK is highly expressed in MM cells and the BTK inhibitor ibrutinib has been reported to be cytotoxic to MM cells via inhibiting the NF-κB pathway (131). BTK is also expressed in osteoclasts and implicated in bone resorption by regulating osteoclast differentiation (132). Tai *et al.* showed that ibrutinib blocked RANKL/M-CSF–induced phosphorylation of BTK and downstream PLC-γ2 in osteoclasts, resulting in reduced bone resorption activity (133). Ibrutinib treatment also significantly inhibited *in vivo* MM cell growth and MM cell–induced osteolysis of implanted human bone chips in SCID mice. These data suggest that BTK can be a target of MM induced osteolytic bone disease.

Endothelial cells and BM vessel formation in MM

It is well established that neovascularization of solid tumors is important for tumor growth and metastasis (134, 135). In the BM, blood vessel circulation is normally induced and maintained by the balanced interplay of cells, cytokines, and growth factors within a complex ecosystem in which endothelial cells and pericytes proliferate in concert with fibroblasts, DCs, inflammatory cells, and hematopoietic stem cells (136). It is now clear that malignant cell growth in the BM upset this balance, leading to increased vascularity and thus tumor progression (136).

The first evidence suggesting that neovascular formation is involved in the MM pathogenesis date back to 1995 (137, 138), when an increase in microvascular density (MVD) was observed in the BM of active MM patients compared to those with inactive MM and MGUS (137). Rajkumar *et al.* (139) then showed a gradual increase in BM angiogenesis along the disease spectrum of plasma cell discrasias from MGUS to smoldering MM, newly diagnosed MM, and relapsed MM, although the expression levels of VEGF, bFGF, and their receptors were similar among MGUS, smoldering MM, and newly diagnosed MM (140). Several other studies have found a significant correlation between BM MVD and clinical parameters of prognosis, including progression free survival (PFS) and overall survival (OS), in MM patients (141).

Increase in PB and BM plasma or serum of the major pro-angiogenic cytokines VEGF, bFGF, HGF, and Syndecan-1 correlate with progression of MM to advanced stage of disease (141). All these observations strongly suggest that BM neovessels and neovessel formation are important pathogenic mechanisms, likely promoting MM progression (142, 143).

Interestingly, endothelial cells (ECs) derived from the BM of MM patients have been shown to be different in terms of transcriptome (144) and proteome (145) profiles from those of MGUS or anemic patients (136). MM derived ECs are endowed of an over-angiogenic phenotype able to support directly the growth, proliferation, and invasion of MM through the direct secretion of several growth factors, including IL-6 (146). For example, a cross-talk between MM cells and ECs has been described, where MM cells secrete VEGF that stimulates ECs to produce IL-6; this cross-talk ultimately determines simultaneous proliferation of both MM cells and ECs (147). MM ECs, but not MGUS ECs, are able to spontaneously secrete several other growth factors, including bFGF and HGF, which also promote MM cell proliferation and survival (148). All these studies show that vessel formation together with functional transformation of BM MM ECs ultimately lead to MM progression in a synergistic way. Although novel anti-MM drugs like thalidomide (148), lenalidomide (145), and borteziomib (149) have anti-angiogenic ability that in part explains their potent anti-MM activity, anti-angiogenic drugs that target the VEGFR2 pathway (such as bevacizumab) have failed to show activity in MM patients (142). This indicates that new and more effective strategies to target MM-associated vasculature are needed to exploit the therapeutic potential of vessel targeting in MM.

The non-cellular compartment

Extracellular matrix

The ECM is a major component of the tumor microenvironment in several cancers, contributing to the regulation of cell survival, proliferation, differentiation, and metastasis (150). Gene-expression profiling has shown that genes encoding for ECM components are dysregulated during tumor progression (151) and in MM ECM components, such as integrins, have been shown to play an important role in drug resistance (152). MM plasma cells directly interact with the ECM via binding of syndecan-1 and very late antigen-4 (VLA-4) to ECM proteins, such as collagen type 1 and fibronectin. These adhesive interactions of MM cells result in upregulation of anti-apoptotic proteins and cell cycle dysregulation (96).

It is increasingly clear, however, that not only does the ECM form an important part of the permissive tumor microenvironment for malignant plasma cells, it also contributes to the premetastatic niche with alterations in the ECM evident at the MGUS phase. A recent proteome profiling study of primary BMSCs from MM patients, MGUS patients, and non-neoplastic control patients revealed a group of ECM proteins, ECM receptors, and ECM-modulating enzymes that are upregulated in a stepwise fashion from MGUS to MM. This includes proteins such as laminin $\alpha 4$, lysyl-hydroxylase 2, integrin $\alpha 5\beta 5$ and matrix metalloproteinase-2 (MMP-2). This indicates that matrix remodeling in MM is already present at the MGUS phase and may provide rationale for ECM directed targeting of the premetastatic niche in MM (153). Preparation of the metastatic niche is potentially mediated by factors such as matrix metalloproteinases and lysl oxidase, with consequent modulation of collagens and elastins at the premetatstatic site. The concept of release of growth factors and cytokines by the tumor cells in preparation for distant site engraftment is now emerging and, for example, bone marrow-derived hematopoietic cells that express VEGFR1 home to tumor specific premetastatic sites and form cellular clusters before the arrival of tumor cells, coincident with the upregulation of fibronectin at these sites providing a permissive niche for arriving tumor cells (154).

As we uncover more about the composition of the ECM in MM and the nature of the complex interactions between MM cells, BMSCs, and ECM components, therapeutic targets are likely to emerge that are not only promising for MM patients, but also for selected targeting of the bone marrow niche in patients with premetastatic disease.

Oxygen concentration

Hypoxia is an imbalance between oxygen supply and consumption that deprives cells or tissues of oxygen. Decrease in oxygen levels are observed in certain types of pathological situations, such as cancer. Hypoxic regions arise in tumors because of rapid cell division and aberrant blood vessel formation (155). In solid tumors, it has been indicated that the hypoxic microenvironment contributes to cancer progression by activating adaptive transcriptional programs, thereby promoting tumor-cell survival, motility, and metastasis leading to worse prognosis (156, 157). It has been shown that the BM of MM mouse models (158, 159) and MM patients (160) are hypoxic compared to healthy controls, so targeting hypoxia niches should be considered as a novel approach for the treatment of MM.

Intratumoral hypoxia promotes metastasis through acquisition of epithelial mesenchymal transition (EMT) features in several models of solid tumors (161–163). Recent studies have suggested that hypoxia activates EMT-related machinery in MM cells, decreases expression of E-cadherin, decreases adhesion of MM cells to the BM, and enhances the egress of MM cells to the circulation (164). In parallel, hypoxia increased the expression of CXCR4, consequently increasing the migration and homing of circulating MM cells to new BM niches. In addition, hypoxia induces immature and stem cell-like phenotypes in myeloma cell lines *in vitro* (165). These data show that hypoxic conditions change MM cells to a different phenotype, which may lead to changes in treatment sensitivity. TH-302, a 2-nitroimidazole-based nitrogen mustard prodrug, was tested as a hypoxic-activated treatment in the 5T33 MM mouse model. TH-302 induced apoptosis of the MM cells within the bone marrow microenvironment (158) and worked synergistically in combination with bortezomib (166). Therefore, targeting the hypoxic microenvironment in combination with other novel anti-MM agents can be a new anti-MM treatment strategy.

Conclusion

This review shows the advances in the understanding of the association between MM cells and the BM microenvironment. Although the response rate and OS of MM have significantly improved due to the introduction of novel agents, MM is still an incurable disease. As MM pathophysiology is supported by a strong interaction between the clonal plasma cells and the surrounding bone marrow microenvironment, treatment targeting this interaction is necessary for the cure of MM. Further understanding of the MM supportive BM microenvironment and development of new drugs or strategies targeting them are needed.

References

1. Kyle RA, Rajkumar SV. Multiple myeloma. Blood 2008;**111**:2962–2972.
2. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. CA Cancer J Clin 2012;**62**:10–29.
3. Kyle RA, et al. 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 2010;**24**:1121–1127.
4. Kumar SK, et al. Improved survival in multiple myeloma and the impact of novel therapies. Blood 2008;**111**:2516–2520.
5. Ghobrial IM. Myeloma as a model for the process of metastasis: implications for therapy. Blood 2012;**120**:20–30.
6. Quail DF, Joyce JA. Microenvironmental regulation of tumor progression and metastasis. Nat Med 2013;**19**:1423–1437.
7. Swann JB, Smyth MJ. Immune surveillance of tumors. J Clin Invest 2007;**117**:1137–1146.
8. Shankaran V, et al. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. Nature 2001;**410**:1107–1111.
9. Bosma GC, Custer RP, Bosma MJ. A severe combined immunodeficiency mutation in the mouse. Nature 1983;**301**:527–530.
10. Broder S, et al. Impaired synthesis of polyclonal (non-paraprotein) immunoglobulins by circulating lymphocytes from patients with multiple myeloma Role of suppressor cells. N Engl J Med 1975;**293**:887–892.
11. Robertson JD, et al. Immunogenicity of vaccination against influenza, Streptococcus pneumoniae and Haemophilus influenzae type B in patients with multiple myeloma. Br J Cancer 2000;**82**:1261–1265.
12. Pratt G, Goodyear O, Moss P. Immunodeficiency and immunotherapy in multiple myeloma. Br J Haematol 2007;**138**:563–579.
13. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. Nat Rev Immunol 2009;**9**:162–174.
14. Almand B, et al. Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer. J Immunol 2001;**166**:678–689.
15. Diaz-Montero CM, Salem ML, Nishimura MI, Garrett-Mayer E, Cole DJ, Montero AJ. Increased circulating myeloid-derived suppressor cells correlate with clinical cancer stage, metastatic tumor burden, and doxorubicin-cyclophosphamide chemotherapy. Cancer Immunol Immunother 2009;**58**:49–59.
16. Walter S, et al. Multipeptide immune response to cancer vaccine IMA901 after single-dose cyclophosphamide associates with longer patient survival. Nat Med 2012;**18**:1254–1261.
17. Solito S, et al. A human promyelocytic-like population is responsible for the immune suppression mediated by myeloid-derived suppressor cells. Blood 2011;**118**:2254–2265.
18. Serafini P, et al. Phosphodiesterase-5 inhibition augments endogenous antitumor immunity by reducing myeloid-derived suppressor cell function. J Exp Med 2006;**203**:2691–2702.
19. Li H, Han Y, Guo Q, Zhang M, Cao X. Cancer-expanded myeloid-derived suppressor cells induce anergy of NK cells through membrane-bound TGF-beta 1. J Immunol 2009;**182**:240–249.
20. Youn JI, Nagaraj S, Collazo M, Gabrilovich DI. Subsets of myeloid-derived suppressor cells in tumor-bearing mice. J Immunol 2008;**181**:5791–5802.
21. Movahedi K, et al. Identification of discrete tumor-induced myeloid-derived suppressor cell subpopulations with distinct T cell-suppressive activity. Blood 2008;**111**:4233–4244.
22. Huang B, et al. Gr-1+CD115+ immature myeloid suppressor cells mediate the development of tumor-induced T regulatory cells and T-cell

anergy in tumor-bearing host. Cancer Res 2006;**66**:1123–1131.
23. Brimnes MK, et al. Increased level of both CD4+FOXP3+ regulatory T cells and CD14+HLA-DR(-)/low myeloid-derived suppressor cells and decreased level of dendritic cells in patients with multiple myeloma. Scand J Immunol 2010;**72**:540–547.
24. Gorgun GT, et al. Tumor-promoting immune-suppressive myeloid-derived suppressor cells in the multiple myeloma microenvironment in humans. Blood 2013;**121**:2975–2987.
25. Ramachandran IR, et al. Myeloid-derived suppressor cells regulate growth of multiple myeloma by inhibiting T cells in bone marrow. J Immunol 2013;**190**:3815–3823.
26. Favaloro J, et al. Myeloid derived suppressor cells are numerically, functionally and phenotypically different in patients with multiple myeloma. Leuk Lymphoma 2014. [Epub ahead of print]
27. Zhuang J, et al. Osteoclasts in multiple myeloma are derived from Gr-1+CD11b+myeloid-derived suppressor cells. PLoS ONE 2012;**7**:e48871.
28. Hideshima T, Mitsiades C, Tonon G, Richardson PG, Anderson KC. Understanding multiple myeloma pathogenesis in the bone marrow to identify new therapeutic targets. Nat Rev Cancer 2007;**7**:585–598.
29. Serafini P, Mgebroff S, Noonan K, Borrello I. Myeloid-derived suppressor cells promote cross-tolerance in B-cell lymphoma by expanding regulatory T cells. Cancer Res 2008;**68**:5439–5449.
30. Meyer C, et al. Chronic inflammation promotes myeloid-derived suppressor cell activation blocking antitumor immunity in transgenic mouse melanoma model. Proc Natl Acad Sci USA 2011;**108**:17111–17116.
31. Noonan KA, Ghosh N, Rudraraju L, Bui M, Borrello I. Targeting Immune Suppression with PDE5 Inhibition in End-Stage Multiple Myeloma. Cancer Immunol Res 2014;**2**:725–731.
32. Whiteside TL. The tumor microenvironment and its role in promoting tumor growth. Oncogene 2008;**27**:5904–5912.
33. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol 2003;**4**:330–336.
34. Nishikawa H, Sakaguchi S. Regulatory T cells in tumor immunity. Int J Cancer 2010;**127**:759–767.
35. Mougiakakos D, Choudhury A, Lladser A, Kiessling R, Johansson CC. Regulatory T cells in cancer. Adv Cancer Res 2010;**107**:57–117.
36. Curiel TJ, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med 2004;**10**:942–949.
37. Bates GJ, et al. Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse. J Clin Oncol 2006;**24**:5373–5380.
38. Lahl K, et al. Selective depletion of Foxp3+ regulatory T cells induces a scurfy-like disease. J Exp Med 2007;**204**:57–63.
39. Klages K, et al. Selective depletion of Foxp3+ regulatory T cells improves effective therapeutic vaccination against established melanoma. Cancer Res 2010;**70**:7788–7799.
40. Teng MW, Ngiow SF, von Scheidt B, McLaughlin N, Sparwasser T, Smyth MJ. Conditional regulatory T-cell depletion releases adaptive immunity preventing carcinogenesis and suppressing established tumor growth. Cancer Res 2010;**70**:7800–7809.
41. Beyer M, et al. In vivo peripheral expansion of naive CD4+CD25high FoxP3+ regulatory T cells in patients with multiple myeloma. Blood 2006;**107**:3940–3949.
42. Feyler S, et al. CD4(+)CD25(+)FoxP3(+) regulatory T cells are increased whilst CD3(+) CD4(-)CD8(-)alphabetaTCR(+) Double Negative T cells are decreased in the peripheral blood of patients with multiple myeloma which correlates with disease burden. Br J Haematol 2009;**144**:686–695.
43. Giannopoulos K, Kaminska W, Hus I, Dmoszynska A. The frequency of T regulatory cells modulates the survival of multiple myeloma patients: detailed characterisation of immune status in multiple myeloma. Br J Cancer 2012;**106**:546–552.
44. MuthuRaja KR, Rihova L, Zahradova L, Klincova M, Penka M, Hajek R. Increased T regulatory cells are associated with adverse clinical features and predict progression in multiple myeloma. PLoS ONE 2012;**7**:e47077.
45. Laronne-Bar-On A, Zipori D, Haran-Ghera N. Increased regulatory versus effector T cell development is associated with thymus atrophy in mouse models of multiple myeloma. J Immunol 2008;**181**:3714–3724.
46. Prabhala RH, et al. Dysfunctional T regulatory cells in multiple myeloma. Blood 2006;**107**:301–304.
47. Gupta R, Ganeshan P, Hakim M, Verma R, Sharma A, Kumar L. Significantly reduced regulatory T cell population in patients with untreated multiple myeloma. Leuk Res 2011;**35**:874–878.
48. Feyler S, et al. Tumour cell generation of inducible regulatory T-cells in multiple myeloma is contact-dependent and antigen-presenting cell-independent. PLoS ONE 2012;**7**:e35981.
49. Lutsiak ME, Semnani RT, De Pascalis R, Kashmiri SV, Schlom J, Sabzevari H. Inhibition of CD4(+) 25+ T regulatory cell function implicated in enhanced immune response by low-dose cyclophosphamide. Blood 2005;**105**:2862–2868.
50. Ghiringhelli F, et al. CD4+CD25+ regulatory T cells suppress tumor immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumors to be curative. Eur J Immunol 2004;**34**:336–344.
51. Sharabi A, Laronne-Bar-On A, Meshorer A, Haran-Ghera N. Chemoimmunotherapy reduces the progression of multiple myeloma in a mouse model. Cancer Prev Res (Phila) 2010;**3**:1265–1276.
52. Galustian C, et al. The anti-cancer agents lenalidomide and pomalidomide inhibit the proliferation and function of T regulatory cells. Cancer Immunol Immunother 2009;**58**:1033–1045.
53. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998;**392**:245–252.
54. O'Doherty U, et al. Human blood contains two subsets of dendritic cells, one immunologically mature and the other immature. Immunology 1994;**82**:487–493.
55. Bell D, et al. In breast carcinoma tissue, immature dendritic cells reside within the tumor, whereas mature dendritic cells are located in peritumoral areas. J Exp Med 1999;**190**:1417–1426.
56. Treilleux I, et al. Dendritic cell infiltration and prognosis of early stage breast cancer. Clin Cancer Res 2004;**10**:7466–7474.
57. Sandel MH, et al. Prognostic value of tumor-infiltrating dendritic cells in colorectal cancer: role of maturation status and intratumoral localization. Clin Cancer Res 2005;**11**:2576–2582.
58. Zou W. Immunosuppressive networks in the tumour environment and their therapeutic relevance. Nat Rev Cancer 2005;**5**:263–274.
59. Conrad C, et al. Plasmacytoid dendritic cells promote immunosuppression in ovarian cancer via ICOS costimulation of Foxp3(+) T-regulatory cells. Cancer Res 2012;**72**:5240–5249.
60. Faget J, et al. ICOS-ligand expression on plasmacytoid dendritic cells supports breast cancer progression by promoting the accumulation of immunosuppressive CD4+ T cells. Cancer Res 2012;**72**:6130–6141.
61. Ramos RN, Chin LS, Dos Santos AP, Bergami-Santos PC, Laginha F, Barbuto JA. Monocyte-derived dendritic cells from breast cancer patients are biased to induce CD4+CD25+Foxp3+ regulatory T cells. J Leukoc Biol 2012;**92**:673–682.
62. Banerjee DK, Dhodapkar MV, Matayeva E, Steinman RM, Dhodapkar KM. Expansion of FOXP3high regulatory T cells by human dendritic cells (DCs) in vitro and after injection of cytokine-matured DCs in myeloma patients. Blood 2006;**108**:2655–2661.
63. Garcia De Vinuesa C, et al. Dendritic cells associated with plasmablast survival. Eur J Immunol 1999;**29**:3712–3721.
64. Jego G, Palucka AK, Blanck JP, Chalouni C, Pascual V, Banchereau J. Plasmacytoid dendritic cells induce plasma cell differentiation through type I interferon and interleukin 6. Immunity 2003;**19**:225–234.
65. Brown RD, et al. Dendritic cells from patients with myeloma are numerically normal but functionally defective as they fail to up-regulate CD80 (B7-1) expression after huCD40LT stimulation because of inhibition by transforming growth factor-beta1 and interleukin-10. Blood 2001;**98**:2992–2998.
66. Brimnes MK, Svane IM, Johnsen HE. Impaired functionality and phenotypic profile of dendritic cells from patients with multiple myeloma. Clin Exp Immunol 2006;**144**:76–84.
67. Chauhan D, et al. Functional interaction of plasmacytoid dendritic cells with multiple

myeloma cells: a therapeutic target. Cancer Cell 2009;**16**:309–323.
68. Ray A, et al. A novel TLR-9 agonist C792 inhibits plasmacytoid dendritic cell-induced myeloma cell growth and enhance cytotoxicity of bortezomib. Leukemia 2014;**28**:1716–1724.
69. Kukreja A, et al. Enhancement of clonogenicity of human multiple myeloma by dendritic cells. J Exp Med 2006;**203**:1859–1865.
70. Groh V, Rhinehart R, Secrist H, Bauer S, Grabstein KH, Spies T. Broad tumor-associated expression and recognition by tumor-derived gamma delta T cells of MICA and MICB. Proc Natl Acad Sci USA 1999;**96**:6879–6884.
71. Famularo G, et al. Natural killer cell frequency and function in patients with monoclonal gammopathies. J Clin Lab Immunol 1992;**37**:99–109.
72. Frassanito MA, Silvestris F, Cafforio P, Silvestris N, Dammacco F. IgG M-components in active myeloma patients induce a down-regulation of natural killer cell activity. Int J Clin Lab Res 1997;**27**:48–54.
73. Sawanobori M, Suzuki K, Nakagawa Y, Inoue Y, Utsuyama M, Hirokawa K. Natural killer cell frequency and serum cytokine levels in monoclonal gammopathies: correlation of bone marrow granular lymphocytes to prognosis. Acta Haematol 1997;**98**:150–154.
74. Pessoa de Magalhaes RJ, et al. Analysis of the immune system of multiple myeloma patients achieving long-term disease control by multidimensional flow cytometry. Haematologica 2013;**98**:79–86.
75. Jinushi M, et al. MHC class I chain-related protein A antibodies and shedding are associated with the progression of multiple myeloma. Proc Natl Acad Sci USA 2008;**105**:1285–1290.
76. von Lilienfeld-Toal M, et al. Reduced immune effector cell NKG2D expression and increased levels of soluble NKG2D ligands in multiple myeloma may not be causally linked. Cancer Immunol Immunother 2010;**59**:829–839.
77. Benson DM, Jr, et al. 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 2010;**116**:2286–2294.
78. Davies FE, et al. Thalidomide and immunomodulatory derivatives augment natural killer cell cytotoxicity in multiple myeloma. Blood 2001;**98**:210–216.
79. Hayashi T, et al. Molecular mechanisms whereby immunomodulatory drugs activate natural killer cells: clinical application. Br J Haematol 2005;**128**:192–203.
80. Tai YT, et al. Immunomodulatory drug lenalidomide (CC-5013, IMiD3) augments anti-CD40 SGN-40-induced cytotoxicity in human multiple myeloma: clinical implications. Cancer Res 2005;**65**:11712–11720.
81. Lioznov M, et al. Lenalidomide as salvage therapy after allo-SCT for multiple myeloma is effective and leads to an increase of activated NK (NKp44 (+)) and T (HLA-DR(+)) cells. Bone Marrow Transplant 2010;**45**:349–353.
82. Cook J, Hagemann T. Tumour-associated macrophages and cancer. Curr Opin Pharmacol 2013;**13**:595–601.
83. Zheng Y, et al. Macrophages are an abundant component of myeloma microenvironment and protect myeloma cells from chemotherapy drug-induced apoptosis. Blood 2009;**114**:3625–3628.
84. Zheng Y, et al. PSGL-1/selectin and ICAM-1/CD18 interactions are involved in macrophage-induced drug resistance in myeloma. Leukemia 2013;**27**:702–710.
85. Kim J, et al. Macrophages and mesenchymal stromal cells support survival and proliferation of multiple myeloma cells. Br J Haematol 2012;**158**:336–346.
86. Hope C, et al. TPL2 kinase regulates the inflammatory milieu of the myeloma niche. Blood 2014;**123**:3305–3315.
87. Berardi S, et al. Multiple myeloma macrophages: pivotal players in the tumor microenvironment. J Oncol 2013;**2013**:183602.
88. Ribatti D, Moschetta M, Vacca A. Macrophages in multiple myeloma. Immunol Lett 2014;**161**:241–244.
89. Ishida Y, Agata Y, Shibahara K, Honjo T. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. EMBO J 1992;**11**:3887–3895.
90. Dong H, et al. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. Nat Med 2002;**8**:793–800.
91. Liu J, et al. Plasma cells from multiple myeloma patients express B7-H1 (PD-L1) and increase expression after stimulation with IFN-{gamma} and TLR ligands via a MyD88-, TRAF6-, and MEK-dependent pathway. Blood 2007;**110**:296–304.
92. Tamura H, et al. Marrow stromal cells induce B7-H1 expression on myeloma cells, generating aggressive characteristics in multiple myeloma. Leukemia 2013;**27**:464–472.
93. Rosenblatt J, et al. PD-1 blockade by CT-011, anti-PD-1 antibody, enhances ex vivo T-cell responses to autologous dendritic cell/myeloma fusion vaccine. J Immunother 2011;**34**:409–418.
94. Hallett WH, Jing W, Drobyski WR, Johnson BD. Immunosuppressive effects of multiple myeloma are overcome by PD-L1 blockade. Biol Blood Marrow Transplant 2011;**17**:1133–1145.
95. Kearl TJ, Jing W, Gershan JA, Johnson BD. Programmed death receptor-1/programmed death receptor ligand-1 blockade after transient lymphodepletion to treat myeloma. J Immunol 2013;**190**:5620–5628.
96. Hideshima T, Bergsagel PL, Kuehl WM, Anderson KC. Advances in biology of multiple myeloma: clinical applications. Blood 2004;**104**:607–618.
97. Markovina S, et al. Bone marrow stromal cells from multiple myeloma patients uniquely induce bortezomib resistant NF-kappaB activity in myeloma cells. Mol Cancer 2010;**9**:176.
98. Hao M, et al. Bone marrow stromal cells protect myeloma cells from bortezomib induced apoptosis by suppressing microRNA-15a expression. Leuk Lymphoma 2011;**52**:1787–1794.
99. Uchiyama H, Barut BA, Mohrbacher AF, Chauhan D, Anderson KC. Adhesion of human myeloma-derived cell lines to bone marrow stromal cells stimulates interleukin-6 secretion. Blood 1993;**82**:3712–3720.
100. Kumar S, et al. Expression of VEGF and its receptors by myeloma cells. Leukemia 2003;**17**:2025–2031.
101. Radtke F, Raj K. The role of Notch in tumorigenesis: oncogene or tumour suppressor? Nat Rev Cancer 2003;**3**:756–767.
102. Nefedova Y, Cheng P, Alsina M, Dalton WS, Gabrilovich DI. Involvement of Notch-1 signaling in bone marrow stroma-mediated de novo drug resistance of myeloma and other malignant lymphoid cell lines. Blood 2004;**103**:3503–3510.
103. Giuliani N, Storti P, Bolzoni M, Palma BD, Bonomini S. Angiogenesis and multiple myeloma. Cancer Microenviron 2011;**4**:325–337.
104. Corre J, et al. Bone marrow mesenchymal stem cells are abnormal in multiple myeloma. Leukemia 2007;**21**:1079–1088.
105. Corre J, et al. Bioactivity and prognostic significance of growth differentiation factor GDF15 secreted by bone marrow mesenchymal stem cells in multiple myeloma. Cancer Res 2012;**72**:1395–1406.
106. Tanno T, et al. Growth differentiating factor 15 enhances the tumor-initiating and self-renewal potential of multiple myeloma cells. Blood 2014;**123**:725–733.
107. Feng Y, et al. Bone marrow stromal cells from myeloma patients support the growth of myeloma stem cells. Stem Cells Dev 2010;**19**:1289–1296.
108. Roccaro AM, et al. BM mesenchymal stromal cell-derived exosomes facilitate multiple myeloma progression. J Clin Invest 2013;**123**:1542–1555.
109. Hideshima T, Chauhan D, Podar K, Schlossman RL, Richardson P, Anderson KC. Novel therapies targeting the myeloma cell and its bone marrow microenvironment. Semin Oncol 2001;**28**:607–612.
110. Mukherjee S, et al. Pharmacologic targeting of a stem/progenitor population in vivo is associated with enhanced bone regeneration in mice. J Clin Invest 2008;**118**:491–504.
111. Azab AK, et al. CXCR4 inhibitor AMD3100 disrupts the interaction of multiple myeloma cells with the bone marrow microenvironment and enhances their sensitivity to therapy. Blood 2009;**113**:4341–4351.
112. McMillin DW, et al. Tumor cell-specific bioluminescence platform to identify stroma-induced changes to anticancer drug activity. Nat Med 2010;**16**:483–489.
113. Karadag A, Oyajobi BO, Apperley JF, Russell RG, Croucher PI. Human myeloma cells promote the production of interleukin 6 by primary human osteoblasts. Br J Haematol 2000;**108**:383–390.
114. Shipman CM, Croucher PI. Osteoprotegerin is a soluble decoy receptor for tumor necrosis factor-related apoptosis-inducing ligand/Apo2 ligand and can function as a paracrine survival

factor for human myeloma cells. Cancer Res 2003;**63**:912–916.
115. Christoulas D, Terpos E, Dimopoulos MA. Pathogenesis and management of myeloma bone disease. Expert Rev Hematol 2009;**2**:385–398.
116. Tian E, et al. The role of the Wnt-signaling antagonist DKK1 in the development of osteolytic lesions in multiple myeloma. N Engl J Med 2003;**349**:2483–2494.
117. Bafico A, Liu G, Yaniv A, Gazit A, Aaronson SA. Novel mechanism of Wnt signalling inhibition mediated by Dickkopf-1 interaction with LRP6/Arrow. Nat Cell Biol 2001;**3**:683–686.
118. Yaccoby S, Ling W, Zhan F, Walker R, Barlogie B, Shaughnessy JD, Jr. Antibody-based inhibition of DKK1 suppresses tumor-induced bone resorption and multiple myeloma growth in vivo. Blood 2007;**109**:2106–2111.
119. Edwards CM, et al. Increasing Wnt signaling in the bone marrow microenvironment inhibits the development of myeloma bone disease and reduces tumor burden in bone in vivo. Blood 2008;**111**:2833–2842.
120. Fulciniti M, et al. Anti-DKK1 mAb (BHQ880) as a potential therapeutic agent for multiple myeloma. Blood 2009;**114**:371–379.
121. Garrett IR, et al. Selective inhibitors of the osteoblast proteasome stimulate bone formation in vivo and in vitro. J Clin Invest 2003;**111**:1771–1782.
122. Deleu S, et al. Bortezomib alone or in combination with the histone deacetylase inhibitor JNJ-26481585: effect on myeloma bone disease in the 5T2MM murine model of myeloma. Cancer Res 2009;**69**:5307–5311.
123. Heider U, et al. Bortezomib increases osteoblast activity in myeloma patients irrespective of response to treatment. Eur J Haematol 2006;**77**:233–238.
124. Swami A, et al. Engineered nanomedicine for myeloma and bone microenvironment targeting. Proc Natl Acad Sci USA 2014;**111**:10287–10292.
125. Bataille R, et al. Mechanisms of bone destruction in multiple myeloma: the importance of an unbalanced process in determining the severity of lytic bone disease. J Clin Oncol 1989;**7**:1909–1914.
126. Roodman GD. Pathogenesis of myeloma bone disease. Leukemia 2009;**23**:435–441.
127. Ehrlich LA, Roodman GD. The role of immune cells and inflammatory cytokines in Paget's disease and multiple myeloma. Immunol Rev 2005;**208**:252–266.
128. Yaccoby S, Pearse RN, Johnson CL, Barlogie B, Choi Y, Epstein J. Myeloma interacts with the bone marrow microenvironment to induce osteoclastogenesis and is dependent on osteoclast activity. Br J Haematol 2002;**116**:278–290.
129. Body JJ, et al. A study of the biological receptor activator of nuclear factor-kappaB ligand inhibitor, denosumab, in patients with multiple myeloma or bone metastases from breast cancer. Clin Cancer Res 2006;**12**:1221–1228.
130. de Weers M, Mensink RG, Kraakman ME, Schuurman RK, Hendriks RW. Mutation analysis of the Bruton's tyrosine kinase gene in X-linked agammaglobulinemia: identification of a mutation which affects the same codon as is altered in immunodeficient xid mice. Hum Mol Genet 1994;**3**:161–166.
131. Rushworth SA, Bowles KM, Barrera LN, Murray MY, Zaitseva L, MacEwan DJ. BTK inhibitor ibrutinib is cytotoxic to myeloma and potently enhances bortezomib and lenalidomide activities through NF-kappaB. Cell Signal 2013;**25**:106–112.
132. Shinohara M, et al. Tyrosine kinases Btk and Tec regulate osteoclast differentiation by linking RANK and ITAM signals. Cell 2008;**132**:794–806.
133. Tai YT, et al. Bruton tyrosine kinase inhibition is a novel therapeutic strategy targeting tumor in the bone marrow microenvironment in multiple myeloma. Blood 2012;**120**:1877–1887.
134. Folkman J, Watson K, Ingber D, Hanahan D. Induction of angiogenesis during the transition from hyperplasia to neoplasia. Nature 1989;**339**:58–61.
135. Carmeliet P. Angiogenesis in life, disease and medicine. Nature 2005;**438**:932–936.
136. Vacca A, Ribatti D. Angiogenesis and vasculogenesis in multiple myeloma: role of inflammatory cells. Recent Results Cancer Res 2011;**183**:87–95.
137. Vacca A, et al. Bone marrow of patients with active multiple myeloma: angiogenesis and plasma cell adhesion molecules LFA-1, VLA-4, LAM-1, and CD44. Am J Hematol 1995;**50**:9–14.
138. Ribatti D, Vacca A, Nico B, Fanelli M, Roncali L, Dammacco F. Angiogenesis spectrum in the stroma of B-cell non-Hodgkin's lymphomas. An immunohistochemical and ultrastructural study. Eur J Haematol 1996;**56**:45–53.
139. Rajkumar SV, et al. Bone marrow angiogenesis in 400 patients with monoclonal gammopathy of undetermined significance, multiple myeloma, and primary amyloidosis. Clin Cancer Res 2002;**8**:2210–2216.
140. Kumar S, et al. Bone marrow angiogenic ability and expression of angiogenic cytokines in myeloma: evidence favoring loss of marrow angiogenesis inhibitory activity with disease progression. Blood 2004;**104**:1159–1165.
141. Jakob C, et al. Angiogenesis in multiple myeloma. Eur J Cancer 2006;**42**:1581–1590.
142. Ribatti D, Mangialardi G, Vacca A. Antiangiogenic therapeutic approaches in multiple myeloma. Curr Cancer Drug Targets 2012;**12**:768–775.
143. Hose D, et al. Induction of angiogenesis by normal and malignant plasma cells. Blood 2009;**114**:128–143.
144. Ria R, et al. Gene expression profiling of bone marrow endothelial cells in patients with multiple myeloma. Clin Cancer Res 2009;**15**:5369–5378.
145. Berardi S, et al. Four proteins governing overangiogenic endothelial cell phenotype in patients with multiple myeloma are plausible therapeutic targets. Oncogene 2012;**31**:2258–2269.
146. Vacca A, et al. Endothelial cells in the bone marrow of patients with multiple myeloma. Blood 2003;**102**:3340–3348.
147. Dankbar B, et al. Vascular endothelial growth factor and interleukin-6 in paracrine tumor-stromal cell interactions in multiple myeloma. Blood 2000;**95**:2630–2636.
148. Vacca A, et al. Thalidomide downregulates angiogenic genes in bone marrow endothelial cells of patients with active multiple myeloma. J Clin Oncol 2005;**23**:5334–5346.
149. Roccaro AM, et al. Bortezomib mediates antiangiogenesis in multiple myeloma via direct and indirect effects on endothelial cells. Cancer Res 2006;**66**:184–191.
150. Cretu A, Brooks PC. Impact of the non-cellular tumor microenvironment on metastasis: potential therapeutic and imaging opportunities. J Cell Physiol 2007;**213**:391–402.
151. Eckhardt BL, et al. Genomic analysis of a spontaneous model of breast cancer metastasis to bone reveals a role for the extracellular matrix. Mol Cancer Res 2005;**3**:1–13.
152. Damiano JS, Cress AE, Hazlehurst LA, Shtil AA, Dalton WS. Cell adhesion mediated drug resistance (CAM-DR): role of integrins and resistance to apoptosis in human myeloma cell lines. Blood 1999;**93**:1658–1667.
153. Slany A, et al. Extracellular matrix remodeling by bone marrow fibroblast-like cells correlates with disease progression in multiple myeloma. J Proteome Res 2014;**13**:844–854.
154. Kaplan RN, et al. VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. Nature 2005;**438**:820–827.
155. Keith B, Simon MC. Hypoxia-inducible factors, stem cells, and cancer. Cell 2007;**129**:465–472.
156. Rohwer N, et al. HIF-1alpha determines the metastatic potential of gastric cancer cells. Br J Cancer 2009;**100**:772–781.
157. Vaupel P. Prognostic potential of the pre-therapeutic tumor oxygenation status. Adv Exp Med Biol 2009;**645**:241–246.
158. Hu J, et al. Targeting the multiple myeloma hypoxic niche with TH-302, a hypoxia-activated prodrug. Blood 2010;**116**:1524–1527.
159. Chesi M, et al. Drug response in a genetically engineered mouse model of multiple myeloma is predictive of clinical efficacy. Blood 2012;**120**:376–385.
160. Martin SK, Diamond P, Gronthos S, Peet DJ, Zannettino AC. The emerging role of hypoxia, HIF-1 and HIF-2 in multiple myeloma. Leukemia 2011;**25**:1533–1542.
161. Cannito S, Novo E, di Bonzo LV, Busletta C, Colombatto S, Parola M. Epithelial-mesenchymal transition: from molecular mechanisms, redox regulation to implications in human health and disease. Antioxid Redox Signal 2010;**12**:1383–1430.

162. Hung JJ, Yang MH, Hsu HS, Hsu WH, Liu JS, Wu KJ. Prognostic significance of hypoxia-inducible factor-1alpha, TWIST1 and Snail expression in resectable non-small cell lung cancer. Thorax 2009;**64**:1082–1089.
163. Liang X, Zheng M, Jiang J, Zhu G, Yang J, Tang Y. Hypoxia-inducible factor-1 alpha, in association with TWIST2 and SNIP1, is a critical prognostic factor in patients with tongue squamous cell carcinoma. Oral Oncol 2011;**47**:92–97.
164. Azab AK, et al. Hypoxia promotes dissemination of multiple myeloma through acquisition of epithelial to mesenchymal transition-like features. Blood 2012;**119**:5782–5794.
165. Kawano Y, et al. Hypoxia reduces CD138 expression and induces an immature and stem cell-like transcriptional program in myeloma cells. Int J Oncol 2013;**43**:1809–1816.
166. Hu J, et al. Synergistic induction of apoptosis in multiple myeloma cells by bortezomib and hypoxia-activated prodrug TH-302, in vivo and in vitro. Mol Cancer Ther 2013;**12**:1763–1773.