

# Targeted Inhibition of Multiple Proinflammatory Signalling Pathways for the Prevention and Treatment of Multiple Myeloma

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## 1. Introduction

Multiple myeloma (MM) is a B cell malignancy involving the post germinal centre B cells. The disease is characterized by the presence of blood and urinary monoclonal proteins, osteolytic bone lesions and infiltration of bone marrow with malignant plasma cells of low proliferative index. Multiple myeloma is mainly a disease of elderly males, but, there is evidence to support that there is increasing incidence in younger individuals as well. American blacks are more prone than American whites. MM is the most common non Hodgkin's haematological malignancy, contributing 13% of all malignancies and 1% of all neoplasias. The median survival is 3-4 years, but with autologous stem cell transplantation and high dose chemotherapy, the median survival has increased to 5-7 years [1].

Most, if not all, multiple myeloma evolve from a premalignant condition known as 'Multiple Gammopathy of Undetermined Significance (MGUS)'. It then progresses via a 'smouldering multiple myeloma' stage, to a full blown disease and finally to an 'extramedullary MM' condition, where the malignant cells are no longer dependent on the bone marrow microenvironment for their proliferation. On a cellular scale, the origin of MM is thought to be post germinal centre B cell or memory B cell, indicated by the presence of hypermutated immunoglobulin gene. Evidence also supports the stem cell origin of the disease, as indicated by activated Wnt and Hedgehog signalling in the subset of cells in MM primary samples [2].

Cornelius Celsus, a Roman physician, first described the features of inflammation (inflammation - to set on fire) with the following signs: heat (calor), pain (dolor), redness (rubor) and swelling (tumor). The main purpose of inflammation is to protect the host

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organism from the microbes and other noxious stimuli. However, when the infection cannot be controlled or when there is a constant presence of the damaging stimuli, inflammatory process gets deregulated, resulting in a condition called chronic inflammation, which is destructive to the host organism. Thus inflammation is aptly termed as a 'double edged sword'. The link between inflammation and cancer was first established in 1897, by a German pathologist named Dr. Rudolf Virchow. He found that leukocytes infiltrate tumour tissue and therefore, termed tumours as 'wounds that do not heal'. Since then there has been much evidence to link inflammation and cancer, so as to be able to add inflammation as one of the hallmarks of cancer [3-5].

In the linking of inflammation and cancer, two pathways are said to exist - extrinsic and intrinsic. In the extrinsic pathway, chronic inflammation leads to autoimmune diseases, which eventually culminate in cancer. For example, *H.pylori* infection in the stomach, Hepatitis B and Hepatitis C infections in the liver, inflammatory bowel diseases and inflammation of the prostate gland (prostatitis); lead to incidences of gastric cancer, hepatocarcinoma, colon cancer and prostate cancer, respectively. In fact, about 20% of all cancers are said to arise in an inflammatory environment. In the intrinsic pathway, activation of oncogenes or inactivation of the tumour suppressor genes, causing both cancer and inflammation, which complement each other [6, 7]. Irrespective of the pathways involved, the perpetrators of the cancer related inflammation are inflammatory cells and inflammatory mediators, such as cytokines, chemokines, growth factors, all of which finally converge on a few transcription factors [8]. Not surprisingly, agents modulating cancer-related inflammation have been tried in cancer therapeutics [9].

MM cells depend largely on a bone marrow microenvironment for their growth and survival, until the last stage of the disease, where they invade other areas to be termed as extramedullary MM. The bone marrow microenvironment can be broadly divided into cellular and non-cellular components. Cellular components include myeloma cells, bone marrow stromal cells or bone marrow fibroblasts, haematopoietic precursor cells, osteoclasts, osteoblasts, endothelial cells and immune cells. Of these, the supportive role of stromal cells in MM has been studied extensively. The interactions between myeloma cells and osteoclasts have also been studied to an extent. The bone marrow stromal cells and osteoclasts provide the myeloma cells with the ability to grow and survive, either by direct adhesion and/or by secreting growth and survival cytokines.

The non-cellular compartment is comprised of the extracellular matrix and the soluble factors. Extracellular matrix consists of various proteins like collagen, fibronectin and laminin. The extracellular matrix not only acts as depots for the growth factors, but also provides the myeloma cells with the ability to resist cell death induced by chemotherapeutic agents. The survival advantage offered by the bone marrow microenvironment to the MM cells is achieved by 1. the soluble growth factors which are secreted by various cellular components, 2. insoluble growth factors that are bound to the extracellular matrix component and 3. adhesion molecules that help MM cells adhere to the extracellular matrix and the cellular compartment. In fact, in a recent study, 22 out of the 51 multiple myeloma growth factor genes that could be interrogated by affymetrix were found to be significantly overexpressed by at least one bone marrow environment population compared to others [10].

The stromal derived factor (SDF/CXCL12), secreted by the bone marrow stromal cells, plays an important role in the homing of MM cells to the bone marrow, which expresses receptor CXCR4. Moreover, adhesion of MM cells to stromal cells or fibronectin, induces chemoresistance in MM cells, mediated by integrins [11]. The adhesion molecules namely, very late antigen (VLA-4), vascular cell adhesion molecule (VCAM-1) and lymphocyte function-associated antigen 1 (LFA-1), intercellular adhesion molecule (ICAM-1), mediate integrin induced chemoresistance [12]. The resistance is mediated partly due to the activation of NF- $\kappa$ B, which upregulates anti-apoptotic gene products. MM samples are found to have various mutations activating both classical and alternative NF- $\kappa$ B. Apart from the mutations, the NF- $\kappa$ B pathway can also be stimulated by B cell growth factors like BAF and APRIL, which are secreted by the bone marrow microenvironment [13].

Adhesion of MM cells to the stromal cells, induces the latter to secrete IL-6. IL-6 is the main growth factor for the MM cells. IL-6 then induces JAK/STAT 3, PI3/AKT and MAPK survival pathways. STAT 3 transcription factor upregulates its targets, namely, cyclin D1 and Mcl-1, which promote cell proliferation and antiapoptosis respectively. In addition to the IL-6 induced activation of STAT 3, DNA methylation is found to silence the negative regulators of STAT 3. On the other hand, IGF secreted by bone marrow stromal cells induces PI3/AKT pathways [14]. AKT promotes cell proliferation by phosphorylating GSK3 $\beta$ , which regulates cyclin D1 proteolysis. Activated MAPK pathway leads to the activation of ERK, promoting MM growth and survival [15]. The following section will elaborate on the very common and important inflammatory player, involved in the progression of MM.

## 2. Role of proinflammatory cytokines and growth factors

### 2.1 Interleukin - 6

Interleukin-6, a pleotropic cytokine, is involved in processes such as haematopoiesis, immunity and inflammation. It was discovered as a factor secreted from mitogen stimulated T cells, which helps mature B cells transform into antibody producing plasma cells [16]. Because of its pleotropic nature, various laboratories were working with its different functions, giving it different names: B cell stimulating factor II (BSF II) as it stimulated B cells to turn into plasma cells and secrete antibodies, interferon- $\beta$ 2 [17] as it was thought to have the properties of interferon but later it was proven that IL-6 does not have properties of interferon, 26 kDa protein - named after its molecular weight, a hybridoma/plasmacytoma growth factor as it induced plasmacytoma in balb/c mice injected with mineral oil [18] and a hepatocyte-stimulating factor as it stimulated hepatocytes to produce acute phase proteins [19].

IL-6 binds to its receptor, which is either membrane bound or in soluble form. It then activates ubiquitously expressed receptor gp130 [20]. Once gp130 gets activated, IL-6 acts by three of the following signalling pathways: JAK-STAT pathway, MAPK-ERK and PI3-AKT pathway. Most of the actions of IL-6 are executed by JAK-STAT pathway [21]. IL-6 is found to be involved in the growth of many solid tumours like prostate cancer and renal cancer. Pathogenesis of Kaposi sarcoma has been proven to be due to the secretion of IL-6 [22-24]. IL-6 is also involved in the growth of many haematological malignancies.

IL-6 is one of the main growth factors in multiple myeloma [25]. In fact, IL-6 knock out mice failed to develop MM [26]. Moreover, the serum level of IL-6 and soluble IL-6 receptor has

been proven to be a prognostic marker for tumour load, disease progression and survival [27-31]. Moreover, serum levels of IL-6 in patients with smouldering MM and monoclonal gammopathy of undetermined significance are comparable with healthy individuals, indicating the important role of IL-6 in the disease progression [32].

Initially, it was thought based on the following findings that myeloma cells secrete and respond to IL-6 in an autocrine manner. Firstly, IL-6 induces *in vitro* growth of freshly isolated MM cells. Secondly, MM cells express the IL-6 receptor (IL-6R). Thirdly, purified MM cells produce IL-6 and lastly, *in vitro* growth of MM cells is inhibited by anti-IL-6 antibodies [33]. But, again controversies prevailed among the research laboratories on the autocrine secretion of IL-6 by myeloma cells. Because, though all myeloma derived cell lines and patients cells express IL-6 receptor, only subsets of cell lines express IL-6 mRNA [34]. It was also found that bone marrow stromal cells are the main source of IL-6 [35-37]. Interestingly, when myeloma cells were co-cultured with bone marrow stromal cells, they tend to adhere to each other tightly and the IL-6 secretion by these cells reaches the peak. But, when the bone marrow stromal cells were fixed by paraformaldehyde, there was no increase in the level of IL-6, confirming that the source of IL-6 was bone marrow stromal cells and not myeloma cells. Moreover, it was found that the stromal cells secrete IL-6 when stimulated by the adhesion of myeloma cells to the stromal cells. This is evident from experimental setup where these cells were cultured in transwell chambers without any physical contact with the myeloma cells. As a result, the bone marrow cells failed to secrete IL-6, emphasising the importance of adhesion molecules in the cross talk between the group of cells and pathophysiology of myeloma [38]. The adhesion mediated secretion of IL-6 was found to be NF- $\kappa$ B dependent [39].

In addition to bone marrow stromal cells, adhesion of myeloma cells to the peripheral blood derived osteoclastic cells protected myeloma cells from serum deprivation induced apoptosis and doxorubicin induced apoptosis. Osteoclasts produced osteopontin (OPN) and IL-6, and adhesion of MM cells to osteoclasts increased IL-6 production from osteoclasts. In addition, IL-6 and osteopontin in combination, enhanced MM cell growth and survival. However, the effects of osteoclasts on MM cell growth and survival were only partially suppressed by a simultaneous addition of anti-IL-6 and anti-osteopontin antibodies and were completely abrogated by inhibition of cellular contact between MM cells and osteoclasts. Osteoclasts enhance MM cell growth and survival through a cell-cell contact-mediated mechanism that is partially dependent on IL-6 and osteopontin [40].

The IL-6 induced survival of myeloma cells is mediated by STAT3, which upregulates anti-apoptotic proteins Bcl-XL and Mcl-1 and cell cycle proteins like cyclin D1, c-Myc and Pim. The IL-6 induced proliferation is mediated by MAPK-ERK pathway [41]. A PI3-AKT pathway mediates proliferation and induces survival by phosphorylating Bad and activating cell cycle proteins and NF- $\kappa$ B. Gene expression profiling studies demonstrated that out of 138 genes shown to be regulated by IL-6 in myeloma cells, 54% regulated cell cycle progression. This finding emphasises the role of IL-6 in myeloma cell proliferation [42]. IL-6 was shown to inhibit Fos induced apoptosis [43]. IL-6 can inhibit dexamethasone induced apoptosis of myeloma cells by gp130 induced activation of SHP2, which deactivates related adhesion focal tyrosine kinase (RAFTK) [44, 45] and activates the PI3/AKT pathway [46]. Partial reduction in the levels of IL-6 can sensitise the myeloma cells to chemotherapeutic agents [47, 48].

Various strategies, including IL-6 antagonist, IL-6 receptor inhibitor (CNTO 328), antisense oligonucleotides against IL-6 and IL-6 super antagonist (SANT7), have been tried for MM, but even after effectively blocking IL-6 receptor by the monoclonal antibody, the results were disappointing in clinical trials [49]. Accordingly, in the presence of bone marrow stromal cells, IL-6 receptor inhibition did not induce apoptosis, indicating the significance of the pleiotropism offered by other growth and survival factors present in the bone marrow microenvironment [50, 51].

## 2.2 TNF $\alpha$

In 1894, William Coley noticed that an injection of bacterial extracts into the tumour, could induce necrosis of tumours [52]. O'Malley et al. demonstrated that serum from mice injected with bacterial endotoxin can induce tumour regression [53]. The factor that can induce anti-cancer activity in vivo and in vitro, present in the sera of mice treated with endotoxin or LPS, was identified as Tumour Necrosis Factor  $\alpha$  [54, 55]. The gene expressing human TNF $\alpha$  was cloned in 1984 [56]. Thereafter, the recombinant TNF $\alpha$  was used for experimental and therapeutic purposes. The therapeutic dose of TNF $\alpha$  induced serious hemodynamic instability and septic shock-like symptoms preclinically. TNF $\alpha$  can induce necrosis of the tumour by selective destruction of the blood vessels, only when injected at higher concentrations loco-regionally [57]. Its induction of apoptosis is highly context dependent. Physiologically, TNF $\alpha$  is an important cytokine regulating inflammation, immunity and haematopoiesis. Its deregulation is involved in lots of inflammatory and autoimmune conditions like rheumatoid arthritis and Crohn's disease. Recent research has realised the potent protumorigenic effect of TNF $\alpha$  [58]. TNF $\alpha$  KO and TNF $\alpha$ -R1 KO mice do not develop chemical carcinogen induced skin cancers [59, 60]. TNF $\alpha$ -R1 KO mice do not develop chemical carcinogen induced liver cancer [61]. TNF $\alpha$  antagonists are in various stages of clinical trials for a variety of cancers.

In MM, TNF $\alpha$  is not a strong growth factor, but it is an important factor secreted from myeloma cells to act on BMSCs to stimulate the secretion of IL-6. TNF $\alpha$  induces the expression of adhesion molecules on both myeloma cells and BMSCs. TNF $\alpha$  secreted by myeloma cells acts both directly and by increasing the adhesion between myeloma cells and the bone marrow stromal cells to secrete IL-6 by an NF- $\kappa$ B mediated mechanism in bone marrow stromal cells. TNF $\alpha$  is very potent when compared to other growth factors [62]. TNF $\alpha$  also participates in transendothelial migration of myeloma cells by acting via TNF-R2 and upregulating the secretion of MCP-1 in myeloma cells [63]. Clinically, the agents which are known to inhibit TNF $\alpha$ ; namely, thalidomide and its derivatives and bortezomib, have significant anti-myeloma activity.

## 2.3 BAFF and APRIL

BAFF and APRIL also belong to the TNF family of cytokines. They act by binding to receptors TACI (transmembrane activator and calcium modulator, and cyclophilin ligand interactor), BCMA (B-cell maturation antigen) and BAFF-R (BAFF Receptor) which is specific for BAFF. Myeloma cells express these receptors in a heterogeneous manner [64]. In fact, patient groups whose myeloma cells had low expression of TACI receptor were less differentiated and showed attenuated dependence on the bone marrow and portending

poor prognosis; whereas patients whose myeloma cells express high levels of TACI receptor showed mature plasma cell signature exhibiting good prognosis [65]. There is evidence for these cytokines being secreted from myeloma cells [64, 66], bone marrow cells [67] and osteoclastic cells [65]. BAFF and APRIL seem to induce myeloma cell growth and inhibit dexamethasone induced apoptosis. BAFF and APRIL activate NF- $\kappa$ B, PI3kinase/AKT, and MAPK pathways in myeloma cells and induce a strong upregulation of the Mcl-1 and Bcl-2 anti-apoptotic proteins [68, 69]. Cell adhesion induced bone marrow cells secrete BAFF, which acts on myeloma cells to regulate their growth and survival [67]. Interestingly, bortezomib has been found to inhibit BAFF and APRIL induce proliferation of myeloma cells [66].

#### **2.4 Insulin-like Growth Factor 1 (IGF-1)**

Recent studies have delineated the role of IGF-1 in MM. IGF-1 was shown to be a strong indicator of prognosis in MM patients [70]. In the bone marrow milieu, IGF-1 is mainly produced and secreted from bone marrow stromal cells and mediates cell growth and survival in MM cells both in vitro [71, 72] and in vivo [73-75]. IGF-1 and its receptor were shown to be acting as growth factors [76] and preferentially expressed in MM cells [77] as compared to B-Lymphoblastoid cell lines.

IGF-1 inhibits Dexametasone-induced apoptosis in MM cell lines [78]. IGF-1 augments the proliferative and anti-apoptotic effects of IL-6 [71, 79]. Although IL-6 has mostly been described as a proliferation factor for MM, it has become clear that IGF-1 has an equally important proliferative and anti-apoptotic effect [80-82]. It could be that IGF-1 plays an even more pivotal role in the survival of MM cells, as IL-6 independent lines still respond to IGF-1 [80, 82]. Another group demonstrates that IGF-1 serves as a chemoattractant for MM cells [73]. In vivo induction of the receptor IGF-1R helps murine multiple myeloma cells in their homing and growth in the bone marrow [83].

IGF-1 transduces its signal by receptor phosphorylation of the insulin response substrate 1 and its activation of PI-3K and subsequently Akt kinase (PI-3K pathway). In fact, IGF-I increases adhesion of MM cell lines to fibronectin (FN) in a time and dose-dependent manner, as a consequence of IGF-1R activation and subsequent activation of  $\beta$ 1- integrin and PI3-kinase/AKT signalling [84]. Several important biological characteristics have been associated with this segment of the PI-3K pathway [85]. Akt subsequently phosphorylates Bad, a member of the Bcl-2 family, producing an anti-apoptotic effect. The second pathway associated with IGF-I stimulation signals through the Shc, Grb-2, Sos complex, resulting in activation of Ras and subsequently the mitogen-activated protein kinase (MAPK) signalling cascade.

IGF-1 is also shown to mediate the activation of NF- $\kappa$ B [86], induce the phosphorylation of FKHR (forkhead) transcription factor, upregulate a series of intracellular anti-apoptotic proteins (including FLIP, survivin, cIAP-2, A1/Bfl-1 and XIAP) and decrease drug sensitivity of MM cells [75]. Caveolin-1, which is usually absent in blood cells, is expressed in MM cells and plays a crucial role in IGF-1-mediated signalling cascades [87]. Specifically, IGF-1 induces HIF-1 $\alpha$ , which triggers VEGF expression [88, 89]; consequently, inhibition of IGF-1 activity markedly decreases VEGF secretion in MM/BMSC co-cultures [75].

Therapies targeting IGF-1, such as inhibitors of IGF-1 receptor, have already shown preclinical anti-MM activity and will soon undergo clinical evaluation [75]. IGF-1R inhibition with neutralizing antibody, antagonistic peptide, or the selective kinase inhibitor NVP-ADW742 has in vitro activity against MM cell types and in orthotopic xenograft MM model had synergistic anti-tumour activity in combination with conventional chemotherapy. Another study [90] reports that IGF-1R inhibition blunts tumour cell response to other growth factors, overcomes the drug resistance phenotype conferred by the bone microenvironment and abrogates the production of proangiogenic cytokines. These sets of studies provide in vivo proof of the principle for therapeutic use of selective IGF-1R inhibitors in cancer.

## 2.5 Fibroblast Growth Factor (FGF)

Besides bone marrow microvessel density (MVD), serum levels of FGF, along with VEGF, are predicted to be prognostic markers of MM disease activity [91, 92]. Expression of bFGF correlates with clinical characteristics of MM and its high level also indicates poor prognosis [93]. However, the levels of bFGF may serve as a predictor for good response to the treatment of MM with Thalidomide [94]. Patients responsive to Thalidomide may have significantly higher concentrations of bFGF than non-responsive patients, but this observation is not consistent even between the same authors [95, 96]. Stimulation of BMSCs with FGF-2 induced a time and dose-dependent increase in IL-6 secretion, a well studied cytokine, which was completely abrogated by anti-bFGF antibodies. Conversely, stimulation with IL-6 enhanced bFGF expression and secretion by myeloma cell lines as well as MM patient cells, suggested a paracrine interaction between the myeloma and the stromal cells with respect to the above cytokines [97].

The FGF receptor 3 (FGFR3) is now recognized as a potential oncogene. Ectopic expression of FGFR3 originates from the translocation t(4;14) occurring in 10-25% of MM patients [98, 99]. Gain of function mutations in FGF receptors, especially FGFR3, have been widely implicated and studied in MM pathogenesis [98]. Suppression of FGFR3 using short hairpin RNAs (shRNAs), lead to apoptosis and anti-tumour effects in MM [100, 101].

FGF binding to the FGFR, results in dimerization of the receptor and autophosphorylation of the FGFR dimer at intracellular tyrosine residues. The activated receptor either binds directly to signalling molecules or recruits adapter molecules to link the activated receptor to downstream targets at the cell membrane.

Three FGF signalling downstream pathways have been identified in MM [102]: the Ras mitogen-activated protein kinase (MAPK) pathway, the phosphoinositol pathway and the signal transducer and activator of transcription (STAT) pathway.

## 2.6 Transforming Growth Factor (TGF- $\beta$ )

Transforming Growth Factor beta (TGF- $\beta$ ) is a growth factor that controls proliferation, cellular growth and differentiation [103], and embryonic development [104]. During tumourigenesis, the TGF- $\beta$  signalling pathway becomes mutated and TGF- $\beta$  no longer controls the cell cycle [105, 106]. The cancer cells along with the surrounding stromal cells (fibroblasts) proliferate unchecked. Both these cells increase their production of TGF- $\beta$ ,

which acts on the surrounding stromal cells, immune cells, endothelial and smooth-muscle cells, causing immunosuppression [106, 107] and tumour angiogenesis, and increasing the invasiveness [108, 109] and motility [110] of cancer.

TGF- $\beta$  also plays a role in the suppression of bone formation in MM bone lesions [111]. Overproduction of TGF-beta 1 in MM patients was reported by Kroning et al. [112]. TGF- $\beta$  is mainly produced by BMSCs, but is also secreted by malignant plasma cells and can regulate interleukin-6 (IL-6) secretion [113]. According to Cook et al., TGF- $\beta$  produced by MM cells plays a significant role in suppressing host T cells and immune responses [114, 115]. TGF- $\beta$  inhibition was able to suppress MM cell growth within the bone marrow while preventing bone destruction in MM-bearing animal models [116].

### 3. Role of chemokines

In MM, chemokines mainly help homing the myeloma cells to the bone marrow microenvironment. Their role in proliferation and survival of myeloma cells is only moderate. This effect can be either direct or mediated indirectly by inducing the secretion of IL-6, VEGF, or any other growth factor involved in the growth and survival of myeloma cells. The role of chemokines, especially that of MIPs, in osteolytic bone lesions is well established. Homing is defined by transendothelial migration of cells from the blood stream towards the chemokine gradient. This involves adhesion of cells to the endothelial layer, transendothelial migration and eventually residing in the microenvironment. So, it is apparent that bone marrow endothelial cells play an active role in the migration of plasma cells. They do so by secreting various chemokines and expressing adhesion molecules; thereby helping myeloma cells to migrate towards them. Upon adherence, MM cells will extravasate using their MMP arsenal to move through the basal lamina of bone marrow sinusoids. This process is also aided by the chemokine gradient in the bone marrow microenvironment because certain chemokine are said to be present in higher concentrations in the bone marrow microenvironment than in bone marrow endothelial cells which make sure that the cells are confined to the bone marrow microenvironment.

#### 3.1 Macrophage Inflammatory protein: (MIP-1, CCL3)

MIP1 belongs to the CC family of chemokine and mainly acts via CCR1, CCR5 and CCR9 receptors. Myeloma cells have been shown to express both the receptors (CCR1, CCR5) and the chemokine [117, 118]. Controversial findings on the effect of growth and survival of myeloma cells could be due to usage of different experimental models and design [118, 119], but its role in migration and homing of myeloma cells, and in the progression of the myeloma bone disease, are clearly demonstrated. SCID mice injected with stable MIP1 knock-down clones of ARH cell line showed comparably less adhesion to the bone marrow, reduced survival and less bone pathology when compared to wild type ARH cell line injected group [117]. Suzanne Lentzsch et al. showed in vitro evidence that MIP1 $\alpha$  can induce myeloma cell migration. Interestingly, they also showed that MIP1 $\alpha$  can induce proliferation and survival of myeloma cells by inducing MAPK/ERK pathway, PI3/AKT pathway [118]. There is a study in which the various effects of MIP1 $\alpha$  on 5TMM has been dissected. MIP1 $\alpha$  induced migration has been attributed to the CCR5 and CCR1 receptor mediated signalling. Both the receptors mediate the MIP1 $\alpha$  induced bone marrow angiogenesis and at least CCR1 mediates this effect directly [119].

### 3.2 MCP1 (or monocyte chemoattractant protein - CCL2)

As mentioned earlier, endothelial cells play an active role in the extravasation of myeloma cells and eventually their homing to the microenvironment. Murine endothelial cells are shown to secrete CCL2 and murine myeloma cells express the cognate receptor CCR2. Myeloma cells migrated towards the endothelial cell conditioned medium and this migration was inhibited by using antibodies against MCP1 [120]. Human bone marrow stromal cells also secrete MCP1, MCP2 and MCP 3, and myeloma cells migrate towards a stromal cell conditioned medium. This effect was inhibited by using antibodies against the MCPs and maximal inhibition was observed when all the three MCPs were blocked together, suggesting the role of various MCPs in myeloma homing [121].

### 3.3 CXC chemokines and CXCR3 receptor involvement in MM

CXCR3 receptor is expressed by activated T cells. It binds to CXC chemokines namely: CXCL11 or Interferon-inducible T-cell Alpha Chemoattractant (I-TAC), Mig (Monocyte/macrophage-activating IFN $\gamma$ -inducible protein)/CXCL9 and IP10 (IFN $\gamma$  - inducible 10 kDa protein)/CXCL10. Myeloma cells derived from patients with myeloma, as well as myeloma derived cell lines, express CXCR3 receptor and they respond to their ligands by inducing tyrosine kinase phosphorylation and secreting MMP2 and MMP9 [122]. Bone marrow endothelial cells also secrete CXC chemokines and certain myeloma cells expressing their cognate receptors migrate in response to these chemokines [123].

### 3.4 Stromal Derived Factor (SDF-1 $\alpha$ /CXCL12)

Stromal derived factor is a member of CXC family of cytokines and its cognate receptor is CXCR4. CXCL12/CXCR4 is the most extensively studied chemokine/receptor system with respect to cancer. It has been implicated in progression, migration, invasion and metastasis of various cancers. The role of CXCL12/CXCR4 has been well established in the homing of haematopoietic progenitor cells. Bone marrow plasma and bone marrow stromal cells secrete this chemokine, with the myeloma cells from the patient sample and myeloma derived cell lines expressing the cognate receptors. The chemokine mediates the secretion of IL-6 and VEGF, and induces proliferation, migration and inhibits dexamethasone induced cell death [124]. In the 5TMM model, bone marrow stromal cells and endothelial cells secrete SDF-1 $\alpha$  and myeloma cells express the receptor. In vitro, SDF-1 $\alpha$  induces moderate proliferation of myeloma cells, which was abrogated by blocking antibodies. 5T myeloma cells migrated towards a stromal cells conditioned medium which was partially inhibited by CXCR4 inhibitor. SDF also stimulated myeloma cells to secrete MMP9, demonstrated by zymography. Accordingly, SDF induces invasion and the CXCR4 inhibitor inhibits SDF induced invasion. In vivo, CXCR4 inhibitor inhibited the tumour burden and the immediate homing to about 40% [125].

When the myeloma cells were mobilized, the CXCL12/CXCR4 axis is downregulated. There is a downregulation of very late antigen (VLA4) in the peripheral blood myeloma cells after mobilization. This results in a suppression of the adhesion of myeloma cells to the bone marrow stromal cells, which can be rescued by induction with IL-6 [126]. Moreover, bone marrow endothelial cells are also shown to secrete CXCL12 and induce migration of myeloma cells towards the bone marrow endothelial cells. Thus, angiogenesis induced

migration of myeloma cells is also mediated by CXCL12 chemokine [123]. The expression of CXCR4 was higher in bone marrow plasma cells of patients with myeloma than patients with MGUS. Moreover, the bone marrow plasma of myeloma patients has higher SDF-1 $\alpha$  levels than that of peripheral blood of myeloma cells and bone marrow plasma of healthy individuals [127]. Consistent with its effect on migration, invasion, homing, proliferation and survival, CXCL12/CXCR4 axis induced MAPK/ERK, AKT, PKC and NF- $\kappa$ B pathways [124, 127].

## 4. Role of proinflammatory transcription factors

### 4.1 STAT3

STAT3 is a member of the STAT family of transcription factors. STAT family proteins were first discovered in the context of the specificity of the IFN signalling [128]. STAT3 was first described as a DNA-binding factor, in IL-6 stimulated hepatocytes, capable of selectively interacting with an enhancer element in the promoter region of acute-phase genes [129].

STAT3 is constitutively phosphorylated in v-Src-transformed cells and has been found to be necessary for the v-Src induced carcinogenesis. Expression of a constitutively active version of STAT3 on its own can lead to fibroblast transformation, showing that STAT3 is an oncogene [130]. Consistent with its role in various cancers, STAT3 regulates various genes involved in different aspects of cancer progression. Genes regulated by STAT3 that are involved in proliferation and growth include c-myc, cyclinD3, cyclin A, cdc25a, p21, cyclinD1, Pim-1 and Pim-2. Genes regulated by STAT3 that are involved in survival include proteins belonging to the family of Bcl-2 and IAPs, namely, Bcl-2, Bcl-xL, Mcl-1 and survivin. STAT3 has also been shown to downregulate the Fas cytokine. STAT3 mediated angiogenesis is mediated by VEGF; STAT3 also regulates MMP family members MMP2 and MMP9 [131]. STAT3 is vital for development, seen from STAT3 knock out mice which succumb to embryonic lethality [132]. However, disruption of STAT3 function either by deleting the gene or by introducing the dominant negative form of STAT3, leads to only a few phenotypical changes [133]. These findings are critical for the development of therapeutic strategies with high therapeutic index. In MM, STAT3 plays an important role in survival. It upregulates anti-apoptotic proteins like Bcl2, Bcl-XL and Mcl-1 [134-136]. Constitutive expression of STAT3 confers myeloma cells resistance to apoptosis [137]. Out of all the anti-apoptotic proteins regulated by STAT3, Mcl-1 seems to be more important. While antisense inhibition of Bcl-xL did not inhibit survival, knock down of Mcl-1 was sufficient to inhibit survival in myeloma cells. Overexpression of Mcl-1 was able to promote proliferation of multiple myeloma cells lines, even in the absence of IL-6 [138].

Knock down of Bcl-2 can augment dexamethasone induced apoptosis [139], but again, the importance of STAT3 in regulating the anti-apoptotic proteins and thereby the survival of myeloma cells remains controversial in the light of a lack of correlation between the constitutive expression of STAT3 and the anti-apoptotic proteins [140]. However, it is clear that STAT3 is not the only factor which regulates the survival of myeloma cells because myeloma cells become independent of a IL-6-gp130-STAT3 pathway in the presence of bone marrow stromal cells [51]. Almost 48% of MM patients have constitutively activated STAT3 [140]. There has been no activating mutations of STAT3 detected in MM. But, there has been epigenetic silencing of negative regulators of STAT3, namely, SHP1 and SOCS in MM. 27 of

34 (79.4%) myeloma samples showed SHP1 hypermethylation. At least in U266 MM cells, methylation of SHP1 may be responsible for constitutive STAT3 activation, because treatment with 5-azacytidine, a DNA demethylator, led to a progressive demethylation of SHP1 and a parallel downregulation of phosphorylated STAT3 [15]. SOCS-1 is hypermethylated in 23 out of 35 (62.9%) MM patient samples and consistently expression of this protein is upregulated after treatment with demethylators. So, it can be concluded that suppression of the expression of negative regulators of IL-6-JAK-S TAT pathway by epigenetic silencing increases the sensitivity of myeloma cells to IL-6 induced proliferation and survival [141]. Moreover, overexpression of SOCS using adenoviral vector inhibited the IL-6 induced proliferation in IL-6 dependent multiple myeloma cells, hinting at another strategy to inhibit IL-6 induced downstream signal transduction pathways [142].

There are lots of therapeutic strategies that are being developed to target JAK-STAT3 pathway in MM. In fact, the novel agents that are being used nowadays namely, thalidomide and its derivatives and bortezomib, act partially to disrupt the NF- $\kappa$ B induced activation of IL-6 and thereby STAT3 activation. Numerous drugs that inhibit IL-6-JAK-STAT3 pathway at various levels induce apoptosis, both in vitro and in vivo [143-175].

#### 4.2 NF- $\kappa$ B pathway

NF- $\kappa$ B is a Rel family of transcription factors consisting of p50, p52, c-Rel, p65/RelA and RelB subunits [176, 177]. It was discovered by Dr. Baltimore and colleagues in 1986 as a DNA binding protein, recognising specific sequences in the immunoglobulin kappa light chain joining (J) segment gene region in B cells [178].

Various inflammatory stimuli activate the NF- $\kappa$ B pathway. There are two pathways involved in the activation of the NF- $\kappa$ B pathway: the classical pathway and the alternative pathway.

NF- $\kappa$ B is a main transcription factor regulating various genes involved in inflammation. NF- $\kappa$ B has been casually implicated in various types of tumours [179]. Selective deletion of NF- $\kappa$ B in hepatocytes or inhibition of TNF- $\alpha$  production by neighbouring parenchymal cells, induced programmed cell death of transformed hepatocytes and reduced the incidence of liver tumours. Paracrine activation of NF- $\kappa$ B in initiated cells was not important in the early stages of liver tumour development, but it was crucial for malignant conversion [180]. In colitis associated cancer model of mice, selective deletion of IKK- $\beta$  in inflammatory cells that are surrounding the enterocytes reduced the mRNA of inflammatory cytokine levels and subsequently decreased the tumour formation. However, selective deletion of IKK- $\beta$  in enterocytes did not reduce inflammatory features, but it induced enhanced cell death in enterocytes leading to a decrease in the incidence of colon cancer [181]. It is quite obvious from these experiments that NF- $\kappa$ B affects both tumour cells and inflammatory stromal cells to induce and promote cancer. NF- $\kappa$ B acts on enterocytes to inhibit apoptosis and also acts on inflammatory cells to stimulate the secretion of various mediators of inflammation which in turn acts on the enterocytes to induce cancer. However, in some tissues, NF- $\kappa$ B acts to prevent cancer. For example, inhibition of NF- $\kappa$ B in keratinocytes leads to squamous cell carcinoma of skin [182]. In MM, patient samples show a constitutive activation of NF- $\kappa$ B to a variable degree [183]. How these cells activate NF- $\kappa$ B in a constitutive manner is still under investigation. Soluble cytokines belonging to TNF $\alpha$  super family including TNF- $\alpha$ , BAFF,

APRIL, lymphotoxin b, are known to activate NF- $\kappa$ B and are present in the bone marrow microenvironment. Adhesion of myeloma cells to the bone marrow stromal cells and osteoclasts also activates the NF- $\kappa$ B pathway in both myeloma cells and osteoclasts, and bone marrow stromal cells.

Moreover, around 15-20% of myeloma samples and 40% of the cell lines show activating mutations in the NF- $\kappa$ B pathway [13, 184, 185]. There could be some unidentified genetic mutations or epigenetic modifications that might explain the constitutive activation in the remaining tumours. Gain of function mutations include ones encoding receptors known to activate NF- $\kappa$ B namely, CD40, LT $\beta$ R, TAC1, NIK (NF- $\kappa$ B-inducing kinase), and direct mutations involving NF- $\kappa$ B1 p50/p105 and NF- $\kappa$ B2 p52/ p100. Loss of function mutations include those that involve negative regulators of NF- $\kappa$ B activation namely, TRAF3, TRAF2, CYLD and cIAP1/cIAP2, inactivation of TRAF3 being the most common. These mutations activate both classical and alternative pathways of NF- $\kappa$ B. CD40, LT $\beta$ R, TAC1 and receptor overexpression may be sufficient to activate the NF- $\kappa$ B pathway or might enhance the sensitivity of MM cells to factors in the tumour microenvironment. Overexpression of NIK or NF- $\kappa$ B1 p105 directly leads to constitutive activation of NF- $\kappa$ B. Deletion of sequences in the p100 I $\kappa$ B-like domain of NF- $\kappa$ B2 promotes processing of p100 to p52 and activation of the alternative NF- $\kappa$ B pathway [184, 185]. Activating mutations of the NF- $\kappa$ B pathway helps the myeloma cells become independent of the bone marrow, as they overcome the need for external cytokines activating the pathway [13].

Activation of NF- $\kappa$ B in myeloma cells induces proliferation, survival and chemoresistance. When compared to chemosensitive myeloma cell lines, chemoresistant myeloma cells express higher levels of NF- $\kappa$ B, suggesting a link between NF- $\kappa$ B and development of chemoresistance [186, 187]. Moreover, dexamethasone induced apoptosis is associated with a decrease in the NF- $\kappa$ B DNA binding activity. Interestingly, NF- $\kappa$ B can also serve as a prognostic indicator for response to dexamethasone. Only patients who responded to dexamethasone, demonstrated decreased NF- $\kappa$ B DNA binding activity in their samples. Enforced ectopic expression of Bcl-2 in myeloma cells conferred resistance to dexamethasone induced apoptosis, and this was also associated with enhanced NF- $\kappa$ B DNA binding [187]. Inhibition of NF- $\kappa$ B by IKK inhibitor abrogates the protective effect of IL-6 on dexamethasone induced apoptosis. It also potentiated TNF $\alpha$  induced apoptosis in myeloma cells. NF- $\kappa$ B inhibition abrogated the TNF $\alpha$  induced upregulation of ICAM-1, both in myeloma cells and in bone marrow stromal cells. It also inhibited the myeloma cell adhesion induced IL-6 secretion by bone marrow stromal cells and resulting proliferation of myeloma cells. These findings indicate that pro-survival functions of the bone marrow microenvironment are abrogated upon NF- $\kappa$ B inhibition. The novel therapeutic agents namely, bortezomib and thalidomide and its derivatives, act at least partially by inhibiting NF- $\kappa$ B [188].

## **5. Role of matrix proteinases, angiogenic and adhesion molecules**

### **5.1 Matrix metalloproteinase**

Matrix metalloproteinase belong to a family of proteases, capable of degrading all kinds of extracellular matrix proteins. In 1962, Gross et al. discovered MMP, when they found collagenase activity in the tail of a tadpole during metamorphogenesis [189]. These proteins function not only to remodel the extracellular matrix, but also are involved in the cleavage

and thereby activation and inactivation of various biologically significant proteins like chemokines and growth factors. In the context of cancer, both the cancer cells and stromal cells secrete MMPs. Their involvement in invasion and metastasis was examined in various clinical models. Recent evidence suggests the role of MMPs in various hallmarks of cancer progression [190]. Culture supernatants of bone marrow derived stromal cells from multiple myeloma patients were found to have higher levels of MMP-1 and MMP-2 than control samples [191]. Moreover, endothelial cells secrete hepatocyte growth factor, which acts on myeloma cells to stimulate the secretion of MMP-9 [192]. 5T MM bone marrow expresses various MMPs, such as MMP2, MMP8, MMP9 and MMP13. Adequate inhibition of these MMPs by a broad spectrum MMP inhibitor SC-964 suppresses angiogenesis, reduces tumour load and osteolytic lesions [193].

## 5.2 Vascular Endothelial Growth Factor (VEGF)

VEGF is a signal protein that stimulates formation of new blood vessels, through vasculogenesis and angiogenesis. The activity of VEGF is mediated through three receptor tyrosine kinases: VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1) and VEGFR-3 [194]. Dysregulation of VEGF has been shown to be a major contributor to tumour angiogenesis as well, promoting tumour growth, invasion and metastasis [195]. Upon stimulation by VEGF, bovine capillary endothelial cells were shown to proliferate and show signs of capillary-like tube structures [196]. Significantly elevated levels of VEGF are observed in a variety of haematologic malignancies [197-201]. Several studies link VEGF inactivation to anti-tumour effects [202]. Angiogenesis appears to play a role in haematological malignancies [203]. There is growing evidence that increased bone marrow angiogenesis occurs in myeloma [204, 205] and is related to disease activity [206, 207]. Angiogenesis in myeloma also appears to be correlated with the Plasma Cell Labelling Index, PCLI [206]. Micro vessel density (MVD) increases five-to-six fold in magnitude with progression from gammopathy of undetermined significance (MGUS) or non-active MM to the active MM [93, 208]. Moreover, after chemotherapy, MVD decreases significantly in patients in complete or partial remission [209]. MM cells release angiogenic factors, such as FGF and VEGF [93, 210], and are shown to induce angiogenesis *in vivo* in the Chick Chorioallantoic Membrane assay [93]. They secrete matrix metalloproteinase-2 and -9 (MMP-2 and MMP-9) and urokinase-type plasminogen activator [93] and cytokines recruiting inflammatory cells, such as mast cells, that then induce angiogenesis through secretion of angiogenic factors in their granules [211]. A better understanding of some of the above angiogenic factors would help in developing novel therapeutic targets against MM. A few of the widely prominent angiogenic factors are reviewed in detail in the following section.

A number of studies implicate dysregulation of VEGF in MM pathogenesis and associated clinical features, including lytic lesions of the bone and immune deficiency. VEGF protein was found in malignant cells from 75% of MM patients studied [212]. Increased serum levels of VEGF have been correlated with a poor prognosis in patients with advanced stages of MM [213]. In fact, Iwasaki T et al. report predicting treatment responses and disease progression in myeloma using serum vascular endothelial growth factor [214]. Another patient study claims that the levels of VEGF, along with FGF, parallel disease activity [210]. VEGF may also affect the immune response in MM patients. Sera from MM patients' bone

marrow inhibits antigen presentation by dendritic cells (DCs); conversely, anti-VEGF antibodies neutralized this inhibitory effect, confirming that VEGF mediates immunosuppression in MM patients [215]. The cytokine is probably involved in the progression of MM to plasma cell leukaemia (PCL) [216]. Not just the ligand, its receptor VEGFR-1 is also widely expressed on both MM cell lines and patient MM cells, confirmed both by reverse-transcriptase polymerase chain reaction (RT-PCR) analyses and immunoprecipitation [217-219]. VEGF is generally present in the bone marrow (BM) microenvironment of patients with MM and associated with neovascularization at sites of MM cell infiltration [220]. The induction of VEGF enhances the microvascular density of bone marrow and accounts for the abnormal structure of myeloma tumour vessels [221]<http://www.nejm.org.libproxy1.nus.edu.sg/doi/full/10.1056/NEJMra1011442-ref12>. VEGF increases both osteoclastic bone-resorbing activity [222] and osteoclast chemotaxis [223], and inhibits maturation of dendritic cells [224]. As marrow neovascularization parallels disease activity in MM, it is reasonable to postulate that the vascular growth factor is acting in an autocrine fashion. However, MM cells express VEGF receptors only weakly, if at all. Therefore, the mechanism may be paracrine and result from a VEGF-induced time and dose-dependent increase in stromal cell secretion of interleukin-6 (IL-6), a known MM growth factor [225]. Another cytokine, TNF $\alpha$ , has been reported to be involved in the control of VEGF production by myeloma cells [226]. Moreover, VEGF directly, or indirectly through its stimulatory activity on TNF- $\alpha$  and IL- $\beta$ 1, stimulates the activation of osteoclasts and thus contributes to the lytic lesions in MM [222].

Other factors modulating VEGF secretion include Interleukins: IL-1 $\beta$  [227], IL-10 and IL-13 [228]; secretion of IL-6 [218, 225, 229] or VEGF by both BMSCs and tumour cells (paracrine/autocrine loop); hypoxia and the presence of mutant oncogenes (i.e., mutant Ras [mutRas] or Bcr-Abl, which up-regulate VEGF expression via HIF-1 $\alpha$  protein); secretion of growth factors, such as insulin-like growth factor-1 (IGF-1) [88, 230], fibroblast growth factor-4 (FGF-4) [231], platelet-derived growth factor (PDGF) [232], TGF- $\beta$  [233], TNF- $\alpha$  [234] and gonadotropins [235]; c-maf-driven expression of tumour integrin  $\beta$ 7 [236]; tumour cell expression of ICAM1 and LFA1 modulating adhesion to ECM and BMSCs, thereby increasing VEGF production and secretion; and CD40 activation, which induces p53-dependent VEGF secretion. Binding of VEGF to MM cells triggers VEGFR tyrosine phosphorylation, activating several downstream signalling pathways, particularly involving phosphatidylinositol-3 kinase [237, 238]. PI3-kinase-dependent cascade mediates MM cell migration on fibronectin, evidenced by using the PI3-kinase inhibitor bis-indolylmaleimide I and LY294002 [237]. This signal transduction pathway is mediated by focal adhesion proteins [239], such as FAK, paxillin and cortactin, which are responsible for the stabilization of focal adhesion plaques and the reorganization of actin fibres [240]. VEGF also regulates MM cell survival by modulating the expression of Mcl-1 and survivin [241].

MAP kinases (MAPK) are the final effectors of the signal to the nucleus, thereby activating genes for proliferation, migration and survival [242]. This increased migration and cell proliferation is because of the activation of VEGFR-2, since it is totally inhibited by a VEGFR-2 blocking antibody [243]. In fact, MEK-extracellular signal-regulated protein kinase (ERK) pathway is shown to mediate MM cell proliferation, evidenced by use of anti-VEGF antibody and PD098059 [217]. Approaches to disrupt the VEGF/VEGF receptor signalling

pathways range from small molecule VEGF/VEGFR inhibitors, anti-VEGF and anti-VEGF receptor antibodies, such as bevacizumab [244, 245], and VEGF transcription inhibitors. Of interest are various kinase inhibitors that block the signal transduction mediated by VEGF. The VEGF receptor tyrosine kinase inhibitor PTK787 is active preclinically and undergoing clinical protocol testing in MM [246, 247]. It acts directly on MM cells to inhibit VEGF-induced MM cell growth and migration, and inhibits paracrine IL-6-mediated MM cell growth in the BM milieu. Pazopanib [248], another VEGF receptor tyrosine kinase inhibitor, has been studied for cancer therapy.

### 5.3 Adhesion molecules

Cell adhesion is a key physiological event involved in morphogenesis and histogenesis. Adhesion molecules mediate cell-cell and cell-ECM interactions [249], and are also involved in intracellular signalling after engagement with their receptors. Broadly, there are five groups of adhesion molecules. They are 1) the integrins-mediating cell-ECM and cell-cell adhesion 2) the cadherin family-mediating homotypic cell-cell adhesion 3) the selectin family-mediating heterotypic cell-cell adhesion 4) the immunoglobulin superfamily-mediating cell-cell adhesion and 5) other transmembrane proteoglycans, such as CD44 adhesion molecules and syndecan that mediate cell-extracellular matrix adhesion [12]. Dysregulated expression or function of adhesion molecules are involved in various steps of cancer progression.

In MM, there is evidence that adhesion molecules mediate homing of MM cells to the bone marrow, secretion of cytokines and growth factors, and development of chemoresistance. Out of all the adhesion molecules, VLA-4 and VLA-5 expressed by the myeloma cells play a crucial role in the myeloma pathogenesis [250]. VCAM-1 and fibronectin are the receptors for VLA. VLA adheres to the bone marrow stromal cells by binding to VCAM, CS-1 fragment and H1 region of fibronectin [251]. Inhibition of VLA using blocking antibodies inhibit the adhesion of myeloma cells to the bone marrow stromal cells and fibronectin [252]. VLA dependent adhesion to the bone marrow is regulated by the CXCL12/CXCR4 axis [253]. This is further supported by the finding that disruption of CXCL12/CXCR4 axis results in downregulation of VLA-4 and decreased adhesive capacity in the mobilised myeloma cells when compared to premobilisation bone marrow myeloma cells [126].

VLA dependent adhesion of MM cells to the bone marrow stromal cells induces secretion of IL-6 by an NF- $\kappa$ B mediated mechanism [38, 39]. Drug-sensitive 8226 human myeloma cells, expressing both VLA-4 and VLA-5 receptors, are relatively resistant to the apoptotic effects of doxorubicin and melphalan, when pre-adhered to FN and compared with cells grown in suspension. Upon exposure to chemotherapeutic agents, myeloma cells expressing high levels of VLA-4 have survival advantage over those that express them at low levels. When the cells were removed from a chronic drug exposure, the VLA-4 expression decreased. However, there was no upregulation of common mediators of drug resistance like anti-apoptotic proteins and drug exporting glycoproteins in the cells. It was concluded that though the survival advantage offered by VLA-4 induced adhesion to fibronectin is less, it is significant in helping them survive the acute drug exposure and gives them adequate time to employ the classic mechanisms of drug resistance [254]. How adhesion of cells to fibronectin is rendering the cells resistance to chemotherapy, is still not completely understood. It was shown that adhesion of myeloma cells to fibronectin activates NF- $\kappa$ B and its regulated gene products, leading to drug resistance [255]. Moreover, it seems that IL-6 and fibronectin collaborate to stimulate STAT3 and fibronectin augments IL-6 induced STAT3 activation [256].

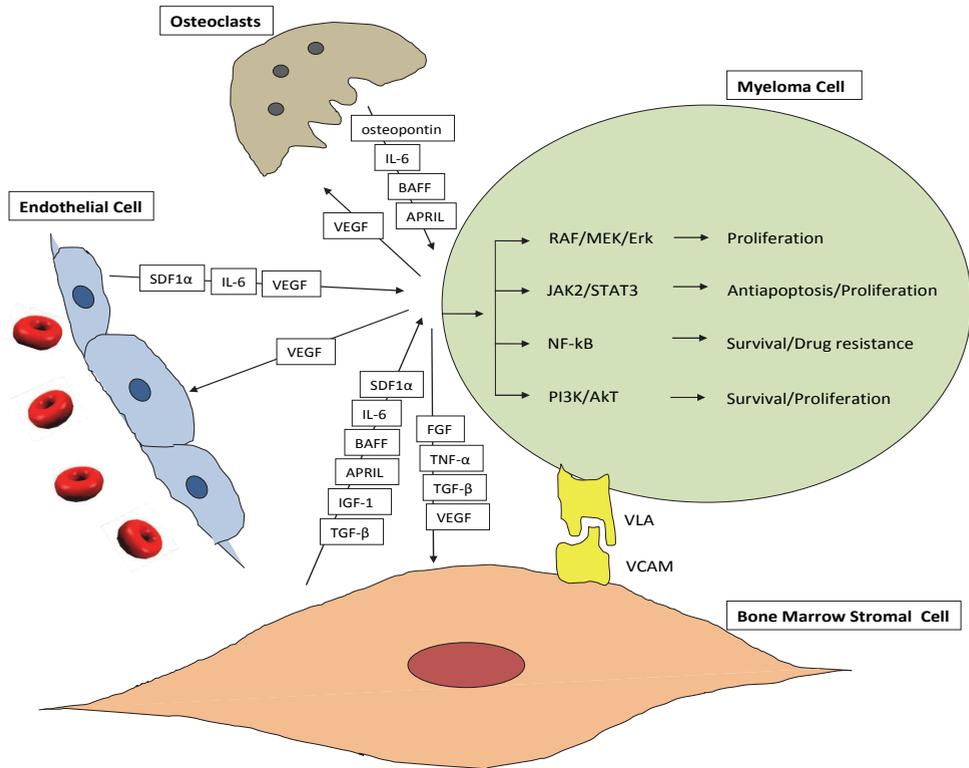


Fig. 1. Comprehensive representation of the role(s) of various inflammatory mediators in MM

	<i>Pharmacological/ Biological Blockers</i>	<i>Mechanism(s) of Action</i>	<i>References</i>
<b>IL-6</b>	1339	high-affinity fully humanized anti-IL-6 mAb	[257]
	6-amino-4-quinazoline Bortezomib	inhibits IL-6 signalling	[166]
	CHIR-12.12 (Human anti-CD40 antagonist antibody)	downregulates gp130 inhibits CD-40 enhanced IL-6 secretion	[258] [259]
	CNTO 328 (siltuximab)	IL-6 neutralizing monoclonal antibody	[260-262]
	ITF2357 histone deacetylase inhibitor	down-modulates the interleukin-6 receptor $\alpha$ (CD126)	[263]
	Novel indolinone BIBF 1000	abrogates stroma-derived IL-6 secretion	[264]
	Sant7	IL-6 receptor superantagonist	[265]

	<i>Pharmacological/ Biological Blockers</i>	<i>Mechanism(s) of Action</i>	<i>References</i>	
<b>TNF<math>\alpha</math></b>	Rituximab	monoclonal antibody	[266]	
	Thalidomide and its analogues	suppresses the expression of TNF $\alpha$	[267]	
<b>BAFF &amp; APRIL</b>	Ataccept	blocks the binding of BAFF and APRIL	[268]	
	CHIR-12.12 (Human anti-CD40 antagonist antibody)	inhibits CD-40 enhanced VEGF secretion	[259]	
<b>VEGF</b>	Bevacizumab	humanized murine anti--VEGF monoclonal antibody	[269]	
	PTK787/ZK222584, SU6668, SU5416	VEGF receptor tyrosine kinase inhibitors	[203, 270-272] [273]	
<b>IGF</b>	Sorafenib	dual Raf kinase/VEGF-R inhibitor		
	$\alpha$ -IR3	neutralizing monoclonal antibody to IGF-1R	[274]	
	JB-1	IGF-1 like competitive peptide antagonist	[275, 276]	
<b>TGF-<math>\beta</math></b>	NVP-ADW742	IGF-1R tyrosine kinase inhibitor	[277]	
	SD-208	TGF- $\beta$ receptor I kinase inhibitor	[278]	
<b>CXCL12</b>	4F-benzoyl-TN14003	CXCR4 antagonist	[279]	
	AMD3100			
	Thalidomide	CXCR4 inhibitor immunomodulator- downregulates CXCL12 and CXCR4	[267, 280] [281]	
<b>STAT3</b>	AR-42	downregulates gp130	[175]	
	Atiprimod	inhibits STAT3 activation	[143]	
	Auranofin	inhibits activation of JAK2	[162]	
	Avicin D	activates protein phosphatase-1	[282]	
	Azaspirane	inhibits STAT3	[283]	
	AZD1480	JAK-2 inhibitor	[174]	
	Baicalein	Inhibits IL-6-mediated phosphorylation of signalling proteins	[157]	
		Betulinic acid	inhibits the activation of Src kinase, JAK1 and JAK2	[165]
		Butein	inhibits the activation of Src kinase, JAK1 and JAK2; and upregulates SHP-1	[164]
		Cantharidin	inhibits phosphorylation of STAT3	[172]
		Capsaicin	inhibits the activation of Src kinase, JAK1	[146]
		Celastrol	inhibits JAK2 and Src kinase phosphorylation	[284]
		upregulates SHP-1		

<i>Pharmacological/ Biological Blockers</i>	<i>Mechanism(s) of Action</i>	<i>References</i>
Compound K	inhibits constitutive and IL-6-inducible	[167]
Curcumin	STAT3 phosphorylation inhibits activation of JAK2	[145]
Decursin	upregulates PTEN	[150]
Embelin	inhibitor of JAK-2	[149]
Emodin	inhibits the activation of c-src	[161]
Genipin	induces protein tyrosine phosphatase	[152]
Guggulsterone	SHP-1 inhibits the activation of c-src and	[172]
Icariside II	JAK-2 and upregulates the expression of SHP-1 and PTEN JAK1/2 selective inhibitor	[151]
INCB16562	Janus kinase inhibitor	[155]
INCB20	forced overexpression of SOCS	[285]
Infectivity-enhanced adenoviral vector of SOCS	inhibits Aurora kinase A, Aurora kinase B, and Janus kinase 2/3	[142]
Multitargeted kinase inhibitor, AT9283	reduces Jak kinase auto- phosphorylation	[173]
Nifuroxazide	induces the expression of the protein tyrosine phosphatase, SHP-1	[163]
Plumbagin	pan-Janus-activated kinase inhibitor	[286]
Pyridone 6	inhibits both constitutive and IL-6	[169]
Resveratrol	induced activation of STAT3 inhibits activation JAK2	[287]
TG101209	inhibits of c-Src and JAK2 activation	[171]
Thymoquinone	JAK2 tyrosine kinase inhibitor	[154]
Tyrphostin AG490	inhibits the activation of Src kinase,	[148]
Ursolic acid	JAK1 and JAK2, and upregulates SHP- 1	[168]
<b>NF-κB</b>		
Azacitidine	inhibits both NF-κB nuclear translocation and DNA binding	[288]
Azaspirane	inhibits IκBα NFκB- p65 phosphorylation TNF-α	[283]
Bay 11-7082	pharmacological NF-κB inhibitors	[289]
Celastrol	inhibits JAK2 and Src kinase phosphorylation	[284]
Curcumin	suppresses NF-κB activation	[183]
Genistein	suppresses constitutively active NF-κB IκB kinase β inhibitor	[290]
MLN120B	suppresses NF-κB activation	[291]
Parthenolide	suppresses constitutively active NF-κB	[292]
Resveratrol	through inhibition of IκBα kinase and	[287]

	<i>Pharmacological/ Biological Blockers</i>	<i>Mechanism(s) of Action</i>	<i>References</i>
		the phosphorylation of I $\kappa$ B $\alpha$ and of p65	
<b>MMPs</b>	Chitosan	a marine phospholipid that inhibits the activity of MMP-2 and MMP-9	[293]
	SST0001	a chemically modified heparin with antiheparanase activity	[294]
<b>Integrins</b>	Anti-alpha4 Ab	monoclonal antibody to alpha4 integrin	[295, 296]
	QLT0267	integrin-linked kinase inhibitor	[297,297]

Table 1. List of various pharmacological/biological agents modulating inflammatory mediators in MM

## 6. Conclusions

Understanding the various growth and survival pathways activated in both myeloma cells and various components of the bone marrow microenvironment is of paramount importance, not only to the basic understanding of the biology of MM, but also to effectively produce efficacious and safer anti-myeloma agents. In essence, myeloma is initiated by the primary genetic abnormalities and supported by the bone marrow microenvironment induced growth and survival. The secondary genetic mutations and epigenetic abnormalities emancipate myeloma cells of their dependence on the bone marrow microenvironment, which is when they progress to extramedullary MM. There are multiple signalling pathways activated, which serve overlapping functions. Combined inhibition of multiple signalling pathways offers better effects.

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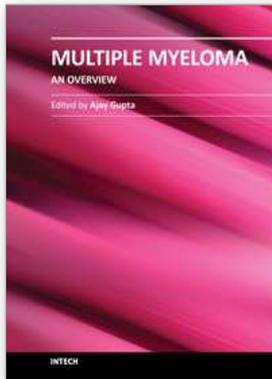
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Multiple myeloma is a malignant disorder characterized by the proliferation of plasma cells. Much insight has been gained into the molecular pathways that lead to myeloma and indeed much more remains to be done. The understanding of these pathways is closely linked to their therapeutic implications and is stressed upon in the initial chapters. Recently, the introduction of newer agents such as bortezomib, lenalidomide, thalidomide, liposomal doxorubicin, etc. has led to a flurry of trials aimed at testing various combinations in order to improve survival. Higher response rates observed with these agents have led to their integration into induction therapies. The role of various new therapies vis a vis transplantation has also been examined. Recent advances in the management of plasmacytomas, renal dysfunction, dentistry as well as mobilization of stem cells in the context of myeloma have also found exclusive mention. Since brevity is the soul of wit our attempt has been to present before the reader a comprehensive yet brief text on this important subject.

### **How to reference**

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