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Review

Mechanism of immunomodulatory drugs' action in the treatment of multiple myeloma

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Although immunomodulatory drugs (IMiDs), such as thalidomide, lenalidomide, and pomalidomide, are widely used in the treatment of multiple myeloma (MM), the molecular mechanism of IMiDs' action is largely unknown. In this review, we will summarize recent advances in the application of IMiDs in MM cancer treatment as well as their effects on immunomodulatory activities, anti-angiogenic activities, intervention of cell surface adhesion molecules between myeloma cells and bone marrow stromal cells, anti-inflammatory activities, anti-proliferation, pro-apoptotic effects, cell cycle arrest, and inhibition of cell migration and metastasis. In addition, the potential IMiDs' target protein, IMiDs' target protein's functional role, and the potential molecular mechanisms of IMiDs resistance will be discussed. We wish, by presentation of our naive discussion, that this review article will facilitate further investigation in these fields.

Keywords immunomodulatory drugs; multiple myeloma; cancer treatment; cereblon; E3 ubiquitin ligase

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Introduction

Multiple myeloma (MM) is a cancer of plasma cells originating in bone marrow (BM). Plasma cells are normally responsible for the production of antibodies [1]. In MM, accumulation of the abnormal plasma cells in bones results in bone lesions whereas accumulation in BM interferes with the production of normal blood cells, such as MM-associated anemia. In addition, MM, in most cases, features the production of a paraprotein, i.e. an ineffective abnormal monoclonal antibody from the clonal plasma cells that can cause kidney problems and interfere with the production of normal antibodies that lead to immunodeficiency [1]. Furthermore, common problems with MM include bone pain, radicular pain, weakness, confusion, fatigue, headache, visual changes, retinopathy, loss of bowel control or loss of bladder control, carpal tunnel syndrome, and other neuropathies.

MM is generally thought to be incurable, but remissions might be induced with steroids, chemotherapy, and stem cell transplants. In fact, the treatment of MM has a long history. The first reported attempt, including rhubarb pill and infusion of orange peel, was published in 1844 [2]. Then, phlebotomy was used as a maintenance therapy for MM [3] and urethane was used to decrease the number of myeloma cells [4]. Prednisone, which was isolated in 1950 and commercially synthesized in 1955, is a synthetic corticosteroid drug that is quite effective as an immunosuppressant drug and widely used to treat many different diseases including MM [5–7]. Dexamethasone (DEX) is another synthetic corticosteroid drug that is 27 fold more potent than the naturally occurring hormone cortisol and six times more potent than prednisone. DEX is used as a direct chemotherapeutic agent in certain hematological malignancies, especially in the treatment of MM, in which DEX is either given alone or in combination with other chemotherapeutic drugs [8–11]. The development of alkylating agent melphalan provides another chemotherapeutic agent to treat MM [12] and the combination of melphalan with prednisone yielded better outcomes than melphalan alone [13]. Bortezomib, a proteasome inhibitor that specifically inhibits the threonine proteases of the 20S proteasome subunit [14–16], was synthesized in 1995 and approved, due to the promising results derived from the study of uncontrolled myeloma managed with proteasome inhibition therapy (SUMMIT) [17], in the United States by the Food and Drug Administration (FDA) for use in the treatment of MM.

The anti-angiogenic activity of thalidomide found in a rabbit cornea micropocket assay prompted investigation of thalidomide as an anti-cancer drug [18]. Based on this finding, Singhal *et al*. [19] evaluated the efficacy of thalidomide in MM patients with refractory disease and found that thalidomide can induce marked and durable responses in some patients with MM, including those patients who relapse after high-dose chemotherapy. Thalidomide, based on its promising effects, was approved by FDA in 2006 in combination with DEX for the treatment of newly diagnosed MM. Due to adverse side-effects of thalidomide, such as dose-limiting toxicities including somnolence, constipation,

neuropathy, and increased incidence of venothromboembolism, more potent and safer analogs, such as lenalidomide and pomalidomide, of thalidomide were developed. Lenalidomide, approved by FDA in 2006, and pomalidomide, approved by FDA in February 2013, are a series of synthetic compounds derived by modifying the chemical structure of thalidomide and have been found that both of them are more potent and safer than thalidomide.

In order to further improve the outcomes of the aforementioned drugs, multiple drug combinations, such as bortezomib+DEX [8–10]; bortezomib+DEX+thalidomide [11]; lenalidomide+DEX [20]; melphalan+prednisone+bortezomib [5,6]; melphalan+prednisone+thalidomide [7], were actively investigated. In this review, we will focus on the molecular mechanism of IMiDs' action in the treatment of MM.

Development of IMiDs

IMiDs, including thalidomide, lenalidomide, and pomalidomide at the moment, are a group of compounds consisting of two portions: phthalimide and glutarimide in which only the phthalimide portion was modified (**Fig. 1**). The first IMiD, i.e. thalidomide, was synthesized by the German pharmaceutical company Chemie Grunenthal in early 1950 and received patent approval in 1954. Thalidomide was prescribed in the 1950s to pregnant women as a treatment for their morning sickness. However, treatment with this sedative drug caused birth defects [21–23]. Due to this infamous teratogenic effect, thalidomide was withdrawn from the market in 1961.

After being removed from the pharmaceutical market, thalidomide has become the subject of the research in many fields, such as the treatment of the patients infected with human immunodeficiency virus [24] and the patients with an autoimmune skin disease actinic prurigo [25]. In 1964, thalidomide was used to treat a patient critically ill with leprosy. This treatment resulted in the discovery of its anti-inflammatory properties in the treatment of the patients with erythema nodosum leprosum (ENL), a complication of leprosy [26]. Subsequently, many years research and practice in this field resulted in the FDA's approval of using thalidomide to treat patients with ENL in 1998.

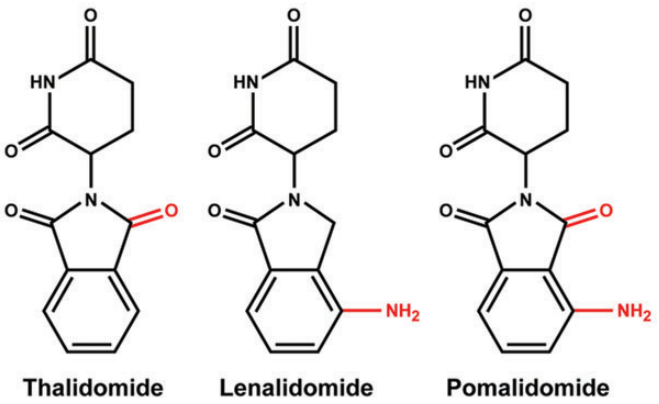

Figure 1. Diagram of immunomodulatory drugs including thalidomide, lenalidomide, and pomalidomide Black shows the common structure whereas red shows the unique carboxyl group or amino group in each of these compounds.

The use of thalidomide did not stop at the treatment of patients with ENL. Further investigation found that thalidomide possesses anti-angiogenic properties [18]. This finding triggered further investigation in the field of cancer treatment. Indeed, the research of thalidomide's effects on relapsed and refractory MM resulted in the discovery of its strong anti-cancer activity [19], leading to the FDA's approval of using thalidomide to treat the newly diagnostic MM patients.

Although thalidomide possesses strong anti-cancer activity, its adverse side-effects, such as teratogenesis, dose-limiting toxicities including somnolence, constipation, neuropathy, and increased incidence of venothromboembolism, cannot be ignored. In order to search for more potent and safer anti-cancer agents, a formal medicinal chemistry program was initiated by the Celgene Corporation. Basically, the structure of thalidomide was moderately modified to yield lenalidomide and pomalidomide (**Fig. 1**). Interestingly, these slightly modified compounds (thalidomide analogous), such as lenalidomide, are not only up to 50,000 fold more potent than thalidomide in terms of tumor necrosis factor α (TNFα) inhibition, but also much more potent than thalidomide in their ability to co-stimulate T-cells [27,28]. In addition, their adverse side-effects are much less severe than thalidomide [29,30]. Based on these criteria, lenalidomide was approved by FDA in 2006 whereas pomalidomide, in February 2013, for their use in the treatment of patients with MM.

Effects of IMiDs in the Treatment of MM

Immunomodulatory activities of IMiDs

IMiDs, such as thalidomide, lenalidomide, or pomalidomide, have a strong capacity to boost immune responses, therefore, being referred to as immunomodulatory drugs. It has been reported that *in vitro* exposure of stem cells to IMiDs resulted in the generation and activation of murine dendritic cells (DCs) [31]. DCs are cells that form part of the mammalian immune system. Immature DCs constantly sample the surrounding environment for pathogens, such as viruses or bacteria, performed through pattern recognition receptors, such as the toll-like receptors. Once the immature DCs phagocytose pathogens, these cells will degrade their proteins into small pieces and send them to their cell surface by using major histocompatibility complex molecules. During this activation process, these DCs up-regulate cell

surface receptors that act as co-receptors, such as cluster of differentiation 80 (CD80), CD86, and CD40, in T-cell activation and also up-regulate chemokine receptor 7 that induces the DC to travel through the blood stream to the spleen or through the lymphatic system to a lymph node. In this process, they act as antigen-presenting cells and activate helper T-cells and killer T-cells as well as B-cells by presenting them with antigens derived from the pathogen, alongside non-antigen-specific co-stimulatory signals. Recent observations suggest that pomalidomide and lenalidomide enhance tumor antigen uptake by DCs with an increased efficacy of antigen presentation [32] and potentiate the immune response by restoring DC function and inhibiting T-cell regulatory activity, leading to the activation of T lymphocytes and natural killer T (NKT) cells by increasing the production of interleukin-2 (IL-2) and interferon gamma (IFN-γ) [33].

It has been reported that thalidomide is a potent co-stimulator of primary human T-cells, synergizing with stimulation via the T-cell receptor complex to increase IL-2-mediated T-cell proliferation and IFN-γ production [34]. Thalidomide and thalidomide analogous co-stimulating effects and induction of IL-2 and IFN-γ production were further confirmed [35–40]. Secretion of IL-2 and IFN-γ increases the number of natural killer (NK) cells, improves their function, and mediates lysis of MM cells. Further investigation indicated that IMiDs-induced augmentation of IL-2 production is mediated by the increase of activator protein 1 (AP-1) transcriptional activity [37–39]. AP-1 is a transcription factor that forms heterodimers with proteins belonging to the c-fos, c-Jun, ATF, and JDP families and regulates gene expression in response to a variety of stimuli, including cytokines, growth factors, stress, and bacterial or viral infections [41]. Suppressor of cytokine signaling 1 (SOCS1) is a member of the signal transduction and transcription (STAT)-induced STAT inhibitor family that functions downstream of cytokine receptors and takes part in a negative feedback loop to attenuate cytokine signaling. Interestingly, the treatment of MM cells with IMiDs down-regulated SOCS1 expression, demonstrating that modulation of SOCS1 may enhance immune response and efficacy of IMiDs in MM [42]. Cytotoxic T-cell antigen 4-immunoglobulin (CTLA-4-Ig) is a protein receptor that inhibits T-cell proliferation, via blocking the B7-CD28 co-stimulation pathway. Interestingly, IMiDs partially overcome the inhibitory effects of CTLA-4-Ig on T-cell proliferation and Epstein–Barr virus or influenza virus triggered IFN-γ secretion [40]. In addition, IMiDs triggered tyrosine phosphorylation of CD28 on T-cells and followed by activation of nuclear factor kappa B (NF-κB) [40]. Furthermore, IMiDs facilitated the nuclear translocation of nuclear factor of activated T cell-2 (NFAT2) and AP-1 via activation of phosphoinositide-3-kinase (PI3K) signaling, resulted in IL-2 secretion and T-cell proliferation [38]. Taking together, these data support the notion that IMiDs may mediate their anti-MM effect, at least in part, by modulating NK cell number and function.

NKT cells are a heterogeneous group of T-cells that recognize lipids and glycolipids presented by CD1d molecules. NKT cells, upon activation, produce large amounts of IFN-γ, IL-4, IL-2, IL-13, IL-17, IL-21, TNF-α, and granulocyte-macrophage colony-stimulating factor. Interestingly, lenalidomide enhances antigen-specific expansion of NKT cells in response to the NKT ligand α-galactosylceramide in both healthy donors and patients with MM [43]. NKT cells, activated in the presence of lenalidomide, have greater ability to secrete IFN-γ. Antigen-dependent activation of NKT cells was greater in the presence of DEX plus lenalidomide than with DEX alone. Therapy with IMiDs also led to an increase in NKT cells *in vivo* in patients with MM and del5q myelodysplastic syndrome [43]. Taking together, these data support the notion that IMiDs may mediate their anti-MM effect by modulating NKT cells.

Regulatory T-cells (Tregs) are a component of the immune system that suppresses immune responses of other cells. In other words, accumulation of Tregs will suppress immune responses whereas decreased Tregs will argument immune responses. Therefore, Tregs play an important role in 'self-check' built into the immune system. Tregs were elevated in patients with MM, leading to suppress the function of naive T-cells [44]. Regulatory function for Tregs is provided by the expression of the forkhead family transcription factor forkhead box p3 (FOXP3). It has been shown that lenalidomide and pomalidomide strongly inhibit Tregs proliferation via decreased FOXP3 mRNA expression [45]. Therefore, IMiDs may be ideal anti-cancer drugs showing features including marked immune stimulatory properties as well as being able to inhibit Tregs. Nevertheless, contradictory results have been observed, i.e. the treatment of the newly diagnosed (untreated) MM patients with IMiDs increased the number of Tregs cells [46,47]. Therefore, further investigation is needed to solve these controversial results.

Anti-angiogenic activities of IMiDs

Angiogenesis is a process of generating new blood vessels. In many cancers, this process can nurture tumor cells and increase the growth and metastasis of tumors. In MM, the interaction between the indigenous bone marrow stromal cells (BMSCs) and MM cells significantly increased the levels of pro-angiogenic cytokines, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) [35,48–54]. VEGF and bFGF are growth factors that, once activated by binding to their receptors, mediate the formation of new blood vessels. In response to these factors stimulation, BMSCs and microvascular endothelial cells produce IL-6 that stimulates the growth of malignant plasma cells. It has been found that IMiDs decreased the expression of VEGF and bFGF [50], thereby inhibiting

new blood vessel formation and decreasing the tumor growth. Indeed, microvessel growth in the IMiDs treated samples was significantly less than in the control [31,55–59]. From this point of view, the inhibition of tumor growth by anti-angiogenic properties of IMiDs is independent of their immunomodulatory effects [18,55,56].

Effects of IMiDs on the interaction between MM cells and BMSCs

MM is a cancer of malignant plasma cells residing in BM microenvironment by adhering to extracellular matrix (ECM) proteins and the proteins on BMSCs. The proteins involved in the interactions between MM cell and ECM or between MM cell and BMSCs include CD44, very late antigen 4 (VLA-4), VLA-5, leukocyte function-associated antigen 1 (LFA-1, CD11a), neural cell adhesion molecule (NCAM, CD56), intercellular adhesion molecule 1 (ICAM-1, CD54), vascular cell adhesion molecule 1 (VCAM-1, CD106), syndecan (CD138), and monocyte chemoattractant protein 1 [60].

The initial homing of MM cells to the BM milieu is mediated by binding of the stromal-derived growth factor (SDF-1α) in BM to its receptor C-X-C chemokine receptor type 4 (CXCR-4, CD184) located on MM cells. The interaction between MM cells and BMSCs promotes MM cell survival via cell–cell contact and cytokines. The interaction between MM cells and BMSCs leads to increased production of IL-6, a myeloma cell growth and survival factor, and other growth factors [61,62]. In addition, SDF-1α also modulates the expression of cell surface adhesion molecules VLA-4, LFA-1, VCAM-1, and ICAM-1 that favors the adhesion between MM cells and BMSCs. Furthermore, adhesion of MM cells to BMSCs enhanced NF-κB activity that further up-regulates IL-6 and VEGF [50,62,63]. TNFα, secreted by MM cells, enhances the expression and secretion of IL-6 from BMSCs [64]. TNFα also activates NF-κB and induces the expression of LFA1, ICAM-1, VCAM-1, VLA-4, and MUC-1 on MM cell lines as well as VCAM-1 and ICAM-1 on BMSCs [64].

It has been reported that IMiDs, such as thalidomide, in contrast to the co-stimulation effects in certain T lymphocytes [31,36,65–67], inhibited production of TNFα [28,68–79], suggesting that the treatment with IMiDs might decrease the production of IL-6 and the cell surface adhesion molecules between MM cells and BMSCs including LFA1, ICAM-1, VCAM-1, and VLA-4. Indeed, the expression of IL-6, upon treatment with IMiDs, was significantly decreased [28,50,80–102]. Furthermore, the expression of cell surface adhesion molecules, upon treatment with IMiDs, is also significantly decreased [103–113], meaning that IMiDs can inhibit the adhesion of MM cells to BMSCs and overcome cell surface adhesion-mediated drug resistance by down-regulating the expression of these adhesion molecules.

Anti-inflammatory effects of IMiDs

It has been found that IMiDs, such as thalidomide, inhibited the production of TNFα [28,68–79]. TNFα is a pro-inflammatory cytokine that affects a wide variety of cells to induce many similar inflammatory reactions, including fever, production of other cytokines, endothelial gene regulation, chemotaxis, leukocyte adherence, and activation of fibroblasts. In fact, IMiDs also inhibit the production of other pro-inflammatory cytokines, such as IL-1, IL-6, and IL-12, and increase the secretion of anti-inflammatory cytokines, such as IL-10 [28,37,114]. Furthermore, IMiDs are able to inhibit the expression of cyclooxygenase 2 (COX-2) [115], but not COX-1, in lipopolysaccharide-TNFα and IL-1β stimulated peripheral blood mononuclear cell (PBMC) [114]. COX-2 is an enzyme that catalyzes arachidonic acids into various pro-inflammatory prostaglandins (PGs). Thus, the decreased expression of COX-2 may lead to decreased production of PGs. Indeed, the treatment of PBMCs with IMiDs decreased the production of PGE2 [114]. Therefore, IMiDs possess significant anti-inflammatory effects.

Anti-proliferation effects of IMiDs

[^{3}H]-thymidine uptake by human MM cell lines or cells derived from MM patients was significantly decreased upon treatment with thalidomide or its analog [51], suggesting that the cell proliferation might be inhibited by IMiDs or the cells might be killed by IMiDs. In considering the fact that: (i) IMiDs inhibit the production of TNFα [28,68–79], a factor that may not directly induce growth of neoplastic cells, but binds to a TNFα response element of the IL-6 promoter in BMSCs and induces expression of IL-6 [28,50,64,80–102], a growth factor for the proliferation of myeloma cells [116]; (ii) IMiDs inhibit the activity of NF-κB [101,102,110,117–137], a factor that is retained in the cytoplasm with IκBα as an inactive form and is activated by a wide variety of stimuli including stress, cytokines, free radicals, ultraviolet irradiation, and bacterial or viral antigens and followed by its translocation to the nucleus where it functions as transcription factor; (iii) IMiDs inhibit the activity of PI3K/Akt pathways [59,88,131,138–144] that plays a key role in multiple cellular processes including cell proliferation; it is most likely that the IMiDs possess anti-proliferation effects in MM cells. In addition, the treatment of MM cells with IMiDs down-regulated CCAAT/enhancer-binding protein β (C/EBPβ), resulting in abrogation of cell proliferation [145]. In fact, IMiDs did not alter C/EBPβ mRNA levels or protein stability, but blocked C/EBPβ translation through interfering eukaryotic translation initiation factor 4E (eIF4E) [145]. IMiD-induced decrease of C/EBPβ protein resulted in decreased production of interferon regulatory factor 4 (IRF4) [145], a transcription factor that is critical for MM cell growth and survival [146]. IMiD-mediated

down-regulation of IRF4 was also observed by other investigators [147–151].

Pro-apoptosis effects of IMiDs

Apoptosis is triggered by either extrinsic signals, including toxins, hormones, growth factors, nitric oxide, or cytokines, or intrinsic signals such as radiation- or hypoxia-caused damage or increased intracellular calcium concentration. Multiple factors are involved in the apoptosis process. For example, pro-apoptotic factors, such as Bcl-2 antagonist of cell death (BAD), Bcl-2 associated X protein (BAX), and Bcl-2 antagonist killer 1 (BAK), can form a pore on mitochondria so that small mitochondria-derived activator of caspases and/or cytochrome c can be released from mitochondria to cytosol where they activate caspases that mediate apoptosis, whereas anti-apoptotic factors, such as B-cell lymphoma protein 2 (Bcl-2), Bcl-2 related protein, long isoform (Bcl-xL), and/or myeloid cell leukemia 1 (Mcl-1) inhibit the pore formation. Protein kinase B (or Akt) phosphorylates pro-apoptotic BAD protein on Ser136 that leads to BAD dissociation from the Bcl-2/Bcl-xL complex, resulted in preventing initiation of apoptotic process. Therefore, decreased Akt activity by IMiD will play a pro-apoptotic role. Akt can also activate NF-κB via regulating IκB kinase. Once NF-κB is activated, it enhances the expression of many genes involved in cell survival, such as inhibitor of apoptosis protein (IAP) [119,152] or cellular FLICE-like inhibitory protein (cFLIP) [119,152]. In addition, IL-6 enhanced the expression of anti-apoptotic factors Bcl-xL [153] and Mcl-1 [154,155]. Thus, the decreased NF-κB activity or the decreased expression of IL-6 by IMiD will also play a pro-apoptotic role. Furthermore, the fact that IMiDs triggered activation of caspase 3 [88,138,156–167], caspase 8 [119,158,162–164,167,168], caspase 9 [138,158,159,162–165,168], and caspase 12 [163], the increased expression of pro-apoptotic factors BAX and BAK [169] and the decreased expression of anti-apoptotic factors Bcl-2 [169], cFLIP, and Bcl-xL [170] indicates that IMiDs possess significant pro-apoptotic effects.

Cell cycle arrest effects of IMiDs

It has been reported that IMiDs up-regulated the expression of cyclin-dependent kinase (CDK) inhibitor 1 (CIP or p21/waf1) [149,164,171,172]. P21/waf1 is a key cell cycle regulator that modulates the activities of CDKs and reduces the phosphorylation of retinoblastoma proteins, thereby, causing cell cycle arrest at the G0/G1 phase. In addition, IMiD-mediated growth inhibition has been found to be associated with the induction of CDK inhibitors p15, p16, and p27 and tumor suppresser genes, such as early growth response protein 1 (Egr1), Egr2, and Egr3 [164]. The increased expression of p21/waf1 has been proved to be associated with a switch from methylated to acetylated histone H3 on p21/waf1 promoter region [172]. Although the mechanism of IMiD-mediated switch from methylated to acetylated histone H3 is unknown, the up-regulation of p21/waf1 correlated well with the inhibition of CDK2, CDK4, and CDK6 activities [171]. The inhibition of these CDKs resulted in cell cycle arrest [171] and this IMiD-mediated cell cycle arrest has also been found in a wide variety of cancer cells [51,142,147,160,165,169,173–181].

IMiDs' effects on cell migration and metastasis

Metastasis is a complex process involving cell dispersion from the primary site, migration, adhesion, and growth in the new sites (organs). It has been reported that the treatment with IMiDs, such as thalidomide or its analogs, inhibits or attenuates cancer metastasis process in animal models [109,124,141,182–186]. The detailed mechanisms for metastasis inhibition remain unclear. However, the effects of IMiDs on the factors involved in cancer cell migration and metastasis may provide a clue. Matrix metalloproteinases (MMPs) are a group of zinc-dependent endopeptidases that are thought to play a major role on cell behaviors including cell proliferation, dispersion, migration, adhesion, differentiation, angiogenesis, apoptosis, and host defense. Focal degradation of ECM, catalyzed by MMPs, is the first step in the invasion of cancer cells. It has been reported that the treatment with IMiDs, such as thalidomide or its analog, decreased the production of MMPs [130,167,187–190], thereby inhibiting the degradation of ECM. NFAT is a transcription factor that is implicated in the process of cell motility at the basis of metastasis formation. One such example is that NFAT3 functions as an inhibitor of cell motility [191]. IMiDs activate NFAT transcriptional pathways [125], thereby inhibiting cancer cell migration. In addition, the treatment with IMiDs decreased the expression of integrin subunits and/or integrin receptors [192–196]. Since integrins are crucial for cell–matrix interactions and mediate cell adhesion to endothelium, decreased the expression of integrins, upon treatment with IMiD, will result in inhibition of cell migration. Other cell adhesion molecules, such as ICAM, VCAM, NCAM, LFA, or VLA, which play an important role in the interactions between cancer cells and stromal cells, also contribute to cancer cell migration and metastasis process. Thereby, upon treatment with IMiDs, the altered expression of the cell adhesion molecules [103–106,108–113,193] will affect the cancer metastasis processes.

IMiDs' Target Protein in MM Cells

As mentioned in the previous section, the expression of many genes is altered upon treatment with IMiDs. We have found that 1036 genes were down-regulated whereas 1236 genes were up-regulated in MM cells upon treatment with lenalidomide [148]. Although the treatment with IMiDs

affected so many genes, the molecular mechanism of the IMiD-mediated gene regulation in MM cells is not well elucidated.

By using thalidomide-conjugated ferrite-glycidyl methacrylate (FG) beads, Ito *et al*. [197] pulled down cereblon (CRBN) and damaged DNA-binding protein 1 (DDB1). They had also found that the development of pectoral fins and otic vesicles in thalidomide-treated zebrafish embryos was disturbed. The embryos injected with an anti-sense oligonucleotide against zebrafish Crbn yielded specific defects in fin and otic vesicle development, which is similar to those of the thalidomide-treated embryos. These defects were rescued by co-injection of zebrafish Crbn mRNA [197]. In addition, thalidomide treatment of zebrafish embryos over-expressing Y374A/W376A-mutated zebrafish Crbn, which prevents thalidomide binding, did not significantly affect otic vesicle size [197]. Thus, CRBN was considered as a direct target protein for thalidomide teratogenicity [197–200].

IMiDs, such as thalidomide, lenalidomide, or pomalidomide, are profoundly active in the treatment of MM and related diseases. What is the direct target protein of IMiDs in the treatment of these diseases? We have found that wild-type CRBN expression is required for the anti-myeloma activity of IMiDs [148]. By using thalidomide-conjugated FG beads, Lopez-Girona *et al*. [149] could pull down CRBN and DDB1 from U266 myeloma cell extracts and pre-incubation of the U266 cell extracts with either lenalidomide or pomalidomide completely blocked the pull-down. Thus, although whether CRBN is the sole IMiDs target protein remains unknown, CRBN is definitely an IMiDs' direct target protein in the treatment of patients with MM.

Functional Role of CRBN

CRBN and DDB1 were pulled down by using thalidomide-conjugated FG beads [149,197], indicating that CRBN directly binds DDB1 protein. The DDB1, CUL4, and really interesting new gene (RING) or ring box 1 (RBX1) or regulator of cullin 1 (ROC1) complex is an identified cullin-RING E3 ubiquitin ligase that regulates virtually all of the aspects of cellular function, such as DNA repair [201–207], DNA replication [208–211], and transcription [212]. Although DDB1 in this complex might directly recruit substrates to the E3 ubiquitin ligase, ubiquitination of several known substrates suggested that this ubiquitination process requires additional cellular factors [204,213]. In searching for additional cellular factors that might participate in ubiquitination in E3 ubiquitin ligase complex, CRBN, along with many other proteins, was identified as a potential factor or substrate receptor contributing to ubiquitination of cellular proteins and named as DDB1-CUL4-associated factor (DCAF) [214,215].

Although DCAF proteins were suggested to be factors serving as the substrate-recruiting modules or substrate receptors for the E3 ubiquitin ligase machinery [214–216], the functional role of CRBN in this complex is still unknown. Given the fact that CRBN also binds to a large-conductance Ca^{++}-activated potassium channel (BK_{Ca}) α-subunit [217,218], a voltage-gated chloride channel-2 (ClC-2) [219], and an α1 subunit of AMP-activated protein kinase (AMPK) [220], it is possible that CRBN might function as a substrate receptor to bind to these proteins for ubiquitination by the E3 ubiquitin ligase machinery, leading to proteasome-mediated degradation.

However, even if it is the case that binding of BK_{Ca}, ClC-2, and AMPK to CRBN leads to ubiquitination and proteasome-mediated degradation, it is still not clear whether CRBN will directly bind to all those factors mentioned in the previous section or not. In other words, regardless of whether there is/are mono-target or multiple targets for IMiDs, the question of how IMiDs regulate so many genes remains unsolved. However, the results derived from IRF4, a transcription factor that is critical for MM cell growth and survival [146], may give us a clue. The treatment with IMiDs down-regulate the expression of IRF4 [147–151], perhaps via IMiD-induced decrease of C/EBPβ protein [145], meaning that CRBN may not directly bind to IRF4. It has been reported that knockdown of IRF4 with IRF4 small hairpin RNA (shRNA) altered the expression of a set of genes that were consistently down-regulated (435 genes) or up-regulated (410 genes) [221]. Thus, it is possible that some of the CRBN direct downstream substrates (**Fig. 2**) could be the factors associated with transcription (activation or suppression), RNA splicing, and/or translation and degradation of these factors, via ubiquitination by E3 ubiquitin ligase and proteasome-mediated degradation, might alter the expression of multiple sets of genes.

IMiDs Resistance in MM Cells

Although treatment with IMiDs has dramatically improved the survival for MM patients, majority of the MM patients treated with IMiDs develop resistance over time by mechanisms that remain unknown [222]. Fortunately, recent work has gradually uncovered the mechanism of the action of IMiDs. As discussed in the previous section, CRBN has been considered as one of the IMiDs' direct target proteins. We have found that the expression of IMiDs' target protein CRBN is required for their anti-myeloma activity [148]. It has also been reported that high expression of CRBN is associated with improved clinical response in patients with MM treated with IMiDs [223,224], further confirming that the expression of IMiDs' direct target protein CRBN is required for their anti-myeloma activity. Notably, CRBN shRNAs decreased the CRBN expression and conferred them

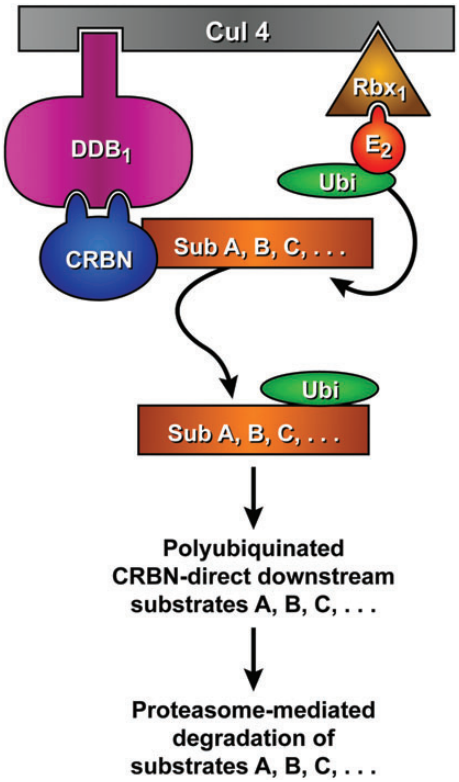

Figure 2. Diagram of E3 ubiquitin ligase complex-mediated degradation of CRBN direct downstream substrates CRBN recognizes its direct downstream substrates and recruit them for E3 ubiquitin ligase-mediated ubiquitination. These mono-ubiquitinated substrates will be further poly-ubiquitinated, leading to proteasome-mediated degradation. Cul 4 represents Cullin 4A or Cullin 4B; Rbx1, ring box 1; E2, E2 ubiquitin-conjugating enzyme; Ubi, ubiquitin; DDB1, damaged DNA-binding protein 1; CRBN, cereblon; Sub A, B, C, CRBN direct downstream substrates A, B, or C.

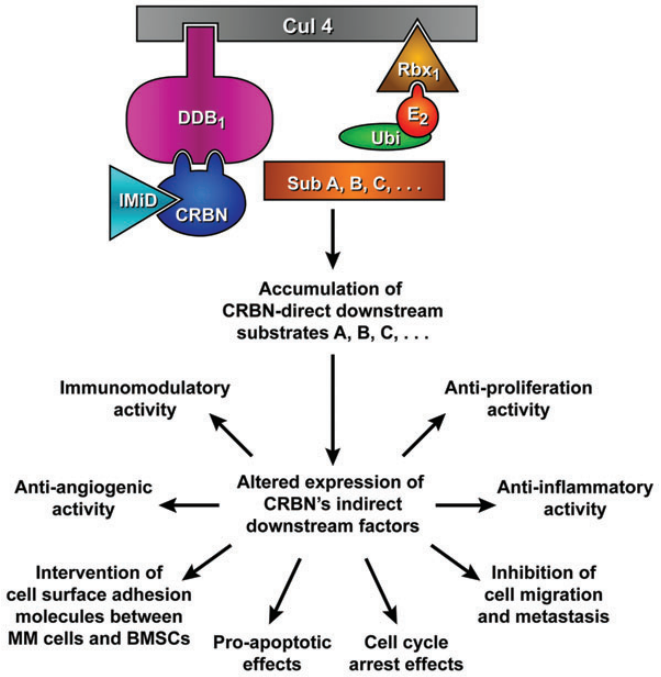

Figure 3. Diagram of immunomodulatory drug (IMiD) effects Upon IMiD binding, the binding of CRBN direct downstream substrates could be altered. If the binding of IMiD to CRBN decreases the binding of CRBN direct downstream substrates, it will prevent ubiquitination of these substrates, leading to accumulation of these CRBN direct downstream substrates. However, if the binding of IMiD to CRBN enhances the binding of CRBN direct downstream substrates (the diagram did not show this enhancement effect), it may facilitate ubiquitination of these substrates, leading to degradation of these CRBN direct downstream substrates. Altered steady state of the CRBN direct downstream substrates may affect the expression of CRBN indirect downstream factors that may elicit variant effects including immunomodulatory activity, anti-angiogenic activity, intervention of cell surface adhesion molecules, pro-apoptotic effect, cell cycle arrest, inhibition of cell migration and metastasis, anti-inflammatory activity and anti-proliferation activity, etc.

resistant to IMiDs [148,149]. However, these cell lines had similar sensitivity to melphalan, DEX, and bortezomib [148], demonstrating a requirement of CRBN for the activity of IMiDs, but not other commonly employed anti-myeloma therapeutics. In addition, a majority of the lenalidomide-resistant MM patients expressed significantly lower CRBN level than the IMiDs sensitive patients [148,223], suggesting that the dysregulation of IMiDs' direct target protein CRBN (**Fig. 3**) confers them resistant to the IMiDs. Furthermore, introducing wild-type CRBN back to the IMiD-resistant MM cells restored their sensitivity to IMiDs (manuscript in preparation), further confirming that the expression of IMiDs' target protein CRBN is required for their anti-myeloma activity.

Of note, CRBN shRNAs decreased CRBN expression and also myeloma cell viability [148]. Interestingly, however, once a CRBN-knocked down cell line is established, the growth rate of these cells is similar to their parental cells, implying that the expression of CRBN direct downstream substrates and/or indirect downstream factors might be altered. This hypothesis is supported by the finding that some of the MM patients have high levels of CRBN, but resistant to IMiDs treatment [148], suggesting that the dysregulation of CRBN's indirect downstream factors (**Fig. 3**) might confer them resistant to IMiDs.

In fact, it has been reported that the over-expression of IRF4, a CRBN indirect downstream factor, confers the activated B-cell-like diffuse large B-cell lymphoma cells resistant to IMiDs [151,221]. We have also found that the over-expression of CRBN indirect downstream factors, such as IRF4 or Myc, in IMiD-sensitive MM cells reduced their sensitivity to IMiDs (manuscript in preparation). Over-expression of C/EBPβ, a transcription factor that is down-regulated by IMiDs, rescued MM cells from IMiD-induced inhibition of proliferation [145]. The treatment of MM cells with lenalidomide significantly increased the expression of β-catenin [225], suggesting that β-catenin might be an IMiDs' target protein's indirect downstream factor. Although the mechanism of lenalidomide mediated up-regulation of β-catenin remains unsolved, the over-expression of β-catenin conferred

them resistant to IMiDs [225]. Furthermore, consequence of the enhanced β-catenin expression resulted in the over-expression of hyaluronan-binding protein CD44 [226] that conferred cell adhesion-mediated drug resistance.

All the data mentioned above support the notion that the dysregulation of IMiDs' direct target protein, such as CRBN, CRBN direct downstream substrates and/or CRBN indirect downstream factors might affect the sensitivity to IMiDs. Interestingly, down-regulation of CUL4A, a CRBN upstream factor that plays a scaffold role in the E3 ubiquitin ligase complex [214], conferred resistance to thalidomide, whereas ectopic CUL4A expression greatly enhanced the sensitivity to this drug [227], implying that the dysregulation of CRBN upstream factors, such as DDB1 and/or CUL4, might also affect the sensitivity to IMiDs.

Clarifying the molecular mechanisms of IMiDs' resistance might provide a possibility to overcome the corresponding IMiD resistance. As such, Bjorklund *et al*. [226] tested this possibility and found that blockade of CD44 with monoclonal antibodies, free hyaluronan, or CD44 knockdown reduced adhesion and sensitized them to lenalidomide. In addition, Wnt/β-catenin suppression by FH535, a reversible dual inhibitor of Wnt/β-catenin, enhanced the activity of lenalidomide [226]. Furthermore, all-trans-retinoic acid down-regulated β-catenin and CD44, reduced adhesion of lenalidomide-resistant myeloma cells, and enhanced the activity of lenalidomide in a lenalidomide-resistant murine xenograft model [226].

Concluding Remarks

The introduction of IMiDs, especially in combining with other anti-cancer drugs, into the MM treatment regimens dramatically improved the outcome of the patients with MM. Unfortunately, majority of the patients treated with IMiDs will eventually develop resistance to these drugs. Therefore, developing a novel therapeutic approach to overcome this drug resistance is urgently needed. Recent work [226] indicates that it is possible to overcome, by blockade of CRBN function, CRBN direct downstream substrates, and/or CRBN indirect downstream factors, the acquired IMiD resistance. Therefore, future dissection of CRBN direct downstream substrates and CRBN indirect downstream factors will help to delineate the underlying mechanisms of IMiD action and identify new biomarkers for prediction of IMiD response/resistance as well as developing a novel therapeutic approach to treat the patients with MM.

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