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Cereblon and Its Role in the Treatment of Multiple Myeloma by Lenalidomide or Pomalidomide

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Abstract Cereblon (CRBN) is part of the cullin 4-containing E3 ubiquitin ligase complex ($CRL4^{CRBN}$) and functions as a target of thalidomide and its analogs (lenalidomide and pomalidomide) known as immunomodulatory drugs (IMiDs). The *CRBN* gene consists of 1329 base pairs, 11 exons, and encodes a protein of 443 amino acids. Exons 10-11 code for the binding site of IMiDs and exons 5-7 for the binding site of DNA damage binding protein 1 (DDB1). CRBN consists of three sub-domains, the amino-terminal domain, the helical bundle domain involved in DDB1 binding and the carboxy-terminal domain harbouring IMiD– binding hydrophobic pocket CRBN in the absence of IMiDs binds to its endogenous substrate proteinsand it leads to ubiquitination of these substrates by the $CRL4^{CRBN}$ and their degradation by proteasomes. However, in the presence of IMiDs, CRBN binds new substrate proteins, transcription factors IKZF1 (IKAROS) and IKZF3 (AILOS), for drug-induced ubiquitination by the $CRL4^{CRBN}$ and next degradation in proteasomes. The administration of IMiDs alters the specificity of the $CRL4^{CRBN}$ and affects simultaneously the levels of two groups of substrate proteins. IMiDs upregulate the levels of endogenous substrates (MEIS2 and CRBN) and decrease the amounts of new substrates (IKAROS family proteins). In the meantime we do not know all possible substrates of the $CRL4^{CRBN}$ because it depends on the cell type and proteins expressed. IMiDs have the anti-proliferative, anti-angiogenic and immunomodulatory activities and are efficient in several hematological malignancies as multiple myeloma, chronic lymphocytic leukemia, mantle lymphoma and isolated del (5q) myelodysplastic syndrome. Targeted knockdown of IKAROS and AILOS causes the decrease of myeloma survival factor IRF4 (interferon regulatory factor 4) and c-myc with the decrease in cell viability in multiple myeloma cells and the increase of interleukin-2 in T-cells and their co-stimulation, both similar to that after IMiDs treatment.

Keywords: *cereblon, cullin 4-containing E3 ubiquitin ligase complex, Ikaros family, immunomodulatory drugs, lenalidomide, multiple myeloma, proteasome*

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1. Introduction

Thalidomide and its derivatives, lenalidomide and pomalidomide, are important immunotherapeutic drugs. Chemical structure of these drugs is shown in Figure 1. Thalidomide, 2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (Thalomid), the first IMiD, was originally synthesized in Germanyfrom α-phtaloylisoglutamine, to be used as sedative and antimetic drug [1]. In 1957, after a short period of preclinical studies, thalidomide was approved for first trimester gestational sickness in pregnant women. The appearance of malformations such as phocomelia in the newborn banned its use three years later [2,3,4,5,6]. Despite its history as a human teratogen, thalidomide is emerging as a treatment of cancer and inflammatory diseases. The US Food and Drug Administration (FDA) approved thalidomide in 1998 for the treatment of erythema nodosum leprosum.On May 26, 2006, FDA granted accelerated approval for thalidomide in combination with dexamethasone for the treatment of newly diagnosed multiple myeloma (MM) patients [7,8,9]. Thalidomide therapy induces response in non-Hodgkin lymphomas [10,11], mainly in relapsed mantle cell lymphoma [12,13], and in chronic lymphocytic leukemia [14,15,16]. A small but consistent fraction of transfusion-dependent MDS patients achieved transfusion independence by treatment with thalidomide [17,18,19,20].

Lenalidomide, 3-(4-amino-1-oxo1,3-dihydro-2H-isoindol-2-yl)piperidine-2,6-dione, and pomalidomide, 4-amino-2-(2,6-dioxo-3-piperidyl)isoindoline-1,3-dione, are analogs of thalidomide with potent immunomodulatory, anti-angiogenic and direct neoplastic cell inhibitory activities [21-33]. Lenalidomide was developed in order to avoid thalidomide side effects (sedation, constipation and peripheral neuropathy), and to increase tumoricidal efficacy [22]. Lenalidomide (CC-5013, Revlimid) shares a number of structural and biological properties with thalidomide but is safer and more potent than thalidomide. Lenalidomide is not teratogenic in rabbit models [22]. Lenalidomide also is a more potent stimulator of T-cell

proliferation and cytokine production (γ-interferon and interleukin-2) [27]. Pomalidomide (originally CC-4047, also known as Pomalyst in the US or its brand name Imnovid) is indicated for patients with MM who have received at least two prior therapies including lenalidomide and a proteasome inhibitor bortezomib and have demonstrated disease progression on or within 60 days of completion of the last therapy [34-41]. Pomalidomide has lower incidence of adverse effects like lenalidomide and appears to be more potent than both thalidomide and lenalidomide with regard to T-cell co-stimulation [23]. IMiDs have multiple effects including immunomodulatory activity, anti-angiogenic activity, intervention of cell surface adhesion molecules, cell cycle arrest, inhibition of cell migration and metastasis, anti-proliferation aktivity, anti-inflammatory activity, and pro-apoptotic activity (Figure 2).

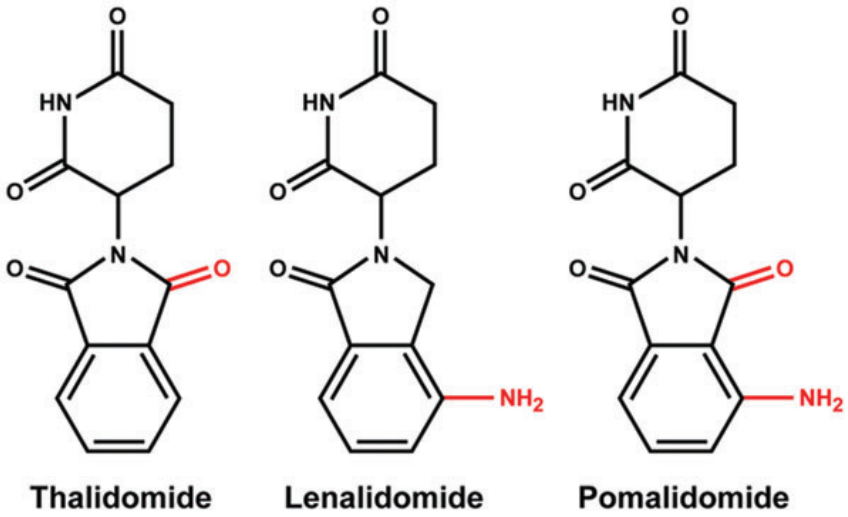

Figure 1. Chemical structures of immunomodulatory drugs (IMiDs) including thalidomide, lenalidomide, and pomalidomide. Lenalidomide and pomalidomide are synthetic compounds derived by modifying the chemical structure of thalidomide

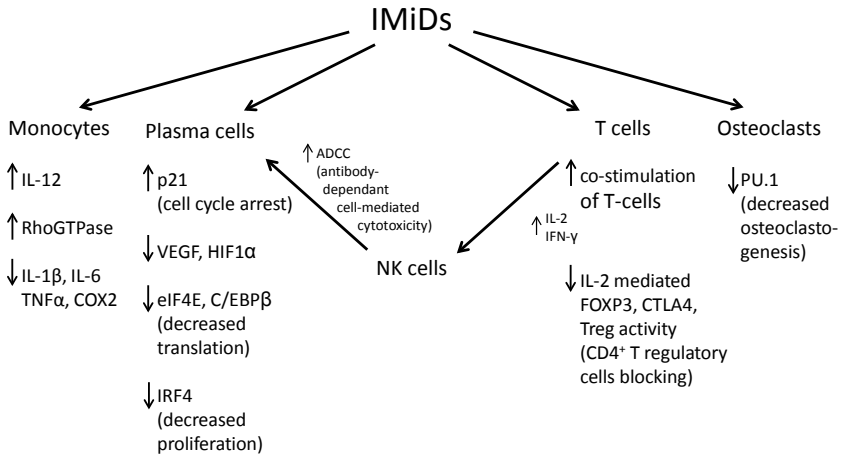

Figure 2. Various mechanisms of theaction of immunomodulatory drugs (IMiDs). IMiDs have multiple effects including immunomodulatory activity, anti-angiogenic activity, intervention of cell surface adhesion molecules, cell cycle arrest, inhibition of cell migration and metastasis, anti-proliferation aktivity, anti-inflammatory aktivity, and pro-apoptotic activity

CRBN functions as a substrate receptor of the E3 ubiquitin ligase complex ($CRL4^{CRBN}$)and is the primary target of thalidomide teratogenicity [42,43,44]. The $CRL4^{CRBN}$protein ligase complex mediates the ubiquitination and subsequent proteasomal degradation of target proteins and is required for cellular protein homeostasis. The ubiquitin-proteasome system (UPS) is the major intracellular pathway for extra-lysosomal protein degradation and plays a major role in cell cycle regulation, cell differentiation, response to stress, transcription regulation, DNA repair and programmed cell death [45-60]. Deregulation of the UPS contributes to the pathogenesis of diseases, such as cancer, neurological, autoimmune, genetic and metabolic disorders [50,57,58,61,62]. The target specificity of the $CRL4^{CRBN}$ protein ligase complex is changed by thalidomide or its analogs binding to CRBN (Figure 2). In the presence of IMiDs, the $CRL4^{CRBN}$ targets proteins IKZF1 and IKZF3but in the absence of IMiDs this E3 targets the homeobox transcription factor MEIS2 for ubiquitination and proteasomal degradation [63-69]. The anti-proliferative effect of IMiDs in MM cells and the immunomodulatory effect in T cells is mediated through CRBN protein and have recently been linked to IKZF1 and IKZF3 ubiquitination and proteasomal degradation. Down-regulation of IKZF1 and IKZF3 by IMiDs is connected with a decrease of the interferon regulatory protein 4 (IRF4) mRNA and protein levels, a decrease of *Myc* expression and with anti-proliferative effect of IMiDs in MM cells [63,64,65,70,71,72]. IKZF3 depletion stimulates *IL2* (interleukin 2) gene transcription in T cells and their proliferation [63,64,65]. IKZF1 level decrease induces both IL2 and IFNγ in T cells [65]. The immune

system plays a key role in controlling cancer initiation and progression. T cellsand natural killer (NK) cells activation and regulatory T cells (Tregs) depletion are central to anti-tumor immune response [23,24,25,26,27]. So far, we do not know additional substrates of $CRL4^{CRBN}$ which might be important for IMiDs effect in another cells (for example del(5q) myelodysplastic syndrome cells).

2. $CRL4^{CRBN}$ as Apartofubiquitin-proteasome System

Ubiquitin is a highly conserved protein of 76 amino acids that is covalently attached to substrate proteins through an energy-dependent enzymatic mechanism and polyubiquitinated proteins are degraded by a multicompartmentalized protease called the 26S proteasome [45,46,47,48]. For the discovery of ubiquitin and its function in non-lysosomal pathway of protein degradation, the 2004 Nobel Prize in Chemistry was awarded to Drs. Avram Hershko, Aaron Ciechanover and Irwin Rose [49,50,51,52].

Schematic representation of the ubiquitin conjugation (ubiquitination, also referred to as ubiquitylation or ubiquitinylation) and of the the ubiquitin-proteasome system is shown in Figure 3. Ubiquitination is a posttranslational modification of proteins. Ubiquitin is activated in an ATP-dependent manner by a ubiquitin – activating enzyme known as an enzyme-1 (E1). Subsequently, ubiquitin is transferred to a ubiquitin-conjugating enzyme-2 (E2). E2, with the help of a ubiquitin-protein ligase (E3) and in some cases in the presence of an accessory ubiquitin chain assembly factor (E4) [73,74], specifically attaches ubiquitin to the protein substrate. Only ten E1 enzymes, but about 40 E2 enzymes and 1000 E3 enzymes exist in human cells [59,74].

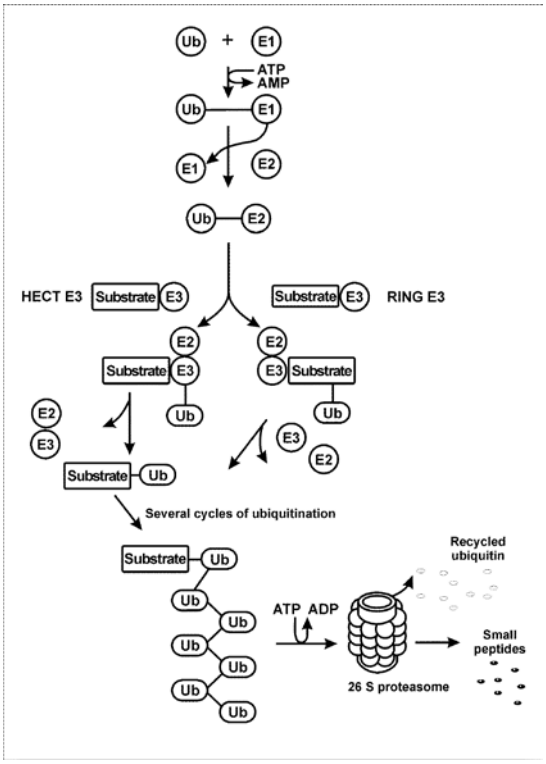

Figure 3. The ubiquitin-proteasome system. Attachment of ubiquitin to the target protein requires three enzymatic steps. Ubiquitin-activating enzymes activate ubiquitin by forming a high energy thiol ester bond between an E1 active site-located cystine residue and the C-terminal glycine residue of ubiquitin. This reaction requires energy provided by the hydrolysis of ATP and forms an activated thiol ester bond to ubiquitin-conjugating enzymes that serve as carrier proteins. Ubiquitin-protein ligases catalyze the covalent attachment of ubiquitin to the target proteinby the formation of isopeptide bonds. Multiple cycles of ubiquitination finally result in the synthesis and attachment of polyubiquitin chains that serve as a recognition signal for the degradation of the target protein by the 26S proteasome

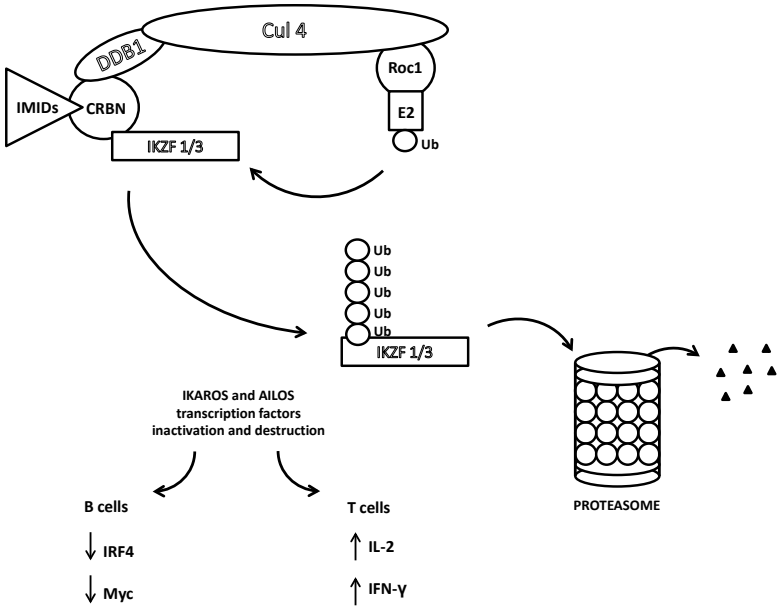

Figure 4. Schematic model of CRBN-directed cullin 4 ring E3 ubiquitin ligase complex (CRL4) action after binding of IMiDs to CRBN. CRBN forms an E3 ubiquitin ligase complex with three other proteins-damaged DNA binding protein 1 (DDB1), cullin 4 (CUL4), and regulator of cullins 1 (Roc1). IMiDs binding to CRBN results in the selective ubiquitination and proteasomal degradation of Ikaros family zinc finger transcription factors IKZF1 and IKZF3. This degradation decreases interferon regulatory factor 4 (IRF4) in plasma cells and increases IL-2 expression in T cells

E3 ubiquitin ligases determine the specificity of protein substrates and are targets for pharmaceutical intervention. There are two major types of E3 ligases: the RING (really interesting new gene) domain-containing E3s and the Hect

(homologous to E6-associated protein carboxyl terminus) domain-containing E3s. RING E3s bring the E2 enzyme in close proximity of the target protein, allowing the E2 to directly ubiquitinate the substrate. However, in the case of Hect E3s, ubiquitin is first transferred onto a conserved cysteine in the Hect domain. Then, Hect E3 enzyme ubiquitinates the substrate protein.

Polyubiquitin chain formation results from a linkage between the C terminus of one ubiquitin and a lysine side chain in another. Generated polyubiquitin chain (at least four attached ubiquitins) functions as signal for the subsequent degradation of protein substrates in the 26S proteasome.

About 400 distinct cullin-RING E3s constitute the large majority of the E3s in mammalian cells [75]. CRL4s contain the cullin 4 scaffold and a large β-propeller protein, DDB1, as a linker to interact with DDB1 and CUL4-associated factors (DCAFs) as substrate receptors [76-85]. CRL4 binds in its complex a small RING protein ROC1 or ROC2 (also known as Rbx or Hrt1) [82]. CRLs are activated via the covalent modification of the cullin protein with the ubiquitin-like protein Nedd8. This results in a conformational change in the cullin carboxy terminus that facilitates the ubiquitin transfer onto the substrate. COP9 signalosome-mediated cullin denedylation is essential for CRL activity *in vivo*. COP9 signalosome inhibits substrate receptor autoubiquitination [86]. Crystal structure of the DDB1-CUL4A-ROC1 ubiquitin ligase complex hijacked by the V protein of simian virus showed that DDB1 uses one β-propeller domain for cullin scafold binding and a variably attached separate double β-propeller fold for substrate presentation [77,78]. Distinct classes of substrate receptors show a broad spectrum of cellular processes regulated by CUL4-DDB1 [79].

CRBN functions as a DCAF for the E3 ubiquitin ligase $CRL4^{CRBN}$. The structure of CRBN revealed that CRBN does not exhibit WD-repeat architecture typical for the majority of DCAFs. WD-repeat is a domain defined at the primary sequence level by a Gly-His dipeptide and a Trp-Asp (WD) dipeptide separated by 20-30 residues [82]. WD-repeats form β-propeller structures. The CRBN structure showed two distinct domains, an N-terminal Lon-like domain (LLD) and C-terminal binding domain (TBD). CRBN interacts with DDB1 via helices between amino acid residues 221-248 in CRBN [68]. The IMiD-compound pocket in TBD of the CRBN is formed by three tryptophan residues, Trp380, Trp386 and Trp400, with a phenylalanine residue at the base (Phe402) [68]. These residues form a small hydrophobic pocket in which the glutarimide portion of lenalidomide is accommodated. Lopez-Girona et al. [43] demonstrated that the binding affinities of lenalidomide and pomalidomide for CRBN are similar, whereas the affinity of thalidomide for CRBN is slightly weaker. Furthermore, glutarimide alone or N-methyl-2-pyrrolidone (NMP) interacted with CRBN and had antimyeloma activity [68,87].

3. Identification of CRBN-binding Proteinsin Multiple Myeloma and the Regulation of Their Levels by Lenalidomide

Co-immunoprecipitation of proteins in MM cell lysates with an anti-CRBN antibody or pull-down using Ni^{+} beads techniques were used for identification of CRBN-binding proteins [69]. Using these procedures, 244 CRBN binding proteins were detected. Their relevance to MM biology was established by changes in their abundance after exposure to lenalidomide. After lenalidomide treatment, the abundance of 46 CRBN binding proteins decreased [69]. Zinc finger transcription factors IKZF1 and IKZF3 are the most downregulated CRBN-associated proteins [63,64,65,69]. Alternatively, 16 CRBN binding proteins underwent an increase in abundance after lenalidomide treatment, including CUL4A, CUL4B, DDB1, SQSTM1 (sequestosome 1/p62/A170) and [69,78-83,88,89,90,91]. Top lenalidomide-regulated CRBN-interacting proteins are shown in Table 1 [67,69]. Lenalidomide decreased the levels of nucleolar protein GLN2 (guanine nucleotide binding protein-like 2 /nucleolar/) [92]; STUB1 (stress-induced phosphoprotein 1 /STIP1/ homology and U-box containing protein 1, also known as CHIP (C-terminus of HSC70 /heat shock 70 kDa protein/-interacting protein, E3 ubiquitin ligase and co-chaperone of heat shock protein 70) [93]; IKAROS family proteins IKZF1 (IKAROS) and IKZF3 (AILOS) [94,95,96,97,98]; ANKRD12 (ankyrin repeat domain-containing protein 12, a 224 kDa nuclear protein) [99]; and KPNA2 (karyopherin alpha 2, that is included in the nuclear import of proteins) [100,101]. On the other hand, lenalidomideincreased the levels of the cullinproteins CUL4A and CUL4B [78-83], DDB1 [78-83,88,89], SQSTM1 [90,91], and MEIS2 (myeloid ecotropic insertion site 2, a member of the TALE /three amino acid loop extension/ superclass of homeodomain proteins) [102,103,104,105].

Table 1. Top lenalidomide-regulated CRBN-interacting proteins

Proteins	Regulation
GNL2	Down
STUB1	Down
IKZF1	Down
IKZF3	Down
ANKRD12	Down
KPNA2	Down
CUL4A	Up
CUL 4B	Up
DDB1	Up
SQSTM1	Up
MEIS2	Up

4. Measuring Cereblon mRNA and Cereblon Levels as a Biomarker of Response or Resistance to Lenalidomide and Pomalidomide

After the exposure of MM cells to lenalidomide, a great down-regulation of the *CRBN* expression (measured by CRBN mRNA or protein levels) is associated with the development of marked IMiDs resistance in human MM cells [43,106-112]. Certain *CRBN* expression is thus required for the anti-myeloma activity of IMiDs. CRBN pre-mRNA undergoes alternative splicing and therefore validated assays are necessary for evaluation of the *CRBN*

gene expression in the clinic [109,110]. Thefull-length coding sequence of the CRBN mRNA has 1329 nucleotides (variant 1). Transcript variant 2 coding sequence has 1326 nucleotides. It lost 3 nucleotides at the end of exon 2. There are 22 CRBN mRNA splice variants, designated 001-022 [110]. CRBN mRNA splice variants 001-004 have an open reading frame and are translated to protein but only variant 001 to the functional CRBN protein. Variant 002 has exon 10 deleted and is not expected to bind IMiDs. Variants 003 and 004 lack a part of the putative DDB1- binding domain or complete this domain. Thus, all these variants 002-004 are non-functional. All other variants contain multiplestop codons in the primary sequences and are not likely to produce translated versions of CRBN protein [110]. There are also variants lacking exon 8 alone or in combination with exon 10. Therefore, the best assay for measurement of CRBN mRNA levels as a potential predictive biomarker of response of MM patients to lenalidomide is the "best coverage" TaqMan assay Hs00372271_m1 (primers in exon 8 and 10); Applied Biosystems, Life Technologies Corp., which measures all CRBN mRNA variants that are translated to functional protein with the exception of variants with removed exon 10 (part of IMIDs binding region).

Measurement of CRBN protein is also associated with a number of assay limitations. Currently available commercial antibodies are neither sensitive nor specific for reliable detection of CRBN protein [110]. Gandhi *et al. [110]* characterized a monoclonal antibody CRBN65 which is highly sensitive and specific in Western blot and immunohistochemical (IHC) analysis. This monoclonal antibody can detect as little as 200 pg of CRBN protein by Western blot.

MM cell lines that acquire resistance to IMiDs through long-term passage showed a decline in both CRBN mRNA and CRBN protein level compared with the parental sensitive MM cells. This decline in CRBN mRNA and CRBN protein levels indicates that loss of CRBN mRNA and CRBN protein may play a role in acquired resistance. Transduction of wild type *CRBN* restored IMiD sensitivity to MM cells with low endogenous *CRBN* expression [111,113]. Transduction of mutant (loss of IMiD binding) *CRBN* didn´t restore IMiD sensitivity to MM cells with low endogenous *CRBN* expression [111,113]. Thus, an IMiD-CRBN complex may still be cytotoxic to MM cells that have achieved CRBN independence. Multiple mechanisms, including miRNA, likely control *CRBN* expression [114,115,116].

Many of the anti-MM activities of IMiDs are thought to involve modulation of the tumor microenvironment. Up to now, *c* was studied in total bone marrow mononuclear cells [108] or $CD138^+$ sorted plasma cells [107,112]. While it has been shown that CRBN and IRF4 levels correlate with lenalidomide responsiveness in patients, *in vitro* investigations, using human myeloma cell lines, didn´t replicate this results [43,108,115]. It will be important to detect CRBN and IRF4 levels in $CD138^-$ cells as well as in $CD138^+$ myeloma cells [43,108,110,117].

5. Therapy for Multiple Myeloma Patients with Low *CRBN* Expression

Schuster *et al*. [112] demonstrated significantly reduced *CRBN* expression in t(4;14) MM, a subgroup known to benefit from proteasome inhibition [111]. MM is a plasma cell malignancy in which significant advances have been observed during the past 15 years. Improvements in its molecular characterization and in treatment with two new classes of active agents, proteasome inhibitors and IMiDs, resulted in a significant improvement in overall survival of MM patients. These novel therapies target both normal plasma cell biology as well as the cancer biology of myeloma [118,119]. Thalidomide and bortezomib were the first examples of these active agents. Combinations of new generation of these agents followed by stem cell transplant result in responses in nearly all MM patients. 20-30% of MM patients survive for over 10 years. It is no longer appropriate to call MM an incurable disease.

6. Conclusions

Zhu *et al*. [106] showed that CRBN is the critical target for anti/mzeloma activitz in preclinical models. The subsequent studies have shown a correlation between *CRBN* expression and IMiD clinical activity [107,108,111,112,113]. Complexes $CRL4^{CRBN}$ are part of the UPS (ubiquitin- proteasome system), however unlike targeting the proteasome, CRBN likely effects the fate of a smaller subset of proteins. Recent findings are consistent with this possibility as two studies demonstrated that lenalidomide binding to CRBN modifies its degron binding and results in the targeting of two zinc finger transcription factors (IKZF1 and IKZF3) that are important for plasma cell maintenance [63,64]. IKZF1 (IKAROS) and IKZF3 (AILOS) regulate the expression of IRF4 and studies have demonstrated that IMiDs downregulate IRF4 in myeloma cells [106,113,120]. IRF4 was identified as a critical factor for MM cell survival [70]. The direct targets of IRF4 are several important genes such as Myc, CDK6 and CASP3 [70]. Both lenalidomide and pomalidomide were demonstrated to inhibit *IRF4* gene expression [106,113,120] and *Myc* gene expression. The role of Myc in myeloma genesis has become clearer through its ability to lead to a myeloma-like disease in a murine model [121,122] as well as the recent discovery of the prevalence of Myc translocations resulting in juxtaposition to superhancers active in myeloma [123,124].

Furthermore, the effect of IMiDs on CRBN also explained the ability of these drugs to alter T cell secretion of TNF-α and IL-2 [33,43,125,126]. IMiDs significantly prevent TNF-α production and pomalidomide is the most potent in this inhibition of TNF-α production [43]. The inhibitory effect of IMiDs on TNF-α production was impaired by CRBN silencing. Thus, the immunomodulatory effect of IMiDs on T cells is mediated by CRBN [33,43,125,126]. IKZF3 depletion stimulates *IL2* (interleukin 2) gene transcription in T cells and their proliferation [63,64,65]. IKZF1 level decrease induces both IL-2 and IFNγ in T cells [65]. Secretion of IL-2 and IFNγ increases the number of natural killer (NK) cells, improves their function, and mediates lysis of MM cells. The immune system plays a key role in controlling cancer initiation and progression. T cells and natural killer (NK) cells activation and regulatory T cells (Tregs) depletion are central to anti-tumor immune response [23,24,25,26,27].

Acknowledgements

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