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Modulation of cereblon levels by anti-myeloma agents

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Abstract
The use of thalidomide derivatives (IMIDs) has improved multiple myeloma prognosis, through an unknown mechanism of action. Recently one molecular target, the cereblon (CRBN) protein, has been identified. CRBN acts by binding to DDB1-CUL4-ROC1 forming a ubiquitin ligase multiprotein complex. We have generated antibodies to different regions of CRBN protein, and analyzed the biological consequences of augmenting or decreasing CRBN levels. CRBN was expressed in all the myeloma cell lines tested, independently of their sensitivity to IMIDs, and the CRBN-DDB1-CUL4 complex was efficiently formed. At the molecular level, long-term treatment with IMIDs induced a slight decrease in CRBN levels and a reduction in the CRBN-DDB1-CUL4 complex. Interestingly, treatment with other anti-myeloma drugs downregulated cellular contents of CRBN, and in a much faster fashion. These results suggest that CRBN is an important mediator of the cellular response to IMIDs, but also critical in the maintenance of cell viability and/or proliferation.

Keywords: Cancer, CRBN, IMIDs, myeloma, thalidomide

Introduction
In 1999, Barlogie and colleagues demonstrated that thalidomide was effective in the treatment of advanced multiple myeloma (MM) [1]. Since then, several clinical trials have confirmed the efficacy of thalidomide used as a single agent [1–3]. Besides, results of phase I and phase II studies of combination therapies of thalidomide with dexamethasone or cytotoxic chemotherapy have shown improved activity of such combinations [3,4]. Due to these promising results, second and third generation derivatives of thalidomide such as lenalidomide or pomalidomide have been developed. Lenalidomide was approved in 2006 by the FDA for its use in combination with dexamethasone in patients with MM who had received at least one prior treatment [5]. Pomalidomide is a new compound with increased in vitro activity when compared with thalidomide or lenalidomide [6]. Importantly, a recent report demonstrated that pomalidomide overcame the refractoriness to prior therapy with lenalidomide and bortezomib, opening new possibilities for those patients who had already developed such resistances [7].

The mechanisms by which thalidomide and its derivatives act have been studied and several hypotheses have been proposed including DNA intercalation, acetylation of macromolecules or the generation of oxidative stress [8–10]. Nevertheless, in 2010 a milestone paper identified the protein cereblon (CRBN) as a target of thalidomide [11]. Human CRBN cDNA encodes a 442 aminoacid protein with an estimated molecular weight of 51 kDa. This protein was initially described to be involved in mental retardation [12]. Shortly after, its interaction with the large conductance calcium-activated potassium BK channel alpha-subunit (KCNMA1) was also reported [13]. In addition, CRBN may play a role in the energy balance by directly interacting with the α1 subunit of the AMP-activated protein kinase (AMPK) [14]. CRBN forms an E3 ubiquitin ligase complex together with the damaged DNA binding protein 1 (DDB1), Cullin4A (CUL4) and Roc1. Within this CRL4 E3 ligase complex, CRBN could function as a novel substrate receptor, competing with other receptors as DDB2 or Cockayne Syndrome A (CSA) [11]. Thalidomide binding to CRBN occurs in a region located to the C-terminus of CRBN [11]. The teratogenic effect of thalidomide is due to its binding to CRBN in this DDB1 complex, and the inhibition of its ubiquitin ligase activity. In fact, using zebrafish as a model, both thalidomide treatment or knockdown of CRBN induced similar defects, including retardation of pectoral fin and otic vesicle (ear) formation in part through the downregulation of growth factors as Fg8 and Fgf10 [11].

Given the importance of thalidomide and its derivatives in myeloma treatment, Zhu and collaborators investigated the role of CRBN as mediator of the lenalidomide and pomalidomide antimumela activity. They demonstrated that cellular depletion of CRBN in myeloma cells was initially cytotoxic, although those few cells surviving such depletion were highly resistant to both lenalidomide and pomalidomide [15]. However, other reports showed contrasting data when demonstrating that reduction of CRBN in MM cells did not have an effect on malignant plasma cell viability [16]. Moreover, the levels of CRBN were lower in lenalidomide
refractory patients than in responding ones [15]. Other studies have also demonstrated that high levels of expression of CRBN in MM patients correlated with an improved clinical response to the combined treatment of lenalidomide and dexamethasone [17].

More recently several independent groups have identified Ikaros and Aiolos as the downstream targets of CRBN-DDB1 complex after IMIDs activation. In fact IMIDs would induce the E3-ubiquitin ligase activity of the complex that would ultimately lead to the degradation of these lymphoid transcription factors, being such loss necessary and sufficient for IMIDs therapeutic effect [18 – 20].

Despite these precedents, the role of CRBN in MM and its relationship to drug sensitivity in this disease remain poorly characterized. One of the reasons is the lack of reagents to accurately measure CRBN protein levels. In fact, and despite the potential interest of using CRBN levels as an indicative predictor of response to IMIDs, most of the studies on CRBN biology are based on qRT-PCR levels. As this is not the best indicator to assess protein levels, and due to the lack of good commercial antibodies to detect CRBN, we have developed several antibodies to CRBN. With the aim of augmenting our knowledge of the importance of CRBN in MM pathophysiology, the level of this protein in different MM cell lines was analyzed and its behavior after treatment with different agents used in myeloma treatment, determined. Moreover, the biological effect of its overexpression or downregulation was studied in these models.

**Material and methods**

**Reagents and antibodies**

Cell culture media, puromycin, dexamethasone and melphalan were purchased from Sigma. Foetal bovine serum and antibiotics from Life technologies, Immobilon P membranes from Millipore Corp and the JetPEI™ reagent from Polyplus-transfections SA. Pomalidomide and lenalidomide were from Selleck, bortezomib from LC Laboratories and doxorubicin from Fluka. Other generic chemicals were purchased from Sigma Chemical Co., Roche Biochemicals or Merck.

The anti-DDB1 and anti-CUL4A antibodies were obtained from Abcam, the anti-PARP and anti-IGF1R from Santa Cruz Biotechnology, and the anti-α-tubulin from Oncogene Science.

Anti-CRBN specific antibodies were generated in rabbits that were manipulated at the animal facility according to Institutional Guidelines for the Use of Laboratory Animals of the University of Salamanca, after acquiring permission from the Ethical Committee for Animal Experimentation of the University of Salamanca, and in accordance with current Spanish laws on animal experimentation. Rabbits were injected with peptide sequences corresponding to the C-Terminus of the molecule (anti-CRBN-CT) or an internal peptide that had already been described (anti-CRBN-NT) [11]. A third antibody was raised against a fusion protein in which aminoacids 223–300 of human CRBN were fused to...
GST. That fusion protein was produced in bacteria, purified, and used to immunize rabbits (anti-CRBN-Mid). The regions of CRBN recognized by these antibodies are schematically represented in Figure 1A. The different anti-CRBN antibodies are available through the Translational Oncopharmacology Unit of our institute (http://www.cicancer.org/en/unidad-de-oncofarmacologia-traslacional).

**Cell lines and culture, transfections, generation of lentiviruses and infection**

The multiple myeloma cell lines were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and antibiotics. 293T and HeLa cells were grown in DMEM supplemented with 5% FBS. All the cell lines were cultured at 37°C in a humidified atmosphere in the presence of 5% CO₂/95% air. The source of all these cell lines has already been described [21].

To overexpress CRBN in MM cells, human CRBN cDNA was amplified with specific primers from total mRNA previously purified from HeLa cells. Using appropriate restriction enzymes the full length cDNA was subcloned into the retroviral vector pLZR-IRES-GFP-puro, and the final construct verified by restriction digest and sequencing. Retroviruses were generated as described [21] and used to infect myeloma cells.

To diminish CRBN levels, knockdown experiments using lentiviruses were performed. The lentiviral vectors containing shRNA for CRBN were obtained from Open Biosystems. For the production of lentiviruses, vectors were cotransfected in HEK-293T cells with the pMDLg/RRE, pRSV-Rev and pMD2.G plasmids by JetPEI™ transfection using a previously described protocol [21]; 48 h after the transfection, the lentivirus-containing supernatants were filtered (0.45 μm) and either used to infect target cells or stored at −80°C in aliquots. For the infection, target myeloma cells were incubated for 10–12 h with the lentiviruses in the presence of 6 μg/ml polybrene. Two days after the infection, cells were selected with puromycin (1 μg/ml).

**Immunoprecipitation, Western blotting and cell fractionation**

To prepare cells for protein analyses, they were harvested by centrifugation, washed in PBS and lysed in ice-cold lysis buffer. Immunoprecipitation experiments, SDS-PAGE and Western Blot were performed as described [21]. Cell fractionation experiments were carried out following previously published indications [22].

**Cell proliferation, cell cycle and apoptosis assays**

To assess cell proliferation, cells were plated in six-well plates and counted at different time points with a Z1 Coulter® Particle Counter (Beckman). Alternatively, conventional MTT assays were used [21]. For cell cycle profiling, cells fixed and permeabilized in 70% ice-cold ethanol were resuspended...
in PBS containing 50 µg of propidium iodide and 200 µg of DNase-free RNase A and incubated for 1 h at room temperature. To determine apoptosis, cells were resuspended in binding buffer (10 mM HEPES/NaOH, 140 mM NaCl, 2.5 mM CaCl2, pH 7.4) containing 5 µl of Annexin V-FITC (BD Biosciences) and 5 µl of 50 µg/ml PI and stained for 15 min [21]. All the stained cells were analyzed in a FACScalibur flow cytometer.

**Statistical analysis**

All data are represented as the mean ± standard error of triplicate wells of a representative experiment that was...
repeated at least three times. The differences among groups were determined by Student’s t-test; *p* < 0.05 was considered significant.

**Results**

**CRBN is expressed in all the MM cell lines tested**

To investigate the role of CRBN in the biology of MM three antibodies to different domains of CRBN (Figure 1A) were raised in rabbits. One of them was raised against a peptide located at the C-Terminus of CRBN (CRBN-CT) and another against an internal peptide close to its N-Terminus (CRBN-NT). The latter matched the amino acid sequence of the thalidomide-binding protein [11]. Besides, a third antibody detecting a larger internal peptide was similarly generated (CRBN-Mid). All three antibodies were affinity purified using the respective antigens.

To establish their capability to recognize CRBN we utilized HeLa cells, which were initially used to characterize CRBN as the thalidomide binding protein [11]. In parallel, we prepared HeLa cells transfected with CRBN to increase its amount and facilitate its detection. Cell extracts were prepared and immunoprecipitated with each antibody, followed by WB with the same antibody (Figure 1B). The anti-CRBN-CT antibody and the anti-CRBN-Mid antibodies identified a band with the expected molecular weight of CRBN and whose amount was increased after transfection of CRBN to cause its overexpression. In addition these antibodies recognized a band with faster migrating properties (labeled with an asterisk) whose amount was unchanged in cells overexpressing CRBN. The anti-CRBN-NT identified a band with a similar molecular weight to CRBN, but whose amount was not increased after transfection (Figure 1B, right panel). Using these overexpression conditions, extracts were immunoprecipitated in parallel with the three antibodies, and the WB was probed with the anti-CRBN-CT antibody. In those conditions only the CRBN-CT and the CRBN-Mid antibodies precipitated CRBN. The anti-CRBN-NT antibody hardly detected it, indicative of a much lower affinity of this antibody for the native CRBN protein. To verify the specificity of the anti-CRBN-CT antibody, another set of samples was immunoprecipitated with the antibody that had previously been preincubated with an excess of the peptide injected to raise it (Figure 1D). Lysates from HeLa cells and the myeloma cell line MM1S were prepared and 1 mg of protein extracts were immunoprecipitated with or without the peptide competition. In those conditions the anti-CRBN-CT detected a band in MM1S cells coincident with CRBN (Figure 1D). As shown above, this antibody detected other bands migrating slightly faster in the HeLa samples (Figure 1B, C and D, asterisk). In those Westerns, a faint band with a higher molecular weight could also be detected in both HeLa and MM1S cells (empty arrowhead). Since this faint band was only present in the anti-CT immunoprecipitates but not in the Mid ones, and its amount was not increased by CRBN transfection, we interpreted that such band was unrelated to CRBN. Together, these results indicated that the Mid or CT antibodies should be used to further characterize CRBN.

We then analyzed the level of CRBN in a panel of MM cell lines representative of various genetic alterations and resistance properties. Protein extracts of the different cell lines were immunoprecipitated with the anti-CRBN-CT antibody and Westerns probed with the same antibody. All the cell lines tested expressed CRBN although at heterogeneous levels (Figure 2A). Preincubation of the antibody with the peptide against which the C-Terminus antibody was raised prevented precipitation of CRBN.

**CRBN forms a cytosolic multiprotein complex in MM cells**

It has been described that the cellular role of CRBN is mediated by its binding to the DDB1-CUL4A complex [11]. To investigate whether CRBN also forms this type of multiprotein complexes in myeloma cells, MM1S cell extracts were immunoprecipitated with the anti-CRBN-CT antibody and Westerns probed with DDB1 and CUL4 antibodies. As shown in Figure 2B, the anti-CRBN antibody pulled down DDB1 and CUL4. As expected, CRBN, DDB1 or CUL4 could not be detected when the immunoprecipitates were performed with an excess of the peptide used to generate the antibody (Figure 2B).

We also explored the cellular site where CRBN and this multiprotein complex locate. To this end, the cellular...
CRBN levels. Thus, we used RNA interference against CRBN in the myeloma cell lines MM1S, OPM2, and RPMI-8226 to diminish their CRBN content. Five shRNA lentiviral vectors carrying different target sequences were tested. All of them produced substantial knockdown of CRBN levels in MM1S cells (Supplementary Figure 1A to be found online at http://informahealthcare.com/doi/abs/10.3109/10428194.2015.1037752). When transduced into MM1S cells, the five shRNA lentiviral vectors provoked strong inhibition of cell proliferation (Supplementary Figure 1B to be found online at http://informahealthcare.com/doi/abs/10.3109/10428194.2015.1037752). The following experiments have been verified for at least three of them, although only two are shown.

**Figure 5. Effect of IMIDs on CRBN levels.** MM1S (A), OPM2 (B) or RPMI-8226 (C) cells were treated for 3 or 5 days with 100 nM pomalidomide (P) or 1 μM lenalidomide (L). Protein extracts were prepared and CRBN immunoprecipitated from them. The amount of CRBN or the co-precipitating DDB1 was determined by WB. Besides, 50 μg total extract were separated by SDS-PAGE to assess total levels of DDB1. (D–F). The proliferation of those same cells was determined in parallel by direct cell counting. (G) CRBN downregulation impairs the response of MM1S cells to pomalidomide. MM1S cells were transduced with empty vector (pLKO), or shRNAs targeting CRBN, treated with 100 nM pomalidomide (Poma) and cell proliferation was assessed by MTT assays. (H) CRBN overexpression and cellular response to IMIDs. The cellular response of the indicated cell lines overexpressing CRBN to 5 days treatment with 100 nM pomalidomide (P) or 1 μM lenalidomide (L) was analyzed by MTT as described, and normalized to the growth of the non-treated cells. The blot shows CRBN levels. Statistical significant differences are shown (*p < 0.05).

**CRBN downregulation leads to myeloma apoptotic cell death**

To gain insights into the potential role of CRBN in myeloma biology we used strategies to augment or down-regulate CRBN levels. Distribution of CRBN was analyzed by subcellular fractionation. CRBN was detected in the cytosolic fraction not only in MM1S cells, but also in HeLa cells, that were used as a control (Figure 2C). Moreover the whole complex was efficiently found in the cytosolic fraction of the cell, indicating a strong association between these proteins (Figure 2C). Blots using antibodies to IFG-1R and α-tubulin were carried out as markers of membrane and cytosolic compartments, respectively.
The efficiency of the knock-down in the three MM cell lines was confirmed by IP and Western blot of CRBN using the anti-CRBN-CT antibody (Figure 3A). The decrease in CRBN levels was accompanied by a concomitant decrease in the amount of DDB1 associated to CRBN (Figure 3A and B). The reduction of CRBN levels decreased cell growth, as indicated by the MTT metabolization assays (Figure 3C). To analyze whether the decrease in MTT metabolization in CRBN knocked-down cells was due to augmented cell death or decreased proliferation and transit through the cell cycle, propidium iodide stainings were performed. When the cell cycle profile was studied by this staining, no major change in

Figure 6. Treatment with drugs causing MM cell death induces CRBN downregulation. (A–C) Time course treatment of MM1S (A), OPM2 (B) or RPMI-8226 (C) for the indicated hours with dexamethasone (DXM, 50 nM), bortezomib (Bort, 10 nM), melphalan (Mel, 5 μM) or doxorubicin (DXR, 1 μM). CRBN was immunoprecipitated in the different conditions as described. The percentage of cleaved PARP on each experimental condition is numerically shown under PARP WB. (D) CRBN downregulation is also dose-dependent. MM1S were treated for 48 hours with the indicated concentrations of bortezomib, and analyzed as before. (E) CRBN degradation is caspase dependent. Prior to bortezomib (Bort) or dexamethasone (DXM) 2 days treatment, MM1S were treated for 1 hour with 50 nM Z-VAD-FMK. Both CRBN levels or co-precipitating DDB1 were determined by IP and WB.
their different phases was observed in living cells (Figure 3D). In contrast, an increase in the subG0/G1 population (M1) was detected, indicative of cell death (Figure 3E). Such death was likely due to apoptosis given the increase in annexin V positive cells observed (Figure 3F).

**Overexpression of CRBN confers increased growth potential to MM cells**

The above data suggested that a decrease in the levels of CRBN may compromise MM cell survival/proliferation. We therefore reasoned that an inverse scenario could favor MM growth. To explore this possibility and since nothing is known about the in vivo effects of CRBN overexpression, we investigated such effects in the myeloma context. With this purpose we subcloned the cDNA coding for CRBN in the bicistronic retroviral vector pLZR-IRES-GFP. The correct insertion of the cDNA was verified by restriction analysis and sequencing. Following standard procedures, MM1S myeloma cells were transduced with the appropriate retrovirus and sorted by GFP to isolate a CRBN-overexpressed enriched population. In parallel, another population was similarly generated with the pLZR-IRES-GFP empty vector (MM1S-Mock). First, the levels of CRBN both in the retrovirus-generating 293T cells as well as the MM1S cells were analyzed by standard IP and WB with the C-terminus antibody (Figure 4A). These studies demonstrated that both the virus-generating 293T and infected MM1S cells overexpressed CRBN when compared with those transduced with the empty vector (Mock).

To test whether augmented CRBN levels increased the formation of the functional complex, we determined the presence of DDB1 and CUL4A co-immunoprecipitating with CRBN in MM1S-Mock and MM1S-CRBN cells lines. In the overexpression conditions, the complex was more efficiently formed (Figure 4B). Moreover, there was the same proportion of overexpressed CRBN than that of DDB1 or CUL4A coprecipitating. The overexpression of CRBN in MM1S cells (MM1S-CRBN) had a positive effect on cell growth, as demonstrated by the higher MTT metabolism values obtained from these cells (Figure 4C).

**IMiDs downregulate the functional CRBN-DDB1 complex**

Prolonged exposure of myeloma cells to high dose lenalidomide has been reported to induce a reduction on CRBN levels [15,16]. In fact, in our experimental model we confirmed that after 5 days of treatment with pomalidomide and lenalidomide a slight decrease in CRBN levels was detected in the three cell lines tested (Figure 5A–C). We determined the presence and stability of the CRBN-DDB1 functional complex by the co-immunoprecipitation of DDB1 with CRBN in MM1S, OPM2 and RPMI-8226. In those conditions the presence of CRBN as well as its interaction with its active partner followed similar kinetics. Thus, the levels of DDB1 co-immunoprecipitating with CRBN decreased after 5 days of treatment with the IMiDs, even though the total levels of DDB1 were not affected. Cell proliferation experiments demonstrated that treatment of MM1S and OPM2 cells with pomalidomide or lenalidomide had a strong inhibitory effect (Figure 5D and E). Interestingly, RPMI-8226 cells were resistant to the treatment with IMiDs (Figure 5F), pointing to other component different to the CRBN-DDB1 complex as the responsible of the primary resistance to IMiDs observed in these cells.

If CRBN is, in fact, the protein responsible of IMiDs action, the modification of cellular CRBN levels should affect the cellular response to these drugs. To test this possibility, MM1S cells with reduced levels of CRBN were treated with pomalidomide and compared with cells with regular levels of CRBN. Pomalidomide was unable to augment the effect of CRBN knockdown (Figure 5G). In fact the reduction of cell proliferation with pomalidomide was similar to that obtained when CRBN levels were reduced (Figure 5G). On the other hand, we would expect that higher levels of CRBN could affect the response to these agents. Thus, when mock or CRBN-expressing MM1S or OPM2 cells were treated with pomalidomide and lenalidomide for 5 days, a better response to the drugs was achieved in cells overexpressing CRBN (Figure 5H). Interestingly, the IMID-unresponsive cell line RPMI-8226 remained insensitive to pomalidomide or lenalidomide even after up-regulation of CRBN levels.

**CRBN levels are diminished when MM cells are committed to die**

We next determined the effect of other drugs used in the therapy of myeloma on CRBN levels. Treatment with bortezomib (Bort) induced a drastic reduction of CRBN levels in the three cell lines analyzed. This effect occurred earlier than in the case of IMiDs treatment (Figure 6A–C). Moreover, the effect of bortezomib was found to be dose-dependent (Figure 6D and data not shown). Such decrease correlated with the apoptotic cell death induced by this drug, as indicated by the cleavage of PARP (Figure 6A–C). In MM1S cells treatment with dexamethasone (DXM), melphalan (Mel) and doxorubicin (DXR) also decreased CRBN levels. Nonetheless, in RPMI-8226 cells, these drugs failed to reduce CRBN levels.

Bortezomib is known to induce a strong apoptotic response in MM cells [23]. In line with this was the stimulation of PARP cleavage observed in the three myeloma cell lines used (Figure 6A–C). To further study the implication of apoptotic pathways in CRBN degradation, MM1S cells were pre-treated with the pan-caspase inhibitor Z-VAD-FMK prior to Bort. Such pre-treatment was able to prevent Bort-induced CRBN downregulation pointing to a role of caspases in such process (Figure 6E, top panel). Moreover, in MM cells in which DXM also had a proapoptotic action, as judged by stimulation of PARP cleavage (Figure 6A), preincubation with Z-VAD-FMK also prevented DXM-induced CRBN downregulation. In those conditions, not only CRBN degradation was prevented, but a similar although milder effect was observed in the amount of coprecipitating DDB1 (Figure 6E, bottom panel).

**Discussion**

CRBN has recently been described as the cellular target of thalidomide and their analogs, generally known as IMiDs [11]. In MM cells recent reports have linked CRBN levels to the response to IMiDs [15,16]. In fact several recent studies...
have described that high CRBN levels could be used as a predictor of clinical response to thalidomide or lenalidomide [24,25], or the combination of lenalidomide and dexamethasone [17]. Besides, low CRBN expression can predict resistance to IMIDs monotherapy being a predictive biomarker for survival [26]. Most of these data have been generated using gene expression profiling or qRT-PCR. However, other reports have described a lack of correlation between CRBN mRNA and protein levels [27], pointing to the absolute requirement of high quality reagents to analyze CRBN levels. Moreover, the lack of CRBN antibodies has hampered the development of knowledge about the biology of CRBN in MM. To advance in this clinically relevant field, we generated antibodies to CRBN. One of them, that had been designed against a region previously used to raise antibodies to CRBN [11], was able to detect a protein with similar molecular weight but did not correspond to CRBN, as suggested by the overexpression experiments and proteomics analysis that were carried out (data not shown). This finding is relevant as it alerts with respect to the use of such sequence to generate anti-CRBN antibodies. The other two anti-CRBN antibodies generated, designed against the C-Terminus or a central region within CRBN protein, both detected CRBN.

The use of both antibodies has given us useful tools to study the biology of CRBN in multiple myeloma cells. Thus, we demonstrated that all the cell lines tested expressed CRBN irrespective of their cytogenetic or drug response properties. In addition, we also observed that CRBN forms a cytosolic multiprotein complex with DDB1 and CUL4. The search of substrates targeted by this complex will be important to better understand resistances to IMIDs. Some substrates for such activity have been recently proposed, as is the case of the transcription factors Ikaros and Aiolos [18–20]. Nevertheless, the identification of other factors targeted by this complex is still needed. On the other hand other molecules downstream CRBN have been proposed, although the ultimate mechanism interconnecting them is not well defined. That is the case of the transcription factor IRF4 [15] or β-catenin [17,24].

CRBN overexpression led to an increased cell proliferation. In those conditions not only CRBN levels were augmented, but also the functional CRBN-DDB1 complex was more efficiently formed, which could sequester DDB1 from other complexes and alter signaling pathways. Besides, since CRBN is the cellular target for IMIDs we would expect that the presence of increased levels of this protein and the complex by which CRBN executes its action should increase cell sensitivity to this kind of drugs. In fact, an increased sensitization to both pomalidomide and lenalidomide was observed in those cells, leaving unaffected the response to other unrelated drugs. Our results fall in line with several recent studies which have linked CRBN expression to clinical response to IMIDs [24,25]. On the opposite side, when CRBN levels were diminished, their cell viability was compromised in the three cell lines tested, independently of their primary sensitivity to IMIDs. Thus the elimination of CRBN induced apoptotic cell death and those cells able to survive without CRBN were no longer sensitive to the treatment with IMIDs.

In line with this observation, there was no clear correlation between CRBN levels and the resistance to IMIDs on the cell lines that we analyzed. For example when CRBN levels were carefully analyzed in the cell line RPMI-8226, primarily resistant to IMIDs treatment, it exhibited CRBN protein levels similar to those found in IMID sensitive cell lines. Moreover, when CRBN levels were knocked down in this cell line, their cell viability was also compromised, similarly to what happened with the sensitive cell lines MM1S or OPM2. Besides, after sequencing CRBN or DDB1 genes in this cell line, no mutation on any of these genes has been identified [28]. These data are important as they indicate that other factors different from CRBN are the cause of the resistance of these cells to IMIDs. A different case seems to be the secondary resistance to IMIDs, acquired after prolonged treatment with IMIDs. In that case, MM cells seem to partially loose CRBN expression ([15,16], and our data not shown). In our cell lines, long term treatment with pomalidomide or lenalidomide induced a slight reduction of CRBN levels. This reduction was observed after 5 days of treatment. However, at shorter treatments of up to 3 days, IMIDs did not decrease CRBN levels. These data are in line with a previous report in which Greenberg and collaborators described lack of CRBN or IRF4 downregulation after 3 days of treatment with lenalidomide in vitro [29].

The availability of the anti-CRBN antibodies that we raised allowed us to explore the effect of other antamyeloma treatments on CRBN levels. Interestingly other treatments able to induce MM cell death were also able to produce CRBN downregulation. Thus, after 24 h of bortezomib treatment most of CRBN protein had disappeared. Such downregulation was caspase-dependent pointing to a close relationship between apoptotic cell death and CRBN levels in this context. If CRBN downregulation is cause or consequence of such cell death must be carefully assessed. In any case, the fact that reduction of CRBN levels by knockdown affected cell viability suggests that drugs which may affect CRBN levels may also use pathways controlled by CRBN in their proapoptotic action.

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Potential conflict of interest: Disclosure forms provided by the authors are available with the full text of this article at www.informahealthcare.com/lal.

References


Supplementary material available online

Supplementary Figure 1.