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Resistance to immunomodulatory drugs in multiple myeloma: the cereblon pathway and beyond

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Title page

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Running title: Non-canonical mechanisms of IMiD resistance

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Abstract

Acquired resistance to immunomodulatory drugs (IMiDs) remains a significant unmet need in the treatment landscape of multiple myeloma (MM). CRBN pathway-dependent mechanisms are known to be vital contributors to IMiD resistance; however, they may account for only a small proportion. Recent research has unveiled additional mechanisms of acquired IMiD resistance that are independent of the CRBN pathway. In this review, we provide a comprehensive overview of the existing work on IMiD resistance in MM, focusing specifically on the emerging evidence of CRBN pathway-independent mechanisms. Finally, we discuss the plausible actionable strategies and outlook for IMiD-based therapies moving forward.

Significance:

CRBN-independent mechanisms are fast becoming an important factor associated with IMiD resistance in myeloma. With the burgeoning research on this topic, it may not be easy for the research community to keep abreast of its latest developments. Here, we provide an up-to-date evidence (at point of writing) on this topic which can serve as an essential reference for the myeloma community (or even beyond) to facilitate their ongoing and future research.

Keywords

Multiple myeloma, Immunomodulatory drugs, Cereblon, Treatment resistance

Introduction

Immunomodulatory drugs (IMiDs) are a major class of drugs that have changed the treatment paradigm for multiple myeloma (MM). Thalidomide, the first-in-class IMiD, was introduced in the late 1950s as a sedative and anti-emetic during pregnancy, but was soon withdrawn due to neuropathy and teratogenicity[1]. However, a breakthrough study in 1999 reported promising efficacy in MM patients, leading to accelerated approval for MM treatment in May 2006[2, 3] (Figure 1). This success spurred the development of thalidomide analogues, namely, lenalidomide and pomalidomide, to enhance therapeutic effectiveness while reducing toxicities. Lenalidomide received U.S. Food and Drug Administration (FDA) approval in June 2006 for use in MM patients who have had at least one prior line of therapy [4]. Pomalidomide was granted FDA approval seven years later for RRMM patients who had undergone at least two prior therapies, including lenalidomide and bortezomib[3]. While thalidomide is now less prescribed, lenalidomide is widely used as the backbone of numerous combination treatments for newly diagnosed MM (NDMM), as post-transplant maintenance, and in relapsed/refractory MM (RRMM), whereas pomalidomide is commonly used for treatment of RRMM, especially for lenalidomide-refractory patients[5].

The clinical benefit of IMiDs in MM is well established, however, their therapeutic efficacy and durability are significantly limited by primary and acquired drug resistance. In MM, approximately 5% patients demonstrate primary resistance to IMiDs, while those who initially responded to IMiD-based regimens eventually acquire resistance over time[6-9]. Moreover, recent analyses suggest age-dependence to efficacy and key subset differences, particularly in older patients where clinical benefit is more limited. There is therefore a significant unmet need in understanding the underlying mechanisms of resistance to IMiDs.

Landmark studies have revealed that IMiDs exert their activity by binding to a specific tri-tryptophan pocket of cereblon (CRBN), a substrate adaptor protein of the $CRL4^{CRBN}$ E3

ubiquitin ligase complex, which consists of DNA damage-binding protein 1 (DDB1), cullin-4A/B (CUL4A/B), and regulator of cullins 1 (ROC1)[10, 11].

Multiple studies on CRBN pathway abnormalities have facilitated our understanding on IMiD resistance[12-14]. Resistance mechanisms beyond the CRBN pathway, on the other hand, are gradually emerging, but their relative significance and how one study is related to another remains incompletely understood. In this current review, we 'deep dive' into the evidence on CRBN pathway-independent mechanisms of IMiD resistance, dissect the details of the studies, and systematically describe the evidence based on how one may be supporting another. Additionally, we discuss areas of future research that may hold promise in advancing our understanding of IMiD resistance and propose plausible therapeutic strategies to overcome IMiD resistance in the clinic.

Mechanism of action of IMiDs

By binding to CRBN, IMiDs redirect the $CRL4^{CRBN}$ E3 ubiquitin ligase machinery to target and induce proteasomal degradation of a range of neo-substrates, including the transcription factors Ikaros and Aiolos, encoded by IKZF1 and IKZF3 genes respectively[10, 11, 15] (Figure 2A). Both IKZF1 and IKZF3 (IKZF1/3) are regulators of B-cell differentiation and were described to be essential genes in MM[16, 17]. IMiD-induced-degradation of IKZF1/3 is, therefore, crucial for the anti-neoplastic effects in MM, mediating the cellular toxicity and/or induction of immunomodulatory responses (Figure 2B).

The direct anti-MM effect is mainly attributed to the downregulation of two MM essential genes, IRF4 and c-MYC, causing disruption of their oncogenic drive and hence cytotoxicity[18, 19]. At the microenvironment level, IMiDs enhance immunomodulatory responses by [1] promoting immune recognition through increased antigen presentation by dendritic cells, [2] increasing production of anti-tumor cytokines such as interferon-γ (IFN-γ) and interleukin-2 (IL-2) to drive T cells expansion and natural killer (NK) cells activation, [3] reducing adhesion

molecules such as VCAM-1 and ICAM-1 on bone marrow stromal cells (BMSCs) to impede tumor cell-BMSC interactions, [4] inhibiting immunosuppressive T-regulatory cells (Tregs), as well as [5] impairing signalling of angiogenic factors, vascular endothelial growth factors (VEGFs) and fibroblast growth factors (FGFs) in the BM niche[20-22].

The distinct clinical efficacy of each thalidomide derivative reflects the differences in its CRBN binding affinity and the subtly different spectrum of neo-substrates degradation[23]. Thalidomide, lenalidomide and pomalidomide share common phthalimide and glutarimide moieties, but all differ respectively in a carboxy and an amino group at the phthalimide ring. These minor but key structural variations lead to significant differences in clinical efficacy, with increased potency observed from thalidomide to lenalidomide and then to pomalidomide[1]. Compared with thalidomide, lenalidomide was documented to be 50-2000 times more potent in inducing T-cell proliferation, and 300-1200 times more potent in augmenting T-cell activity, due to increased IL-2 and IFNγ production. Pomalidomide is 10 times more efficient than its predecessor in stimulating T-cells and inducing pro-inflammatory cytokines from Th1 cells, while reducing anti-inflammatory cytokines from Th2 cells[24].

IMiD resistance associated with the CRBN pathway, and its paradox

Owing to the core function of CRBN in the activity of IMiDs, disruption to the $CRL4^{CRBN}$ E3 ubiquitin ligase components has been the most commonly reported mode of resistance to this group of drugs (Table 1). Here, we document the key findings from previous studies on the genomic and non-genomic abnormalities of CRBN and its pathway genes, and their association, or not, with patients' responses to IMIDs[25-28].

Genomic alterations in the CRBN gene that have been reported in MM patients include most commonly, single nucleotide variation (SNV) and copy number loss, while the non-genomic events involved epigenetic and transcriptomic aberrations that affect its stability and expression, including the abnormal exon 10 splicing[25, 26, 29-31]. Somatic SNVs in *CRBN* are

infrequent among newly diagnosed MM (<1%), however, their prevalence significantly increases to 9-12% of IMiD-refractory patients[25, 26]. Genetic mapping identified that these SNVs were predominantly located within the IMiD-binding domain[26], and their ectopic introduction into MM cell lines obliterated responses to lenalidomide[26, 32]. There was also an increase in the frequency of CRBN copy number loss, from 1.5% in NDMM to 7.9% in Len-refractory and a significant 24% in Pom-refractory patients[25]. In addition, higher levels of alternative splicing of exon-10 in *CRBN,* which prevents IMiD-binding, has an incidence reaching up to 10% of Len-refractory patients, and has been consistent in predicting poor responses to IMiDs[25, 33]. These reports underscore the biological role of genomic and non-genomic lesions of *CRBN* in IMiD resistance. However, it is noteworthy that they represent only a small proportion of IMiD-refractory patients (up to 20% for lenalidomide and up to 30% for pomalidomide), suggesting that the majority of acquired IMiD resistance cases (i.e. >70-80%) are unaccounted for by *CRBN* abnormalities. Studies from smaller independent cohorts have not reported changes in the frequency of *CRBN* mutations in Lenalidomide-refractory vs NDMM patients [34-36], but are mainly small and/or limited to SNV detection.

Pre-clinical modelling of acquired IMiD-resistance demonstrated that resistant cell lines had depleted CRBN expression[29]. This is consistent with clinical observations, whereby high CRBN expression correlated with improved progression-free survival (PFS) in IMiD-treated-patients, while the IMiD-non-responders exhibited reduced CRBN expression[30, 37]. However, it is also notable in other studies that CRBN levels were not predictive for IMiD responses [34, 38]. Importantly, amongst all types of CRBN abnormalities described in IMiD-RRMM, there was no one mechanism that rendered a complete loss of CRBN expression[25, 29, 31]. For instance, neither did the cases with CRBN copy loss nor those with an aberrant exon 10 splicing demonstrated changes/reduction in CRBN expression compared to their counterparts without these aberrations[25]. More interestingly, a substantial proportion of the IMiD-RRMM (32%) paradoxically exhibited increased CRBN expression with no loss-of-function variant detected[39]. These data suggest that in the cases without genetic loss-of-

function, the activity of the CRBN pathway was putatively retained in the IMiD-resistant MM cells. This supports the observation that lenalidomide-refractory patients were responsive to subsequent pomalidomide treatment[5, 40, 41], suggesting that low but intact CRBN expression does not abolish the functional $CRL4^{CRBN}$ E3 ubiquitin ligase activity and residual CRBN signalling may still mediate responsiveness to the more potent pomalidomide.

Investigations into CRBN axis genes have also yielded inconclusive results. For instance, IMiD-refractory disease had an increased mutation frequency in *IKZF1*, *IRF4* and *CUL4B* compared to NDMM[26], but another study showed no difference in the mutation status of *DDB1*, *CUL4A*, *CUL4B*, *IKZF1*, *IKZF2*, and *IKZF3*[36]. High IKZF1/3 expression has been associated with poorer PFS in lenalidomide-treated-MM patients[42], and in contrasting data, with a favourable response to lenalidomide and better PFS[43, 44]. In another patient cohort, IKZF1/3 protein levels were non-prognostic[45]. Further upstream in the CRBN pathway, COP9 signalosome (CSN) and E2 ubiquitin ligase proteins (UBE*)* are required for the maintenance of $CRL4^{CRBN}$ E3 ubiqitin ligase activity. Whole-genome sequencing of MM patients (n=522) revealed increased incidence of copy number loss of chromosomal 2q37, the region containing CSN members (*COPS7B* and *COPS8)* in the lenalidomide-refractory, and lenalidomide-then-pomalidomide-refractory patients compared to the NDMM patients[28]. Importantly however, the enrichment of this abnormality was again observed only in a small subset of the refractory patients (10-16%) and mutational analysis revealed low frequency for CSN and UBE members, implicating that once those with CRBN aberrations also removed, >60% of IMiD-refractory patients are still unaccounted for by the aberrancy in these CRBN pathway proteins[28].

In summary, CRBN pathway abnormalities are not a universal event in IMiD-refractory patients and the mechanism of resistance is likely to extend beyond this canonical IMiD-pathway.

IMiD resistance independent of CRBN pathway

Supporting this notion, evidence on IMiD resistance mechanisms independent of the CRBN pathway are gradually emerging. These have been described to include myeloma cell-intrinsic factors and myeloma cell-extrinsic factors (Table 2).

(a) Intrinsic mechanisms

The main myeloma cell-intrinsic mechanisms of IMiD resistance in MM involve the dysregulation of various oncogenic mediators, including known MM essential genes and other under-studied MM drivers. Some of these studies reported direct mechanistic evidence, while others showed clinical association without clear mechanisms (Figure 3).

IL-6/STAT3 signalling, a crucial MM driver, was found to be upregulated in an acquired lenalidomide resistant MM cell line, XG1 (XG1-LenRes)[46, 47]. The authors identified autocrine production of IL6 in the XG1-LenRes, which was further enhanced in the presence of lenalidomide treatment. Stimulation of parental-XG1 cells with IL6-induced lenalidomide resistance, whereas inhibition of STAT3 with a selective STAT3 inhibitor (PB-1-102) re-sensitized its isogenic resistant counterpart to lenalidomide. Constitutive IL6/STAT3 activity in XG1-LenRes was associated with sustained expression of IRF4. Notably, XG1-LenRes did not have any accompanying abnormalities in CRBN and its downstream components. There was no change in CRBN expression and neither was there any differential effects on lenalidomide-induced IKZF1/3 degradation. In concordance, introduction of exogenous CRBN also failed to restore lenalidomide sensitivity in XG1-LenRes[47], indicating the involvement of CRBN pathway-independent mechanisms.

Dysregulation of another oncogenic pathway, the Wnt/β-catenin, was also observed in lenalidomide-resistant MM cells. Through gene expression profiling of lenalidomide-resistant-U266, -ANBL-6, -KAS-6 and -MM1.S vs. their parental cells, several Wnt/β-catenin intermediates (Wnt-3, Fzd-4, β-catenin) were found to be upregulated. This increase in

Wnt/β-catenin activity led to stabilization of cytoplasmic β-catenin and upregulation of MM drivers CyclinD1 and c-Myc. Knocking down β-catenin, in turn, restored MM cell sensitivity to lenalidomide[48]. Another report showed that CD44, a downstream transcriptional target of β-catenin, was also associated with IMiD resistance. The authors found that increased CD44 expression in the lenalidomide-resistant cells enhanced MM cell adhesion to BMSCs to promote cell survival. Inhibition of β-catenin, and consequently CD44, with all-trans retinoic acid (ATRA) successfully re-sensitized resistant MM cells to lenalidomide[49].

The main players in the oncogenic MAPK pathway such as NRAS, KRAS and to a lesser extend BRAF, are the most frequently mutated genes in MM patients, with NDMM and RRMM cases bearing a high 20-50% and 45-80% frequency, respectively[26, 50-52]. BRAF/KRAS/NRAS are upstream mediators of the MEK/ERK kinases and activating mutations of BRAF/KRAS/NRAS genes trigger these kinases to upregulate a series of proliferative and cell cycle signals[51]. A mouse xenograft study bearing MM1.S plasmacytomas with acquired IMiD resistance showed that resistance onset was accompanied by hyperactivity of MEK1/ERK pathway (increased pMEK1/2 and pERK1/2). The addition of selumetinib, a small-molecule MEK inhibitor, effectively reinstated IMiD sensitivity, both in and ex vivo[53], hence suggesting the role of the BRAF/KRAS/NRAS /MEK/ERK signalling cascade in mediating IMiD resistance. Nevertheless, it should be noted that BRAF/KRAS/NRAS gene mutations are a general predictor of poor clinical outcome and are observed widely in all RRMM states[50]. There is a diverse range of SNVs reported[54] and the functional impact of these different BRAF/KRAS/NRAS point mutations on the activation of MEK/ERK pathway leading specifically to IMiD resistance, requires more study.

The biological role of MEK/ERK signalling was further demonstrated in a genome-wide CRISPR-Cas9 knockout screen in pomalidomide-treated MM.1s cells, in which *TRAF2* appeared as a modulator of resistance[55]. In cells co-cultured with BMSCs or BMSC supernatants, the authors identified that IL-6 directly activates MEK/ERK signalling while

triggering proteasomal degradation of *TRAF2 t*o stimulate NF-κB and ERK signalling. MM1.S cells with *TRAF2* knockout exhibited significant resistance to lenalidomide and pomalidomide, alongside activation of NF-κB and MEK/ERK pathways, independently of the CRBN-IKZF1/3 axis. Consistent with the former study, inhibition of MEK with selumetinib effectively overcame IMiD resistance in *TRAF2* knockout MM cells. However, the authors also identified that *TRAF2* knockout conferred higher resistance to dexamethasone and melphalan treatments, indicating that *TRAF2* knockout-induced drug resistance may not be specific to IMiDs[55].

Epigenetic alterations are widely implicated in cancer drug resistance and in the case of IMiDs in MM, [1] a global increase in DNA methylation, with [2] a reciprocal decrease in chromatin accessibility and [3] a dominance of gene downregulation, were observed in acquired IMiD-resistant-OPM2 and -H929, with the main components of the CRBN pathway (*CRBN*, *IKZF1/3*, and *IRF4)* being unaffected. This is consistent with reports that promoter silencing of CRBN and its pathway genes were not associated with CRBN pathway deficiency and IMiD resistance[39, 56, 57]. In this study, the authors, instead, identified SMAD3 (a transcription factor and cell signalling regulator), as the novel gene commonly downregulated in the resistant counterpart of both the cell lines. Treatment with a combination of 5-azacytidine (Aza) and the EZH2 inhibitor (EPZ-6438) reverted chromatin repression, increased SMAD3 expression and ultimately re-sensitized the resistant cells to IMiDs[57]. In support of this pre-clinical finding, Kalff et al. have reported some clinical efficacy in combining Aza (oral) with lenalidomide-dexamethasone (Rd) in heavily treated LEN-resistant RRMM patients (ORR 37.5%, clinical benefit rate 50%)[58]. Although Khouri et al.'s Rd-Aza (subcutaneous) treatment protocol in another patient cohort yielded a lower response rate (ORR 22%, clinical benefit rate 32%), the authors propose that Rd-Aza may overcome some IMiD refractoriness with careful regime optimisation and correct patient selection[59].

Further evidence of epigenetic involvement in IMiD resistance was demonstrated in a ChIP-seq study across 16 MM cell lines, comparing lenalidomide-resistant vs. lenalidomide-sensitive cells[60]. At the gene specific level, the authors reported that in the lenalidomide-resistant cells, the promoter regions of *ANKRD30B* and *SLAMF6* exhibited the highest occupancy of the active H3K4me3 mark, while the promoter regions of *GPR15* and *NKX6-1* demonstrated a marked depletion. Among the CRBN pathway genes, only *CUL4B* displayed enriched H3K4me3 at its promoter region in the lenalidomide-sensitive cells. Nonetheless, the underlying mechanism by which IMiDs induce epigenetic reprogramming and the extent to which changes in epigenetics contribute to IMiDs' lack of function was not described, and shall remain an imperative work moving forward.

Furthermore, NCOR2, an epigenetic remodelling gene, has been implicated in multi-drug resistance in MM, including to IMiDs[61]. The authors identified that NCOR2 was interacting with nucleosome remodeling and deacetylase (NuRD) complex, to repress the expression of CD180 by directly binding to its promoter and resulted in the downregulation of MYC. They showed in IMiD-resistant cells that low NCOR2 and CD180 expression was associated with increased MYC expression. There was no change in CRBN and IKZF1 expression, and thus they concluded that high MYC in IMiD-resistance was induced by deregulation of NCOR2-CD180 pathway, independently of CRBN. It is noteworthy that in this study, NCOR2 knockout also led to resistance to BET and HDAC inhibitors, thus the therapeutic implication of loss of NCOR2 in MM was not specific to IMiDs.

A more specific epigenetic dissection of IMiD resistance was recently reported; out of 48 MM cell lines challenged with pomalidomide, 44 (92%) remained viable, despite significant depletion of IKZF1/3. These MM cells displayed high growth rate with most of the IMiD-resistant cell lines retaining high levels of MYC and IRF4 expression[62]. Further investigations using ATAC-Seq revealed reduced chromatin accessibility for IKZF1-binding in the pomalidomide- vs DMSO-treated cells. Notably, the sites that lost chromatin accessibility for

IKZF1 were enriched for BATF, IRF4 and FOX bHLH binding motifs. RNA-sequencing of 66 MM cell lines revealed that the inherently IMiD-resistant cell lines (e.g. KMS-12BM, RPMI-8226) expressed high levels of BATF. They identified that BATF heterodimerization was compensating for the IMiD-induced loss of IKZF1/3 to sustain IRF4 overexpression, ultimately leading to IMiD resistance. In the analysis of paired NDMM and RRMM patients treated with IMiDs (n=35), a significant upregulation of BATF upon relapse was observed. Cross referencing these findings in the CoMMpass dataset, the authors identified that high BATF expression indeed conferred poorer survival outcome (n=484) in IMiD-treated patients[62].

IKZF1/3 redundancy in IMiD responses was also highlighted in a complementary study whereby two other factors, EP300 and BRD4, compensated for the IMiD-mediated loss of IKZF1/3[63]. On ChIP-seq analysis, half the chromatin-bound IKZF1/3 sites overlapped with EP300 and BRD4 binding sites. While lenalidomide universally depleted chromatin-bound IKZF1 in both IMiD-sensitive MM1.S and IMiD-resistant RPMI-8226 cells, the IMiD-resistant MM cells maintained BRD4 and P300 super-enhancer occupancy. Further interrogations revealed that this was acting through transcription factor *ETV4*, which co-binds the enhancers with IKZF1 to induce IMiD resistance. They also reported that *ETV4* expression was associated with poorer PFS and OS for CoMMpass patients treated with IMiDs, and for POLLUX (NCT02076009) patients treated with Rd. Analysis of 36 paired CoMMpass patients and 14 paired POLLUX patients showed that *ETV4* was significantly upregulated at relapse, whereas no change was observed in the expression of *IKZF1*, *IKZF3*, *IRF4* or *MYC*[63]. Alongside the immediate former publication[62], this work identifies that CRBN-mediated-degradation of IKZF1/3 can be bypassed in sustaining the oncogenic IRF4-MYC axis to drive IMiD resistance.

In patients, high-risk MM markers, specifically, t(4;14), t(14;16), del(17p), and gain/amp(1q21) have been associated with early relapses following IMiD-based therapy[64]. A

longitudinal genomic analysis of RRMM patients (n=386) highlighted the enrichment of gain/amp(1q21) and del(17p) in IMiD-refractory cases, underscoring the potential impact of genes upregulated in chr1q and deleted in chr17p on IMiD responses[65]. Concordantly, the Myeloma XI trial (n=556) revealed in their multivariate analysis that isolated gain(1q21) and double-hit cases (defined as two concomitant high-risk features) derived no survival benefit from lenalidomide maintenance[66]. We note however that 1q21(gain/amp) and 17p13(del) are poor predictive markers for a broad range of MM therapeutics, and therefore interactions between mechanisms specific to IMiDs versus general drivers of resistance/ early relapse needs to be further interrogated. Notably, Adenosine Deaminase Acting on RNA (ADAR1), encoding an RNA editing enzyme, is located in the amplified chr1q21 region. Our work has shown a close association between high ADAR1 expression and hyperedited MM transcriptome with reduced responsiveness to IMiDs[67], implicating the involvement of aberrant RNA editing in the mechanism of IMiD resistance. Another study has also shown that ADAR1-mediated editing of glioma-associated oncogene homolog 1 (GLI1), a Hedgehog pathway transcriptional activator and self-agonist, promotes malignant regeneration and IMiD resistance in MM[68]. In view of the growing interest and the biological relevance of RNA abnormalities in MM, our team is currently interrogating the mechanism by which ADAR1 and its aberrant activity regulate IMiD responses in MM. We have identified a novel mechanism involving the ADAR1-regulated-dsRNA sensing pathway in modulating IMiD resistance (manuscript in revision).

Further evidence of RNA-related aberrations in IMiD resistance was reported in a recent circular RNAs (circRNAs) profiling study[69]. A total of 200 and 277 differentially expressed circRNAs were observed, in H929-lenalidomide-resistant and H929-pomalidomide-resistant cells, respectively, compared to their sensitive counterpart. The authors identified ciRS-7 to be consistently downregulated, while circIKZF3 was commonly upregulated in the lenalidomide- and pomalidomide-resistant cells. The depletion of ciRS-7 correlated with

increased methylation levels of the promoter CpG island of its host gene, LINC00632. Combination treatment of an EZH2 inhibitor (EPZ-6438) and a DNA methyl transferase inhibitor (5-azacytidine) partially restored the expression of LINC00632 and ciRS-7 and the IMiD sensitivity of the cells. Nevertheless, silencing ciRS-7 in sensitive parental cells did not increased resistance to IMiDs, potentially suggesting indirect modes of action. In the case of circIKZF3, its underlying mechanism remains elusive, due to the challenges faced by the authors in knocking down circIKZF3 in both lenalidomide- and pomalidomide-resistant cells [69].

Integration of the proteomics and RNA-sequencing analyses of RRMM patients treated with lenalidomide-based therapy has identified CDK6 upregulation as a driver of IMiD resistance [70]. Overexpression of CDK6 in IMiD sensitive MM cell lines resulted in reduced IMiD sensitivity, while the inhibition of CDK6 through Palbociclib or CDK6-specific PROTACs (BSJ-03-123 or CST528) demonstrated synergy with IMiDs both in vitro and in MM1.S xenografts. In their patient cohort, they did not detect any CRBN pathway abnormalities (RNA, protein and phosphoprotein), genetic alterations to the other genes in the $CRL4^{CRBN}$ E3 ligase complex, or association between CRBN and CDK6 protein expression. CRISPR/Cas9-knockout of CRBN in MM lines rendered no change in the expression of CDK6. . Although CDK6 inactivation in conjunction with IMiDs resulted in significant inhibition of MYC, downstream functions of CDK6 in RRMM remain unclear[70].

CD138 is a marker for terminally differentiated plasma cells during normal B-cells development and serves as a specific surface antigen for MM cells[71]. A significant increase in CD138-negative MM cells has been observed in relapsed or progressive patients (n=15) compared to NDMM patients (n=90)[72]. Characterization of two MM cell lines (KYMM-1 and KYMM-2) established from a single patient showed that the cell population with decreased CD138 surface expression had higher lenalidomide resistance. The downregulation of IRF4 and upregulation of BCL6 was suggested as the mechanism for this resistance[72], citing

another study that documented high IRF4 expression were correlated with increased lenalidomide sensitivity[73].

In another study on surfaceome, glycoprotein cell surface capture (CSC) proteomics on Len-resistant OPM2 and H929 showed a common signature of increased CD33 and CD45/PTPRC, when compared to their sensitive counterparts[74]. Analysis of the Multiple Myeloma Research Foundation (MMRF) CoMMpass dataset from paired diagnosis and first-relapse tumor cells (verIA14, n=50, where 94% of patients had received lenalidomide and dexamethasone with a PI as part of their induction regimen) revealed that both *CD33* and *PTPRC* transcripts were significantly increased at first relapse[74]. The authors noted that plasma cell expression of either of these markers has been associated with poor prognosis for NDMM, who exhibited more aggressive disease upon lenalidomide resistance [75, 76]. They, however, did not describe nor further interrogate the basic/plausible mechanisms underlying CD33 and PTPRC associations with disease progression.

Lastly, various other genes have appeared in genome-wide CRISPR-screens in cell lines as regulators of IMiD sensitivity, for example *TOP2B, EDC4, RARA, SNRNP25, OTUB1, PLAA, DEPDC5, SRP14, XRN1, EIF4A1, ARID2, MBTPS1/2 and SCAP*[77-79]. Whether they have any relevance to clinical IMID resistance, and if so how, remains a topic for future research.

(b) Extrinsic mechanisms

The interaction of MM cells with BM components such as secreted growth factors/cytokines, BMSCs and immune cells can promote growth, survival and drug resistance of MM cells (Figure 4).

Characterisation of the MM TME landscape in patients from the MANHATTAN trial (NCT03290950, n=49) who did not achieve minimal residual disease (MRD) negativity and have shorter PFS, demonstrated TME dysregulations including low population of

CD14^{+}monocytes, increased frequency of T-cell exhaustion, persistence of IFNγ-expressing NK cells and decreased T cell receptor (TCR) diversity[80]. These were observed alongside genomic defects such as high *APOBEC* mutational activity, 1p22 (*RPL5*) deletions and *IKZF3* loss, suggesting an interplay between tumor genomic features with the composition of TME in driving drug resistance.

In a prospective observational clinical trial, immune-profiling of lenalidomide-RRMM, non-refractory MM and non-MM-healthy individuals revealed that the lenalidomide-RRMM patients had a significant expansion of effector T cell populations that express elevated levels of checkpoint molecules, LAG3 and PD-1. Their frequency was positively correlated with increased serum inflammatory cytokines, IL6, IL17 and TNFα[81]. High levels of PD-1 and LAG3-positive T cells were predictive of inferior survival and clinical outcomes in these RRMM patients, as in previous studies[82, 83].

The chemokine CCL20 was found to be downregulated in an acquired lenalidomide-resistant MM cell line (U266-Len-resistant), as well as in the MM cells and plasma of lenalidomide-treated RRMM patients (n=5). At both the in vitro and in vivo level, the addition of CCL20 was able to re-sensitive MM cells to lenalidomide[84]. The authors postulated that CCL20 plays a role in increasing lymphocyte chemotaxis to the tumor areas and in assisting the cell-mediated immunity.

Our group has recently reported metabolic reprogramming, with the release of metabolic waste product such as lactate into the TME, to be associated with IMiD resistance[85]. Elevated lactate secretion is known to promote acidosis in the TME, driving metastasis, angiogenesis and drug resistance[86]. t(4;14) is a high-risk MM marker with a prevalence rate of 15-20% that drives overexpression of the histone methyltransferase, NSD2[87]. NSD2 promotes plasma cell transformation by catalysing the active histone mark H3K36me2. We identified that protein kinase C alpha (PKCα) is an epigenetic target of NSD2[85]. Through

metabolomics analysis, we found that lactate was a differential metabolite associated with PKCα. High lactate levels were associated with reduced responsiveness to lenalidomide. Knockdown of PKCα resulted in reduced intracellular and extracellular lactate levels, consequently increasing cellular sensitivity to lenalidomide, independent of the CRBN-IKZF1/3 axis. Clinically, t(4;14) MM patients demonstrated elevated plasma lactate levels compared to non-t(4;14) patients and did not derive significant benefits from lenalidomide-based regimens[85].

MM cells with an IMiD-resistance phenotype have also been associated with increased secretion of extracellular vesicles (EV) and enhanced adherence abilities[88]. Through a comprehensive transcriptomic analysis of acquired lenalidomide-resistant MM cell lines (KMS-21, KMS-27, KMS-34), core regulatory genes governing EV secretion, including SORT1 and LAMP2, were found to be significantly upregulated compared to their sensitive counterparts. Knockdown of SORT1 or LAMP2 reduced EV secretion, decreased cell adhesion and restored lenalidomide sensitivity in lenalidomide-resistant cells without affecting CRBN expression. Further analysis of publicly available clinical data revealed that high SORT1 and LAMP2 expression were associated with poor survival in MM patients (GSE19784, n=300) and in patients treated with lenalidomide (GSE136324, n>200)[88].

Taken together, the above findings suggest that aberrations at the TME may impinge on the efficacy of IMiDs in MM, further highlighting that IMiD resistance is likely to involve an interplay of many biological factors.

Future perspectives

The discovery of CRBN as a pivotal target of IMiDs has been instrumental in advancing our understanding of the molecular mechanism of these therapeutic agents[10, 11]. Given the inevitable occurrence of IMiD-resistance in MM, the identification of biomarkers that can

accurately predict for IMiD responses is of paramount importance to increase the prospects of therapeutic efficacies.

At present, exploring CRBN status as a potential biomarker for predicting IMiD responses and resistance seems a conceivable strategy; however, as reviewed in the earlier sections, the results reported hitherto have been rather inconclusive. In light of this, several critical limitations should be addressed to ensure the reliability of using CRBN as a biomarker. Firstly, standardized assays for quantifying functional levels of CRBN expression in clinical samples are currently lacking[89]. The development of robust and reproducible measurement techniques is crucial if CRBN expression was to have any role as a biomarker in clinical practice. Next, determining the optimal approach for assessing functional CRBN levels—whether at the mRNA or protein level or both—and evaluating their genomic aberrations are equally important. Identifying the cancer clonal fraction (CCF) harbouring these genomic events at relapse vs. diagnosis will provide insights into the clonal selection of IMiD resistant subclones and whether longitudinal exposure to the therapies could drive clonal selection. In addition, determining the threshold level at which the CCF is deemed prognostic in the patients will be essential in guiding personalized treatment strategies. Considering that CRBN expression may be downregulated but not completely abrogated in RRMM, it will also be paramount whether a threshold of expression, and which/how different transcript splice variants should be measured to identify a non-functional CRBN activity, could predict treatment failure. It is likely any such biomarker parameters would also vary between different IMiD and CELMoD agents.

Similarly, the approach to measure other CRBN pathway proteins, such as IKZF1/3, should be undertaken with greater detail. For instance, the rate, rather than magnitude, of IKZF1/3 degradation was found to be the more important determinant for modulating IRF4 expression, and thus, the efficacy of IMiDs[90]. This highlights the need to investigate the timepoint and protein level at which IKZF1/3 cease to be sufficient to support transcription of

the downstream IRF4 oncogenic events, and whether any compensatory mechanisms such as BATF and ETV4 may assume the transcriptional regulation roles of IKZF1/3. Further, IMiD-bound CRBN has binding affinity for IKZF1/3 and other reported competing neo-substrates, which, at variable levels between different myelomas, may be differentially responsible for response and resistance. These are some of the interesting questions that could form the basis of future research.

High-risk copy number alterations in gain/amp(1q21) and del(17p) involve a large number of genes. It is plausible that some of these genes may play a role in driving IMiDs resistance. For instance in chromosome 17p, a few IMiD-response pathway genes (*UBE2G1*, *NCOR1* and *COPS3*) reside close to tumor suppressor *TP53*. The functional impact of the loss of these genes in del(17p) MM has not been interrogated. Whether IMiD resistance might be driven by these genes independently or by their co-deletion with *TP53,* awaits further investigation.

The growing body of evidence on the significance of non-canonical pathways and CRBN-independent mechanisms in regulating the efficacy of IMiDs, have been demonstrated mostly in cell line models (Table 2), which means that clinical evidence is not well-established. This highlights the need to determine how these alternative genes/modes of action are translationally relevant, given that many of the CRBN-independent abnormalities seem to be enriched also in patients resistant to non-IMiD treatment regimens. The proposed associations should, therefore, be validated in clinical samples, alongside the well-studied CRBN pathway genes, to determine the prognostic value of any associations found, and if they are worthwhile to be further explored as a novel predictor and specific biomarker for IMiDs treatment.

As described, there are also quite a number of studies on clinical samples that report associations with IMiD response but do not yet have direct mechanistic explanations.

Greater effort into elucidating these mechanisms and how they modulate IMiD responses in MM is of paramount importance moving forward to identify information that can be used to aid clinical decision making.

Previous genome-wide CRISPR/Cas9-mediated studies were conducted with the typical aim of identifying genes that regulate IMiD sensitivity[77-79, 91]. It is also worthwhile to validate these findings in a converse manner, i.e., CRISPR studies on IMiD-resistant cells to decipher their novel dependencies and therapeutic vulnerabilities. This might identify genes that, when knocked out or activated, will result in the re-sensitisation or killing of IMiD-resistant MM cells.

Tumor heterogeneity leads to the development of multiple mechanisms of resistance to IMiDs[92]. Rapid advancements in single cell profiling technology have enabled us to dissect the heterogeneity of cells at both single and spatial resolution. Spatial single-cell transcriptomics is a burgeoning tool to decipher tumor architecture and TME [93, 94], and studies utilising this cutting-edge technology in MM is still at its infancy. Given the current lack-of-knowledge surrounding the association between cell-intrinsic and -extrinsic mechanisms in IMiDs resistance, it will be pertinent to adopt spatial single-cell technology into future investigations to enable detailed characterisation of the MM cells-TME interaction.

It is becoming evident that the mechanisms underpinning IMiD resistance in MM involves a complex interplay of genomic, transcriptomic, and proteomic events both in tumour and immune cells. Future approaches to overcoming IMiD resistance may therefore call for targeting a combination of CRBN pathway-dependent and -independent mechanisms. This would entail adopting personalised multi-drug strategies that concurrently target individually-relevant pathways. With the immense array of possible target combinations, predicting the optimal drug combination for an individual patient presents a future trajectory for optimal

therapy. In this era of artificial intelligence, it is likely to require deep machine learning techniques to effectively map individualized drug-drug interactions, rank different drug combinations and determine a more accurate drug dosage for optimal clinical efficacy. Such an approach has been successfully adopted in our previous proof-of-concept study in MM and lymphoma[95, 96].

Next-generation cereblon targeting agents, CELMoDs (iberdomide and mezigdomide), are in ongoing clinical trials in MM as a means to overcome IMiD resistance. They promise higher potency, more robust degradation of known targets and an expanded repertoire of neo-substrates targets[97, 98]. Compared to IMiDs, the binding affinity of Iberdomide for CRBN was 20-fold higher (IC50 for CRBN binding was ~3uM and ~0.15uM, respectively) which leads to a more rapid IKZF1/3 degradation and therefore, enhanced treatment efficacy[99]. Iberdomide and mezigdomide have both shown meaningful clinical outcome in heavily pre-treated IMiD-refractory MM patients with ORR 26% for the former and 40% for the latter[100, 101]. However, this also implies that up to 60% of IMiD-refractoriness cannot yet be rescued by next generation counterparts. Given that CELMoDs share the same mechanisms of action as IMiDs, it remains to be seen whether any particular CRBN-dependent or -independent mechanisms also contribute to CELMoDs' therapeutic insufficiency[102-104]. The comprehensive understanding of the range of biology of IMiD resistance therefore holds the key in facilitating the successful integration of these new agents into clinical practice.

Conclusion

IMID resistance associated with CRBN pathway has been extensively reviewed previously[12-14]. In this review, we extended our discussion into several other important aspects including potential CRBN pathway-independent IMiD resistance mechanisms, ranging from the cell intrinsic IMiD-resistance to the extrinsic components in the TME. The transformation of IMiDs from a teratogenic "dark remedy" to the pioneering standard-of-care treatment today in MM marks a significant shift in treatment paradigms, but lasting responses are inevitably

hindered by acquired resistance which remains an unmet need. As we move forward, a clearer understanding of which resistance mechanisms are clinically relevant and why, will lead us to new avenues for personalized and effective therapeutic interventions as we manoeuvre the challenging IMiD landscape, with IMiD resistance and the differential impact of new agents across age gaps[41] in particular, and so better translates these promising results from clinical trial to real world practice[105].

Abbreviations

ADAR1: Adenosine deaminase acting on RNA

ARID2: AT-rich interactive domain 2 (ARID2)

ASCT: Autologous stem cell transplantation

ATAC-Seq: Assay for transposase-accessible chromatin with sequencing

ATRA: All-trans retinoic acid

BM: Bone marrow

BMSCs: Bone marrow stromal cells

C2H2: Cys2-His2

CCF: Cancer clonal fraction

CCL20: Chemokine (C-C motif) ligand 20

CDK6: Cyclin dependent kinase 6

CELMoDs: Cereblon E3 ligase modulators

ChIP-seq: Chromatin immunoprecipitation sequencing

CircRNAs: Circular RNAs

CRBN: Cereblon

CSC: Cell surface capture

CSN: COP9 signalosome

CUL4A/B: Cullin-4A/B

DDB1: DNA damage-binding protein 1

DFCI: Dana-Farber Cancer Institute

ERK: Extracellular signal-regulated kinase

EV: Extracellular vesicles

FDA: Food and Drug Administration

FGFs: Fibroblast growth factors

GL1: Glioma-associated oncogene homolog 1

IFM: Intergroupe Francophore du Myelome

IFN: Interferon

IKZF1: Ikaros

IKZF3: Aiolos

IL-2: Interleukin-2

IL-6: Interleukin-6

IMiDs: Immunomodulatory drugs

KPNA2: Karyopherin subunit alpha 2

LAG3: Lymphocyte activating 3

LAMP2: Lysosomal associated membrane protein 2

LenRes: Lenalidomide-resistant

LOF: Loss-of-function

MEK1: Mitogen-activated protein kinase 1

MM: Multiple Myeloma

MMRF: Multiple Myeloma Research Foundation

MRD: Minimal residual disease

NF-κB: Nuclear factor κB

NK: Natural Killer

NSD2: Nuclear receptor binding SET domain protein 2

OS: Overall survival

PD-1: Programmed cell death 1

PFS: Progression-free survival

PI: Proteasome inhibitors

PKCα: Protein kinase C alpha

PROTACs: Proteolysis-targeting chimaeras

ROC1: Regulator of cullins 1

RRMM: Relapsed/Refractory Multiple Myeloma

SALL4: Sal-like protein 4

SDC1: Syndecan-1

SMAD3: SMAD family member 3

SORT1: Sortilin 1

TCR: T cell receptor

Th1: Type 1 helper

Th2: Type 2 helper

TME: Tumor microenvironment

TNF-α: Tumor necrosis factor alpha

TRAF2: TNF receptor-associated factor 2

Tregs: T-regulatory cells

UBE: E2 ubiquitin ligase protein

VEGFs: Vascular endothelial growth factors

WGS: Whole-genome sequencing

ZNF91: Zinc finger protein 91

ZNF827: Zinc finger protein 827

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Table 1. Immunomodulatory drugs (IMiD) resistance associated with Cereblon (CRBN) pathway

No.	Study details	Key findings	Ref.
1.	Gene-expression profiling of MM patients (HOVON-65/GMMG-HD4 trial) on thalidomide maintenance (n = 96)	**Decreased *CRBN*** gene expression was significantly associated with poorer PFS	[37]
2.	qPCR of paired MM patients' samples at pre-treatment and at lenalidomide resistance (n = 9)	20-90% **reduction of CRBN** expression in 8 patients and 2-fold increase in 1 patient	[29]
3.	Targeted sequencing of paired MM patients' samples at pre- and post-lenalidomide treatment (n = 25)	68% of post-lenalidomide patients showed **reduced CRBN** expression while 32% showed increased expression	[39]
4.	Immunohistochemistry of paired MM patients' samples at diagnosis and at lenalidomide-refractory (n = 55)	77% lenalidomide-refractory patients had **reduced CRBN** expression with a median decrease of 53.1% (range: 6.6% - 99.2%) whereas 23% patients had no decrease in CRBN expression **IKZF1, IKZF3 and IRF4** protein expression: **Unchanged** at lenalidomide-refractory **c-Myc** protein expression: Slight **increase** at lenalidomide-refractory	[31]

5.	• **WGS** (N=455): Newly diagnosed (n=198), lenalidomide-refractory (n=203) and pomalidomide-refractory cohorts (n=54) • **RNA-seq:** Newly diagnosed (n=437), lenalidomide-refractory (n=176) and pomalidomide-refractory (n=42)	Overall incidence of ***CRBN*** abnormalities: 20.7% of lenalidomide-refractory cases 29.6% of pomalidomide refractory cases Breakdown of ***CRBN*** **abnormalities** • *CRBN* **mutations**: Newly diagnosed (0.5%), lenalidomide-refractory (2.2%), pomalidomide-refractory (9%) • *CRBN* **gene copy loss**: Newly diagnosed (1.5%), lenalidomide-refractory (7.9%), pomalidomide-refractory (24%) • **Exon 10 splicing:** Increased ratio of spliced transcript/full length from newly diagnosed to lenalidomide- and pomalidomide-refractory patients	[25]
6.	Targeted sequencing of IMiD-refractory patients (n = 50)	Genomic mutations in ***CRBN*** (12%), ***IKZF1*** (2%), ***IRF4*** (4%) and ***CUL4B*** (6%)	[26]
7.	WGS (N=522) : Newly diagnosed (n=198), lenalidomide-refractory	Mutation or copy loss in **CSN members**	[28]

	(n=269) and pomalidomide-refractory cohorts (n=55)	• ***COPS3:*** newly diagnosed (14%), lenalidomide-refractory (26%), pomalidomide-refractory (22%) • ***COPS4:*** newly diagnosed (10%), lenalidomide-refractory (14%), pomalidomide-refractory (11%) • ***COPS5:*** newly diagnosed (5%), lenalidomide-refractory (8%), pomalidomide-refractory (3%) • ***COPS6:*** newly diagnosed (1%), lenalidomide-refractory (2%), pomalidomide-refractory (3%) • ***COPS7A:*** *n*ewly diagnosed (17%), lenalidomide-refractory (20%), pomalidomide-refractory (19%) • ***COPS7B:*** newly diagnosed (6%), lenalidomide-refractory (12%), pomalidomide-refractory (19%) • ***COPS8:*** newly diagnosed (8%), lenalidomide-refractory (16%), pomalidomide-refractory (22%) Mutation or copy loss in **UBE2:** • ***UBE2GD3:*** newly diagnosed (5%), lenalidomide-refractory (9%) • ***UBE2G1:*** newly diagnosed (14%),	

		lenalidomide-refractory (28%), pomalidomide-refractory (25%)	

CRBN*:** Cereblon; ***IMiDs*:** Immunomodulatory drugs; ***MM*:** Multiple Myeloma; ***qPCR: Quantitative Polymerase Chain Reaction; ***WGS:*** Whole-genome sequencing

Table 2 Cereblon (CRBN) pathway-independent evidences in Immunomodulatory drugs (IMiD) resistance

No.	CRBN pathway-independent mechanisms/features	Types of IMiD	Study model/cohort	Key findings	Ref.
Intrinsic mechanisms					
1.	IL-6/STAT3 pathway	Lenalidomide	aCGH, mRNA-seq, (XG1 parental vs acquired-Len-resistance)	High IL-6/STAT3 signalling led to sustained expression of IRF4 in len-resistant cells Introduction of exogenous CRBN failed to restore lenalidomide sensitivity	[47]
2.	Wnt/β-catenin signalling	Lenalidomide	Affymetrix microarray GEP Lenalidomide-refractory (n=26) vs IMiD-naive	**Increased Wnt/β-catenin activity** resulted in upregulation of Myc **Significant increase in CD44** (downstream target of Wnt/β-catenin)	[48] [49]

			(n=13).	surface expression in lenalidomide-refractory patients	
3.	MEK/ERK pathway	Lenalidomide; Pomalidomide	Mouse xenograft model	Lenalidomide- or pomalidomide-resistant plasmacytomas showed **robust stabilisation of pERK1/2** compared to responsive tumors	[53]
		Lenalidomide; Pomalidomide	Genome-wide CRISPR-Cas9 KO screen	TNF-α and IL-6 in the BM milieu induced proteasome degradation of **TRAF2** and activation of the MEK/ERK and NF-κB pathways. *TRAF2* knockout showed no effect on CRBN expression and degradation of IKZF1/3 and IRF4.	[55]

			IHC of newly diagnosed vs refractory to single-agent lenalidomide maintenance therapy (n = 6)	Lower expression of TRAF2 protein at the time of relapse.	
		Lenalidomide	RNA-seq of MM patients (n=69) at first relapse	Nearly universal ERK pathway activation at relapse on lenalidomide maintenance therapy	
4.	Epigenetic alterations	Lenalidomide; Pomalidomide	Genome-wide methylation array; RNA-seq	Acquired lenalidomide- and pomalidomide-resistant-OPM2 and -H929 displayed global **increased DNA methylation, and reduced chromatin accessibility** and gene expression with SMAD3 being	[57]

				commonly downregulated IMiD-resistant cell lines No alteration in chromatin accessibility or DNA methylation profile of *CRBN*, *IKZF1*, *IKZF3* and *IRF4*.	
		Lenalidomide	ChIP-seq in 16 MM cell lines: Lenalidomide-resistant (AMO1, JJN3, KMS-12-BM, SKMM2, XG1, XG5, XG7, XG12, XG20 and XG21) vs. Lenalidomide-sensitive (OPM2, RPMI8226, XG2, XG6, XG13 and XG19)	Lenalidomide-resistant cells displayed mixture of **enrichment/ depletion of the active H3K4me3 marks** Differential H3K4me3 sites enriched in lenalidomide-resistant HMCLs were associated to interferon signaling and cytokine signaling	60

				CUL4B gene, but not other CRBN complex genes, in lenalidomide-sensitive cells was enriched with H3K4me3 mark	
		Lenalidomide; Pomalidomide	WES	-Continuous IMiDs treatment induced loss-of-function mutation and **downregulation of NCOR2 leading to MYC upregulation, via increased CD180 expression,** independent of CRBN mechanisms	[61]
5.	IKZF1/3 transcription factor redundancy for maintenance of c-MYC and IRF4 expression	Lenalidomide; Pomalidomide	RNA-seq, ATAC-Seq, ChIP-seq	92% of the pomalidomide-treated MM cell lines remained highly viable despite significant downregulation of	[62]

				IKZF1/3. Several inherently IMiD-resistant cell lines expressed **high levels of the AP-1 factor BATF.** BATF heterodimerization sustained IRF4 expression, compensating for IMiD-induced loss of IKZF1/3	
			Paired newly diagnosed vs RRMM patients treated with IMiD (n = 35)	Significant upregulation of BATF upon relapse.	
			MMRF CoMMpass dataset (n = 484)	BATF expression is associated with poorer survival outcomes.	
		Lenalidomide	ChIP-seq, ATAC-seq,	**ETV4** bound to the same enhancers as	63

			RNA-seq	IKZF1 ETV4 maintained **BRD4** and **P300** occupancy and oncogenic enhancer function to compensate for the IMiD-mediated loss of IKZF1/3 in IMiD-resistant cells to drive MYC overexpression.	
			MMRF CoMMpass (36 paired samples) and POLLUX (14 paired samples)	*ETV4* expression was associated with poorer PFS and overall survival. *ETV4* was significantly upregulated at the time of relapse. No change in the expression of *IKZF1*, *IKZF3*, *IRF4* or *MYC*.	
6.	High-risk MM	Lenalidomide;	Longitudinal	Significant	[65, 66]

	markers (gain/amp(1q21) and del(17p)	Pomalidomide	genomic analysis of relapsed MM patients (n = 386)	increasing trend of **gain/amp(1q21) and del(17p)** in IMiD-refractory MM cases **High ADAR1 and hyperedited transcriptome in 1q21(gain/amp)** is associated with reduced response to IMiDs	[67]
7.	Differential expression pattern of genome-wide circRNAs	Lenalidomide; Pomalidomide	RNA-sequencing	More **upregulated than downregulated circRNAs** in IMiD-resistant vs -sensitiv cells Major overlap in the specific circRNAs upregulated upon acquired lenalidomide and pomalidomide resistance	[69]

8.	CDK6 activity	Lenalidomide; Pomalidomide	Proteomics and RNA sequencing (pre-treatment and relapsed patient samples)	**CDK6:** **-Upregulated in IMiD- based-RRMM** patients CRBN: -No change in RNA, protein or phosphorylation levels -No genetic alterations in the $CRL4^{CRBN}$ E3 ligase complex	[70]
9.	Modulation of cell surface expression	Lenalidomide	RRMM (n = 15) vs newly diagnosed (n = 90)	**Loss of CD138** surface expression Significant increase in CD138-negative cells in RRMM patients Ex vivo cultured lenalidomide-RRMM cells from a single patient had low CD138 expression that was associated	[72]

				with an immature phenotype, downregulation of IRF4 and increased BCL6 expression.	
		Lenalidomide	Glycoprotein cell surface capture (CSC) proteomics analysis CoMMpass dataset (IA14): Paired diagnosis-first-relapse samples (lenalidomide-based induction regimens)	Acquired lenalidomide-resistant-OPM2 and -H929 showed **increased CD33 and CD45/PTPRC expression.** *CD33* and *PTPRC* mRNA expression was significantly increased at first relapse	74
Extrinsic mechanisms					
1.	Tumor-TME crosstalk	Lenalidomide	WGS	Failed MRD	80

			(MANHATTAN clinical trial (n = 49)	negativity was associated with shorter PFS, high APOBEC mutational activity, 1p22 (*RPL5*) deletions, *IKZF3* loss, **low CD14^{+}monocyte, T cell exhaustion, persistence of IFNγ-expressing NK cells and decreased TCR diversity**	
2.	Exhausted T cell phenotype	Lenalidomide	Immune-profiling on lenalidomide-resistant MM patients	Significant expansion of exhausted effector T cell populations expressing **elevated levels of LAG3 and PD-1.**	[82]
3.	Downregulation of chemokine, CCL20	Lenalidomide	Illumina Gene expression microarray	**Downregulation of CCL20** in U266 Len-resistant cells and in lenalidomide	[84]

				relapsed/refractory MM cases (n = 5). Addition of CCL20 increases MM cell sensitivity to lenalidomide	
4.	Elevated lactate level in the TME	Lenalidomide	Metabolomics	High-risk MM t(4;14) demonstrated **elevated plasma lactate levels** Lactate is a differential metabolite associated with **PKCα.** Knockdown of PKCα increases the sensitivity to lenalidomide, independent of the CRBN-IKZF1/3 axis	[85]
5.	Increased extracellular vesicles	Lenalidomide	RNA-seq	Increased expression of	[88]

	secretion and enhanced adherence abilities			**SORT1 and LAMP2**, core regulatory genes governing **extracellular vesicles** secretion in Len-resistant cells. Knockdown of SORT1 or LAMP2 reduced EV secretion, decreased cell adhesion and restored lenalidomide sensitivity in resistant cells without affecting CRBN expression. High SORT1 and LAMP2 expression are associated with poor survival in patients treated with lenalidomide	
			Analysis of GSE19784, n = 300 and GSE136324, n > 200		

aCGH: Array Comparative Genome Hybridisation; ***ATAC*-Seq:** Assay for transposase-accessible chromatin with sequencing; ***ChIP-seq*:** Chromatin immunoprecipitation sequencing; ***CircRNAs*:** Circular RNAs; ***CRBN*:** Cereblon; **GEP:** Gene Expression Profiling; ***IMiDs*:** Immunomodulatory drugs; ***Len:*** Lenalidomide; ***MMRF*:** Multiple Myeloma Research Foundation; ***MRD*:** Minimal residual disease; ***RRMM*:** Relapsed/Refractory Multiple Myeloma; ***TME*:** Tumor microenvironment; ***WGS*:** Whole-genome sequencing

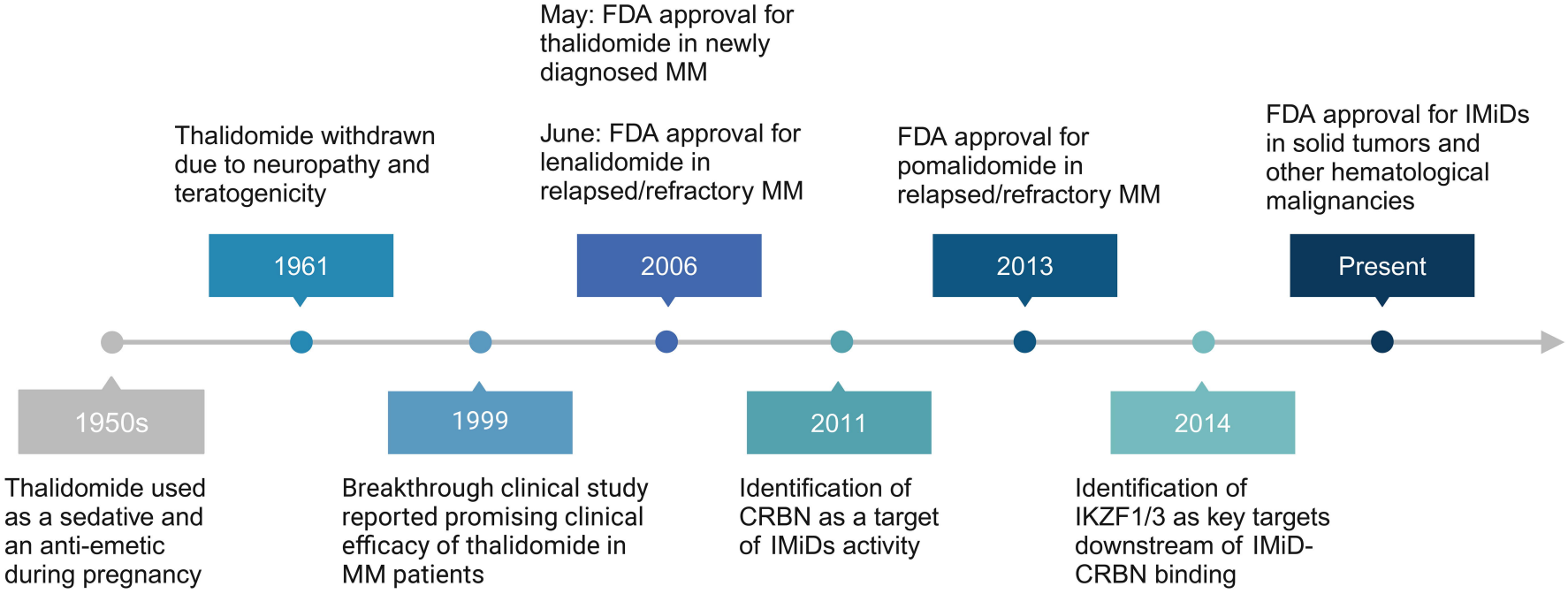
Figure legends

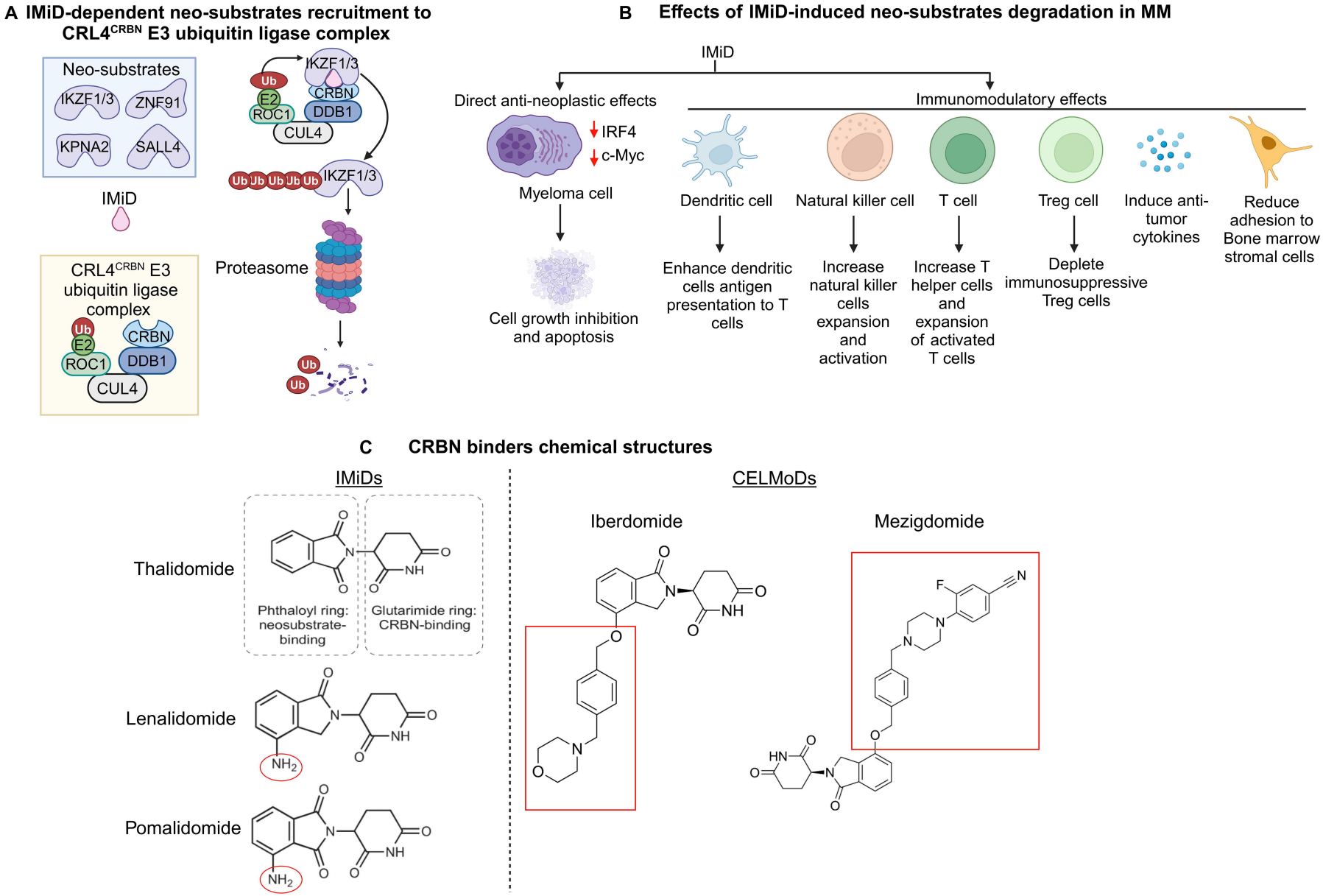
Figure 1. Timeline of the development of immunomodulatory drugs (IMiDs). The historical timeline of IMiDs development over the years from multiple myeloma (MM) to its therapeutic application in different malignancies.

Figure 2. Modes of actions of IMiD. A, IMiD act as a 'molecular glue' to mediate recruitment of neo-substrates to the $CRL4^{CRBN}$ E3 ubiquitin ligase which results in neo-substrate ubiquitylation and proteasome degradation. **B**, Summary of the effects of IMiD-induced neo-substrates degradation in MM which includes direct anti-neoplastic and immunomodulatory effects.

Figure 3. Intrinsic CRBN-independent IMiD resistance mechanisms. Intrinsic factors that contribute to IMiD resistance beyond the CRBN pathway include aberrant activation of signalling pathways (such as IL-6/STAT3, Wnt/β-catenin and MEK/ERK), epigenetic alterations, transcription factors redundancy, high-risk prognostic markers (such as gain/amp(1p21) and del(17p)), differential expression of circRNAs, CDK6 overexpression and dysregulated cell surface receptors.

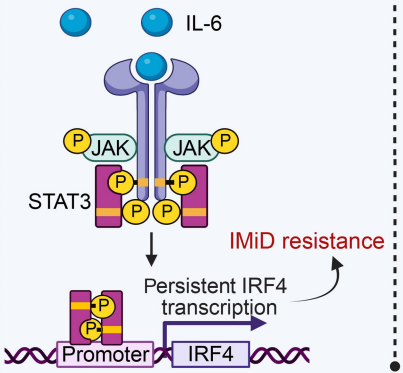
Figure 4. Extrinsic mechanisms independent of CRBN pathway. Potential tumor microenvironment (TME) mechanisms of IMiD resistance which include composition of the immune cells in the TME with tumor-acquired genetic features, expansion of exhausted T cell population, downregulation of CCL20, elevated lactate levels in the TME and increased secretion of extracellular vesicle (EV)-mediated MM cell adhesion and resistance.


1950s
Thalidomide used as a sedative and an anti-emetic during pregnancy
1961
Thalidomide withdrawn due to neuropathy and teratogenicity
1999
Breakthrough clinical study reported promising clinical efficacy of thalidomide in MM patients
2006
May: FDA approval for thalidomide in newly diagnosed MM
June: FDA approval for lenalidomide in relapsed/refractory MM
2011
Identification of CRBN as a target of IMiDs activity
2013
FDA approval for pomalidomide in relapsed/refractory MM
2014
Identification of IKZF1/3 as key targets downstream of IMiD-CRBN binding
Present
FDA approval for IMiDs in solid tumors and other hematological malignancies

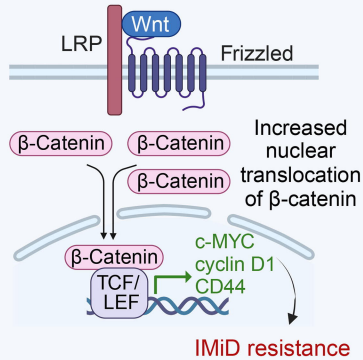

A IMiD-dependent neo-substrates recruitment to CRL4CRBN E3 ubiquitin ligase complex
Neo-substrates
IKZF1/3
ZNF91
KPNA2
SALL4
IMiD
CRL4CRBN E3 ubiquitin ligase complex
Ub
E2
ROC1
CUL4
CRBN
DDB1
IKZF1/3
Proteasome
B Effects of IMiD-induced neo-substrates degradation in MM
IMiD
Direct anti-neoplastic effects
IRF4
c-Myc
Myeloma cell
Cell growth inhibition and apoptosis
Immunomodulatory effects
Dendritic cell
Enhance dendritic cells antigen presentation to T cells
Natural killer cell
Increase natural killer cells expansion and activation
T cell
Increase T helper cells and expansion of activated T cells
Treg cell
Deplete immunosuppressive Treg cells
Induce anti-tumor cytokines
Reduce adhesion to Bone marrow stromal cells
C CRBN binders chemical structures
IMiDs
CELMoDs
Thalidomide
Phthaloyl ring: neosubstrate-binding
Glutarimide ring: CRBN-binding
Lenalidomide
Pomalidomide
Iberdomide
Mezigdomide

Aberrant signaling pathways:

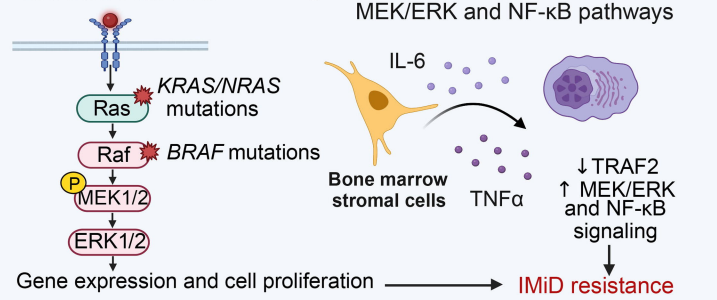
IL-6/STAT3 signaling



Wnt/β-catenin signalling

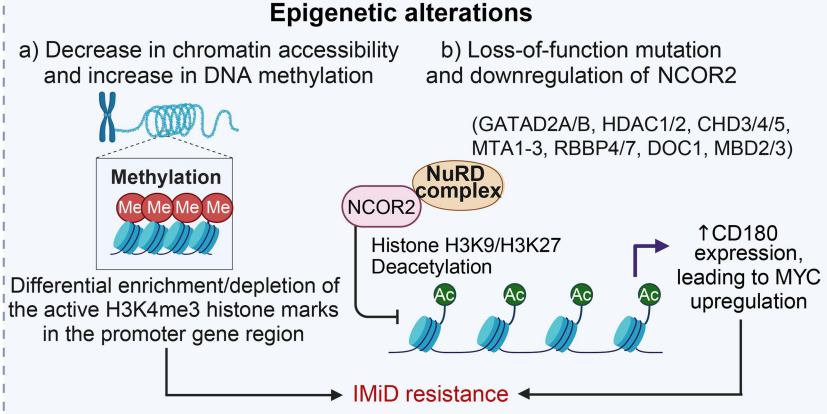


MEK1/ERK pathway

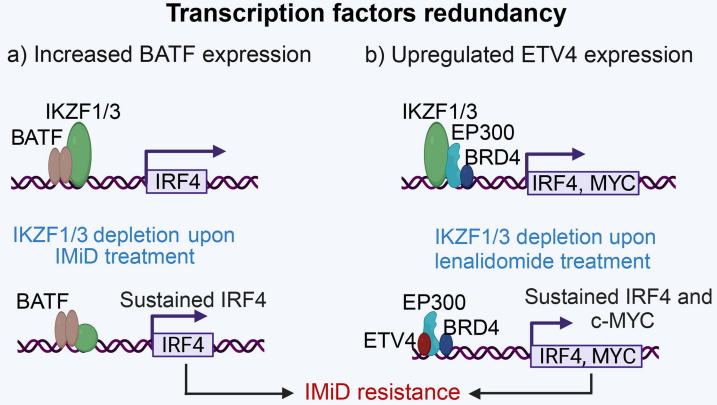


Gene regulatory factors:

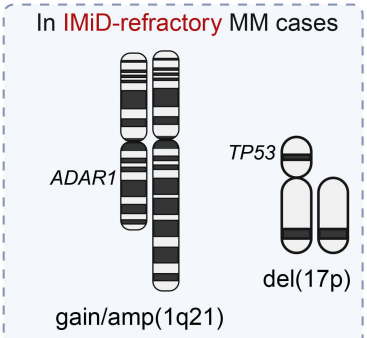
Epigenetic alterations



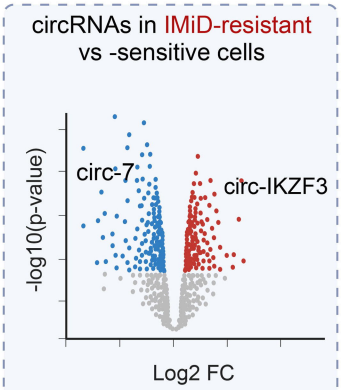
Transcription factors redundancy



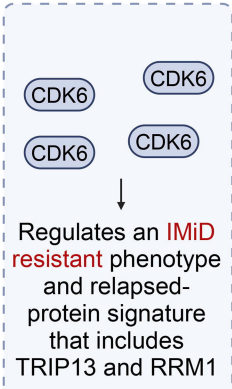
High-risk MM markers



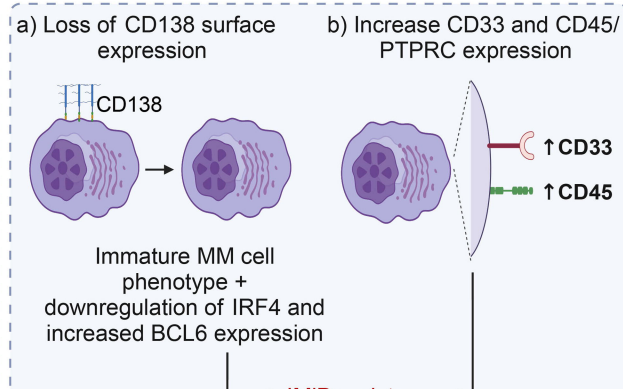
circRNAs expression

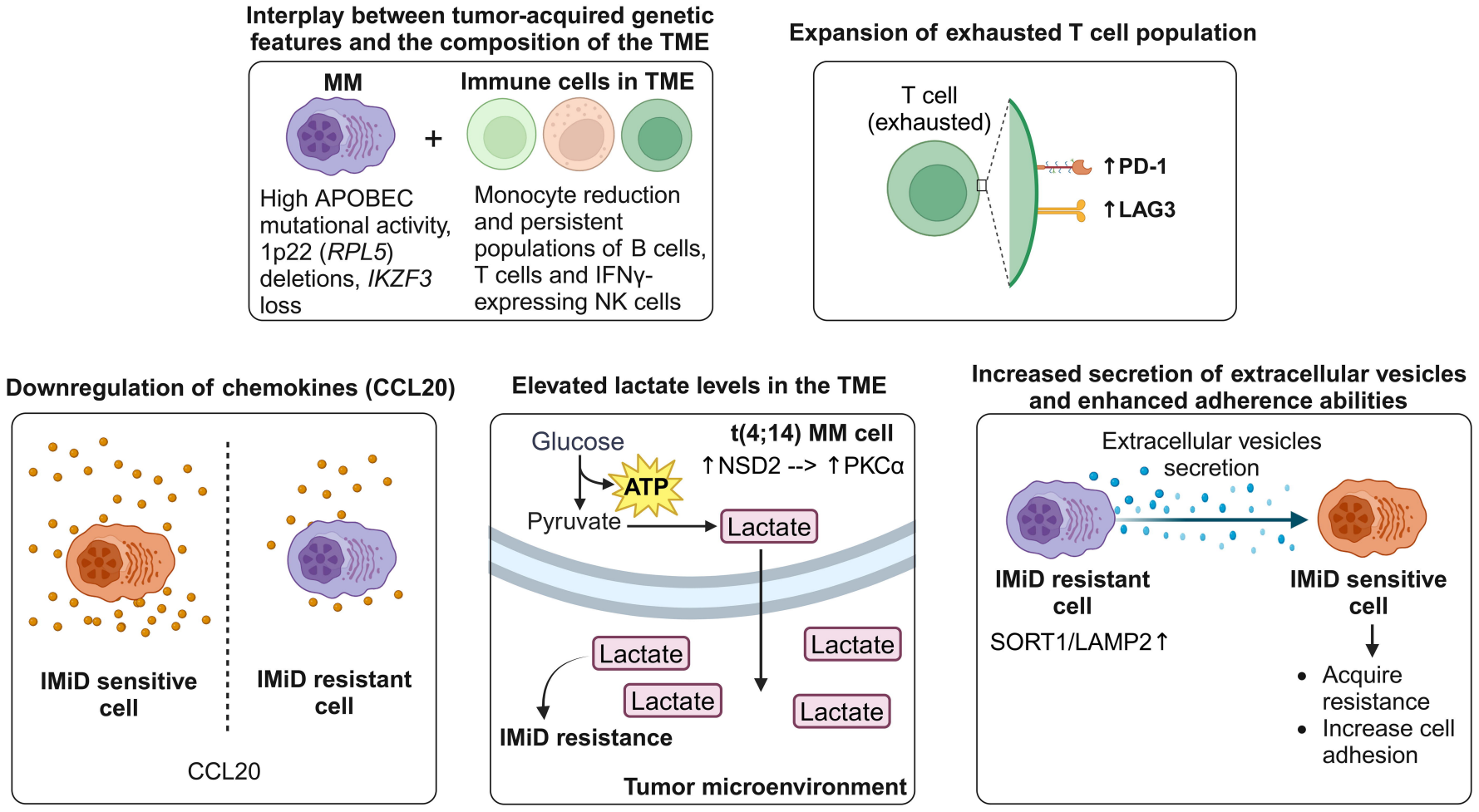


CDK6 upregulation



Modulation of cell surface receptors:




Interplay between tumor-acquired genetic features and the composition of the TME
MM
Immune cells in TME
+
High APOBEC mutational activity, 1p22 (RPL5) deletions, IKZF3 loss
Monocyte reduction and persistent populations of B cells, T cells and IFNγ-expressing NK cells
Expansion of exhausted T cell population
T cell (exhausted)
↑PD-1
↑LAG3
Downregulation of chemokines (CCL20)
IMiD sensitive cell
IMiD resistant cell
CCL20
Elevated lactate levels in the TME
Glucose
ATP
Pyruvate
Lactate
t(4;14) MM cell
↑NSD2 --> ↑PKCα
Lactate
Lactate
Lactate
Lactate
IMiD resistance
Tumor microenvironment
Increased secretion of extracellular vesicles and enhanced adherence abilities
Extracellular vesicles secretion
IMiD resistant cell
SORT1/LAMP2↑
IMiD sensitive cell
Acquire resistance
Increase cell adhesion