Chapter

Programmed Cell Death Deregulation in BCR-ABL1-Negative Myeloproliferative Neoplasms

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Abstract

BCR-ABL1-negative myeloproliferative neoplasms are classically represented by primary myelofibrosis, polycythemia vera, and essential thrombocythemia. These entities are stem cell-derived clonal disorders characterized by hematopoietic progenitor autonomy or hypersensitivity to cytokines, most of them presenting mutations in Janus kinase 2 (*JAK2*), calreticulin (*CALR*), or myeloproliferative leukemia virus oncogene (*MPL*). Deregulation of pro- and antiapoptotic genes is also claimed as an important mechanism involved in cell resistance to cell death and accumulation of myeloid cells in myeloproliferative neoplasms. Apoptosis, as one of the best-characterized types of programmed cell death, has a clear role in hematopoiesis control. However, the exact pathways affected in BCR-ABL1-negative myeloproliferative neoplasms have not yet been fully clarified. This chapter will explore the modifications affecting programmed cell death pathways involved in myeloid proliferation and how these alterations might be exploited in single or combined targeted therapeutic strategies.

Keywords: apoptosis, programmed cell death, cancer stem cells, hematological disorders, cell death mechanisms, molecular interactions, cytokine signaling, cell-cycle inhibitors

1. Introduction

Hematopoiesis is a highly controlled process that ensures the differentiation of the hematopoietic stem cells (HSCs) into lymphoid and myeloid common progenitors and further into all lineages of blood cells [1].

Programmed cell death (PCD) is one of the fundamental mechanisms of an organism's life cycle that controls every system, including hematopoietic system, based on a precisely tuned signaling network. Apoptosis, the most important type of PCD, maybe because it is the most analyzed type of death to date, is well

described in hematopoietic differentiation [2]. Its deregulation in pathological circumstances is potentially deleterious and may influence the fate of the entire organism. Although different other types of PCD were described, apoptosis remains one of the most important processes involved in differentiation and cell survival regulation, while mechanisms as autophagy and necroptosis look like "backup" mechanisms that share some "key players" and diverged from apoptosis at a certain point, to assure the elimination of the malfunctioning system in case of "internal" defect (mutations) or pathogens that inhibit the components of the apoptotic network [3, 4].

Primary myelofibrosis (PMF), polycythemia vera (PV), and essential thrombocythemia (ET) are classic BCR-ABL1-negative myeloproliferative neoplasms (MPNs) that are stem cell-derived clonal disorders characterized by hematopoietic progenitor autonomy or hypersensitivity to cytokines, driven by acquired somatic mutations in critical pathways, resulting in pathological expansion of the myeloid lineages. In their natural course, MPNs could be exacerbated and transformed into secondary acute myeloid leukemia (sAML) associated with treatment resistance and poor clinical outcome [5].

This chapter will explore the most important modifications affecting programmed cell death pathways involved in myeloid proliferation, and how these alterations might be exploited in single or combined targeted therapeutic strategies in a classic BCR-ABL1-negative MPN.

2. Intrinsic and/or extrinsic apoptotic pathways involved in MPN disease entities

Overall, the hematopoietic homeostasis requires a precise balance between blood cell formation and maintenance of an adequate number of mature cells. Although apoptosis is necessary to prevent the excessive accumulation of cells, the hematopoietic progenitors need to be protected and preserved. A disruption of the homeostatic balance in the hematopoietic system is relevant for many hematological disorders, an increased cell death being involved in the etiology of immune deficiencies and anemia, while an inappropriate resistance to apoptosis might lead to hematological malignancies [6], such as MPNs.

As a particular form of PCD, apoptosis is activated via two convergent pathways: the intrinsic and the extrinsic [7]. The intrinsic signaling pathway is triggered at mitochondrial level in response to various stimuli such as genotoxic agents or growth factor deprivation, and it is mainly regulated by the members of BCL-2 protein family that contain one or more BCL2 homology (BH) domains [8]. These proteins are structurally and functionally classified into three groups. The first group includes the critical effectors of the intrinsic pathway, namely BCL-2 antagonist killer 1 (BAK) and BCL-2-associated X protein (BAX). The second group is represented by the prosurvival BCL-2 proteins (BCL-2, BCL-xL, MCL-1, BCL-W, and A1) that hinder BAK and BAX activation, while the third group comprises several structurally different proteins, known as "BH3-only" proteins (BIM, BID, BAD, BIK, PUMA, and NOXA), which share solely a sequence called BH3-domain [8, 9]. Cellular stress signals are sensed by the "BH3-only" proteins that directly activate BAK and BAX or neutralize the prosurvival proteins. Once activated, through conformational changes, BAK and BAX induce the permeabilization of the mitochondrial membrane with subsequent release of apoptogenic factors, such as cytochrome c and second mitochondrial activator of caspases/direct IAP binding protein with low pI (SMAC/DIABLO). Cytochrome c binds to the apoptotic protease activating factor-1 (APAF-1) and forms the apoptosome, a heptameric

complex that activates the initiator caspase-9, followed by activation of the effector caspase-3, caspase-6, and caspase-7 that trigger final events of apoptosis [10, 11].

In the extrinsic apoptotic pathway, caspase activation is elicited at the level of "death receptors" (DR), transmembrane proteins of the tumor necrosis factor (TNF) receptor superfamily typically represented by FAS (CD95), TNF receptors, and TNF-related, apoptosis-inducing ligand (TRAIL) receptors. Through interaction with their corresponding ligands—FASL, TNF-α, and TRAIL, respectively—DR become activated, leading to the recruitment of a death adaptor protein, such as FAS-associated death domain (FADD) or TNFR-1-associated death domain (TRADD). Death adaptors generate a death-inducing signaling complex (DISC), in which procaspase-8 is recruited and activated, the death signal being subsequently transduced to the effector caspases [2, 10].

A very early apoptosis event is the global and rapid mRNA degradation by a mechanism that is not yet completely characterized [12].

Various factors associated with intrinsic and extrinsic apoptotic pathways have been involved in the control of adult hematopoiesis under physiological as well as pathological conditions [2]. In this respect, BCL-2 protein family members play different roles across individual hematopoietic lineages during differentiation and maturation. At the level of HSCs and early myeloid progenitors, MCL-1 is an essential prosurvival factor, being upregulated by stem cell factor and interleukin-3 via JAK/STAT (Janus-activated kinase/signal transducers and activators of transcription) and AKT signaling pathways [13, 14]. During erythropoiesis, erythropoietin (EPO) ensures erythroid progenitor survival, proliferation, and differentiation by acting on its cognate receptor (EPO-R) and inducing JAK2-STAT5 activation that leads to upregulation of BCL-xL [10]. The development, maturation, and survival of megakaryocytes (MKC) is strictly dependent on the presence of both BCL-xL and MCL-1 proteins that are induced by thrombopoietin (TPO) signaling and restrain intrinsic apoptosis, while platelet life span seems to be dictated only by BCL-xL levels [7, 15, 16]. Similarly, MCL-1 is essential for granulocyte progenitor survival and differentiation [16]. On the other hand, the receptor/ligand interactions of the TNF family represent physiological mechanisms that exert a negative regulation in the terminal stages of the hematopoietic differentiation, controlling in this way the size of the expanding hematopoietic clones and maintaining heterogeneity in response to various demands [17].

PV is characterized by erythrocytosis accompanied by a suppressed endogenous EPO production. It often associates thrombocytosis and/or leukocytosis with panmyelosis at bone marrow examination. The pattern of driver mutations is strikingly dominated by *JAK2* V627F that is present in more than 95% of patients, the rest being represented by *JAK2* exon 12 mutations [18].

A study that analyzed gene expression profile of granulocytes isolated from PV patients showed an upregulation of protease inhibitors with affinity for proteases inducing apoptosis in neutrophils (e.g., cystatin F and secretory leukocyte protease inhibitor), as well as of several antiapoptotic and survival factors (e.g., p38 MAPK), compared to granulocytes obtained from healthy subjects [19]. Also, unlike the granulocytes of ET patients or normal controls, the granulocytes of PV patients were found to express an increased amount of heat shock protein 70 (HSP70), which counteracts apoptosis at different levels by preventing BAX translocation to mitochondria, inhibiting APAF-1 and procaspase-9 recruitment to apoptosome, and reducing caspase activation. As shown in primary cell cultures, an HSP-70 inhibitor was able to induce apoptosis in the erythroid lineage [20].

Concerning the extrinsic apoptosis pathway, it was found that erythroblasts isolated from PV patients carrying *JAK2* V617F mutation exhibited an increased resistance to death receptor-induced apoptosis being able to generate elevated red

blood cell counts in the presence of CD95 and TRAIL receptor stimulation. In addition, the *JAK2* mutation was correlated in PV erythroblasts with an overexpression of c-FLIPshort, a potent cellular inhibitor of extrinsic apoptosis [21]. Also, Tognon et al. reported a dysregulated expression of genes related to extrinsic apoptosis (*FAS*, *FASL*, *FAIM*, *C-FLIP*, *TRAILRl/DR4*, and *TRAILR2/DR5*) in bone marrow CD34+ cells and peripheral blood leukocytes obtained from patients with different MPN phenotypes, including PV [22].

ET is defined by thrombocytosis associated with normocellular bone marrow and hyperplasia of enlarged MKC. The molecular profile of ET consists of *JAK2* V617F mutation (in 60–65% of patients), *CALR* exon 9 indels (in 20–25% of patients), *MPL* exon 10 mutations (in about 4–5% of patients), and very rare non-canonical *MPL* mutations (in less than 1% of patients). About 10% of ET patients lack these mutations being considered triple-negative cases [18].

Before the discovery of *JAK2* V617F, in order to gain insight into the molecular mechanisms of ET megakaryopoiesis, Tenedini et al. have employed microarray technology to study the gene expression profiles of bone marrow CD34-derived MKC from ET and healthy individuals. They found in ET a downregulation of the proapoptotic genes *BAX*, *BNIP3*, and *BNIP3L*, as well as of the genes encoding for components of the mitochondrial permeability transition pore complex, along with the upregulation of the antiapoptotic and survival genes *IGF1R*, *CFLAR (C-FLIP)*, and *SDF1*. Also, ET MKC exhibited in cell cultures an increased resistance to apoptosis, relative to their normal counterparts [23].

In a study that aimed to characterize the immunophenotypic apoptotic profiles of MKC on bone marrow biopsy samples obtained from MPN patients, it was observed that ET MKC displayed an antiapoptotic pattern, characterized by an overexpression of BCL-xL and a lower expression of BAX, compared to those of PMF patients [24]. Furthermore, Treliński et al. confirmed by flow cytometry the antiapoptotic profile of ET MKC and bone marrow mononuclear cells (BMMC). As opposed to controls, previously untreated ET patients presented significantly lower percentages of apoptotic MKC and BMMCs, when assessed for the number of annexin-V+ and caspase-3+ positive cells. These findings were accompanied by markedly lower BAX levels and BAX/BCL-2 ratios, especially in *JAK2* V617F-negative cases [25].

Compared to PV and ET, PMF is a more heterogeneous disease, being characterized by clonal myeloproliferation, abnormal cytokine expression, early bone marrow fibrosis, anemia, splenomegaly, extramedullary hematopoiesis, constitutional symptoms, and a lower overall survival rate. On the other hand, during the natural course of the disease PV and ET patients might suffer a conversion into secondary myelofibrosis (MF) that resembles PMF [26, 27]. PMF shares with ET a similar profile of mutations in *JAK2*, *CALR,* and *MPL* [18].

Initially, it was suggested that bone marrow MKC in PMF might undergo an increased apoptosis that could be responsible for the release of fibrogenic cytokines [28]. However, further studies have demonstrated that PMF MKC displayed a high proliferative capacity and resistance to apoptosis, explained by the overexpression of BCL-xL [29]. Also, the gene expression analysis of laser-microdissected MKC from PMF patients indicated a tendency toward an overall downregulation of apoptosis-associated genes, especially of *BNIP3* [30].

Chronic inflammation sustained by the continuous release of proinflammatory cytokines and chemokines and subsequent bone marrow microenvironment alterations are considered key factors in PMF pathogenesis. The abnormal production of cytokines that occurs both in malignant and nonmalignant cells was related to an increased JAK2-STAT3 activation and was found responsible for the inhibition of apoptosis and increased myeloproliferation, creating an environment that favors

MPN clone maintenance and expansion [31, 32]. Recently, it was shown that MF cells downregulated the expression of X-linked inhibitor of apoptosis (XIAP) and mitogen-activated protein kinase 8 (MAPK 8), a necessary component of TNF-mediated apoptosis, via a TNF/TNFR2-dependent autocrine loop. This was considered a mechanism to escape an apoptotic response and to increase NF-κB signaling involved in inflammatory cytokine expression [33].

Overall, these data show the importance of the participation of both intrinsic and extrinsic apoptosis pathways in the pathogenesis of MPNs.

3. Key PCD players in BCR-ABL1-negative MPN entities

Modifications occurred in the regulation of apoptosis, especially in expression of pro- and antiapoptotic genes, have great contribution to the myeloaccumulation in MPNs. Concerning the involvement of other types of PCD in myeloproliferations, few data are available. Some key players are involved in apoptosis regulation and also in autophagy or necroptosis. More often, it is a continuous process from apoptosis, autophagy to necroptosis. Increased death signals and stress levels can switch cell death types in the attempt of eliminating the malfunctioning cells [34, 35].

BCL-2 family of proteins is a very important regulator of apoptosis and, at the same time, is also a negative regulator of BECN1/Beclin-1, a key regulator of autophagy [36, 37]. Autophagy was shown to be a major contributor to chemotherapy resistance in AML [38].

BCL-xL promotes cell survival, such as survival of erythroid cells and platelets, and regulates their lifespan at a steady state. Inhibition of BCL-xL induces profound thrombocytopenia by triggered BAK/BAX-mediated mitochondrial damage, caspase activation, and premature death of MKC [39, 40]. In MPNs, a concerted effect resulted from antiapoptotic BCL-xL over-expression and proapoptotic BNIP-3 downregulation was clearly documented [41].

Bcl-2-associated death promoter (BAD) inhibits antiapoptotic proteins BCL-2 and BCL-xL and is involved in initiating the apoptosis process. In unphosphorylated form, BAD forms heterodimers with BCL-2 and BCL-xL, inhibiting their antiapoptotic functions, and facilitates BAX/BAK activation in response to apoptotic stimuli [42, 43], promoting apoptosis. After activation by phosphorylation, BAD forms a heterodimer with 14-3-3 proteins, releasing BCL-2 that is free to block apoptosis. BAD is a substrate of various kinases, such as AKT, protein kinase A (PKA), and c-Jun NH2-terminal kinase (JNK).

Gene expression studies on CD34+ cells and peripheral leukocytes isolated from ET and PMF patients indicated that mRNA levels of *BAX*, *BAD,* and *BIK* were lower in *JAK2* V617F-positive cases than in negative ones and, additionally, displayed a negative correlation with the *JAK2* V617F mutational load. Also, *A1, MCL1, BCLW,* and *BCL-XL* genes have an increased expression in ET and PMF patients compared to controls. As such, Tognon et al. hypothesized that deregulated expression of apoptosis-related genes is linked to myeloaccumulation and pathogenesis of these two disorders [44].

Studies focused on the apoptosis deregulation in PV identified an increased expression of *A1* and *MCL-1* and a reduced expression of proapoptotic *BAD* and *BAX* genes in PV CD34+ cells compared with controls. *A1* expression was also increased, whereas *BAD* and *BAX* mRNA levels were decreased in the leukocytes of PV patients versus healthy subjects [45]. Rubert et al. evaluated the roles of proapoptotic BIM and antiapoptotic MCL-1 in regulating *JAK2* V617F-positive cell survival. JAK2 inhibition modified Bim-EL Ser69 phosphorylation, as well as decreased MCL-1 level, inducing apoptosis. On the other side, MCL-1 depletion

compromised cells' viability and sensitized *JAK2* V617F-positive cells to JAK2 inhibition [46]. Also, mutant JAK2 inhibits the BCL-xL deamidation pathway and the apoptotic response to DNA damage in primary cells from patients PV [47]. *JAK2* V617F-positive ET patients presented markedly higher activation of caspase-3, as well as higher BAX expression than *JAK2* V617F-negative ones [25]. Recent data pointed out that in ET, the MKC exhibit a more proliferative profile, while in MF, they display, in a larger proportion, a defective proapoptotic mechanism [24, 48].

Survivin is one of the inhibitors of apoptosis proteins (IAPs) that regulate cell death through mitochondrial route by restricting the IAP-inhibitor DIABLO protein and preventing it from activating caspase-9. A greater proportion of myeloproliferative MKC express survivin compared to its reciprocal inhibitor, DIABLO. Survivin seems to be the key mediator of the MKC survival signature in the MPNs and might be a potential therapeutic target [41]. Recently, new evidence suggested that survivin may be involved in the evasion of cell death by manipulation of autophagy [49].

BNIP-3 (BCL2/adenovirus E1B 19 kDa protein-interacting protein 3), a pro-apoptotic mitochondrial protein belonging to the BCL-2 family, is activated under hypoxic conditions with hypoxia-inducible factor (HIF-1α) in normal and cancer tissues. BNIP-3 is involved in the induction of hypoxic necrosis in tumors because it activates caspase-independent necrosis-like cell death by opening the mitochondrial permeability transition pore. In MPNs, BNIP-3 expression is reportedly low and this might indicate that the increased bone marrow cellularity is not only because of proliferative signaling but also due to decreased apoptosis [50, 51].

CALR is a multifunctional endoplasmic reticulum (ER) chaperone involved in the quality control of N-glycosylated proteins, calcium storage, and immune responses [27, 52]. In relation to apoptosis, CALR is implicated in the specific activation of caspase-8, BAX, and BAK, and also in the BCL-2 cleavage [53].

Caspase-8, a key factor in the extrinsic pathway, together with caspase-9, a key factor in the intrinsic pathway, is implicated in regulating MKC turnover [41]. *CALR* mutations particularly affect the MKC lineage as indicated by the higher mean number of endogenous MKC colonies in *CALR*-mutant MPNs than those found in *JAK2* V617F-positive and triple-negative cases [54]. Immunohistochemical studies proved that *CALR*-mutated MKC displayed a dysregulated apoptosis with significant reductions in proapoptotic BNIP-3 that could explain the higher platelet number observed in *CALR*-positive MPNs, in contrast to other molecular subtypes [48]. Caspase-8 just like caspase-9 regulates MKC turnover in the MPNs. Overexpression of caspase-8 induces *TP53* gene transcription to produce p53, which stimulates apoptotic cascade [41]. Caspase-8 uses proteolytic and nonproteolytic functions to change cell behavior. Activated caspase-8 triggers caspase-3 activation that commits cell to death [55]. (How does this relate to MPN? Is it upregulated or downregulated?).

Immunohistochemistry studies showed that the percentage of MKC positive for caspase-8 is higher in MPNs in comparison with controls, suggesting that MKC in MPN tend to counteract the survival advantages acquired through inhibition of the intrinsic apoptotic pathway by activating the caspase-8-mediated extrinsic apoptotic cascade[41].

Caspase-9 is an inducible proapoptotic molecule, which acts relatively late in apoptosis signaling becoming less susceptible to inhibition by apoptosis inhibitors [56]. Caspase-9 is an apoptotic initiator caspase in MKC and platelets being necessary for their efficient death, and it is not required for platelet generation and function, as it was previously thought. Thus, caspase-9 loss is associated with an increased MKC proliferative capacity. In MPNs, especially in the *CALR*-mutated molecular subtype, caspase-9 dysfunction could play a role in the enhanced thrombocytosis [40, 41].

SMAC/DIABLO controls apoptosis by negatively regulating IAPs and by activating caspases. Recently, it was shown that silencing of SMAC/DIABLO caused decreased levels of phospholipids, suggesting that besides proapoptotic functions, SMAC/DIABLO have nonapoptotic lipid synthesis-related function essential for cancer growth and development. Therefore, it was assumed that SMAC/DIABLO could be a promising therapeutic target in cancer [57]. On the other hand, SMAC/DIABLO downregulation was found to be associated with progressive disease and poor survival rate in hematologic malignancies, and DIABLO/SMAC mimetics were proposed as a potential adjunct therapy to enhance DIABLO levels in MPN MKC [41]. More studies are necessary to establish the proper therapeutic options in the light of the new role of SMAC/DIABLO in the phospholipid synthesis.

The tumor suppressor gene *TP53* plays many roles in apoptotic landscape by suppressing or activating a large number of checkpoint and apoptotic genes [58]. Alterations in *TP53* have not been linked to MKC hyperplasia although mutations targeting *TP53* do occur during leukemic transformation of MPNs. Mutated p53 could inhibit the wild-type p53 function or gain new oncogenic functions through protein-protein interactions [59]. As a result, poor prognosis in hematologic malignancies is correlated with mutations in *TP53* due to mutant's stability [58]. Elevated levels of MKC p53-positive are present more in PMF than in PV and ET [41]. According to Malherbe et al., *CALR*-mutated cases compared to *JAK2* V617F-positive cases present more p53, but not caspase-8 positivity. It seems that CALR lesions disrupt alternative apoptotic effectors and affected MKC attempt, a remedial prodeath response dominated by overexpression of p53. Also, *CALR*-mutated MKC display a minor caspase-9 upregulation that is unlikely to induce apoptosis due to concurrent survivin overexpression, but rather promote thrombocytosis [41].

Cell surface death receptor-ligand interaction, such as FASL binding FAS, TRAIL binding death receptor 5 (DR5) or TNFα binding TNFR1, executes extrinsic pathway apoptosis.

The two major necroptotic death effector complexes, the necrosome and ripoptosome, are induced by TNFR1 and toll like receptor 3 (TLR3) signaling, respectively [60]. IFN-R activation, primarily by type-I IFN, is believed to involve a caspase- and FADD-independent, receptor-interacting protein kinase (RIP) 3-dependent mode of cell death via the formation of the necrosome. Following IFN-R activation, JAK/STAT signaling and the activity of RNA-responsive protein kinase (PKR), upstream of RIP1/RIP3 necrosome formation is essential. TNFα binding to TNFR1 causes recruitment of TRADD and RIP1 via their death domains resulting in the prosurvival complex I, which is stabilized by TNFα-bound TNFR2-TRAF2. Internalization of the TNFR1-TRADD-RIP1 complex is required for recruitment of caspase-8 and FADD, necessary for apoptosis. This is therefore a major cell death checkpoint as the absence of NF-κB activation and prosurvival signaling results in proapoptotic complexes or, alternatively, the pronecroptotic complex known as the necrosome. The ripoptosome consists of FADD, cFLIP and caspase-8 and allows necroptosis to prevail if active cleavage of RIP1 by caspase-8 is prevented by cFLIPL. In MPN, it was shown that blocking TNFR2 but not TNFR1 selectively inhibits MPN cells over normal ones and the process involves XIAP, cIAP, and MAPK8 as key mediators of these differential responses to TNF. These data support the potential therapeutic use of cIAP inhibitors and selective TNFR2 inhibitors in the treatment of MF [33].

The TLR3-induced pathway converges with the TNFR1-induced pathway at the necrosome. The execution phase of necroptosis starts with interaction between RIP1 and RIP3. Following stabilization of the RIP1-RIP3 complex, mixed-lineage kinase domain-like protein (MLKL) is recruited to form a functional necrosome. MLKL activated upon phosphorylation by RIP3 results in the translocation of the

MLKL necrosome to the plasma membrane, necroptotic membrane disruption, and release of liposomes containing phosphatidylinositol phosphates (PIPs). This permeabilization, combined with MLKL-mediated calcium or sodium influx ion-pore dysregulation, characterizes the model proposed for necroptosis execution [60]. Human cancers, including MPN and their exacerbated form, sAML, are known for eluding apoptosis; therefore, therapeutic induction of necroptosis may represent a better strategy for an efficient treatment. A series of compounds have been shown to trigger necroptosis, particularly inhibitors of RIP1, RIP3 or MLKL, in leukemia cells; however, a deeper understanding of the signaling network that regulates this type of PCD is still necessary [35].

4. Major signaling pathways involved in apoptotic failure in molecular subgroups of MPN

The constitutive activation of JAK-STAT pathway is a common feature of MPNs irrespective of driving mutation, being observed even in so-called "triple-negative MPNs" that lack known *JAK2*, *MPL,* and *CALR* mutations [41, 48, 61]. In contrast to the transient activation of the JAK-STAT signaling that occurs in the physiological conditions, MPNs are characterized by a hyperactive JAK2 signaling through dimeric myeloid cytokine receptors (EPO-R, TPO-R, and G-CSFR) even in the absence of the ligand that promotes myeloproliferation and resistance to apoptosis (**Figure 1**) via the JAK-STAT, PI3K (phosphatidylinositol 3-kinase)-AKT signaling pathways, and ERK/MAPK pathways [62, 63]. While the most prevalent MPN driver mutation, *JAK2* V617F, induces the activation of all three myeloid cytokine receptors, *MPL* and *CALR* mutations activate only TPO-R. This provides

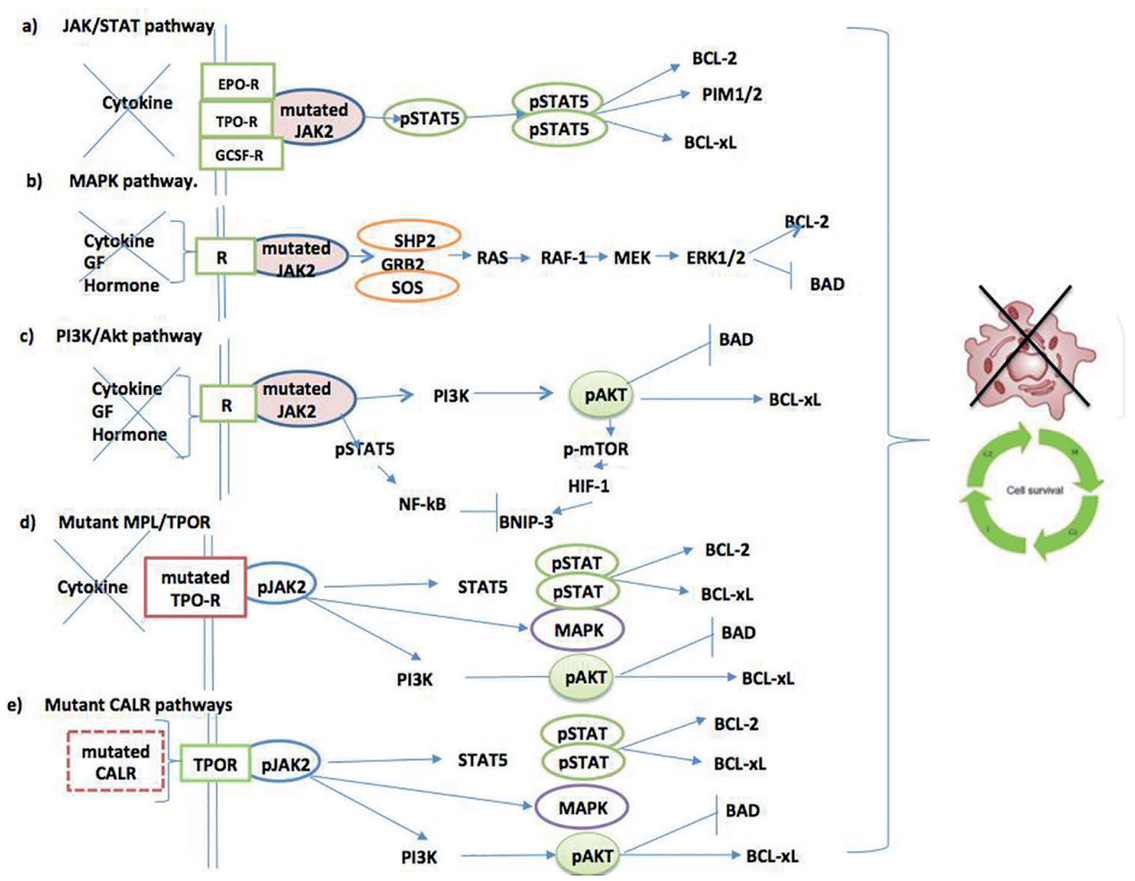

Figure 1.
Major signaling pathways involved in apoptotic failure in molecular subgroups of MPN.

an explanation for the association of *JAK2* V627F mutation with all classical MPN phenotypes (PV, ET, and PMF) and also for the preferential occurrence of *MPL* and *CALR* mutations in ET and PMF.

In addition, loss-of-function or neomorph mutations in genes that are involved in epigenetic regulation, splicing, and signaling can act as disease modifiers by cooperating with MPN driver mutations [52].

The JAK/STAT is the major pathway (**Figure 1a**) involved in MPN pathology [64–66]. *JAK2* V617F mutation promotes constitutive activation of JAK-STAT signaling, erythrocytosis and MKC proliferation, extensive cellular hyperplasia, and abrogated apoptosis [67, 68]. In response to *JAK2* V617F mutation, extensive proliferation conduces to accumulation of irreparable DNA damage. As a consequence, internal apoptotic cascade is triggered with higher BNIP-3 positivity that stimulates MKC apoptosis [69, 70]. This is counteracted by the antiapoptotic effects conferred by excess BCL-XL expression induced by phosphorylated STAT5 (pSTAT5) and pSTAT3. BCL-xL expression is essential to maintain megakaryoblast lineage survival and platelet production, preventing lethal hemorrhage [15].

The MAPK/ERK signaling pathway activation (**Figure 1b**) is required in MKC differentiation, with TPO as signal for induced maturation via MPL receptor [71]. *JAK2* V617F can activate MAPK signaling pathway via receptor tyrosine kinase-Grb2-SOS, continuing with RAS GTPase and RAF-1, which activates MEK, followed by ERK activation [72]. Phosphorylated extracellular signal-regulated kinase (ERK) activates BCL-2 and BAD, both of which have the effect of inhibiting apoptosis.

An increased activation of RAS/RAF/ERK pathway was showed in MPN patients, especially in erythroid precursor cells and MKC (**Figure 1b**). It was shown that ERK is constitutively activated by the *JAK2* V617F mutation. Laubach JP et al. demonstrated an increased activation of RAS/ERK pathway in PV, associated with a dysregulated erythropoiesis and apoptosis resistance of erythroid precursor cells [73]. ET, PV, and PMF patients with *JAK2* V617F mutation demonstrated an increase of ERK phosphorylation level in MKC. Phosphorylated ERK activates BAD, which release apoptosis inhibitor BCL-2, resulting in an overall inhibition of apoptosis that may be the cause of MKC hyperplasia and bone marrow hypercellularity [50].

The PI3K/AKT signaling pathway (**Figure 1c**) may be activated by the *JAK2* V617F mutation or pSTAT5. PI3K/AKT pathway is involved in several cellular processes including cell proliferation and differentiation, protein synthesis, and apoptosis. AKT pathway is known to be active in AML [74, 75]. Dai C et al. showed that increased erythroid progenitor proliferation from PV is associated with increased phosphorylation of AKT [76]. Khan I et al. confirmed that PI3K/AKT signaling pathway was activated in MPN by the *JAK2* V617F and *MPL* W515 L mutations [77]. Moreover, Koopmans et al. demonstrated an increase of pAKT level in the cytoplasm and nucleus of immature myeloid cells and in MKC of MPN patients. Immunohistochemical staining showed that pAKT expression was significantly higher in MKC of ET compared to PV and PMF. Recently, the AKT activation was demonstrated to be a feature of *CALR*-mutant myeloproliferative neoplasms [78]. The higher platelet counts reported in MPN with *CALR* mutations may be due to greater dysregulation of MKC apoptosis [48]. Consequently, AKT was considered a potential target in MPN therapy. Several studies have shown that targeting AKT with specific inhibitors reduced cell growth *in vitro* [77] and induced prolonged survival of the immunodeficient mice injected with *JAK2* V617F-mutated cells, along with reducing spleen size [79].

The activation of AKT upregulates BCL-xL and inactivates BAD, suppressing apoptosis and promoting cell survival. This was observed in MPN patients, where

the activation of pAKT was higher in MKC and associated with the inhibition of MKC apoptosis [50]. pAKT is also known to induce activation of BNIP-3 and caspase-9 through mammalian target of rapamycin (mTOR)—a serine/threonine kinase that is an effector protein of AKT—via activation of HIF-1. Data related to BNIP-3 expression are conflicting; some groups reported a reduced BNIP-3 expression [30], whereas others have shown its upregulation in MPNs [50]. In the study of Koopmans et al., the immunohistochemical expression of BNIP-3, with proapoptotic function, was lower in total bone marrow cells of ET, PV, and PMF patients, compared with the control group. This suggests that a decreased apoptosis might also contribute to the increased bone marrow cellularity observed in MPNs. However, in contrast to total bone marrow cells, the MKC of MPN patients were found to display a high level of BNIP-3 [50]. On the other hand, the most pronounced reductions in BNIP-3 were observed in PMF, suggesting a loss of proapoptotic potential during progression to the "accelerated" phase of MPNs [80].

JAK2 exon 12 mutations, exclusively associated with PV, consist of deletions/insertions, duplications, and point mutations, which affect a conserved region in the proximity of JAK2 pseudokinase domain (residues F537 through E543) and have functional consequences similar to those induced by *JAK2* V617F, however with some quantitative and qualitative differences [81]. Thus, exon 12 mutations cause a constitutive activation of EPO-R (**Figure 1a**) with erythroid hyperplasia, and lesser involvement of other hematopoietic lineages [82, 83]. Unlike *JAK2* V6217F, that is commonly homozygote in PV, at least in a proportion of colonies, exon 12 mutations are predominantly heterozygous, suggesting a stronger activation of JAK2 signaling [82, 84, 85]. As indicated by the levels of pAKT in the erythroid colonies, *JAK2* exon 12 mutations are associated with a weaker activation of AKT signaling compared to *JAK2* V617F. Both *JAK2* V617F and *JAK2* exon 12 mutations block the DNA damage-mediated apoptosis through inhibition of the BCL-xL deamidation pathway [81].

MPL exon 10 mutations induce an increased TPO-R signaling (**Figure 1d**) resulting in the activation of STAT5, STAT3, ERK, and AKT with associated thrombocytosis [86]. The most prevalent *MPL* mutations include substitutions of the juxtamembrane tryptophan W515, mainly by leucine (W515 L) or lysine (W515K) and rarely by alanine (W515A) or arginine (W515R). As tryptophan W515 is part of the amphipathic helical motif RWQFP that prevents TPO-R self-activation, these substitutions cause a cytokine-independent activation of the receptor [87, 88]. In this respect, in vitro expression studies of MPL mutants indicated that *MPL* W515K/L mutations were able to induce spontaneous cell proliferation and activation of JAK/STAT, RAS/MAPK, and PI3K/AKT pathways. In addition, an antiapoptotic effect was observed after cytokine withdrawal in MPL W515K/L-expressing cell lines, characterized by high levels of BCL-xL expression [89]. A second type of exon 10 *MPL* driver mutations, S505 N [52], was initially described as a germline mutation in a Japanese family suffering from hereditary thrombocytosis. Functional studies revealed that cell lines expressing S505 N presented growth factor-independent survival capacity accompanied by a constitutive phosphorylation of MEK1/2 and STAT5b. Furthermore, an autonomous phosphorylation of MEK1/2 was also noticed in the platelets obtained from the affected family members [90]. Later on, the same *MPL* mutation was reported as somatic in less than 1% ET cases [91].

Noncanonical *MPL* mutations (outside exon 10) of somatic or germline origin have been identified by whole-exome sequencing (WES) in approximately 10% of triple-negative ET or PMF cases [92]. By performing several functional assays, it was shown that all *MPL* mutations were gain-of-function resulting in activation of JAK2-STAT5 signaling. In a different study, the *MPL* somatic mutation S204P could induce TPO-independent growth, resistant to cytokine deprivation and constitutive STAT activation, although less efficient than *MPL* W515K mutation [93].

CALR mutations are located in the exon 9 of the gene and induce a + 1 base-pair frameshift, resulting in a new C-terminus that loses the ER retention motif (KDEL). Although more than 50 *CALR* mutations have been described so far, the mutation profile is dominated by a 52-bp deletion (type 1 mutation) and a 5-bp insertion (type 2) [94]. Functionally, both type 1 and type 2 CALR mutants bind to the extracellular domain of TPO-R and cause its activation (**Figure 1e**), leading to constitutive JAK2/STAT/PI-3 K and MAPK signaling and protecting cells from apoptosis [95]. Recently, by using gene expression analysis of K562 cells that lack TPO-R and stably express either CALR WT or the two most common CALR mutants, Salati et al. have identified a novel potential role of *CALR* mutations in MPN development, independent of TPO-R activation. Thus, CALR mutants seemed to diminish the proapoptotic signals downstream the unfolded protein response, generating the accumulation of misfolded proteins in ER and promoting resistance to ER stress-induced apoptosis [96].

Taking into account the above-mentioned cellular and molecular effects of MPN driver mutations, we can assume that megakaryocytic and erythroid progenitor expansion in MPNs results from a combination of increased proliferation and attenuated apoptosis.

5. PCD resistance in myeloid proliferation exploited in single or combined targeted therapeutic strategies

As shown previously, deregulation of the JAK/STAT pathway is central to MPN development and is driven in most cases by activating mutations in *JAK2*, *CALR*, or *MPL*. Signaling through other pathways (RAS/RAF/MEK/ERK, PI3K/AKT/mTOR, and LNK) and alterations in other cellular processes such as DNA methylation (e.g., *TET2* and *DNMT3A* mutations), histone modification (*ASXL1* and *EZH2* mutations), and RNA splicing (*U2AF1*, *SF3B1*, and *SRSF2* mutations) further contribute to initiation, progression of myeloproliferation, and resistance to apoptosis [52, 97].

Understanding molecular mechanisms of MPN pathogenesis has stimulated drug development in the field.

Reduction of thrombotic risk is the major goal of therapy in patients with PV and ET, and hydroxyurea (HU) is normally the first-line drug for achieving cytoreduction [98, 99]. In addition, most patients should receive aspirin, if they have no contraindications. In PV, maintaining hematocrit values <45% is an important therapeutic target. Second-line drugs of choice are interferon-α (IFNα) and busulfan [99].

The clinical efficacy of IFNα has been reported since 30 years ago and was improved with the development of pegylated forms [100, 101]. Furthermore, significant reductions of the *JAK2* V617F allele burden (% *JAK2* V617F) was observed in IFNα-treated patients [102, 103], suggesting that IFNα is able to target the malignant clone. The mechanism of action of IFNα in MPNs is not clearly elucidated, but several studies confirmed a targeted effect against *JAK2* V617F mutant clones. Ropeginterferon alpha-2b (Ropeg) is a long-acting pegylated-IFNα-2b, recently shown to be safe and well tolerated in phase 1–2 studies in PV patients. Both hematological and molecular responses have been reported in a phase 2 trial [104]. The discovery of *JAK2* V617F mutation and its role in constitutive activation of downstream signaling pathways and MPN pathogenesis triggered the search for specific JAK2 kinase inhibitors as potential targeted therapy. One of the first molecules approved for targeted treatment of MPN patients, ruxolitinib/jakafi (RUX), a selective JAK2 and JAK1 inhibitor, is currently used in PV resistant or intolerant to HU, and in intermediate and high risk PMF [99, 105]. RUX, approved in 2014 based

on the results of the RESPONSE trial [106, 107], inhibits ATP-binding catalytic site of JAK kinase domain (both in mutant and wild type JAK), thus leading to a reduction of phosphorylation level of STAT-3/5, ERK, and AKT [108, 109]. Initial studies showed that RUX treatment of *JAK2* V617F-positive Ba/F3 cells inhibited cell proliferation and induced apoptosis [108]. The drug was able to cause apoptosis by decreasing BCL-xL, as well as proviral integrations of Moloney virus (PIM) 1 and 2 at transcriptional level, and consequently by inhibiting BAD phosphorylation [110].

RUX showed efficiency in spleen size reduction and symptomatology alleviation, improving quality of life, and overall survival; however, no significant decrease in allele burden was achieved [11, 111, 112]. RUX effects on the malignant clone are modest, side effects (such as anemia and thrombocytopenia) are reported, and drug resistance may appear. Other therapeutic strategies have been developed; they include the discovery of new inhibitors that target specifically mutant JAK2 and the combination of current therapies with other molecules that inhibit components of signaling pathway [105].

Early studies provided some evidence for the increased resistance to apoptosis of PV erythroid progenitor cells: overexpression of BCL-xL in the absence of EPO and a higher sensitivity to the antiapoptotic growth factor IGF-1 [113]. Moreover, Zeuner et al. have shown that erythroid precursors in PV patients with average and high *JAK2* V627F mutational load often expressed elevated levels of BCL-2 and BCL-xL and were very susceptible to the apoptosis induced by the BH3 mimetic ABT-737 (a small-molecule that inhibits BCL-2, BCL-xL, and BCL-W and causes apoptosis of the leukemic cells) compared to *JAK2* V617F-low or normal erythroblasts [114]. Later, the combination of ABT-737 with a JAK inhibitor proved to be efficient in reducing the number of PV *JAK2* V617F+EPO-dependent and independent erythroid colonies, and BIM was identified as a key mediator of apoptosis induced by JAK2 inhibition [115].

In susceptible cells, apoptosis is caused by exposure to a JAK inhibitor, which leads to dephosphorylation of BAD, enabling BAD to bind and sequester the antiapoptotic protein BCL-xL. On the other side, in potent cells, RAS effector pathways keep BAD phosphorylation in the presence of JAK inhibitors, maintaining a specific dependence on BCL-xL for survival. So, downstream regulation of BCL-xL, more precisely BCL-xL inhibition, might be the key against resistance to JAK inhibition by either co-inhibition of JAK and RAS effector in AKT and ERK pathways or by direct inhibition of BCL-xL inducing apoptosis [116].

At present, there are over 1500 clinical trials evaluating various drug effects on myeloproliferative neoplasms registered at clinicaltrials.gov. Some of them might be successful due to targeting different apoptotic pathways or by targeting simultaneously different types of PCD.

Plitidepsin is a synthetically produced anticancer agent [117], a cyclodepsipeptide related to didemnins, commercialized as Aplidin® (PharmaMar, S.A., Madrid, Spain). Plitidepsin induces dose-dependent cell-cycle arrest and an acute apoptotic process. These effects rely on the induction of early oxidative stress, the rapid activation of Rac1 GTPase, and the sustained activation of JNK and p38/MAPK, which finally result in caspase-dependent apoptosis [118, 119]. JNK phosphorylation can be seen as early as 5–10 minutes after exposure to the compound. The activation of JNK and p38/MAPK is associated with increase in reactive oxygen species and a decrease in reduced form of glutathione [120].

Recent studies have led researchers to hypothesize that the primary target of plitidepsin could be the eukaryotic elongation factor 1A2 (eEF1A2), which is overexpressed in tumors and supports tumor cell proliferation while inhibiting apoptosis [121]. eEF1A2 seems to be an interesting target for therapy and may also be a biomarker predicting drug sensitivity. Aplidin®/Plitidepsin was investigated for

its effect (safety and tolerability) on bone marrow or peripheral blood cells as well as assessed the response rate in patients with PMF, post-PV MF, or post-ET MF, in phase II/open label single agent clinical trial (NCT01149681). Although the drug was well tolerated, the trial was prematurely terminated due to the low response rate [122].

Navitoclax is an orally active, synthetic small molecule and an antagonist of the apoptosis suppressor proteins BCL-2, BCL-xL, and BCL-w, which are frequently overexpressed in a wide variety of cancers, including myeloid ones, and are linked to drug resistance. Inhibition of these apoptosis suppressors prevents their binding to the apoptotic effectors BAX and BAK proteins, thereby triggering apoptotic processes in cells overexpressing BCL-2, BCL-xL, and BCL-w. This eventually reduces tumor cell proliferation. Navitoclax (ABT-263) and RUX are currently evaluated in combination for efficacy, safety, and tolerability on spleen volume as assessed by magnetic resonance imaging (MRI) in participants with MF in a phase II/open label clinical trial (NCT03222609).

Obatoclax (GX15–070) is a BH3-mimetic designed to target and counteract anti-apoptotic BCL-2 proteins. Obatoclax is an MCL-1 antagonist [123] and downregulates p53, and it has a dual mechanism of action, being capable to induce apoptosis or autophagy [124]. On the other side, obatoclax accumulates in lysosomes inducing their alkalinization and inhibiting their function [125]. Parikh et al. conducted a multicenter, open-label, noncomparative phase II study (NCT00360035) of obatoclax in patients with MF. Unfortunately, obatoclax exhibited no significant clinical activity at the tested dose and schedule [126].

Other phase I trial (NCT02436135) investigated the combination of RUX with idelalisib, a PI3K delta inhibitor, as therapy for intermediate to high-risk PMF, post-PV MF, or post-ET MF with progressive or relapsed disease [127].

PIM inhibitors have shown preclinical synergy with JAK inhibitors, as well as the ability to overcome JAK inhibitor resistance in MPN cell lines. PIM regulate JAK/STAT signaling and are involved in oncogenesis through phosphorylation of cell cycle regulators, activation of antiapoptotic proteins, and enhancement of MYC expression [97]. A phase 1b study of RUX plus PIM inhibitor PIM447, or RUX plus CDK4/6 inhibitor ribociclib (LEE011), or the combination of all three is underway in several non-U.S. countries (NCT02370706).

As PI3K/AKT/mTOR signaling is markedly activated in MPNs, small molecule inhibitors of the proteins involved in this pathway have been tested in MF with promising results. Thus, mTOR inhibitor everolimus, as single therapeutic agent, was able to induce responses, in terms of reducing constitutional symptoms and the degree of leukocytosis, thrombocytosis, and anemia, in 23% of patients in a phase I/II clinical trial. Due to the preclinically proved synergic effects of PI3K/AKT/mTOR inhibitors and JAK inhibitors, several clinical studies were initiated: PI3K inhibitor TGR-1202 in combination with RUX (NCT02493530), PI3K inhibitor buparlisib with RUX (NCT01730248), PI3K inhibitor INCB050465 and RUX (NCT02718300), and selective PI3Kδ inhibitor TGR-1202 and RUX (NCT02493530). Preliminary results of buparlisib and RUX phase 1b study indicated that this drug association was well tolerated, and ≥ 50% reduction in splenomegaly was observed in 70% of JAK-inhibitor naive patients and 54% of patients who did not previously respond to JAK2 inhibitor monotherapy [97, 127].

RAF/MEK/ERK pathway is another signaling cascade activated in MPNs by the increased JAK/STAT signaling. Therefore, MEK inhibitors were tested in different murine models, either alone or in combination with JAK inhibitors, showing a decrease in bone marrow fibrosis, inhibition of malignant cell growth, and HSC function recovery, associated with a prolonged survival. Moreover, a new trial that combines the MEK inhibitor selumetinib with the DNA hypomethylating agent azacitidine is soon expected [97].

Preclinical studies reveal a central role for tumor necrosis factor alfa (TNF-α) in promoting clonal dominance of *JAK2* V617F-expressing cells in MPN [101]. *JAK2* V617F appears to confer TNF-α resistance to a preneoplastic TNF-α-sensitive cell, while creating a TNF-α-rich environment at the same time.

SMAC-mimetics are novel apoptosis-inducing agents that stimulate the ubiquitinylation and proteasomal degradation of cellular inhibitors of apoptosis (IAPs) [102], proteins that play an important role in tumor cell resistance to cytotoxicity mediated by TNF superfamily cytokines. These agents have been shown to sensitize cancer cells to TNF family-induced apoptosis [103]. Results from a phase II trial of the SMAC-mimetic LCL-161 in patients with intermediate or high-risk MF intolerant of, ineligible for, or relapsed/refractory to JAK inhibitors were recently presented. Six of sixteen evaluated patients (38%) had objective responses, obtaining clinical improvement and in one case cytogenetic remission [128].

Resistance of hematologic malignancies to PCD significantly limits the efficacy of chemotherapy. As the majority of chemotherapeutic drugs trigger apoptosis, the observed resistance may indicate that novel therapeutic strategies to reactivate nonapoptotic PCD or at least combined therapeutic strategies able to attack simultaneously different mechanisms might be better approaches to eradicate malignant cells.

6. Conclusion

Deregulation of pro- and anti-PCD genes involved in cell resistance to cell death and accumulation of myeloid cells in MPNs is continuously clarified by intense exploration of the modifications affecting different types of PCD pathways involved in myeloid proliferation. At the same time, comprehension of the network of signaling pathways involved in etiology and drug resistance of these disorders facilitate a more efficient exploitation of the knowledge, using combined and synergic, targeted therapeutic strategies.

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Notes

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