Curr Hematol Malig Rep (2013) 8:71–80
DOI 10.1007/s11899-012-0150-1

CHRONIC LEUKEMIAS (J GOLDMAN, SECTION EDITOR)

Signal Transduction in the Chronic Leukemias: Implications for Targeted Therapies

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Published online: 10 January 2013

Abstract The chronic leukemias, including chronic myeloid leukemia (CML), the Philadelphia-negative myeloproliferative neoplasms (MPNs), and chronic lymphocytic leukemia (CLL), have been characterized extensively for abnormalities of cellular signaling pathways. This effort has led to the elucidation of the central role of dysregulated tyrosine kinase signaling in the chronic myeloid neoplasms and of constitutive B-cell receptor signaling in CLL. This, in turn, has stimulated the development of small molecule inhibitors of these signaling pathways for therapy of chronic leukemia. Although the field is still in its infancy, the clinical results with these agents have ranged from encouraging (CLL) to spectacular (CML). In this review, we summarize recent studies that have helped to define the signaling pathways critical to the pathogenesis of the chronic leukemias. We also discuss correlative studies emerging from clinical trials of drugs targeting these pathways.

Keywords Chronic myelogenous leukemia · Tyrosine kinase inhibitor · BCR-ABL1 · JAK2 · Imatinib · Dasatinib · Nilotinib · Bosutinib · Ibrutinib

Introduction

The discovery of the BCR-ABL1 fusion tyrosine kinase as the causative agent of chronic myeloid leukemia (CML) spurred the development of imatinib mesylate, an ATP-competitive tyrosine kinase inhibitor (TKI) of ABL1, for the treatment of CML [1]. Imatinib and other drugs in its class (dasatinib, nilotinib, bosutinib, ponatinib) have since revolutionized the treatment of CML. The majority of CML patients who initiate TKI therapy for their disease achieve a cytogenetic remission [2], and those who do, enjoy a normal life expectancy [3], emphasizing the central role of BCR-ABL1 kinase activity in the pathogenesis of the disease. However, TKI therapy does not appear to cure CML in the majority of patients [4], and a substantial proportion develop required resistance to TKI therapy through ABL1 mutations and other mechanisms [5]. In part, the failure of TKIs to eradicate CML has been attributed to so-called leukemic stem cells (LSCs), pluripotent BCR-ABL1$^+$ progenitors that are largely quiescent [6] and resistant to killing by ABL1 inhibitors [7]. This has rekindled interest in understanding the molecular signaling pathways that mediate the leukemia phenotype and the survival of CML stem cells. By analogy to highly active anti-retroviral therapy in HIV/AIDS, targeting multiple signaling pathways in the leukemic cell might prevent or overcome acquired TKI resistance and lead to a permanent cure.

With the discovery of somatic activating mutations in the JAK2 tyrosine kinase in 2005 [8], new light was shed on the pathogenesis of the Philadelphia-negative myeloproliferative neoplasms. The JAK2 mutations are found in virtually every patient with polycythemia vera (PV), and about half of the patients with essential thrombocythemia (ET) and primary myelofibrosis (PMF) [9–11]. Parallel discoveries of activated fusions of PDGFRα and PDGFRβ in some cases of chronic eosinophilic leukemia [12, 13] and chronic myelomonocytic leukemia [14], respectively, solidified the concept that dysregulated tyrosine kinase signaling underlay the pathogenesis of most of the classic MPNs and myeloid neoplasms with features overlapping MPN and myelodysplasia. While rapid progress ensued on analysis of dyregulated TK signaling in these diseases [15], the development of JAK2 inhibitors was close behind. However, the clinical debut of this class of TKIs has been somewhat disappointing. While specific JAK2

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inhibitors such as ruxolitinib decrease splenomegaly and improve symptoms and quality of life in patients with myelofibrosis [16, 17], as a class they do not appear to reduce marrow fibrosis, improve cytopenias, or decrease or eliminate the malignant clone, and, hence, may have limited impact on survival [18]. A better understanding of the molecular pathogenesis of these diseases is a prerequisite to improving upon TKI therapy, specifically to develop strategies to eliminate leukemic stem cells in CML and to reverse the pathological hematopoiesis underlying the Ph-negative MPNs.

Here, we review current knowledge about the signaling pathways underlying the pathogenesis of the chronic leukemias, with a focus on chronic myeloid leukemia, the Ph-negative myeloproliferative neoplasms, and chronic lymphocytic leukemia. The emphasis will be on recent insights and implications for clinical trials of targeted therapies in these diseases.

Chronic Myeloid Leukemia

The BCR-ABL1 Fusion Kinase

Studies in mouse models 22 years ago defined BCR-ABL1 as the direct cause of CML [19]. Since then, mutagenesis studies have clarified some of the mechanisms and pathways through which BCR-ABL1 induces the CML phenotype (Fig. 1). Among the many functional domains of this fusion protein, two key motifs in the BCR portion of the protein are absolutely required for leukemogenesis. The first of these is a coiled-coil oligomerization domain at the extreme N-terminus of the protein [20], which mediates heterodimerization, cross-phosphorylation, and activation of the fusion protein, all of which are necessary to overcome the autoinhibited conformation of the kinase and stimulate cellular signaling [21]. The second key motif is Tyr177, which is stoichiometrically phosphorylated in leukemia cells and serves as a binding site for the GRB2 adapter protein [22]. The GRB2 protein, in turn, couples to the GAB2 scaffolding protein and activates the phosphatidylinositol 3-kinase (PI3K) and SHP2 signaling pathways [23], both of which contribute to leukemogenesis. A recent study suggested that GAB2 signaling protects CML cells from various BCR-ABL1 TKIs by amplifying the input of BCR-ABL1 into the RAS/ERK and PI3K/AKT/mTOR pathways (discussed below). These results suggest that GAB2 could be a therapeutic target in TKI-resistant disease [24].

PI3K/AKT/mTOR Pathway

The BCR-ABL1 kinase activates the PI3K/AKT pathway through GAB2, and subsequently many downstream targets of the PI3K/AKT pathway have been shown to participate in BCR-ABL1-induced leukomogenesis. The PI3K/AKT activation leads to BAD phosphorylation, which prevents binding to and inactivation of the antiapoptotic protein BCL-2. The FOXO transcription factors, other downstream targets of PI3K/AKT involved in cell-cycle arrest and apoptosis, are phosphorylated by AKT, inhibiting FOXO3a activity [25].

Recent research also suggests that AKT and TGF-β signaling play a distinct role in the maintenance of LSC in CML. Whereas BCR-ABL1 activates PI3K and AKT in myeloid progenitors leading to phosphorylation and cytoplasmic retention of the FOXO3a transcription factor [26], Naka et al., recently demonstrated that the most primitive CML stem cells in the retroviral mouse model display inactive AKT, nuclear FOXO3a, and nuclear phospho-SMAD2/3 (a hallmark of TGF-β signaling) [27]. When CML was induced in HSC from *Foxo3a*$^{-/-}$ donors, the phenotypic LSC population and efficiency of leukemia

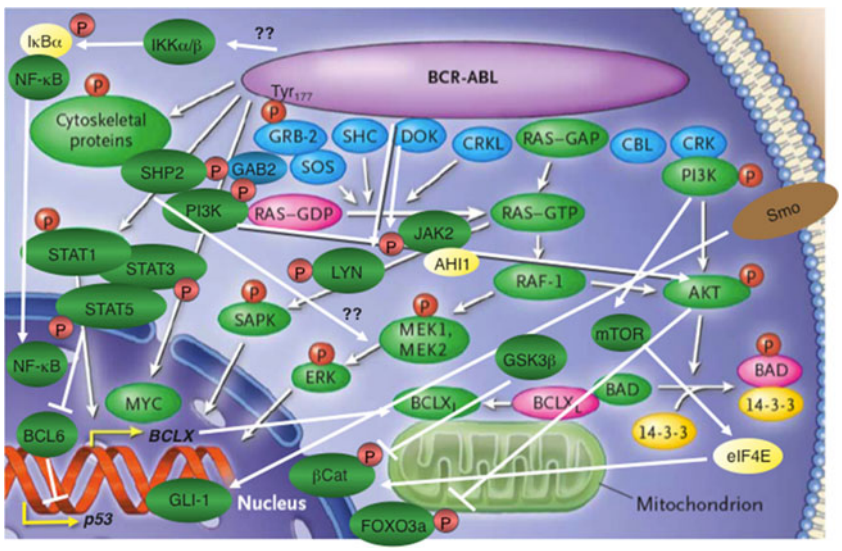

Fig. 1 BCR-ABL1-induced signaling pathways in CML. Schematic representation of the myriad signaling pathways that are activated in CML cells. Phosphorylation is represented by *red* "*P*" *symbols*. *Arrowheads* indicate activation of a downstream molecule, while a *bar at the end of a line* indicates inhibition of a downstream molecule. (Adapted from Goldman and Melo, N. Eng. J. Med 2003;349:1451)

induction declined upon serial transplantation, the most rigorous test of LSC function. Their data suggest that TGF-β signaling (predominantly TGF-β1), through an unknown mechanism, may suppress AKT inhibition of FOXO3a in CML LSCs, which may be critical for LSC maintenance. Another group also described paradoxical nuclear FOXO3a in the most primitive ($Lin^-CD34^+CD38^-$) human CML progenitors [28]; although this paper was subsequently retracted, the data on nuclear FOXO3a are valid (Tessa Holyoake, personal communication). Müschen and co-workers subsequently showed that BCL6 may mediate some of the LSC survival effects of nuclear FOXO through repression of p53, and that targeting BCL6 with a peptidomimetic drug could decrease CML LSCs in the mouse model [29].

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase downstream of the PI3K/AKT pathway that plays a crucial role in regulating mRNA translation in mammalian cells, controlling cell growth and proliferation. The mTOR protein functions as the catalytic subunit for two distinct protein kinase complexes, mTORC1 and mTORC2. Previous studies established that TORC1 and TORC2 play important roles in growth and survival of BCR-ABL1-transformed cells [30, 31]. Mohi et al. [31] demonstrated that the combination of the allosteric mTOR inhibitor rapamycin and imatinib prolonged survival in a murine CML model and was effective against disease induced by imatinib-resistant mutants of BCR-ABL1. More recently, ATP-competitive dual mTORC1/2 inhibitors (PP242 and OSI-027) have exhibited potent growth inhibition in a number of $BCR\text{-}ABL1^+$ cell lines and primary patient samples [32, 33].

In addition to the direct inhibition of TORCs, the strategy of indirect suppression of the mTOR function by modulating the AMP-activated protein kinase (AMPK) pathway has been explored [34]. The AMPK regulates mTOR signaling both directly and indirectly. Once activated, AMPK phosphorylates and activates the TSC1/2 complex, which in turn suppresses Rheb activity, a small G-protein with regulatory functions on mTOR activation [35]. In addition, AMPK directly phosphorylates the Raptor subunit on Ser792, resulting in inactivation of the TORC1 complex [36]. Furthermore, modulation of AMPK by resveratrol, a naturally occurring substance found in grapes, has been shown to exhibit antileukemic effects in both imatinib-sensitive and imatinib-resistant CML cells, including leukemic cells harboring the T315I BCR-ABL1 mutation [37]. Induction of AMPK causes mTOR inhibition irrespective of TKI sensitivity, raising the prospect of using AMPK activators in the treatment of Ph^+ leukemias refractory to TKIs [34]. Another target of resveratrol in CML is SIRT1, a novel class of NAD(+)-dependent protein deacetylases implicated in aging [38, 39]. Pharmacologic inhibition of SIRT1 increased apoptosis in LSC of chronic phase and blast crisis CML and reduced their growth both in vitro and in vivo.

RAS/RAF/MAP Kinase

As discussed above, phosphorylation of tyrosine 177 within the BCR region of BCR-ABL1 mediates the SH2-dependent binding of GRB2 [22]. In addition to GAB2, another effector of GRB2 is SOS (Son of Sevenless), a guanine nucleotide exchange factor of RAS that mediates RAS activation [40]. There is extensive biochemical evidence to suggest that BCR-ABL1 activates ERK through the activation of the RAS/RAF/MEK/ERK pathway [41]. The ERK1/2 pathway is constitutively activated in embryonic stem cells transformed by BCR-ABL1, and ERK2 activation may be involved in resistance to imatinib [32]. Inhibition of the RAS pathway by farnesyltransferase inhibitors exhibit synergy with MEK/ERK inhibitors in suppressing growth and survival in K562 CML cells and in primary chronic phase $CD34^+$ CML cells [42].

Unlike the MEK/ERK pathway, the role of the JNK pathway in the pathogenesis of BCR-ABL1-induced leukemogenesis is still controversial. Some research suggests that activation of the JNK pathway promotes pro-apoptotic signals in BCR-ABL1-expressing cells in response to various agents, such as arsenic trioxide and ceramide. Constitutive BCR-ABL1 kinase activity suppresses JNK activation while TKIs like imatinib restore phosphorylation of JNK, thus driving cells toward apoptosis [43]. However, others have reported that the activation of the JNK pathway is essential for BCR-ABL-induced leukemogenesis, and that inhibition of JNK prevents BCR-ABL1-mediated transformation in vitro [44]. Furthermore, induction of autophagy in CML by resveratrol treatment involves JNK-dependent accumulation of p62, as JNK inhibition or p62 knockdown reversed resveratrol-mediated autophagy and antileukemic effects [37]. Together, these data suggest that the JNK pathway promotes diverse, and possible opposing, effects in BCR-ABL1 leukomogenesis and its exact role, if any, remains to be clarified.

JAK/STAT Pathway

Constitutive activation of human STAT5 has been found in many hematologic malignancies as well as in BCR-ABL1-expressing myeloid and lymphoid leukemia cells [45]. Constitutively active mutants of STAT5a induce MPN-like leukemia in primary mouse hematopoietic cells [46]. Furthermore, inhibition of STAT5 by dominant-negative mutants impairs the survival of BCR-ABL1-expressing cell lines [47], whereas siRNA against STAT5 inhibits myeloid colony formation by primary CML progenitors [48]. More recently, genetic studies have shown that loss of STAT5 abolishes the CML-like leukemia induced in mice by BCR-ABL1, validating STAT5a/b and the genes they activate as targets for therapy in CML [49, 50].

The role of JAK kinases in CML pathogenesis is less clear despite extensive study. The JAK kinases, including JAK2, are clearly activated in BCR-ABL1-expressing cells but JAK2 is not the kinase responsible for STAT5 activation [45, 51]. The JAK2 protein complexes with BCR-ABL1 via the ABL1 C-terminus and contributes to activation of the SRC kinase LYN [52, 53], but neither the JAK2 binding site on BCR-ABL1 [54] nor JAK2 itself [55] are required for induction of CML-like leukemia in mice by BCR-ABL1. Although JAK2 kinase inhibitors can decrease the proliferation and survival of cultured BCR-ABL1-expressing cells [52], JAK2-deficient progenitors are equally sensitive to these drugs [55], indicating that targets other than JAK2 are responsible. However, a role for JAK2 in the maintenance of CML LSC has not been excluded, and JAK2 signaling from the BM microenvironment might contribute to resistance to TKI therapy in CML [56]. Hence, further investigation of JAK2 inhibitors in CML therapy is warranted.

Hedgehog Signaling

The Hedgehog (Hh) pathway plays a critical role in the self-renewal of somatic stem cells and controls response to stress, injury, healing and regeneration [57]. Binding of Hh ligands to their receptor, Patched (PTCH), releases its inhibitory effect on Smoothened (SMO), resulting in SMO activation. The activation of SMO promotes the nuclear translocation of the GLI family of transcription factors (GLI1, 2, 3). The GLI family controls cell proliferation and survival through controlling the expression of genes such as Cyclin D, c-*MYC* and *BCL2* [58].

The role of the Hh pathway in CML (reviewed in [59]) was first investigated by Dierks et al. [60] who reported an increased expression of SMO, as well as the downstream targets GLI1 and PTCH1, in progenitors from mice with BCR-ABL1-induced CML-like MPN and in CML patient cells, the latter in the CML stem cell compartment as well as in BCR-ABL1^{+} cells in both the chronic phase and blast crisis. Subsequent studies in the retroviral mouse model of CML, using donor cells deficient in *Smo* and the SMO antagonist cyclopamine, showed that *Smo* deficiency attenuated BCR-ABL1-induced CML-like leukemia and decreased the efficiency of secondary transplantation of the disease, while cyclopamine treatment also caused significant prolongation of survival, reduction in CML stem cells and reduction of disease onset in secondary transplant recipients. Complementary to these results, Zhang et al., reported that the small-molecule SMO antagonist LDE225 (Novartis) caused a significant reduction in secondary colony formation and replating efficacy in primary CML cells in vitro and improved survival after drug discontinuation in mice [61]. Preliminary clinical results with one SMO antagonist (P-04449913, Pfizer) suggest significant activity against CML and several other myeloid neoplasms [62].

WNT/β-Catenin

The WNT/β-catenin pathway was first connected to CML by a seminal study that discovered aberrant constitutive nuclear β-catenin in granulocyte-macrophage progenitors (GMP) in patients with CML myeloid blast crisis [63]. Normally restricted to the HSC compartment, it was hypothesized that nuclear β-catenin was associated with abnormal self-renewal in the malignant GMPs. The WNT pathway was further implicated in CML disease progression by gene expression analysis that showed increased expression of several WNT target genes, including c-*MYC*, cadherin, *ROK13A*, *MDI1*, prickle 1, and *FZD2*, in accelerated phase and blast crisis [64]. Subsequent studies in the mouse retroviral CML model demonstrated that deletion of β-catenin impaired development of CML-like MPN induced by BCR-ABL1 [65, 66], and a recent study suggested that the requirement for β-catenin is selective to CML stem cells vs normal HSC, representing a valid pharmacologic target in this disease [67].

Philadelphia-Negative Myeloproliferative Neoplasms

The Ph-negative myeloproliferative neoplasms (MPNs) including polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (MF), are prevalent blood disorders that significantly impact the health, quality of life, and overall survival of patients [15]. The MPNs are clonal disorders involving the multipotent hematopoietic stem cell and characterized by increased proliferation of one or more of the myeloid, erythroid, or megakaryocytic lineages, variable progression to acute leukemia, and abnormalities of hemostasis and thrombosis [68]. In 2005, the discovery of a somatic mutation in the JAK2 tyrosine kinase in most patients with Ph-negative MPN [8–11, 69] offered a unifying hypothesis about the pathogenesis of MPNs as resulting from dysregulated tyrosine kinase (TK) signaling [70]. While there is abundant evidence that mutations in other genes, including epigenetic regulators such as *TET2* and *ASXL1*, contribute to the clinical course of MPN [71] and perhaps predisposition [72] to these diseases, it is clear that TK signaling is a central and common feature of MPNs that can be mined for potential targets for therapy.

JAK2 and MPL

The JAK2 kinase is a key regulator of signaling from many cytokine receptors in the myeloid lineages such as the erythropoietin (EPO) receptor, the thrombopoietin (TPO) receptor and the G-CSF receptor. The *JAK2*^{V617F} mutation occurs

in exon 14 and abrogates the inhibitory effect of the pseudokinase domain, causing constitutively active JAK2. Although the pseudokinase domain was presumed to be catalytically inactive and function as a negative regulator, recent studies have shown that the pseudokinase domain has a dual Ser/Tyr kinase activity that modulates the basal activity and signaling of JAK2 through autophosphorylation [73].

The *JAK2*^{V617F} mutation is detected in more than 95 % of patients with PV, 50 %–70 % with ET, and 40 %–50 % with MF, as well as in some cases of atypical MPN [74]. Subsequently, another mutation was detected in exon 12 of *JAK2* in some of the *JAK2*^{V617F}-negative PV patients [75]. Several mutations of the thrombopoietin receptor (MPL) have also been detected in 6–7 % of patients with *JAK2*^{V617F}-negative ET or MF, resulting in the substitution of Trp 515 to Ala, Asp, Leu, or Lys that causes constitutive activation of MPL signaling [76].

Mouse Models of Ph-Negative MPN

As they have done with CML, mouse models of the Ph-negative MPNs have yielded important insights into the signaling mechanisms underlying these diseases. Shortly after the discovery of the *JAK2*^{V617F} mutation, several groups expressed this mutant kinase in mouse hematopoietic stem cells using the retroviral BM transduction/transplantation approach [77–80]. Expression of JAK2^{V617F}, but not JAK2^{WT}, induced non-fatal polycythemia characterized by increased hematocrit and hemoglobin, reticulocytosis, splenomegaly, low plasma erythropoietin (Epo), and Epo-independent erythroid colonies. The JAK2^{V617F} kinase also induced leukocytosis and neutrophilia that was much more prominent in Balb/c mice than in B6 [77, 79]. Platelet counts were not affected in either strain despite expression of JAK2^{V617F} in megakaryocytes and markedly prolonged tail bleeding times [79]. The polycythemia tended to resolve after several months and could progress to frank anemia, coincident with increased spleen and marrow fibrosis resembling post-polycythemia MF. These findings demonstrated that JAK2^{V617F} induces EPO-independent expansion of the erythroid lineage in vivo, and the fact that the central erythroid features of PV are recapitulated by expression of JAK2^{V617F} argued that it is the primary and direct cause of human PV. Similar studies also demonstrated that retroviral expression of the mutant MPL receptor in BM of Balb/c mice caused a fulminant and rapidly fatal MPN characterized by marked leukocytosis and thrombocytosis, hepatosplenomegaly, and marrow fibrosis [76, 81].

Following the development of the retroviral JAK2 models of MPN, several groups reported mouse models wherein JAK2^{V617F} was expressed from a chromosomal transgene. These fell into two general classes, those where JAK2 is expressed from an exogenous promoter, and those where JAK2 is expressed from the native mouse *Jak2* gene promoter ("knock-in" models). Currently, there are three published traditional transgenic strains [82–84] and four knock-in strains [85–88]. All of these strains exhibit some clinicopathological features of MPN, but there are numerous differences between the individual models that collectively make it difficult to draw definitive conclusions about some aspects of MPN pathogenesis (for an excellent review, see [89]). Some important pathogenetic information has certainly emerged from these *JAK2*^{V617F} transgenic models. For example, several models support the hypothesis that the ratio of expression of the mutant JAK2 kinase to endogenous JAK2 determines whether the phenotype will be predominantly one of polycythemia (JAK2^{V617F} ≥ endogenous JAK2) or thrombocythemia (JAK2^{V617F} < endogenous JAK2) [82, 87], which fits with the clinical observation of frequent homozygosity of the *JAK2*^{V617F} mutation in PV but not ET [90, 91]. In addition, studies in one of the *JAK2*^{V617F} knock-in models confirm that the disease-initiating cell population in PV is the phenotypic HSC [88]. However, there are also many discrepancies between the various models. One knock-in model demonstrated that expression of JAK2^{V617F} has a negative effect on self-renewal and repopulation by HSCs [87], another suggested a positive effect [85], while a third found no effect on HSC frequency or function [88] (of note, a recent study of human MPN patients found no effect of *JAK2* mutations on the size or in vitro function of HSCs [92]). Furthermore, two knock-in strains exhibited progressive development of MF [85, 86], another developed MF with very low penetrance [87], while a fourth strain did not develop MF at all [88]. The reasons for these differences are not clear, but may include variable use of human or mouse JAK2.

Downstream Pathways: STAT5 and TGF-β

Mouse models have been used to better define the signaling pathways downstream of JAK2 in the Ph-negative MPNs. For JAK2^{V617F}, genetic deletion of *Stat5a*/*b* in the mouse retroviral model reversed the polycythemia phenotype in recipient mice, with normalization of hematocrit, reticulocytes, and spleen size. However, unlike with BCR-ABL1, where loss of *Stat5* normalized the histology of BM, liver, and spleen, *Stat5* deletion did not eliminate completely myelofibrosis of the BM or infiltration of the liver/spleen with myeloerythroid progenitors in JAK2^{V617F} recipients. Hence, STAT5-independent signaling pathways contribute to the pathogenesis of MPN and MF associated with mutant *JAK2*. By contrast, Mohi et al., found no residual MF at 6 months following *Stat5* deletion in a knock-in transgenic *JAK2*^{V617F} model, but these investigators did not study *JAK2*^{V617F} homozygotes, which may better reflect human PV patients [93].

In human MF, the fibroblasts are polyclonal, do not carry the *JAK2*^{V617F} mutation, and are not part of the malignant clone [94]. However, considerable evidence suggests that one or more cytokines secreted by the clonal megakaryocytes are

the cause of fibroblast proliferation and fibrosis in MF, including PDGF, FGF, and TGF-β. Human MF patients have consistent increases in TGF-β1 in their sera, $CD34^+$ cells, and megakaryocytes [95]. Overexpression of TPO in mouse BM through retroviral transduction and transplantation induces fatal MF [96, 97] with increased plasma levels of TGF-β1 and PDGF [98], whereas donor cells with mutations in the *Tgfb1* gene do not cause MF following overexpression of TPO [99]. These results suggest a possible role for TGF-β1 in the pathogenesis of MF.

Lessons from JAK2 Inhibitors

There is great interest in whether molecularly targeted drugs aimed at the JAK2 signaling pathway may be effective treatments for MPN and MF. Several groups have reported preclinical testing of potent and relatively selective JAK2 inhibitors in mouse models of Ph-negative MPN. Three structurally different JAK2 inhibitors, CYT387 [100], TG101348 (now called SAR302503) [88], and AZD1480 [101] all reversed completely the polycythemia phenotype induced by $JAK2^{V617F}$, with normalization of leukocyte count and hematocrit, reduction in splenomegaly, and substantial restoration of normal splenic and hepatic histology. However, JAK2 inhibitor treatment failed to impact several clinicopathological features of the MPN induced by $JAK2^{V617F}$, as there was no significant decrease in overall extent of marrow fibrosis, in the percentage of GFP^+ cells in blood, spleen and BM, and EPO-independent endogenous erythroid colonies were detected at similar frequency to vehicle-treated mice. Early clinical experience with the sole FDA-approved JAK2 inhibitor ruxolitinib (INCB018424) in patients with MF confirm that this drug is relatively ineffective at reducing MF or the $JAK2^{V617F}$ allele burden [17]. Ruxolitinib was shown to decrease MF in the mouse retroviral model of MPN induced by MPL^{W515}, but this was a short-term study of 2 weeks of treatment beginning 2 weeks post-transplant [102], and it is likely that the results reflect prevention of MF rather than reversal of established MF.

One of the central questions in contemporary MPN research is why JAK2 inhibitors are so different from ABL1 inhibitors, which are very effective at decreasing the *BCR-ABL1* allele burden in CML patients and in mouse models. Levine and colleagues recently described heterodimeric JAK1-JAK2 and TYK2-JAK2 complexes in cell lines selected for cross-resistance to multiple JAK2 inhibitors and in granulocytes from patients treated chronically with ruxolitinib on clinical trials [103]. For unclear reasons, although ruxolitinib is a potent JAK1 inhibitor, inhibition of the JAK1-JAK2 complex by ruxolitinib in vitro was inefficient, and the authors proposed that such heterodimeric JAK complexes may contribute to the persistence of $JAK2^{V617F}$-positive cells in treated patients. Although interesting, several features of this model are difficult to reconcile. First, it is clear that JAK2 inhibitors dramatically reduce the total burden of $JAK2^{V617F}$-positive cells in treated patients, as the spleens are returned to near normal size, but some physiologic response prevents further specific reduction in the malignant cells with continued treatment. Calling this persistence may beg the question, as the production of $JAK2^{V617F}$-positive neutrophils in ruxolitinib-treated patients is nearly normal and represents an enormous and ongoing proliferation of malignant cells in the marrow. Second, it is unclear what stimulus triggers formation of the active heterodimeric JAK complexes, as Levine et al., observed no difference in total JAK1-JAK2 heterodimers in treated patients, only an increase of phospho-JAK2 in the complex [103]. Hence, addition investigation into the molecular mechanisms regulating the formation, catalytic activity, and inhibitor sensitivity of heterodimeric JAK complexes is needed.

Chronic Lymphocytic Leukemia

Chronic lymphocytic leukemia (CLL) is the most common lymphoid malignancy in adults over the age of 65 and represents 11 % of all blood cancer [104]. Conventional chemoimmunotherapy, such as the combination of fludarabine, cyclophosphamide, and rituximab, is often effective at eliminating CLL cells from the peripheral blood. However, CLL cells in lymph nodes and bone marrow are usually resistant to such treatment due to signaling interactions with the lymph node microenvironment [105], which makes CLL incurable with chemotherapy [106]. This has focused much recent attention on the signaling pathways that mediate survival in CLL.

BCR Signaling

The B cell receptor (BCR) signaling pathway plays a crucial role in the pathogenesis of CLL [107]. In a normal B cell, the engagement of the BCR by antigen triggers a signaling pathway controlling proliferation, differentiation and antibody production. Activated BCR recruits kinases such as spleen tyrosine kinase (SYK) and the SRC kinase LYN that phosphorylate ITAM motifs on the cytoplasmic domains of the Ig co-receptors CD79a and CD79b. Such phosphorylation recruits and activates Bruton's tyrosine kinase (BTK) and phosphoinositide 3-kinase (PI3K), subsequently activating many downstream targets including AKT/mTOR, NF-κB, and ERK. Interestingly, BCR activation can occur in a ligand-independent manner. The latter mechanism is especially important, as it is thought to be involved in B cell malignancies including CLL [108]. The major mouse model of dysregulated BCR signaling in CLL is the Emu-TCL1

transgenic mouse strain, which spontaneously develop clonal mature B-cell malignancies resembling CLL [109]. Based on a better understanding of the molecular basis of BCR signaling pathways in CLL, extensive effort has been focused on targeting different components of these pathways [110]. Of particular interest are strategies targeting both the malignant CLL cell and the lymph node microenvironment [105].

SYK/BTK/PI3Kδ: from BCR Signaling to the Clinic

The SYK tyrosine kinase is essential to normal B cell development, as lack of SYK induces severe B cell lymphopenia in mice [111]. The SYK kinase contributes to the initiation and amplification of BCR signaling upon BCR activation by antigen binding and also during ligand-independent activation [112]. In vitro and in vivo studies showed that SYK inhibition impaired BCR signaling, which decreased CLL cell migration, ERK activation and chemokine secretion [113]. These findings strongly implicate SYK as a valid target for therapy in B cell malignancies. A SYK inhibitor, fostamatinib, demonstrated significant activity against CLL in a phase I trial [114]. Second-generation, more selective SYK inhibitors have shown potent effects on CLL cell survival and migration in preclinical studies [115].

Bruton's tyrosine kinase (BTK) was discovered as the product of a gene mutated in human X-linked agammaglobulinemia and in XID immunodeficient mice, both of which exhibit defects in B-cell development and signaling at the pro-B cell level. The BTK is activated downstream of SYK/LYN following BCR stimulation, and in turn leads to activation of the PI3K/AKT and NF-κB pathways. The first clinical inhibitor of BTK is ibrutinib (formerly PCI-32765), a novel irreversible inhibitor of BTK that forms a covalent bond with a cysteine residue in the BTK catalytic domain [116]. Preclinical studies with ibrutinib showed inhibition of BTK phosphorylation and downstream pathways in CLL cells, concomitant with decreased proliferation, increased apoptosis, and blockade of stromal-mediated pro-survival signals [117, 118]. In a phase Ib/II clinical trial, ibrutinib was well-tolerated and showed clinical responses in the majority of CLL patients treated, including those with mutant p53 status [119].

Downstream of BTK, PI3K is critical for BCR signaling in both normal B cells and CLL cells [120]. The predominant PI3K catalytic p110 subunit isoform in hematopoietic cells and lymphocytes is the delta (δ) isoform. Mice lacking p110δ have normal hematopoiesis except for the B-lymphoid lineage, where there is a reduction both in mature B cells and in BCR signaling [121]. The drug CAL-101 (now GS-1101) is a reversible small molecule inhibitor of PI3Kδ [122]. It showed a potent ability to kill CLL cells in vitro while inhibiting chemokine-mediated chemotaxis and migration [123, 124]. In phase I trials, GS-1101 induced lymph node responses in 84 % of CLL patients, independent of high-risk genetic features such as 11q deletions or p53 mutation [125]. Interestingly, both GS-1101 and ibrutinib appear to mobilize CLL cells from the lymphatic niches to the circulation, resulting in significant and sustained leukocytosis that probably reflects disruption of signaling that mediates adhesion of CLL cells to the microenvironment, including the CXCL12-CXCR4 axis.

Conclusions and Future Directions

The chronic leukemias have served as a paradigm for targeted therapies in cancer, as a better understanding of the cellular signaling pathways governing proliferation, survival, and self-renewal has led directly to molecularly targeted drugs that have dramatically impacted the natural history of the disease. In CML, the current interest is focused on preventing disease progression in the vulnerable first year on ABL1 TKI therapy, and on strategies to eliminate CML stem cells and allow TKI discontinuation without relapse. In the Ph-negative MPNs, the challenge is to expand the clinical responses to JAK2 inhibitors to alleviate more symptoms and clinical features of these diseases, including MF, cytopenias, elimination of the malignant clone, normalization of hemostasis and thrombosis, and prolongation of survival. In CLL, the excitement generated by the early clinical results with BCR signaling inhibitors is tempered by the realization that these therapies alone will probably not cure patients. Collectively, these clinical needs should stimulate a renaissance of research into signaling in the chronic myeloid and lymphoid leukemias, where cell biological and mouse modeling studies can be expected to yield new approaches to treatment.

Acknowledgments Supported in part by NIH grants T32 CA009429 (W.A.) and R01 HL089747 (R.A.V.).

Disclosure W. Ahmed received a grant from T32 CA009429; R. Van Etten received a grant from R01 HL089747.

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