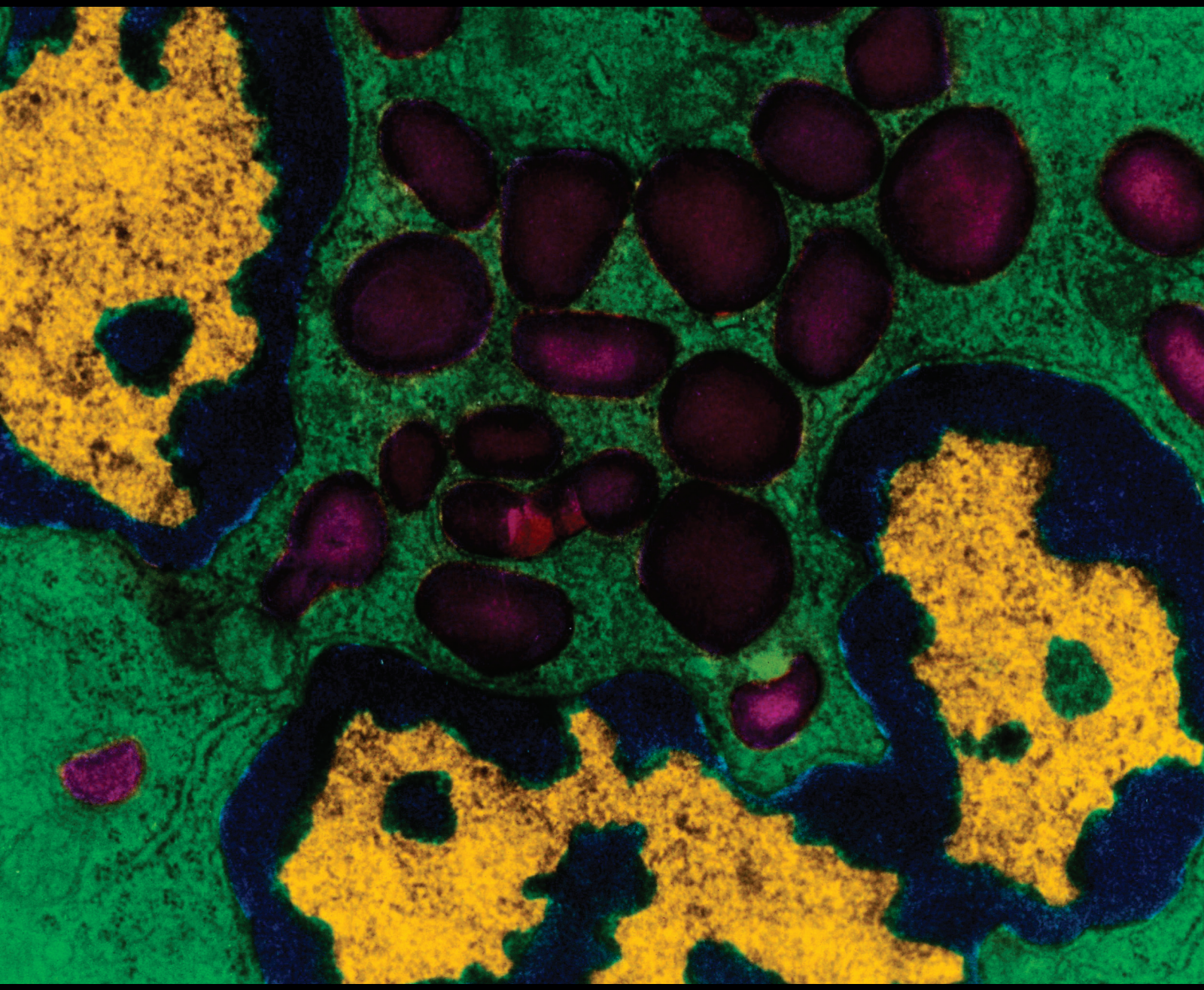
Mediators of Inflammation in Myeloproliferative Neoplasms: State of the Art

Guest Editors: Sylvie Hermouet, Hans C. Hasselbalch, and Vladan Čokić



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Editorial

Mediators of Inflammation in Myeloproliferative Neoplasms: State of the Art

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Myeloproliferative neoplasms (MPNs) are a heterogeneous group of chronic clonal diseases which are characterized by the excessive production of mature cells of one or more of the myeloid lineages. The three main MPN subtypes are very different diseases of distinct clinical presentation, evolution, and variable severity, ranging from mild (essential thrombocythemia (ET)) to moderate (polycythemia vera (PV)) or severe (primary myelofibrosis (PMF)). Recent advances in the biology of MPNs have greatly facilitated the molecular diagnosis of MPNs, since a large majority of MPN patients present with mutation in one of three genes: *JAK2, CALR,* and, more rarely, *MPL*. However, important questions remain regarding the role of these mutations in the pathogenesis of MPNs. While it is established that mutations in the *MPL* and *JAK2* genes result in increased activation of the JAK2/STAT5, STAT3, and STAT1 pathways and hypersensitivity of myeloid progenitors to hematopoietic cytokines, the consequences of *CALR* mutations are not fully understood. Moreover, the reasons why patients present with ET, PV, or PMF when the *JAK2*-V617F mutation is found in all subtypes of MPNs, and the *CALR* and *MPL* mutations are present in ET as well as in PMF, are still not elucidated.

It has long been known that MPN patients present with elevated levels of numerous inflammatory cytokines in blood and bone marrow. Over time several groups established links between inflammatory cytokine levels and MPN phenotype, clinical symptoms, and certain complications. More recently, JAK inhibitors were shown to efficiently decrease the production and signaling of major inflammatory cytokines, leading to significant reduction of spleen size and other invalidating inflammation-linked clinical symptoms in PMF patients while the *JAK2*-mutated MPN clone remained unaffected, as assessed by quantification of the *JAK2* mutant allelic burden. Thus a better knowledge of the causes and molecular mechanisms that underlie inflammation in MPNs could improve understanding of the pathogenesis of MPNs and the treatments proposed to MPN patients.

To help achieve this aim, we invited authors who could shed light on the contribution of inflammation (cytokines and other inflammation markers) to mutation acquisition, clone proliferation, biological parameters, clinical presentation, disease evolution, and response to treatment in MPNs. Major investigators in the field thus described, analyzed, and summarized the biological and clinical findings accumulated in regard to inflammation in MPNs. Our hope is that integrating inflammation in MPN pathophysiology can provide the rationale for novel therapeutic strategies for MPNs that target inflammation in addition to the main mutations.

In the paper entitled "Circulating Cytokine Levels as Markers of Inflammation in Philadelphia Negative Myeloproliferative Neoplasms: Diagnostic and Prognostic Interest", J. Mondet et al. discuss the importance of cytokines in the development of MPNs and, subsequently, the interest of determining the circulating levels of cytokines at the

time of diagnosis. This extensive review presents the current knowledge on cytokine profiles in the different subtypes of MPNs and the role of altered cytokine expression in the pathogenesis of myelofibrosis. Phenotypic correlation, prognostic value of inflammation cytokines in MPNs, and the impact of JAK inhibitors on inflammation cytokine levels are discussed. The authors conclude that circulating cytokines levels could be useful diagnostic and prognostic markers in MPNs. They suggest that cytokine assays could be useful in monitoring treatment efficacy, since a significant cytokine reduction could serve as an indirect marker for therapeutic compliance and efficacy.

In their paper "Impact of Inflammation on Myeloproliferative Neoplasm Symptom Development", H. L. Geyer et al. describe inflammation markers characteristic of MPNs, the upstream sources stimulating their development, their prevalence within the MPN population, and the role they play in symptom development. They present Patient Reported Outcome (PRO) tools designed for evaluating the potential associations between symptoms and inflammation. The authors report clear relationships between individual MPN symptoms (fatigue, abdominal complaints, microvascular symptoms, and constitutional symptoms) and inflammatory cytokines (interleukin- (IL-) 1, IL-6, IL-8, and tumor necrosis factor- (TNF-) α). Information is also provided on the role symptoms paradoxically play in cytokine production, as in the case of fatigue-driven sedentary lifestyles. H. L. Geyer et al. conclude that increased attention should be paid to how inflammation markers differ between MPN subtypes, change with disease progression, and relate to transformation and anticipate novel inflammation-targeting therapies high potential for symptomatic benefit.

The paper of G. Hoermann et al., entitled "Cytokine Regulation of Microenvironmental Cells in Myeloproliferative Neoplasms," reviews the role of MPN-related oncogenes in cytokine expression and release by neoplastic cells and the modulation of microenvironmental cells by these cytokines. The authors describe common as well as distinct pathogenic mechanisms underlying the microenvironmental changes observed in the bone marrow and other organs of MPN patients. Targeting of the microenvironment and of related cytokines (or their receptors) as an attempt to improve therapies in MPNs is also discussed, as such therapies may enhance the efficacy and overcome resistance to established tyrosine kinase inhibitors treatment in MPN patients. For instance, the VEGF/VEGFR, HGF/c-MET, and SDF-1/CXCR4 axes are presented as potential targets in MPNs. The authors conclude that increasing knowledge of the leukemic stem cell- (LSC-) niche interactions will assist in the development of new improved treatment approaches in MPN patients.

In the paper entitled "Inflammation as a Keystone of Bone Marrow Stroma Alterations in Primary Myelofibrosis," C. Desterke et al. discuss bone marrow stroma alterations in PMF, as evidenced by myelofibrosis, neoangiogenesis, and osteosclerosis, and the involvement of altered stromal cells in PMF pathogenesis. The authors propose that the stroma may be inflammatory-imprinted *in vivo* by clonal hematopoietic cells and then becomes "independent" of hematopoietic cell stimulation, rendering this inflammatory loop unbreakable without the association of stroma-targeted therapies to the current protocols.

M. E. Bjørn and H. C. Hasselbalch in "The Role of Reactive Oxygen Species in Myelofibrosis and Related Neoplasms" describe how reactive oxygen species (ROS) are involved in MPN disease initiation and progression throughout the biological continuum from early cancer stage (ET/PV) to advanced cancer stage (myelofibrosis, or MF). Excess ROS, oxidative stress, as a result of chronic inflammation with consequent double stranded DNA breaks in combination with a germline predisposition with impaired DNA repair might account for genetic susceptibility in a subset of individuals implying an increased risk of acquiring an MPN when suffering from chronic inflammatory diseases. The authors hypothesize that the excess production of ROS, by the malignant clone itself, in addition to carcinogenesis also provides an escape from innate and adaptive tumor-immune-surveillance, mainly by blocking interferon signaling. How targeting of ROS with N-Acetyl-Cysteine (NAC) has been a success in other inflammation-driven diseases and why NAC-treatment should be pursued in MPNs as well are discussed. Furthermore, the systemic excess production of ROS also provides a link between the MPNs and MPN-associated comorbidities, in particular the cardiovascular disease-burden.

The paper of J. S. Jutzi and H. L. Pahl, with its provocative title "The Hen or the Egg: Inflammatory Aspects of Murine MPN Models," addresses the contribution of inflammation and other changes in the bone marrow niche in the genesis and maintenance of MPNs. They compare data obtained in gastrointestinal tumors with observations in MPN patients and models and describe novel murine MPN models that may be used to address fundamental questions regarding the role of inflammation in the pathogenesis of MPNs.

In the paper entitled "MPNs as Inflammatory Diseases: The Evidence, Consequences, and Perspectives," H. C. Hasselbalch and M. E. Bjørn describe the evidence for considering the MPNs as inflammatory diseases, "A Human Inflammation Model of Cancer Development," and the role of cytokines in disease initiation and progression. The consequences of this model are discussed, including the increased risk of second cancers and other inflammation-mediated diseases, emphasizing the urgent need for rethinking our therapeutic approach. Early intervention with interferon-alpha 2, which as monotherapy has been shown to induce minimal residual disease in a subset of PV patients, in combination with potent anti-inflammatory agents such as JAK-inhibitors and statins is foreseen as the most promising new treatment modality in the years to come.

The paper of S. Hermouet et al. titled "Pathogenesis of Myeloproliferative Neoplasms: Role and Mechanisms of Chronic Inflammation" discusses the role played by the *JAK2*, *CALR,* and *MPL* mutations in the pathogenesis of the different subtypes of MPNs. The authors review the different aspects of inflammation in MPNs, the main molecular mechanisms involved, and the role of specific somatic or germline genetic defects in the production of inflammatory cytokines. They present evidence that certain inflammatory cytokines present in excess in MPNs are produced independently

from the *JAK2*-V617F mutation. The authors also discuss several inflammation markers that have been identified in MPNs as predictive markers independently of the *JAK2*-V617F mutation and drugs that already exist and target these markers. They also describe possible nongenetic causes of inflammation.

A. G. Fleischman, in her paper "Inflammation as a Driver of Clonal Evolution in Myeloproliferative Neoplasm," provides evidence that the impact of chronic inflammation goes far beyond its role as a driver of constitutional symptoms. The author shows that inflammatory response to the neoplastic clone may be responsible for different pathologic aspects of MPNs and that the *JAK2*V617F-mutated progenitor cells are resistant to the suppressive action of certain inflammatory cytokines, which gives the neoplastic clone a selective advantage and justifies targeting inflammation as a logical therapeutic approach in MPNs.

Last but not least, M. Sevin et al. in "HSP90 and HSP70: Implication in Inflammation Processes and Therapeutic Approaches for Myeloproliferative Neoplasms" report that heat shock proteins (HSP) are key players during inflammation, through their chaperone activity. Notably, HSP90 stabilizes many oncogenes including JAK2 and also key components of the Nuclear Factor-kappa B (NF-κB) signaling pathway, which plays critical roles in the inflammatory response. HSP70 represents another HSP to consider since it tightly regulates the NF-κB pathways. As expected, several HSP90 inhibitors generated as anticancer agents allow the degradation of oncogenes and inhibit the inflammatory response. M. Sevin et al. also discuss the emergence of HSP inhibitors in new protocols designed for the therapy of MPNs.

Altogether, the present special issue highlights the different aspects and the importance of the contribution of inflammation cytokines as crucial regulators of the MPN clone and as mediators of clinical symptoms and complications. The special issue also describes the effects of current drugs or combinations of drugs acting on inflammation in MPNs and proposes new lines of research and novel therapeutic strategies targeting inflammation in MPNs.

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Review Article

Inflammation as a Keystone of Bone Marrow Stroma Alterations in Primary Myelofibrosis

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Primary myelofibrosis (PMF) is a clonal myeloproliferative neoplasm where severity as well as treatment complexity is mainly attributed to a long lasting disease and presence of bone marrow stroma alterations as evidenced by myelofibrosis, neoangiogenesis, and osteosclerosis. While recent understanding of mutations role in hematopoietic cells provides an explanation for pathological myeloproliferation, functional involvement of stromal cells in the disease pathogenesis remains poorly understood. The current dogma is that stromal changes are secondary to the cytokine "storm" produced by the hematopoietic clone cells. However, despite therapies targeting the myeloproliferation-sustaining clones, PMF is still regarded as an incurable disease except for patients, who are successful recipients of allogeneic stem cell transplantation. Although the clinical benefits of these inhibitors have been correlated with a marked reduction in serum proinflammatory cytokines produced by the hematopoietic clones, further demonstrating the importance of inflammation in the pathological process, these treatments do not address the role of the altered bone marrow stroma in the pathological process. In this review, we propose hypotheses suggesting that the stroma is inflammatory-imprinted by clonal hematopoietic cells up to a point where it becomes "independent" of hematopoietic cell stimulation, resulting in an inflammatory vicious circle requiring combined stroma targeted therapies.

1. Introduction

Hematopoiesis is orchestrated through a tightly regulated network of events including cell-cell interactions, cytokines, chemokines, proteases, and extracellular matrix components within an environment where oxygen level and calcium concentration are monitored. At steady state, adult HSCs reside in the BM in specialized niches made up of bone and vascular and nervous structures [1, 2]. Within these niches, the balance between HSC quiescence, self-renewal, and differentiation is controlled by a sophisticated dialogue between HSCs, stromal and neural cells in a "seed (stem cells) and soil (stroma)" relationship. This equilibrium must be tightly controlled since its disruption can participate in the emergence/development of hematological malignancies such as myelodysplastic and myeloproliferative disorders [3–5].

Primary myelofibrosis (PMF) is a clonal myeloproliferative neoplasm (MPN) of the elderly whose severity as well as treatment complexity is mainly attributed to the fact that PMF is a long lasting disease and to the presence of profound changes in the bone marrow (BM) stroma evidenced by myelofibrosis, neoangiogenesis, and osteosclerosis [6]. Despite new therapies targeting the myeloproliferation, PMF is still regarded as an incurable disease except for patients who are successful recipients of allogeneic stem cell transplantation.

This may, in part, be due to the fact that current therapies are unable to influence the altered stroma and to

reestablish efficient hematopoiesis requiring the elimination of neoplastic hematopoietic cells. Actually, with the exception of ruxolitinib in case reports, most JAK2 inhibitors, despite being effective in alleviating constitutional symptoms, have no or very few effects on bone marrow fibrosis [7]. Whereas there is no study analyzing the direct effect of JAK2 inhibitors on stromal cells, these inhibitors have been mainly designed to suppress the cytokine signalling cascade caused by the constitutive activation of JAK2. However, by providing significant improvements in splenomegaly, associated clinical manifestations, and disease related constitutional symptoms, their clinical benefits have been associated with a marked reduction in serum proinflammatory cytokines produced in particular by the hematopoietic cells, demonstrating the importance of inflammation in the pathological process [8]. More recently, preclinical studies have observed that ruxolitinib causes a rapid and prolonged decrement of T regulatory cells and impairs the normal function of dendritic cells suggesting that JAK2 inhibitors can also act via an immunosuppressive effect [9–11].

The development of novel more effective therapies will also depend on a better understanding of the disease pathogenesis. Although current knowledge about the role of mutations in hematopoietic cells partially explains myeloproliferation, functional involvement of stromal cells in PMF pathogenesis remains poorly understood. Up to date, the dogma is that stromal changes, including myelofibrosis that is the hallmark of the disease, are secondary to the cytokine "storm" created by hematopoietic cells from the clone and especially by pathological megakaryocytes (MKs) [14]. This assumption is mainly based on the lack of information on molecular anomalies in stromal cells and does not take into account the possibility for stromal cells to acquire functional abnormalities within the inflammatory process that is developed during the course of the disease. Actually, an increasing number of results from our laboratory suggest the role of an altered dialogue between hematopoietic and stromal cells in the pathogenesis of PMF at the origin of our "bad seeds in bad soil" concept [6, 15–18]. Hence, during the long lasting process of PMF, hematopoietic, immune, and mesenchymal stromal cells could be both effective and responsive cells, creating a vicious circle that is difficult to break by current therapies.

Understanding the mechanisms by which the "bad soil" (stromal cells) contributes and responds to the inflammatory process participating in making the bed for the "bad seeds" (clonal hematopoietic cells) would therefore help in the development of new immune- and cell-based therapies. By targeting inflammation and restoring stroma homeostasis, these new treatments will synergize with the current drugs mainly focused on eradicating the malignant hematopoietic clones.

In this review, based on hypotheses from our group, we will consider arguments concerning the role of inflammation as a driving mechanism for "intrinsic" (i.e., HSC-independent) alterations of mesenchymal stromal cells in PMF patients. We will bring some controversies on the pathogenesis of this no longer "forgotten myeloproliferative disorder" [19], but still misunderstood neoplasm.

2. Myeloproliferation and Myelofibrosis: The Dual Complementarity of Primary Myelofibrosis?

According to the 2008 WHO classification, primary myelofibrosis belongs to Philadelphia negative myeloproliferative neoplasms [20]. Together with Polycythemia Vera (PV) and Essential Thrombocythemia (ET), PMF shares features of myeloproliferative diseases that is the expansion of clonal hematopoietic stem/progenitor cells. PMF is characterized by a shortened life expectancy, myelofibrosis, osteosclerosis, and extramedullary hematopoiesis [14, 21]. Diagnosis relies on clinical, biological, molecular, and bone marrow biopsy analysis. Clinical and biological data demonstrate splenomegaly, dacryocytosis, basophilia, or leukoerythroblastosis.

Several molecular mechanisms and other clues suggest the clonal nature of the disease and that mutational clonal evolution in PMF is dependent on multiple hematopoietic clones [22–24]. The pathological hematopoietic stem cells harbor genetic mutations conferring the proliferative phenotype of the disease. The *JAK2* V617F and *MPL* 515 mutations, present in about 50% and 5% of PMF cases, respectively, result in a permanent activation of the JAK/STAT signalling pathways, conferring *in vitro* altered sensitivity/independence of clones to growth factors [25–27]. The recently discovered Calreticulin mutations complete the scope of PMF mutations, occurring in 25% and 88% of patients without *MPL* and *JAK2* mutations [28]. Finally, less than 10% of patients are "triple-negative" [29]. It is suggested that, as *JAK2* and *MPL* mutations, the most frequent Calreticulin mutation (Exon 9 Calreticulin type 1 mutation) confers a relative independence of the clonal cells to growth factors [30]. Calreticulin protein is involved in intracytoplasmic protein trafficking and mutations could alter membrane expression of receptors participating in the proliferative abilities of clonal cells [31, 32]. Other mutations can occur less frequently and participate in the activation of the JAK/STAT pathways: for instance, LNK, an adaptor protein which negatively regulates TPO signalling, is mutated in some patients [33] or promoters of tumor-suppressor genes like SOCS-3 which are hypermethylated [34]. Apart from the abovementioned mutations, others such as *NRAS* and *NF1* mutations in the MAP-kinase pathways are associated with worse prognosis [35, 36]. Mutations can also occur in epigenetic regulator genes such as *TET-2* [37], *DNMT3A* [38], or *ASXL1* [39]. Recently, stem cell populations from PMF patients identified by the expression of CD133 have been investigated and after transplantation into mice were able to recapitulate major PMF parameters, revealing that CD133 marks a stem cell population that drives PMF [24]. However, despite numerous mutations, none are able, as the *BCR-ABL* mutations in chronic myeloid leukemia, to fully recapitulate the disease in an animal model or to entirely explain the pathophysiological features of PMF.

To decipher the natural course of the disease, clonal cells must be replaced in their environment and time scale should be considered. The concept that hematopoietic stem cells are intimately dependent on interactions with their environment has emerged in the late 70s [40] and became

preeminent in the last few years [41]. Actually, as described in the introduction, HSC cell fate is highly dependent on cell-to-cell connections, matrix-to-cell interactions, and chemokine stimulation. Those cellular and noncellular elements are key components of the so-called "hematopoietic niches" [42]. Three "distinct" niches are conceptually identified. The first one is the endosteal niche, which is located close to the endosteum and whose main component is the Shaped N-Cadherin positive osteoblast [43] and where HSC quiescence is maintained [44]. In contrast, the vascular niche and the CXCL-12 abundant perivascular cells [45] would be the place of differentiation and proliferation [46]. A third niche would be the link between those specialized areas, integrating signals from nervous system through Schwann cells [47]. The mesenchymal stromal cells (MSCs) would be the prominent components of this niche [48]. Through their ability to differentiate into fibroblasts, osteoblasts, and adipocytes and to produce extracellular matrix elements, MSCs are reported be milestone regulators of hematopoiesis, questioning their potential role in hematopoietic malignancies.

In recent years, abnormalities in the BM microenvironment have appeared as critical promoters of myeloid malignancies. In murine models, genetic ablation of the retinoic acid receptor gamma (*Rar-γ*) or retinoblastoma (*Rb*) genes in BM stromal cells have been reported to promote MPN development [49, 50], whereas inactivation of the microRNA-processing enzyme dicer in immature OSTERIX-(OSX) expressing osteoprogenitors caused myelodysplastic syndrome (MDS) [4]. Interestingly, Wei et al. have shown that the murine microenvironment determines the lineage outcome of the human biphenotypic MLL-AF9 leukemia stem cells when graphed in immunodeficient mice [51]. In humans, evidences are scantier. One of the most intriguing piece of data is the development of donor cell leukemia in recipients of hematopoietic stem cell transplantations, with the same phenotype of the former disease, strongly suggesting the role of recipient microenvironment in the onset of the disease [52]. Analysis of beta-catenin expression in osteoblasts of patients with myelodysplastic syndrome or myeloid leukemia also revealed that the microenvironment might interact with hematopoietic cells in the development of the disease [5].

In PMF, several evidences argue for an impaired microenvironment in relation with inflammation. As previously mentioned examination of BM biopsies represents a key step in the PMF diagnosis. Besides the myeloproliferation, especially megakaryocytic proliferation with abnormal morphological features, PMF is characterized by myelofibrosis, neoangiogenesis, and osteosclerosis. Megakaryocytes [12] and monocytes [53] derived from the malignant clones produce high levels of Transforming Growth Factor-beta1 (TGF-β1) [54], Platelet-Derived Growth Factor (PDGF), basic Fibroblast Growth Factor (bFGF) [55], and Vascular Endothelial Growth Factor (VEGF) [56]. Particularly, TGF-β1 exerts profibrotic effects on fibroblasts and favors ossification by osteoblasts. Concomitantly, osteoprotegerin production by fibroblasts inhibits osteoclastogenesis and enhances bone marrow osteosclerosis. Neoangiogenesis associated with morphological modification of vessels and of pericytes is present in the bone marrow of PMF patients [57]. Endothelial cells of spleen vessels harbor *JAK2* mutation [58] and are known to increase cellular adhesion [59], demonstrating that bone marrow modifications are not the sole elements of the microenvironment alterations in PMF. Actually, one of the features that distinguishes PMF from ET and PV is the extramedullary hematopoiesis in spleen and liver and high number of $CD34^+$ circulating cells [60]. Disruption of the CXCL12-CXCR4 axis involved in this phenomenon is related to the abnormal methylation of the CXCR4 promoter [61] and with metalloproteinase deregulation in the bone marrow [62]. In the spleen of PMF patients, $CD34^+$ cells are able to give rise to extramedullary hematopoiesis in a remodeled niche as evidenced by specific properties of fibroblasts isolated from patients [17, 18]. Altogether, these data demonstrate a wide disruption in the crosstalk between hematopoietic stem cells and their stromal microenvironment in PMF (Figure 1).

3. Inflammation: A Pathophysiologically Important Component of MPN Pathogenesis

Inflammation is a key pathophysiological component of a wide range of diseases [63], including PMF and the other Philadelphia-negative chronic MPNs [64]. Inflammation is a protective reaction in response to injury and its objective is to eliminate harmful stimulus or promote repair of damaged tissue, a phenomenon observed during wound healing [1]. It is important to distinguish between acute and chronic inflammation. The acute inflammatory response is a complex and coordinated sequence of events involving a large number of molecular and cellular changes. It begins with the production of soluble mediators including chemokines and cytokines secreted by resident cells and ends with the resolution or "switching off" of the inflammatory response leading to restoration of normal tissue homeostasis. Although the acute inflammatory response is critical for survival [63], dysregulation of this process may predispose certain individuals to the development of chronic inflammation. A prerequisite for inflammation resolution is to switch off or eradicate the primary stimulus that initiated it [63]. Failure to eradicate the initial trigger may lead to chronic inflammation as exemplified by MPNs, which is hypothesized to result from a sustained inflammation exacerbated by continuous release of proinflammatory cytokines and chemokines [64].

3.1. What Triggers Inflammation in MPNs? It is believed that MPNs arise from mutant hematopoietic stem cells implying that these disorders are clonal hematologic diseases [2]. However, if MPNs are clonal stem cell diseases and *JAK2* mutation in the myeloproliferative disorders is not in the germ line but, rather, is acquired [2], then what is the nature of the primary trigger that causes the initial genetic defect? We know that inflammation in general occurs in response to something that destabilizes local homeostasis; in MPNs, identification of that "something" has been proven elusive. The precise nature of the initial trigger may remain unknown,

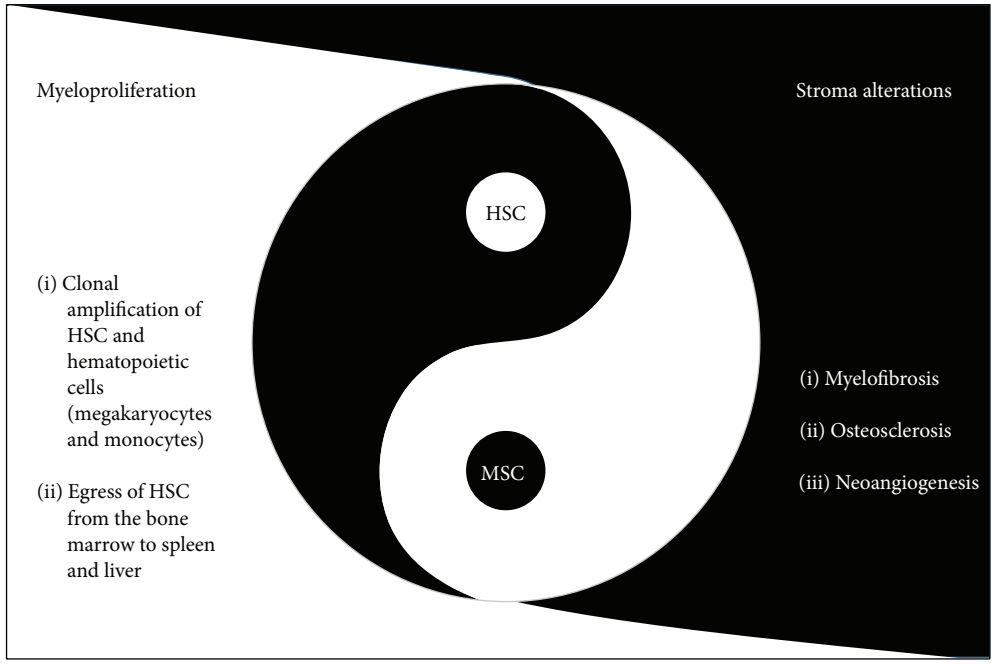


Figure 1: Primary myelofibrosis: the dual complementarity of hematopoietic and stromal stem cells. PMF is characterized by medullar and extramedullary clonal expansion of hematopoietic stem cells (HSCs) and dystrophic megakaryocytes (MKs), altogether with myelofibrosis and osteosclerosis involving fibroblasts and osteoblasts, as well as neoangiogenesis. These elements stand together by growth factors and inflammatory cytokines mediated interactions [6].

but what remains certain is that the MPNs are associated with a chronic inflammatory state which is referred to as a "human inflammation model" with "inflamed bone marrow," "inflamed stem cell niche," and "inflamed circulation" [64].

3.2. Chronic Inflammation in PMF: What Can We Learn from Other Inflammatory Disorders? Could a chronic inflammatory state that is triggered initially by a process other than infection, tissue injury, or autoimmunity be causing genomic instability and fibrosis in PMF? If the answer is yes, then it is tempting to compare PMF with atherosclerosis—class of diseases with nonresolving inflammation. PMF and atherosclerosis share two common characteristics. First and foremost, both atherosclerosis and PMF lack the potential for removing the inflammatory stimulus which would normally occur in most cases of infection or injury [65]. Secondly, both diseases are often associated with aging. Important advances in the treatment of atherosclerosis have been made [66]; hence in this context, what can we learn from the advances made in diseases in which inflammation is an important driving force? More importantly, how might the inflammatory nature of atherosclerosis lead to better understanding of pathological inflammation and new therapeutic opportunities in MPNs? The understanding of the pathology of nonresolving inflammation, which is typically initiated by pattern recognition receptors such as toll-like receptors (TLRs) that recognize pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) [67], leads to discovery of a class of anti-inflammatory drugs known as disease-modifying agents of rheumatoid diseases (DMARDs) [68], which are distinguished by their ability to reduce or prevent tissue damage caused by the inflammatory attack, especially when used early in the course of the disease. Just as in other inflammatory diseases including atherosclerosis [67], TLRs couple to signal transduction pathways that activate latent transcription factors that include members of the NFκB and AP-families [65], which happen to be increased in hematopoietic cells and stroma cells, exposing these cells to a constant oxidative stress [64]. These factors in turn induce the expression of a large number of genes that aid chemokine release, which in turn regulate the recruitment of additional immune cells [64]. Increased TLR activity could result in augmented production of cytokines and chemokines activating leukocytes in the bone marrow to make TNF-α and IL-6. IL-6 is known to increase NFκB and STAT3 causing inhibition of apoptosis and increased myeloproliferation, hence creating an environment favorable to malignant transformation and expansion [64, 69].

4. How Does TGF-β Contribute to Fibrosis in the Context of Inflammation?

TGF-β, the most critical regulator of pathological fibrosis, is overexpressed in all fibrotic tissues and it induces collagen production in cultured fibroblasts, regardless of their origin [70]. TGF-β is part of a superfamily of 33 members that includes BMPs, activins, inhibins, growth differentiation factors, and myostatin [71]. The three TGF-β isoforms are encoded by different genes; TGF-β1, TGF-β2, and TGF-β3, which are secreted as latent proteins, interact with the same

receptor heterodimers, TGFR-1 (TGF-β receptor type-1, also known as ALK-5) and TGFR-2 (TGF-β receptor type-2) [70]. All three isoforms exert TGF-β signalization mainly via its canonical SMAD pathway, although TGF-β can also activate other pathways that are collectively referred to as noncanonical TGF-β pathways [72].

Bone marrow is a heterogeneous organ containing diverse cell types. In the BM of MPNs patients, TGF-β is believed to be produced by hematopoietic cells, including necrotic and viable megakaryocytes [15]—important source of latent TGF-β stored within the alpha-granules of these bone marrow cells [15]. An increasing number of niche components have now been identified revealing a complex network of cell and matrix interactions and signalling pathways, which together create a unique microenvironment with TGF-β being an integral part of this environment. Cell-cell and cell-matrix interactions with the BM are critical components of the orchestrated process of activation of latent TGF-β. Interaction between BM nestin$^+$ MSCs and BM Schwann cells was identified as contributing to MPN pathogenesis [73]. Actually, nonmyelinating BM Schwann cells promote TGF-β activation by exposing the growth factor to proteolytic cleavage by metalloproteinases [73].

TGF-β production correlates with the progression of fibrotic diseases and TGF-β inhibition has been shown to reduce fibrotic processes in many experimental models [74]. TGF-β is unequivocally a prominent stimulus and regulator of extracellular matrix formation. It mediates fibroblast and endothelial cell proliferation, suggesting their involvement in the stromal reaction and reinforcing the hypothesis of a connection between fibrosis and angiogenesis as suggested in various fibrotic diseases including pulmonary and eye fibrosis as well as systemic sclerosis [15, 75]. TGF-β has been also implicated in the development of fibrosis associated with hematological disorders including hairy cell leukemia, acute megakaryoblastic leukemia, and PMF [15]. In PMF and other MPNs the stromal cells and fibroblasts responsible for the increased fibrosis, angiogenesis, and formation of new bone are not derived from the myeloproliferative clone [2]. BM microenvironment and its interactions with TGF-β have been proposed to contribute to myelofibrosis [76]. The question of how latent TGF-β becomes activated in the bone marrow of MPN patients is, therefore, central to the understanding and the treatment of fibrotic diseases. Although integrins [77] and thrombospondin-1 [78] have been known to activate latent TGF-β in other fibrotic disease models such as skin [70] and liver fibrosis [78], it is possible that this pattern of activation may also function in PMF (see Section 6).

Recently, based on transcriptomic analysis, Ciaffoni et al. have suggested that fibrosis in PMF may result from an autoimmune process triggered by dead megakaryocytes through activation on noncanonical TGF-β signaling [79]. The interesting assumption of autoimmunity as a possible cause of marrow fibrosis in PMF is reminiscent to historical articles such as those from Lang et al. [80] and Rondeau et al. [81] describing the presence of autoantibodies, their levels being related to the degree of fibrosis. However, whereas the parallel between apoptosis and fibrosis is of interest, the signification of the presence of autoantibodies in PMF patients as a "cause" or a consequence of the pathological mechanism is not clear. Since many recent studies suggest a positive association between autoimmune and inflammatory diseases and subsequent neoplasia development, this concern would merit extensive studies in an attempt to better combine immunomodulatory therapies to current treatments [82].

5. When Data-Mining Identifies MSCs as a Piece of the Inflammation Puzzle!

In PMF, the huge deregulation of inflammatory/fibrogenic cytokines is suggested to contribute to the clinical phenotype, including bone marrow fibrosis, increased angiogenesis, extramedullary hematopoiesis, constitutional symptoms, and cachexia. It has been suggested by Tefferi's group that plasma cytokine signature provides a useful laboratory tool for predicting and monitoring treatment response [83]. Interestingly, a two-cytokine (IL-8/sIL-2Rα) based risk categorization stratified on a large cohort of patients has been shown to delineate different groups within specific DIPSS plus risk categories [83]. In patients, growth factors have been suggested to be mainly produced by dystrophic megakaryocytes and monocytes; however, recent data from our group also identified PMF MSCs, endothelial cells, and T lymphocytes as important sources of inflammatory cytokines [16, 84].

To characterize inflammation in the altered bone marrow stroma from patients, we query information from the literature by data-mining using inflammation, fibrosis, macrophage, mesenchymal stromal cells, and immunomodulation as keywords (Figure 2). A total of 253.585 connections were collected between Pubmed and gene databases (Figure 2(a)). This collected information was crossed with the gene expression profile of BM-MSCs we performed in PMF patients (GSE44426) [85] in R software [86]. The inflammatory predictive signature allows performing an unsupervised classification and identified two distinct clusters of BM-MSC samples: PMF patients and healthy donors, demonstrating that BM-MSCs from PMF patients have a typical inflammatory gene expression profile which is different from their normal counterparts (Figure 2(b)). This data-mining analysis identified several altered pathways in PMF-MSCs that would be part of the pathophysiological process. Among them, inflammatory response, oncostatin M and TGF-β signalling pathways, focal adhesion, senescence, and autophagy are the most significant within the stromal niche context (Figure 2(c)).

Oncostatin M (OSM), an interleukin-6-like inflammatory cytokine, is reported to play a role in a number of pathological processes including cancer. In MPNs such as PMF, activation of the JAK/STAT signalization resulting from the presence of the *JAK2 V617F* or *MPL 515* mutations in the hematopoietic lineage is known to stimulate OSM production by pathological megakaryocytes [87, 88]. In PMF-MSCs, the altered expression of genes such as *STAT1*, *SOCS3*, *MMP1,* and *SERPINE1* participating in the OSM signalling pathway suggests that they could be activated by OSM (Figure 3). The overexpression of *STAT1* (fold change = 2.21), an effector of signal transduction able to activate the expression of VEGF in response to OSM stimulation [89], evidences a

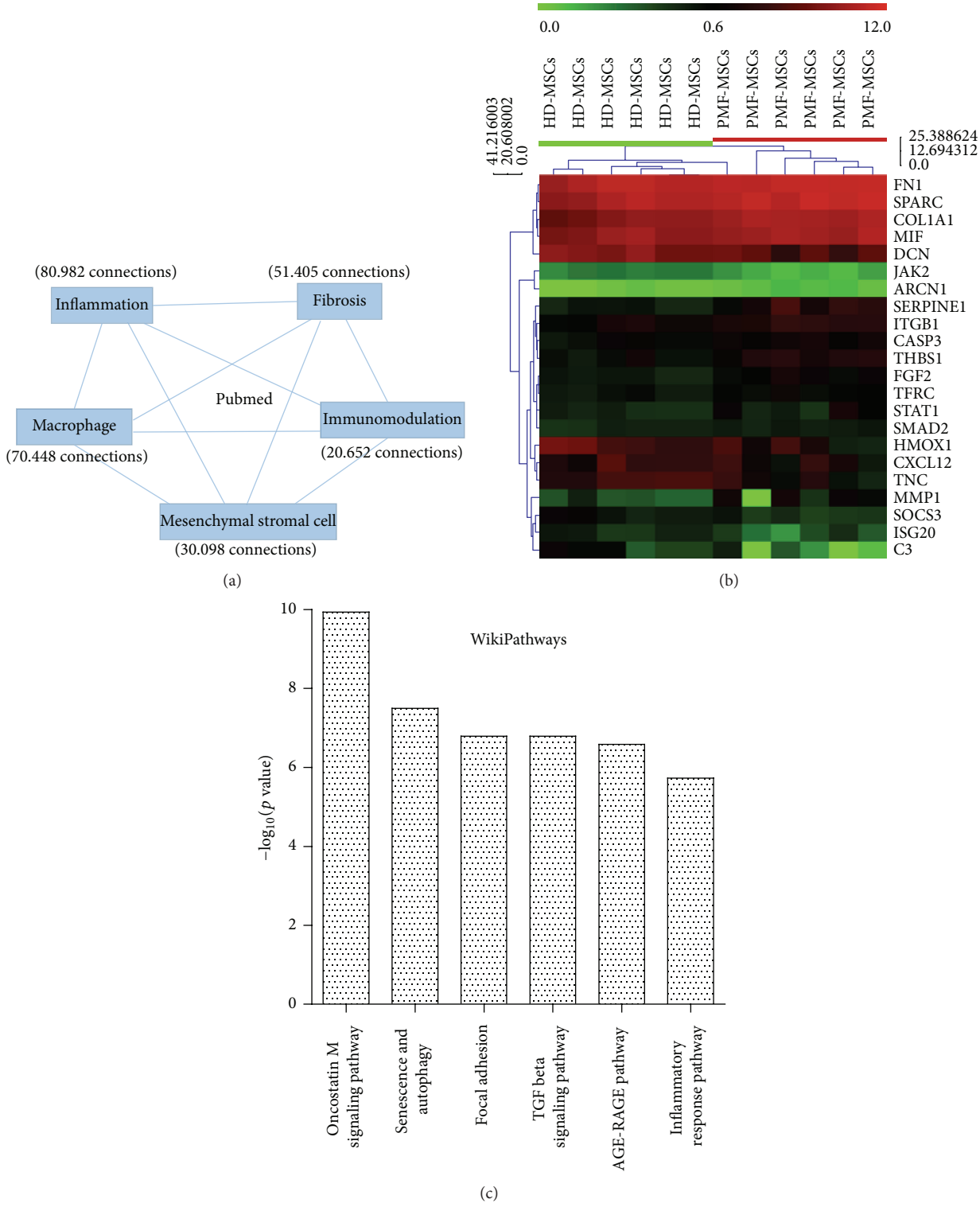
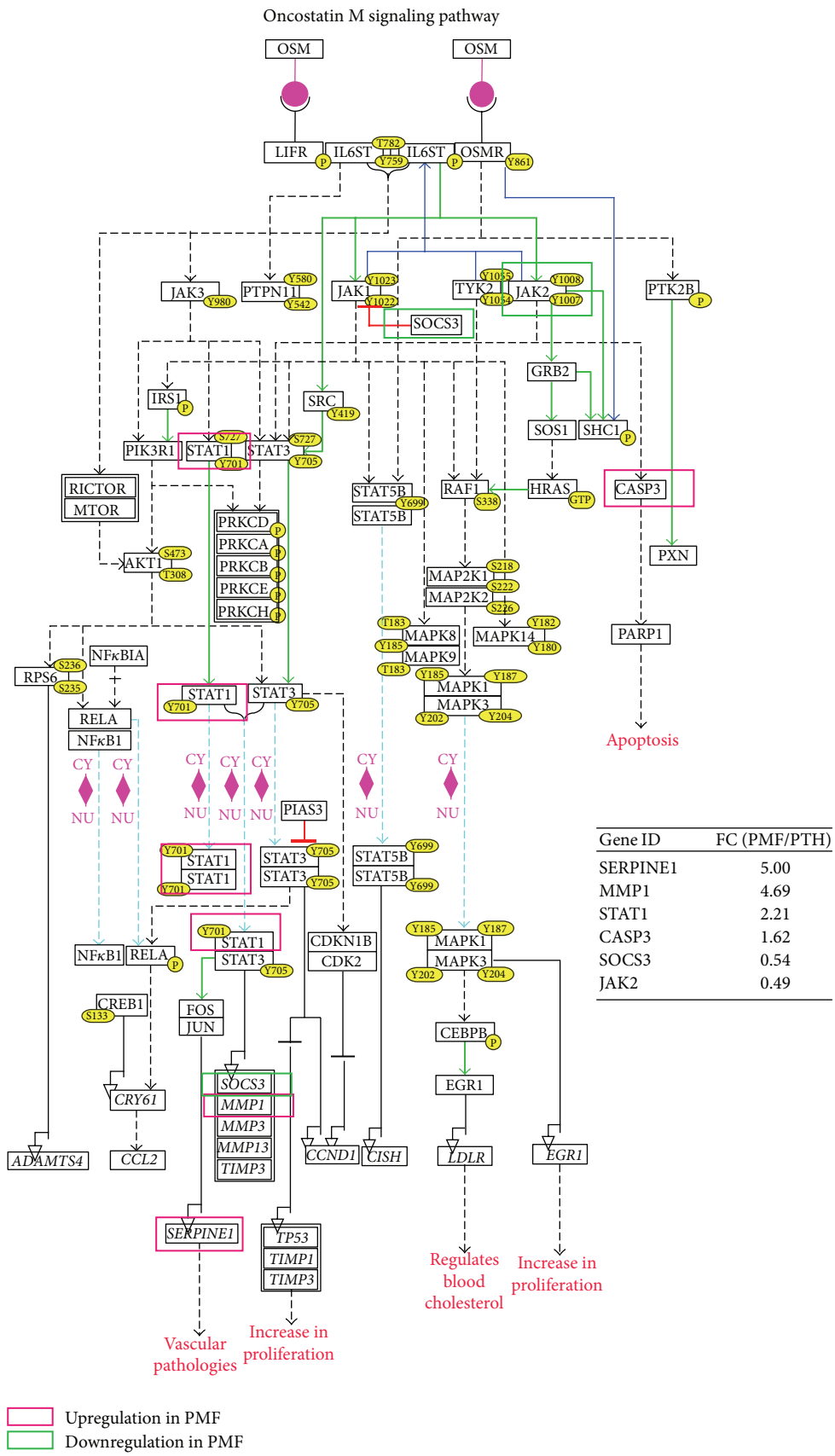


Figure 2: Data-mining prediction of inflammatory gene expression profile in BM-MSCs from PMF patients. (a) Keywords used during data-mining to link the scientific information between inflammation and altered niche in primary myelofibrosis; (b) unsupervised classification on data from inflammation prediction of gene expression profile from PMF BM-MSCs (transcriptome GSE44426); (c) functional enrichment on WikiPathway database for inflammation signature prediction of BM-MSCs from PMF patients.



Gene ID	FC (PMF/PTH)
SERPINE1	5.00
MMP1	4.69
STAT1	2.21
CASP3	1.62
SOCS3	0.54
JAK2	0.49

Figure 3: Oncostatin M signalization in mesenchymal stromal cells from PMF patients. WikiPathway diagram of oncostatin M signalization with drawing of genes deregulated in BM-MSCs from PMF patients (transcriptome GSE44426).

possible link between BM-MSCs, OSM, and the increased VEGF expression [87] (Figure 3). Actually, a paracrine effect of oncostatin M could induce production of VEGF by the bone marrow stromal cells [87]. The massive neoangiogenesis [90] observed in association with the myelofibrosis in PMF patients is in agreement with such hypothesis. This is also confirmed in other Phi-negative myeloproliferative disorders such as PV and ET [91] where the plasma level of VEGF is correlated with the BM microvessel density [92].

SERPINE1 is also highly upregulated (fold change = 5.0) in BM-MSCs from PMF patients. This molecule, also named Plasminogen Activator Inhibitor-1 (PAI-1), is known to be deregulated in PMF [93, 94] and to be associated with a bad prognosis in diverse cancers [95, 96]. Its role in vascular alterations [97], extracellular matrix reorganization [98], and metalloproteases regulation [99] through a TGF-β1 dependent mechanism [100] strengthens its potential participation to the stromal reaction and to the egress of hematopoietic progenitors from the BM observed in patients [62].

Inflammatory expression profile of PMF BM-MSCs highlights alterations of the senescence pathway regulation involving genes such as *SPARC*, *THBS1*, *FN1,* and *COL1A1*. In MPNs, expression of SPARC in BM stromal cells correlates with the degree of stromal changes and the severity of BM failure [101]. In a murine model of thrombopoietin-induced myelofibrosis using Sparc(−/−) mice and BM chimeras, SPARC contributes to the development of significant stromal fibrosis [101]. However, whereas in this thrombopoietin-induced myelofibrosis murine model, THBS1 is not required for TGF-β1 activation [102], it is suggested to be a mediator which discriminates PMF from ET patients within a profibrotic environment [103].

Together with thrombospondin and SPARC, tenascin forms a family of matrix proteins that caused a dose-dependent reduction in the number of focal adhesion-positive cells. Tenascin, observed in myelofibrosis with megakaryocytic hyperplasia, has a strong impact on chronic inflammation and on TGF-β activation and signalling [104]. This is in line with the notion that tenascin synthesis in BM fibroblasts is stimulated by TGF-β also produced by MK cells [105]. Connections between focal adhesions and proteins of the EMC involve integrins. Actually, integrin β1 participates in (1) mediating activation of latent TGF-β via ECM contraction and (2) modulating collagen production via a focal adhesion kinase/rac1/nicotinamide adenine dinucleotide phosphate oxidase (NOX)/reactive oxygen species (ROS) pathway. Therefore, multiple alterations of ECM and focal adhesion components like integrins observed in the BM could participate in activation of the TGF-β signalization in PMF patients.

Altogether results from this data-mining analysis suggest that chronic inflammation present in BM environment of PMF patients could induce a hypersensibility of MSCs to inflammatory molecules participating in creating a vicious circle. Additionally to TGF-β signals, BM-MSC hyperresponsiveness resulting from inflammation could result in liberation/activation of effectors contributing to (i) fibrosis (collagens, fibronectin, and tenascin C), (ii) extracellular matrix modeling (SERPINE1, MMP1), (iii) angiogenesis (oncostatin M signalling pathway), and (iv) hematopoietic progenitor homing/egress (CXCL12). Interestingly, as a demonstration of the role of "inflamm-aging" in BM stromal alterations, MSCs from patients also harbored changes linked to aging such as senescence, hypoxia, and AGEs/RAGE signalling pathways (Figure 3).

6. Inflammation as a Keystone of Bone Marrow Stroma Alterations in PMF

In PMF, bone marrow stroma alterations occur at cellular and noncellular level. Inflammation impacts cellular components of the hematopoietic niche: fibroblasts, osteoblasts, endothelial cells, and MSCs. Basic FGF is able to induce MSC proliferation and to act as an angiogenic growth factor [55, 106]. Interleukine-1 can also modulate fibroblastic abilities [107]. PDGF induces proliferation of fibroblastic cells [108], major producers of matrix components. In association with TGF-β1, this results in an increase of proteoglycans, fibronectin, and collagens. TGF-β1 is a powerful inducer of matrix-associated genes expression [109]. Concomitantly, it inhibits matrix proteases, leading to deep changes in the extracellular matrix (ECM) properties [110]. ECM remodeling could participate in alterations of hematopoiesis: megakaryopoiesis is stimulated by glycosaminoglycans [111], and some heparan sulfate proteoglycans are involved in myeloproliferation [112]. TGF-β1 is a potent inducer of GAG expression by osteoblasts [110], and on the other hand, GAGs could interact with Bone Morphogenic Proteins (BMPs) and induce osteogenic differentiation of MSCs [113]. Remodeling of ECM is of major importance, since matrix to cell interactions can modulate cell fate. For instance, modification of physical traction forces in the ECM can participate in the shift of TGF-β1 from its latent to its active form [13]. Modifications in the ECM composition could modify such tractions forces and participating in a feedback loop to TGF-β1 stimulation on microenvironment cells. GAGs are involved in local concentrations of cytokines and growth factors and reciprocally, TGF-β1 enhance GAGs production [114]. Inflammation is responsible for the creation of acidic microenvironment, which may enhance the release of lactates by hematopoietic cells from the clones [115] and activate latent TGF-β1 [116], hence further adding to the inflammatory storm in the bone marrow. Another key actor of inflammation and pathogenesis of PMF is neoangiogenesis. VEGF is overproduced in patients [117] and, apart from its role in fibrosis, it plays a pivotal role in the increased vascularization of PMF bone marrow [118]. Taken together, these data strongly suggest that chronic inflammation plays a role in the physiopathology of PMF.

The origin of inflammatory cytokines is mainly represented by pathological clonal cells and remodeling of the microenvironment in a pathological niche clearly involves these clonal cells [6]. Nevertheless, some data raise the question of the role of the inflammatory stimuli in the natural history of PMF. The current concept advocates for a dependence of stromal alterations to cytokines production by the hematopoietic clones. This concept suggests that when clonal disease would be cured, inflammation should stop and

will allow an *ad integrum* restitution of the hematopoietic niche. This approach leads to therapy targeting the clonal hematopoietic cells, neglecting other potential target. If evidences are still lacking to attest the nonclonal nature of stromal cells in PMF, some data must be discussed. Cytogenetic-based analyses of bone marrow fibroblasts or MSCs isolated from PMF patients are ancient and based on low-sensitive technics [119–121]. Recently, some data suggested that MSCs from patients could display cytogenetic modification, before culture [122]. Secondly, there is no clear correlation between TGF-β1 level and fibrosis: patients without bone marrow fibrosis could exhibit higher level of inflammatory cytokines than patients with marked myelofibrosis [123]. The clinical features of PMF, particularly fibrosis, prominently involve bone marrow but seem to bypass other organs such as the liver or spleen. Could this be due to the presence of activation pathways "exclusive" to bone marrow? The last point questioning this purely reactive conception of bone marrow alterations is the course of fibrosis under therapy. Remissions have been reported in PMF patients after hematopoietic stem cell transplantation [124]. However, its timing is crucial and should be performed before the disease has developed to a very advanced stage. This limitation could explain why the reduction of fibrosis could be significant [124], slow and incomplete [125, 126], or inexistent [127]. Intriguingly, decrease of fibrosis is not correlated with megakaryocytes that are the main source of profibrotic cytokines [126]. Regarding osteosclerosis, data are more homogenous: no improvement is observed under therapy [126, 128, 129]. So, eradicating the hematopoietic clones is not systematically associated with a cure of stromal alterations, keeping open the question of the mutual instructions between hematopoietic and stromal cells.

7. Do and How Stroma Alterations Become "Independent" of the Inflammatory Hematopoietic Cell Stimulation?

The concept of MSCs being important effector cells which have the ability to influence the hematopoietic niche has helped to develop new hypothesis and further the current understanding of PMF pathophysiology. Do and how the microenvironment could follow a natural history independently of malignant hematopoietic cells stimulation? Chronic inflammation is typically associated with sustained myeloproliferation and the activation of a number of cellular pathways, which ultimately may trigger DNA damage in hematopoietic cells through ROS accumulation [130]. During inflammation-mediated cells harboring DNA damage may ultimately acquire mutations [131]. Genome wide analysis performed on single MSCs may bring answer to this question. DNA methylation of gene promoters can be promoted by oxidative stress or cytokines like interleukin-6, interleukin-1β, or TNF-α [132, 133]. Analysis of bone marrow biopsy from PMF patients revealed that hypomethylation of PDGF-β gene could be correlated with prognosis and fibrosis [134]. Even if cells harboring methylation modifications cannot be inferred, this data provides evidences of epigenetic modifications occurring during PMF natural history. Inflammation can especially exert its effects on MSCs. Actually, recent results from our lab show that their differentiation abilities could be permanently affected, even in absence of *in vitro* malignant hematopoietic cells stimulation [16]. Mechanisms of these epigenetic modifications are still unclear but may involve inflammation. One form of DNA damage is of particular interest: halogenated cytosine residues. These inflammation damage products have been detected in human leukocytes [135]. The methyl-binding proteins cannot distinguish methylated and halogenated DNA; thus DNMT could be deceived and lead to the accumulation of these analogues within the genome [136]. An initial halogenation, triggered by inflammation, could direct methylation of the complementary DNA strand, resulting in heritable alterations in methylation patterns. In rheumatoid arthritis, synovial fibroblasts exhibit epigenetic alterations thought to be in relation with chronic inflammation, performing an imprinting of their proinflammatory state [137]. Methylation includes not only CpG islands, but also large partially methylated domains and DNA methylation valley (DMV), identified in hematopoietic stem cells [138]. In a mouse model, methylation of this domain can be related to inflammatory exposure resulting in a coordinate aberrant DMV methylation [139]. TGF-β1 is a key regulator for DNA methylation through an increase in DNMTs expression and is able to promote methylation in cancer [140, 141]. In renal fibrosis, TGF-β1 can induce overproduction of collagen and sclerostin through H3K4 methylation of their promoter [142]. TGF-β1-induced profibrotic changes in cell phenotype are accompanied by significant alterations in miR expression profile [143]. In association with TGF-β1 challenging, time course of the disease must be taken into account. PMF develops through decades, exposing cellular components to aging, and patient's median age is over 60 years [144]. Analysis of microRNA expression in inflammatory and senescence situation leads to the concept of "inflamm-aging," involving aberrant expression of microRNA involved in several functions including TGF-β1 regulation [145]. MicroRNA expression alteration might occur in MSCs from PMF patients and promote, for instance, modification of TGF-β1 expression, osteogenic differentiation, or MSCs trafficking. Altogether, alterations of epigenetic profile of PMF patient's stroma could be promoted by inflammation, resulting in MSC imprinting. With time, inheritance of these modifications could lead to an "autonomous" behavior of MSCs from clonal hematopoietic cells and participate in the disease in a distinctly different manner. Indeed, persistence of a pathologic inflamed stroma, in "absence/decrease" of clonal cells cured by targeted therapies, may explain relapse or drug resistance.

8. Conclusion and Perspectives

In conclusion, as elegantly proposed by Hasselbalch [64, 131], chronic inflammation may be both an initiator and a driver of clonal evolution in patients with MPNs. In PMF, we suggested that once activated, the stroma is progressively inflammatory-imprinted by clonal hematopoietic cells to an "autonomous" state where it becomes independent of hematopoietic cell stimulation. Therefore, at advanced stage

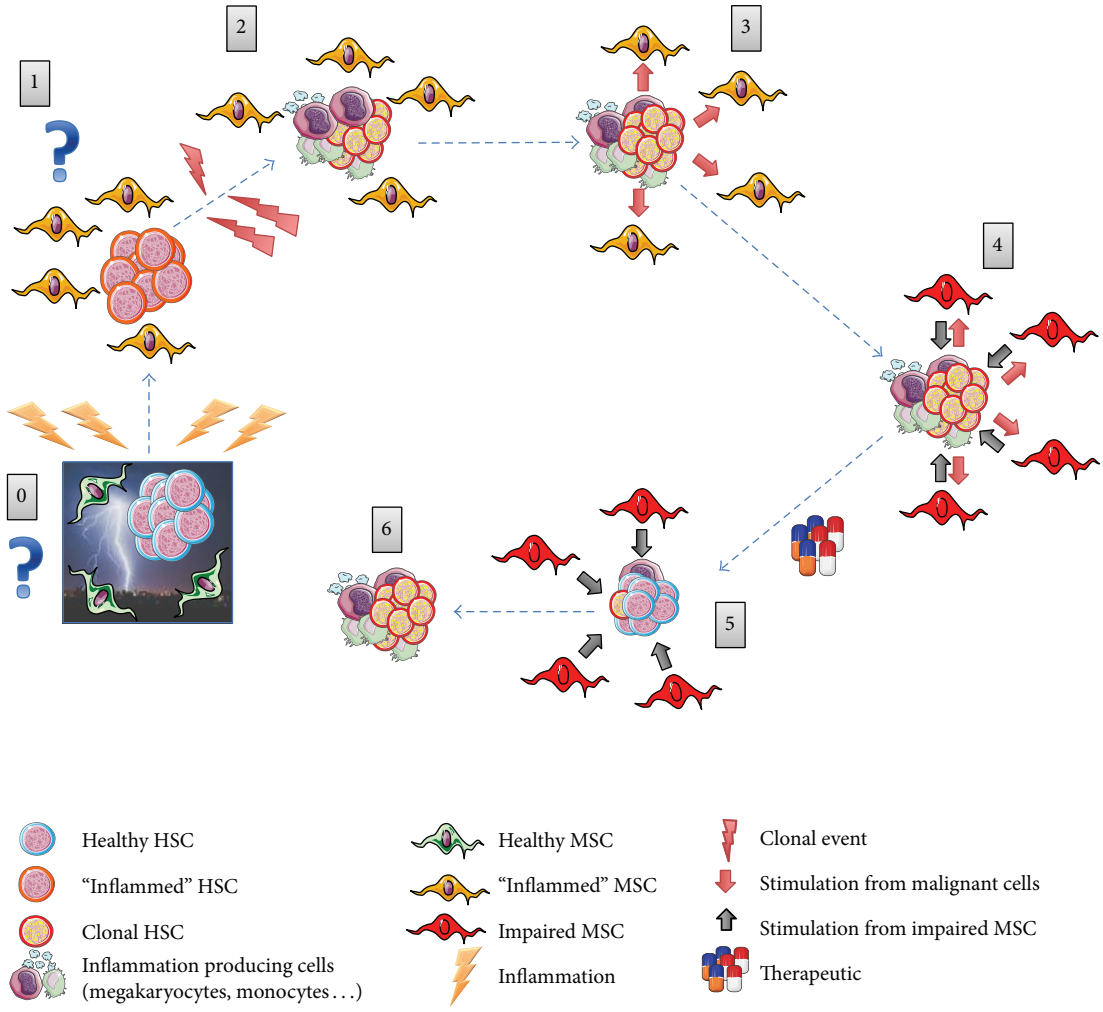


Figure 4: Proposal of a natural history of hematopoietic and stromal cell interactions in bone marrow from PMF patients. The highly inflammed bone marrow environment in PMF is compared to being hit by a "storm" of cytokines [12] (0). (1) This inflammatory environment could involve hematopoietic stem cells (HSCs) and/or mesenchymal stromal cells (MSCs). (2) Clonal events (that would be favored/driven by inflammation (?) [13]) would give rise to clonal hematopoietic cell(s) which will further differentiate into megakaryocytes and monocytes and produce large amount of inflammatory cytokines. (3) These cytokines would modify the bone marrow microenvironment, leading (4) to a permanent impairment of MSCs and a deterioration of the hematopoietic niche. (5) Impaired MSCs would influence malignant hematopoietic cells in an altered crosstalk and over time, MSCs would become inflammatory imprinted. (5) As a result of treatment, the number of malignant hematopoietic cells will reduce. However, the influence of inflammatory imprinted MSCs that would have acquired inherited impaired functions will continue. Unless treated, disease MSCs may continue interacting with HSC and contribute to relapse of the disease (6).

of the disease, this inflammatory vicious circle will become difficult/impossible to break without combined stroma targeted therapies (Figure 4).

The past two decades have provided a wealth of information on how nonresolving inflammation drives a number of widespread chronic diseases including MPNs. Although this knowledge has the potential to open up vast opportunities for new therapeutic advances, the nature of the inflammatory response as a complex system that is critical for normal physiology renders this approach challenging.

Nonetheless, new knowledge about inflammatory signaling, particularly in the setting of MPNs, may provide the promise for new therapeutic options that can successfully meet these challenges. Each of the aspects of pathogenic processes leading to MPNs has unique therapeutic opportunities and challenges. Links between inflammation, JAK2 mutation, and MPNs development have provided a framework for understanding the complex nature of MPNs. However, despite achieving important milestones in the area of MPN research more questions remain unanswered. Important lingering questions include the following: What key triggers lead to activation of inflammation in MPNs? What are the primary danger signals, disease amplifiers, and processes governing sustained chronic inflammation? Can we identify

therapeutic targets that are efficacious yet specific enough to avoid unwanted side effects? Does combination therapy where anti-inflammatory drugs are used in combination with JAK inhibitors show greater effectiveness than JAK inhibitors alone, which themselves have anti-inflammatory effects? Given the role for stroma-derived cytokines in protecting the malignant clones against JAK2-directed therapy [146], how could the stromal niche be manipulated to target the clone and to restore normal hematopoiesis? Answering these questions should increase our understanding about the pathogenesis of MPNs and should provide exciting targets and new treatment options.

Another important question concerns the timing of when to begin the treatment of patients? To be efficient, inflammatory/antifibrotic strategies must not only limit the progression of inflammation/fibrosis by eliminating the source of promoting agents but also counteract damaged BM stroma by promoting repair processes. Similarly to stem cell transplantation, such treatments must be as early as possible, before the disease has developed to a very advanced stage, to avoid the "irreversible" inflammatory imprinting of the stroma and to be given in combination with drugs aiming at prohibiting the hematopoietic clone. In addition to treatments such as JAK inhibitors and anti-inflammatory agents (including immunomodulatory agents such as Interferon-alpha), as monotherapy or in combination, epigenetic modifiers have also been proposed. From a mechanistic viewpoint, it seems plausible that epigenetic therapy directed against DNA methylation, histone acetylation, and microribonucleic acids (microRNAs) might indeed improve clinical outcomes and alleviate PMF-related symptoms [149]. Recently, Tibes and Mesa suggested the concept of targeting Sonic hedgehog (Shh) signalling in PMF since inhibitors of this pathway (sonidegib) have shown preliminary activity (including reduction of fibrosis) as single agents or in combination with ruxolitinib in preclinical and clinical studies [150]. It has been recently reported that Shh signalling from bone marrow-derived mesenchymal stromal cells of MDS patients plays a role in the survival advantage of myelodysplastic cells by modulating DNA methylation [151]. Taking into account the role of the Sonic Hedgehog signalling in modifying the expression of genes modulated in PMF MSCs (our data), targeting Shh in stromal cells could be a promising approach to reduce inflammation in PMF and, in association (or not?) with JAK2 inhibitors, to better control the hematopoietic clonal proliferation.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Christophe Desterke and Christophe Martinaud have equally participated in the redaction and in the authors' work included in the review.

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Review Article

MPNs as Inflammatory Diseases: The Evidence, Consequences, and Perspectives

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In recent years the evidence is increasing that chronic inflammation may be an important driving force for clonal evolution and disease progression in the Philadelphia-negative myeloproliferative neoplasms (MPNs), essential thrombocythemia (ET), polycythemia vera (PV), and myelofibrosis (MF). Abnormal expression and activity of a number of proinflammatory cytokines are associated with MPNs, in particular MF, in which immune dysregulation is pronounced as evidenced by dysregulation of several immune and inflammation genes. In addition, chronic inflammation has been suggested to contribute to the development of premature atherosclerosis and may drive the development of other cancers in MPNs, both nonhematologic and hematologic. The MPN population has a substantial inflammation-mediated comorbidity burden. This review describes the evidence for considering the MPNs as inflammatory diseases, *A Human Inflammation Model of Cancer Development*, and the role of cytokines in disease initiation and progression. The consequences of this model are discussed, including the increased risk of second cancers and other inflammation-mediated diseases, emphasizing the urgent need for rethinking our therapeutic approach. Early intervention with interferon-alpha2, which as monotherapy has been shown to be able to induce minimal residual disease, in combination with potent anti-inflammatory agents such as JAK-inhibitors is foreseen as the most promising new treatment modality in the years to come.

1. Introduction

Recent studies have provided evidence that the chronic myeloproliferative neoplasms (MPNs), essential thrombocythemia (ET), polycythemia vera (PV), and myelofibrosis (MF), may be preceded by or accompanied by chronic inflammation and also may imply an increased risk for the development of other cancers [1–3]. In these neoplasms morbidity and mortality are massively influenced by cardiovascular and thromboembolic complications [1, 4, 5]. The advanced myelofibrotic stage is typically characterized by transfusion-dependent anemia, large spleen, severe bone marrow fibrosis, and steadily increasing white blood cell counts or severe pancytopenia and end-stage development of acute leukemia, seen in up to 20% of patients with MF [1, 5]. The incidence of MPNs is low, but the prevalence is high and comparable with lung cancer. In 2005, a unique breakthrough was described by the identification of the JAK2V617F mutation in almost all patients with PV and about half of patients with ET and MF [1]. It is possible to monitor the "tumor burden" when analyzing the *JAK2* allelic burden by qPCR. In 2013 the calreticulin mutations were described in a large proportion of the JAK2V617F negative ET and MF patients [6, 7]. The clinical implications of these mutations are being described elsewhere in this Theme Issue.

Chronic inflammation is an important risk factor for the development of atherosclerosis which occurs prematurely in patients with chronic inflammatory diseases, including rheumatoid arthritis, systemic lupus erythematosus, psoriasis, and type II diabetes mellitus. In these diseases, in vivo activation of leukocytes, platelets, and endothelial cells contributes significantly to the increased risk of thrombosis. The

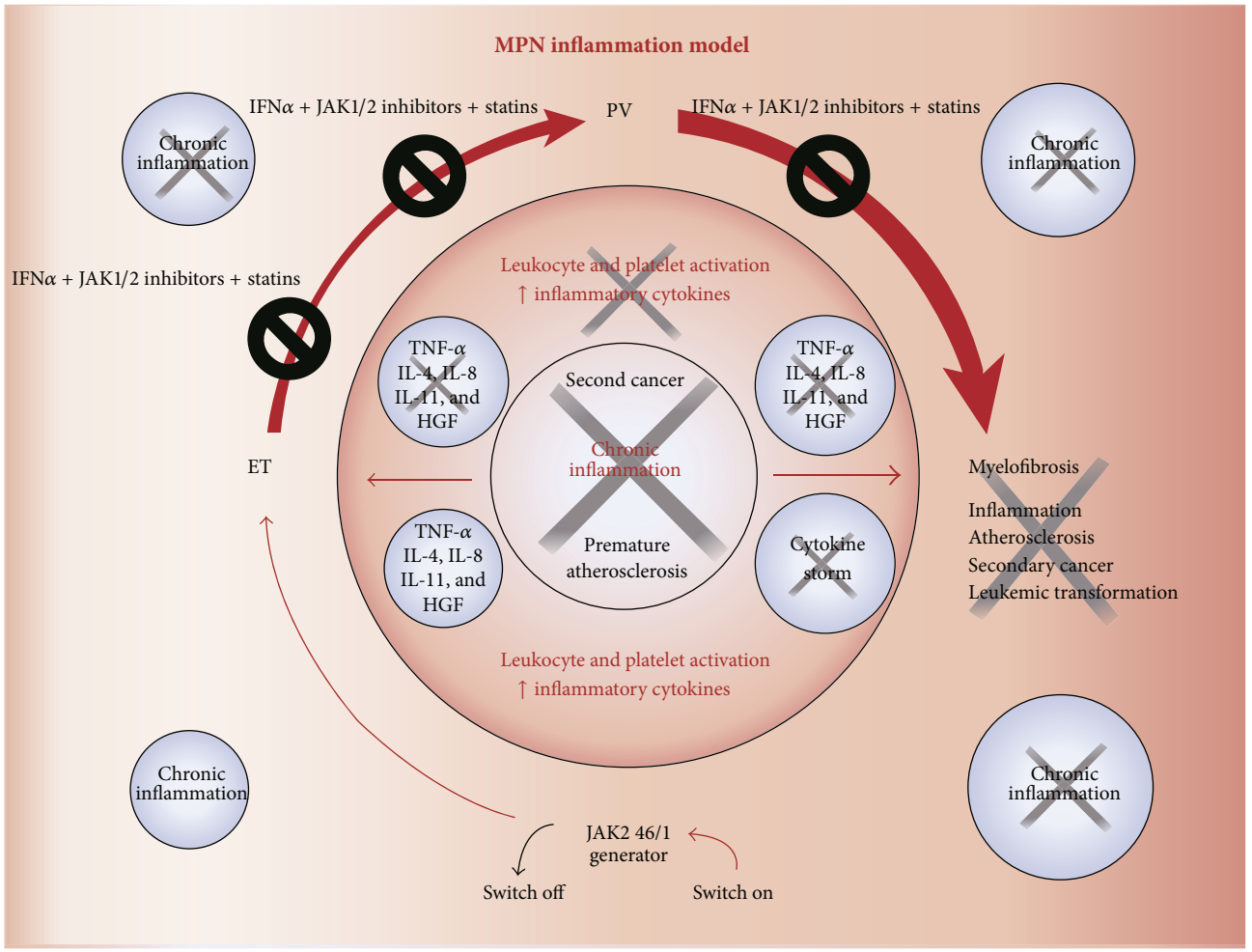

Figure 1: Vicious cycle of inflammation in the biological continuum of ET, PV, and MF. Chronic inflammation is proposed as the trigger and driver of clonal evolution in the biologic continuum from early disease state (ET/PV) to a more advanced disease state (MF). It is possible that combination therapy, using low doses of agents such as interferon-alpha, Janus kinase inhibitors, and statins at the early disease stage, will positively influence the vicious cycle of disease progression. HGF: hepatocyte growth factor; IL: interleukin; MPN: myeloproliferative neoplasm; and TNF: tumor necrosis factor.

same thrombophilia-generating mechanisms are operative in ET, PV, and MF, in which chronic inflammation has recently been described as a potentially very important facilitator not only of premature atherosclerosis, but also of clonal evolution and second cancer [8]. Thus, the chronic MPNs are both "model diseases" for studies of the relationship between chronic inflammation and premature atherosclerosis development in the biological continuum from ET over PV to myelofibrosis and "model diseases" for cancer development from the early cancer stage (ET, PV) to the advanced metastatic cancer stage (MF with myeloid metaplasia) [9–13].

Based upon experimental, clinical, and epidemiological studies we herein argue for the MPNs as inflammatory diseases in accordance with the "Human Inflammation Model for Cancer Development." In the following we will describe the evidence for MPNs as chronic inflammatory diseases and discuss the consequences of chronic inflammation in MPNs in terms of disease progression due to inflammation-mediated clonal expansion and defective tumor immune surveillance. In this context we argue for dampening chronic inflammation at the earliest disease stage (ET/PV), when the tumor burden is minimal, the clone is homogenous (prior to subclone formation and/or acquisition of additional driving mutations), and accordingly the outcome of treatment is logically most favorable (Figure 1).

2. The Evidence of a Link between Chronic Inflammation and Cancer

About 30 years ago Dvorak described cancers as "wounds that do not heal," a concept updated most recently and since 1986 being increasingly recognized [14, 15]. In their seminal contribution from 2000 Hanahan and Weinberg identified the six hallmarks of cancer and recently chronic inflammation was added as the seventh hallmark, emphasizing the huge impact of chronic inflammation on cancer development and progression ("oncoinflammation") [16, 17]. Accordingly, today chronic inflammation is considered of major importance in the development of cancer and several molecular and

cellular signaling circuits have been identified linking inflammation and cancer [18–22]. Indeed, this concept was already described by Virchow in the 19th century when he suggested that chronic inflammation might give rise to malignancy [21]. Regardless, not until more recently, the link between inflammation and cancer has been acknowledged, partly due to epidemiologic studies, which have generated data on chronic infections and inflammation as major risk factors for various types of cancer. In hematological malignancies a link between chronic inflammation and malignant lymphomas has been well described whereas chronic inflammation as a potential initiating event and a driver of clonal evolution in myeloid cancers including MPNs has not been focused upon until very recently [8, 9, 11–13, 23–25].

3. The Evidence of MPNs as Inflammatory and Immune Deregulated Diseases

3.1. What Is the Epidemiological Evidence? An increased risk of autoimmune and/or inflammatory conditions has been documented several years ago in patients with myeloid malignancies and recently a large Swedish epidemiologic study concluded that chronic immune stimulation might act as a trigger for the development of the myelodysplastic syndrome (MDS) and acute myelogenous leukemia (AML) [26, 27]. In regard to MPNs, another Swedish study has shown that inflammatory diseases may precede or develop during the course of ET, PV, and MF. In this Swedish study, a prior history of any autoimmune disease was associated with a significantly increased risk of a myeloproliferative neoplasm. The "inflammatory" diseases included, among others, Crohn's disease, polymyalgia rheumatica, and giant cell arteritis, and the "autoimmune" diseases included immune thrombocytopenic purpura and aplastic anemia [2]. The 46/1 haplotype is present in 45% of the general population and is associated with a predisposition to acquire the *JAK2*V617F mutation and accordingly MPNs but also predisposes to MPNs with no mutation of *JAK2* and to MPNs with mutation in *MPL* [28–31]. Importantly, epidemiological studies have shown that the frequency of the *JAK2* 46/1 haplotype is increased in inflammatory diseases, including Crohn's disease [32, 33].

Risk factors for developing atherosclerosis, a chronic inflammatory disease, have been investigated in a large Danish epidemiological study of 49 488 individuals from the Copenhagen General Population Study. It was discovered that those harboring the *JAK2*V617F mutation had a 2.2-/1.2-fold risk (prevalent/incident) of ischemic heart disease [34].

3.2. What Is the Histomorphological Evidence? Already about 40 years ago it was speculated if autoimmune bone marrow damage might be incriminated in the pathogenesis of "idiopathic myelofibrosis" (IMF). Several observations seem to support the participation of immune mechanisms in the development of bone marrow fibrosis. Thus, histopathological findings of "Fibrin-Faser-Stern" figures, increased numbers of plasma cells and lymphocytes with plasmacytoid appearance, the demonstration of a parallel increase in interstitial deposits of immunoglobulins and the extent of bone marrow fibrosis, and the development of bone marrow fibrosis after repeated antigen injections in animal models all render immune-mediated bone marrow fibrosis possible [35–41]. Importantly, the findings of "Fibrin-Faser-Stern" figures and lymphoid aggregates in bone marrows from MPNs patients have been variably interpreted as evidence of immune activity in the marrow with deposition of immune complexes [35–38]. Immune activity in the bone marrow with an increase of lymphoid nodules has been found to be most prominent in the early stage of IMF [37, 38]. A most recent study investigated the mechanism of bone marrow fibrosis in patients with MF by comparing TGF-β1 signaling of marrow and spleen cells from patients with MF and of nondiseased individuals. The expression of several TGF-β1 signaling genes was altered in the marrow and spleen of MF patients, respectively. Abnormalities included genes of TGF-β1 signaling, cell cycling, and Hedgehog and p53 signaling. Pathway analysis of these alterations predicted an increased osteoblast differentiation, ineffective hematopoiesis, and fibrosis driven by noncanonical TGF-β1 signaling in the marrow and increased proliferation and defective DNA repair in the spleen. The hypothesis that fibrosis in MF might result from an autoimmune process, triggered by dead megakaryocytes, was supported by the findings of increased plasma levels of mitochondrial DNA and anti-mitochondrial antibodies in MF patients. It was concluded that autoimmunity might be a plausible cause of marrow fibrosis in MF [42]. Finally, the clinical observations of a favorable outcome of immunosuppressive therapy in some MF patients with evidence of autoimmune activity support the concept that autoimmunity, immune dysfunction, and chronic inflammation may be important factors in pathogenesis [43–48].

3.3. What Is the Clinical Evidence?

3.3.1. The Inflammation-Mediated Cardiovascular and Thromboembolic Disease Burden. Patients with MPNs have a massive cardiovascular disease burden with a high risk of thrombosis (Figure 2), which is partly explained by excessive aggregation of circulating leukocytes and platelets due to in vivo leukocyte-platelet and endothelial activation in combination with a thrombogenic endothelium [1, 4]. In addition MPNs are associated with a procoagulant state, which has recently been elegantly reviewed by Barbui et al. [49]. The hyperactivation of circulating cells in MPNs has been thought to be attributed to the clonal myeloproliferation. Thus, the *JAK2*V671F mutation per se has been shown to induce leukocyte and platelet activation and several clinical studies have demonstrated that *JAK2*V617F positivity is a thrombogenic factor in MPNs [49–52]. Of note, Barbui et al. have recently shown that the level of C-reactive protein (CRP) is elevated in patients with ET and PV and correlates significantly with the *JAK2*V617F allele burden [53]. Furthermore, elevated CRP levels have also been associated with shortened leukemia-free survival in myelofibrosis [54]. It was speculated if sustained inflammation might elicit the stem cell insult by inducing a state of chronic oxidative stress with elevated levels of reactive oxygen species (ROS) in the bone marrow, thereby creating a high-risk microenvironment for induction of mutations in hematopoietic cells [9].

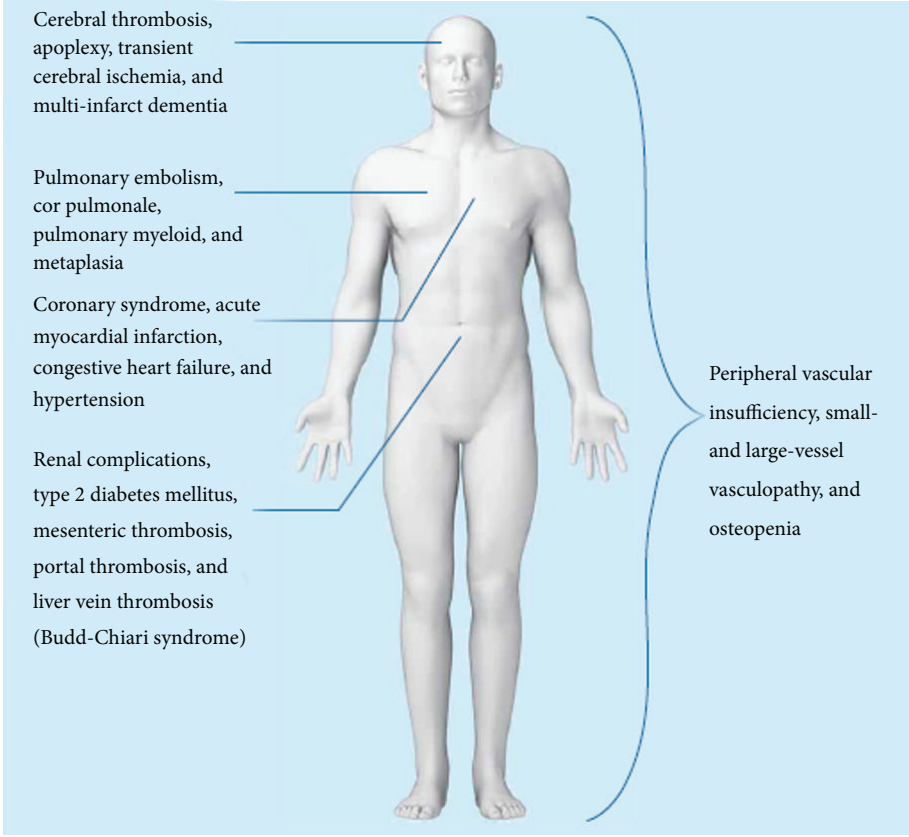

Figure 2: Patients with MPNs have a massive cardiovascular and thromboembolic disease burden.

Being a sensitive marker of inflammation and influencing, for example, endothelial function, coagulation, fibrinolysis, and plaque stability, CRP is considered to be a mediator of vascular disease and accordingly a major vascular risk factor as well [55–57]. This association has recently been demonstrated in a meta-analysis, showing continuous associations between the CRP concentration and the risk of coronary heart disease, ischemic stroke, and vascular mortality [58]. For decades it has been known that atherosclerosis and atherothrombosis are chronic inflammatory diseases [59, 60]. Several studies have reported that chronic inflammatory diseases (e.g., rheumatoid arthritis, psoriasis, systemic lupus erythematosus, and diabetes mellitus) are associated with accelerated atherosclerosis and accordingly development of premature atherosclerosis (early ageing?) [61–65]. In addition, considering the association between atherosclerosis and venous thrombosis, chronic inflammation indirectly predisposes to venous thrombosis and pulmonary thromboembolism as well [66]. In the context of the associations between inflammation and CRP in ET and PV, inflammation might be considered to be a secondary event elicited by clonal cells [53]. However, elevated leukocyte and platelet counts in MPNs may not only reflect clonal myeloproliferation but also reflect the impact of chronic inflammation per se on the clonal cells. In particular, this interpretation is intriguing when considering that one of the hallmarks of MPNs is inherent hypersensitivity to growth factor and cytokine stimulation [8]. In this perspective, chronic inflammation in MPNs may also have a key role in promoting premature atherosclerosis and all its debilitating cardiovascular and thromboembolic complications, the common denominators for their development being elevated leukocyte and platelet counts, elevated CRP levels, and in vivo leukocyte-platelet and endothelial cell activation, taking into account that platelet-leukocyte interactions link inflammatory and thromboembolic events in several other inflammation-mediated diseases [67].

3.3.2. Inflammation-Mediated Chronic Kidney Disease. Uncontrolled chronic inflammation is associated with organ dysfunction, organ fibrosis, and ultimately organ failure [68]. This development is classically depicted in patients with the metabolic syndrome progressing to type II diabetes mellitus (DM) which, without adequate treatment to normalize elevated blood glucose levels, may rapidly develop organ failure due to accelerated atherosclerosis (e.g., hypertension, ischemic heart disease, stroke, dementia, peripheral arterial insufficiency, venous thromboembolism, and chronic kidney disease). The progressive deterioration of multiple organs in uncontrolled DM consequent to elevated blood glucose levels with in vivo leukocyte-platelet and endothelial activation and development of premature atherosclerosis is in several

aspects comparable to the multitude of systemic manifestations in patients with uncontrolled MPNs, the common denominators being a huge cardiovascular disease burden and thromboembolic complications [10]. Importantly, similar to patients with type II DM, it has been demonstrated that patients with MPNs have an increased risk of developing chronic kidney disease [69]. It was concluded that progressive renal impairment may be an important factor in MPNs contributing to the comorbidity burden and likely to the overall survival. In addition it was speculated whether chronic inflammation with accumulation of ROS might be a driving force for impairment of renal function and accordingly supportive of early intervention in order to normalize elevated cell counts and reduce the chronic inflammatory drive elicited by the malignant clone itself [69].

3.3.3. Inflammation-Mediated "Autoinflammatory" Diseases. As outlined above, patients with MPNs may have an increased risk of various autoimmune, "autoinflammatory," or inflammatory diseases. Thus, associations have been reported with systemic lupus erythematosus, progressive systemic sclerosis, primary biliary cirrhosis, ulcerative colitis, Crohn's disease, nephrotic syndrome, polyarteritis nodosa, Sjögren syndrome, juvenile rheumatoid arthritis, polymyalgia rheumatica/arteritis temporalis, immune thrombocytopenic purpura (ITP), and aplastic anemia. In large epidemiological studies these associations have only been significant for Crohn's disease, polymyalgia rheumatica/arteritis temporalis, and ITP [2]. Interestingly, a particular subtype of myelofibrosis, "primary autoimmune myelofibrosis," has been described. This subtype has been considered to be a nonclonal and nonneoplastic disease, featured by anemia/cytopenias and autoantibodies suggesting systemic autoimmunity. Most patients have no or only mild splenomegaly and the bone marrow biopsy exhibits MPN-like histology with fibrosis, hypercellularity, and megakaryocyte clusters. In addition, bone marrow lymphoid aggregates are prominent [46]. It remains to be established if this subset of MF actually exists or if these patients indeed should be categorized within the MPNs disease entity, taking into account that autoimmunity and chronic inflammation today are considered to have a major role in MPNs pathogenesis.

3.3.4. Inflammation-Mediated Osteopenia. A recent Danish registry study has shown that patients with ET and PV have an increased incidence of fractures compared with the general population [70]. Taking into account that chronic inflammation has been suggested to explain the initiation of clonal development and progression in chronic myeloproliferative neoplasms and other chronic inflammatory diseases that are associated with an increased risk of osteopenia it has been speculated if chronic inflammation might induce osteopenia in MPNs and by this mechanism also predispose to the increased risk of fractures [12, 70–72].

3.3.5. Inflammation-Mediated Second Cancers. As noted previously patients with MPNs have been shown to have an increased risk of second cancers [3, 5]. In the perspective that chronic inflammation may be a driving force for clonal evolution in MPNs it is intriguing to consider if chronic inflammation may contribute to the development of second cancers in MPNs as well, taking into account the close association between inflammation and cancer [8, 9, 11–13, 17–22]. In this regard a defective "tumor immune surveillance" consequent to immune deregulation, which has been demonstrated in MPNs in several recent studies and most recently comprehensively reviewed, might be of importance [42, 73–75]. Of note, the increased risk of second cancers has also been recorded prior to the MPNs diagnosis emphasizing that the MPNs may have a long prediagnosis phase (5–10–15 years) with a chronic inflammatory state promoting mutagenesis, defective tumor immune surveillance, and immune deregulation [9, 76–78]. This concept is compatible with the most recent observations of additional mutations that are already present at the time of diagnosis likely induced by a sustained inflammatory drive on the malignant clone several years before diagnosis [7, 9, 78, 79] (Figure 4).

3.4. What Is the Biochemical Evidence? As outlined above MPNs are associated with a low-grade inflammatory state as assessed by slightly elevated CRP in a large proportion of patients with ET and PV [53]. The CRP levels are steadily increasing when patients enter the accelerated phase towards leukemic transformation [54]. Considering the close association between CRP and other inflammatory markers, the leukocyte and platelet counts, it is most relevant to speculate if leukocytosis and thrombocytosis in MPNs are also attributed to the chronic inflammatory drive per se with sustained generation of inflammatory products that fuel the malignant clone in a vicious self-perpetuating circle [8, 11]. Similar to CRP, plasma fibrinogen and plasma D-dimers levels are slightly elevated in several patients and may indeed be more sensitive inflammatory markers than CRP (unpublished observations). Proinflammatory cytokines are elevated in a substantial proportion of patients with MPNs, a topic which has recently been reviewed and thoroughly described by Fleischman and others in this Theme Issue [11].

The hypothesis and the concept of MPNs and the advanced MF stage being elicited and perpetuated by autoimmune/inflammatory mechanisms were intensely investigated and discussed already 30 years ago. Some of the clinical and histomorphological issues with associations between MPNs and autoimmune/inflammatory states have already been addressed above. In addition, several studies from that period reported biochemical evidence of autoimmunity/inflammation in MPNs, such as elevated levels of antibodies to RBCs, antibodies to platelets, anti-nuclear and anti-mitochondrial antibodies (ANA and AMA), rheumatoid factor, lupus-like anticoagulant, low levels of complement, complement activation, increased levels of immune complexes (ICs), and increased levels of interleukin-2 soluble receptors (s-IL2R) [38, 43, 80–85]. It was debated whether deposition of immune complexes in the bone marrow, either formed in situ or trapped from the circulation, might be followed by complement activation with subsequent local inflammatory reaction, an interpretation fitting very well with the findings of complement activation in MF patients [80, 81]. Of note, circulating immune complexes were predominantly found in

the early disease stage. Since circulating ICs were in some studies mainly found in MF patients with a short duration of disease from diagnosis it was hypothesized that potential immune-mediated bone marrow damage might indeed occur in the early phase of the disease and the late, fibrotic stage with undetectable IC representing the "burnt out" phase of the disease [81, 84]. Today, 30 years after the detection of IC in MPNs, their significance in MF and related neoplasms remains unsettled. With the renaissance of the concept of autoimmune bone marrow damage and chronic inflammation as driving forces for disease evolution and progression further studies on circulating ICs and their pathogenetic and clinical relevance are highly relevant and timely. Indeed, their detection may reflect ongoing inflammatory immune reactions in the circulation and in the bone marrow, being likely most pronounced in the initial disease phase and possibly related to a more acute course of the disease [81]. Most recently, a comprehensive study of autoimmune phenomena and cytokines in 100 patients with MF, including early stage MF, has added further proof of the concept that autoimmune and inflammatory mechanisms may be highly important in the pathogenesis of MPNs [86]. Importantly, organ/non-organ-specific autoantibodies were found in 57% of cases, without clinically overt disease, and mostly in low-risk/intermediate-risk-1 and MF-0/MF-1. Furthermore, TGF-β and IL-8 were increased in MS-DAT positive cases, and TGF-β and IL-17 were elevated in early clinical and morphological stages, while IL-8 increased in advanced stages. It was concluded that autoimmune phenomena and cytokine dysregulation may be particularly relevant in early MF [86].

Several studies have shown that circulating YKL-40 levels are elevated in a number of different diseases, including cancer, diabetes mellitus, and cardiovascular diseases, in which YKL-40 serves as an excellent marker of the disease burden. Importantly, a state of chronic inflammation is shared by them all, and YKL-40 also has a major impact upon the severity of chronic endothelial inflammation, which today is considered of crucial importance for the development of atherosclerosis. Considering the MPNs as chronic inflammatory diseases and accordingly with an increased risk of development of premature atherosclerosis we hypothesized that circulating YKL-40 might be an ideal marker of the integrated impact of chronic inflammation in MPNs and accordingly might display correlations with conventional markers of inflammation and disease burden in MPNs. Indeed, we have recently shown that circulating YKL-40 is a potential novel biomarker of disease activity and the inflammatory state in myelofibrosis and related neoplasms [87, 88]. These studies have demonstrated a steady increase in YKL-40 from early cancer stage (ET) over PV to the advanced cancer stage with myelofibrosis, which exhibited the highest YKL-40 levels of them all. Highly interesting, we also found a significant correlation between YKL-40 and several markers of inflammation and disease burden, including neutrophils, platelets, CRP, LDH, and the *JAK2*V617F allele burden. Accordingly, circulating YKL-40 may be a novel marker of inflammation, disease burden, and progression in MPNs [87, 88].

3.5. What Is the Molecular Evidence? The concept of chronic inflammation leading to clonal evolution in MPNs is also supported by gene expression profiling studies (Figure 3), which have unraveled deregulation of several genes that might be implicated in the development and phenotype of the MPNs [89–92]. Using whole-blood transcriptional profiling and accordingly obtaining an integrated signature of genes expressed in several immune cells (granulocytes, monocytes, B cells, T cells, and platelets), we have shown that the MPNs exhibit a massive upregulation of IFN-related genes, particularly interferon-inducible (IFI) gene IFI27 and severe deregulation of other inflammation and immune genes as well. Indeed, several genes (e.g., IFI27) displayed a stepwise upregulation in patients with ET, PV, and PMF with fold changes from 8 to 16 to 30, respectively. The striking deregulation of IFI genes may likely reflect a hyperstimulated but incompetent immune system being most enhanced in patients with advanced MF. In this context, the massive upregulation of the IFI27 gene may also reflect an exaggerated antitumor response as part of a highly activated IFN system, including enhanced IFN gamma expression, which might also imply activation of dendritic cells. IFI27 is also upregulated during wound repair processes, which may be of particular relevance when considering the Dvorak thesis on "Tumors: wounds that do not heal" [14, 15]. Thus, it is tempting to argue that MPNs are "wounds in the bone marrow that will not heal," owing to the continuous release from clonal cells of growth factors and matrix proteases with ensuing extracellular remodeling of the bone marrow stroma. In this scenario, one might speculate whether the high expression of IFI27 may reflect these processes as well, IFI27 cooperating with distinct genes of potential importance for egress of $CD34^+$ cells from the bone marrow niches into the circulation [93]. In the context of matrix remodeling during cancer metastasis (which in MPNs consists of egress of $CD34^+$ cells from the bone marrow niches into the circulation) it is of particular interest to note that IFN-inducible genes, including IFI27, have been shown to be associated with the so-called metagenes in patients with breast cancer, accurately identifying those patients with lymph node metastasis and accordingly predictors of outcomes in individual patients [94]. Thus, the highly upregulated IFI27 gene in MPNs may reflect progressive clonal evolution with "metastasis" (extramedullary hematopoiesis) despite an exaggerated yet incompetent IFN-mediated antitumor response by activated dendritic cells and T cells. In this regard a hyperstimulated immune system might also contribute to the increased risk of autoimmune diseases in MPNs. Accordingly the interferon signature may reflect MF as the terminal stage of chronic inflammation with a huge burden of oxidative stress, genomic instability, and accumulation of additional inflammation-induced mutations, the ultimate outcome being leukemic transformation [8–12]. During this evolution from early cancer stage to the metastatic stage with MF, the interferons are important cytokines for immunity and cancer immunoediting [95]. For this and several other reasons IFN is, today and in the future, considered the cornerstone in the treatment of MPNs which, when instituted in the very early disease stage, may be able to quell the fire and accordingly induce "minimal

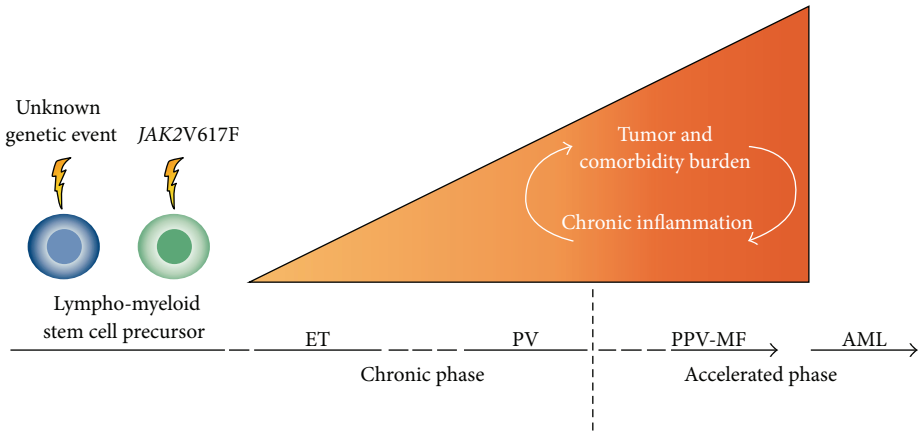

Figure 3: Chronic inflammation as the driving force for clonal evolution in MPNs. Tumor burden and comorbidity burden are illustrated for patients with *JAK2*V617F positive MPNs. Comorbidity burden increases from early disease stage (ET/PV) to the accelerated phase with myelofibrotic and leukemic transformation. With permission: H. C. Hasselbalch [12]. AML: acute myeloid leukemia; ET: essential thrombocythemia; JAK: Janus kinase; PPV-MF: post-polycythemia vera; and PV: polycythemia vera.

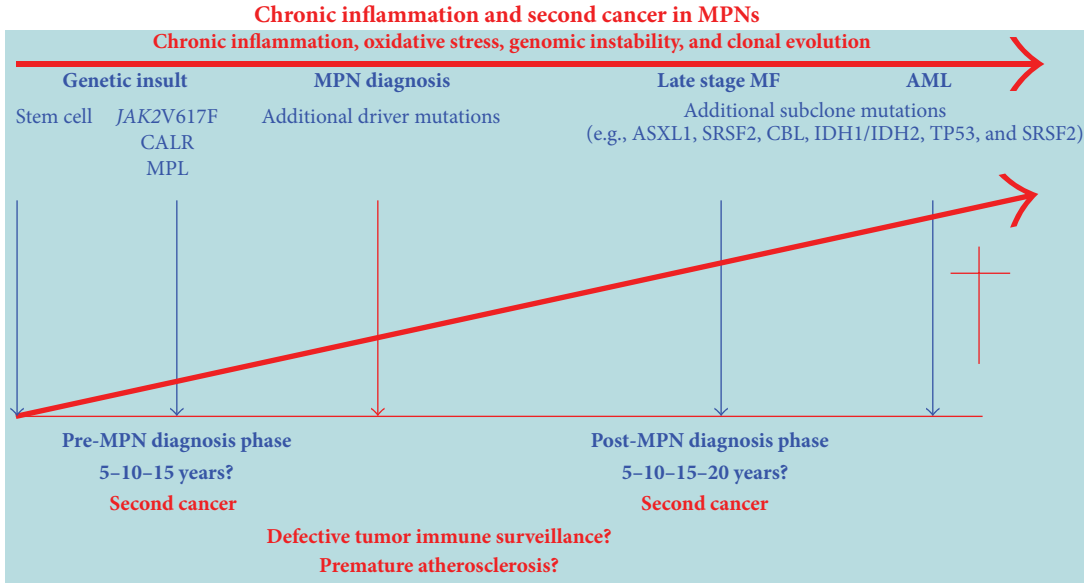

Figure 4: Patients with MPNs have an increased risk of second cancer not only after the MPNs diagnosis but also in the pre-MPNs diagnosis phase, which may last several years in which the patients are at an increased risk of severe cardiovascular and thromboembolic events. According to this model, the initial stem cell insult has occurred 5–10–15 years before the MPNs diagnosis.

residual disease" and in some patients likely cure as will be discussed below [96–103]. Supporting chronic inflammation as the driving force for clonal evolution is also the most recent whole-blood gene expression studies, showing a marked deregulation of oxidative stress genes in MPNs [104]. This issue is extensively described by Bjørn and Hasselbalch in the chapter on "The Role of Reactive Oxygen Species in Myelofibrosis and Related Neoplasms."

3.6. What Are the Consequences of Chronic Inflammation in MPNs?

3.6.1. The Bone Marrow Is Burning. In MPNs chronic inflammation may elicit a "cytokine storm," "a wound that does not heal," due to the continuous release of proinflammatory cytokines that in a self-perpetuating vicious circle drives the malignant clone. Importantly, in this inflammatory

micromilieu, reactive oxygen species (ROS) are steadily accumulating, giving rise to increasing genomic instability, subclone formation with additional mutations, and ultimately bone marrow failure as a consequence of inflammation-mediated ineffective myelopoiesis (anemia, granulocytopenia, and thrombocytopenia), accumulation of connective tissue in the bone marrow, and ultimately leukemic transformation [8, 9, 11–13]. The impact and consequences of ROS for disease progression have been thoroughly described elsewhere by Bjørn and Hasselbalch and the impact of chronic inflammation on bone marrow stroma has been reviewed by Marie Caroline Le Bousse Kerdiles and coworkers.

Chronic inflammation in the bone marrow microenvironment may enhance in vivo granulocyte activation with ensuing release of a vast amount of proteolytic enzymes from neutrophil granules, thereby facilitating egress of $CD34^+$ cells and progenitors from bone marrow niches into the circulation ("metastasis").

3.6.2. The Spleen Is Burning. A common complaint in MPNs patients with enlarged spleens is a "burning" spleen, which on clinical examination may also be extremely painful. Although spleen infarction may occasionally explain the spleen pain, it is in the large majority of patients attributed to inflammation as evidenced by a remarkable relief when being treated with high-dose glucocorticoids and, in particular, during treatment with JAK2 inhibitors which within a few days is associated with a reduction in spleen size and a concomitant improvement in spleen pain as well. Accordingly, the rapid reduction in spleen size during, for example, treatment with ruxolitinib, is primarily consequent to its very potent anti-inflammatory effects as also evidenced by the rapid decrease in circulating inflammatory cytokines [11, 12].

3.6.3. The Circulation Is Burning. As outlined above circulating levels of a large number of inflammatory cytokines are elevated in patients with MPNs [11, 105, 106]. These cytokines activate circulating leukocytes and platelets and also activate endothelial cells as well, giving rise to aggregation of leukocytes and platelets with the formation of microaggregates that compromise the microcirculation in several organs [48, 51] (Figure 5). Taking into account that a large proportion of the circulating leukocytes and platelets are activated per se due to their clonal origin the additional impact of chronic inflammation upon in vivo activation of these cells may profoundly worsen the microcirculation in several organs with ensuing tissue ischemia and associated symptoms, including, for example, CNS-related symptoms (headaches, visual disturbances, dizziness, infarction, and dementia), pulmonary symptoms (dyspnoea due to pulmonary embolism, inflammation due to sequestration of leukocytes and platelets and megakaryocytes in the microcirculation with release of a large number of inflammatory products), symptoms of ischemic heart disease (angina, infarction, and congestive heart failure), or symptoms of peripheral vascular insufficiency [4, 5, 12, 34, 107–112] (Figure 5).

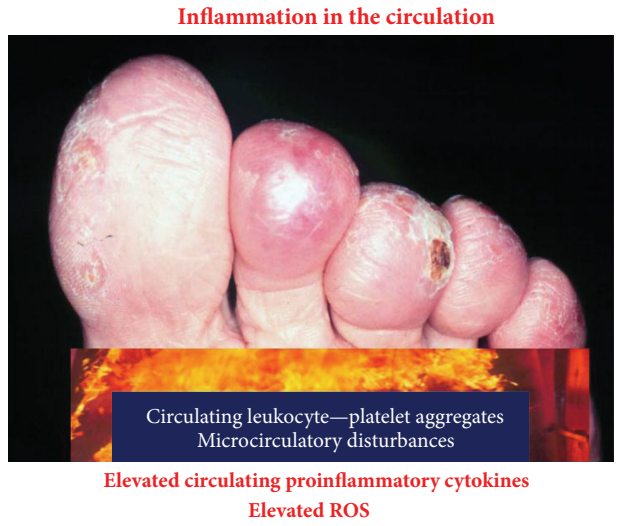

Figure 5: Inflammation in the circulation elicits in vivo leukocyte and platelet aggregation giving rise to circulating microaggregates with ensuing impairment of microcirculation, tissue ischemia, and ultimately development of ulcers on toes and fingers which may terminate with gangrene. Treatment with aspirin momentarily resolves microaggregation with improvement in microcirculation.

4. Discussion and Perspectives

The perspectives of the MPNs as "A Human Inflammation Model for Cancer Development" being driven by chronic inflammation in a self-perpetuating vicious circle from early cancer stage (ET/PV) to the advanced "metastatic" stage with severe MF and egress of $CD34^+$ cells from bone marrow niches to the circulation (metastasis to the spleen and liver and elsewhere) are several [8–13, 96–103].

Firstly, this novel concept calls for the urgent need of a fundamental change in our therapeutic attitude from the conventional "watch-and-wait strategy" to "the early intervention concept" using interferon-alpha2 (IFN) as the cornerstone in the early treatment from the time of diagnosis [96–103] (Figures 1 and 6). However, since access to IFN for the routine use in patients with MPNs is highly variable, a prerequisite for such a change is that opinion leaders within the international MPNs scientific community realize that the time has come to rethink when, how, and who we should treat with IFN. Today the world is divided into two: in one world, not having access to IFN and accordingly its MPNs experts no or only modest experience with the use of IFN most ET and PV patients are followed according to the "watch-and-wait strategy," receiving only cytoreductive treatment with hydroxyurea (HU) for elevated cell counts if they have suffered a prior thrombosis, the platelet count being $>1500 \times 10^9$/L or if they are elderly (>60 years) [113–120]. This risk stratification therapy is partly based upon the concept "do no harm to the patient," since HU treatment implies an increased risk of skin cancer and an increasing concern in regard to an increased risk of other cancers as well, including myelodysplasia and acute myelogenous leukemia

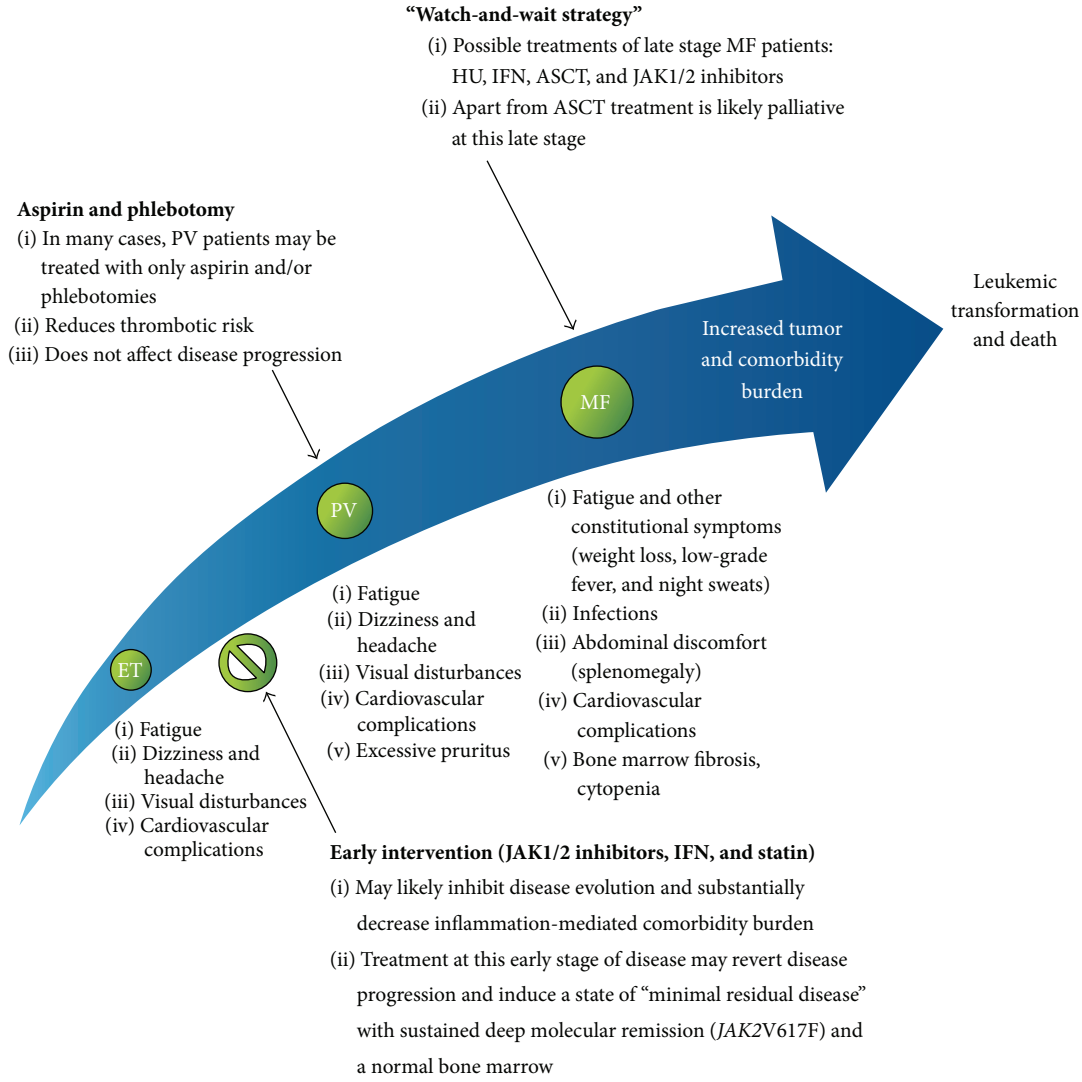

Figure 6: The MPNs care pathway and the effect of early intervention. It is suggested that ET, PV, and MF form a biological continuum and, thus, early intervention with combination therapies including JAK1/2 inhibitors, IFN, and/or statins is likely to result in the inhibition of disease evolution. ASCT: allogeneic stem cell transplantation; ET: essential thrombocythemia; HU: hydroxyurea; IFN: interferon; JAK: Janus kinase; MF: myelofibrosis; and PV: polycythemia vera (with permission: H. C. Hasselbalch [12]).

[98, 100, 102, 121–125]. Accordingly, in this part of the world, HU is avoided in younger patients with ET and PV, who then may not receive cytoreductive treatment for elevated leukocyte counts or elevated platelet counts (>1500 $\times$ 10^9/L) unless they experience the catastrophe, thrombosis or major hemorrhage and consequent *sequelae*. In the other world, having access to IFN, most newly diagnosed patients with ET, PV, and hyperproliferative myelofibrosis are treated routinely with low-dose IFN as described in several studies and reviews during recent years [96–103].

Secondly, we, the MPNs scientific community, and health authorities (Food and Drug Administration (FDA) and EMA (European Medical Agency)) also need to rethink if optimal treatment of MPNs is only determined by the randomized trial or if optimal treatment might also be determined by several single-arm studies proving safety and efficacy of oncology drugs in orphan diseases [102, 123]. In this regard IFN in MPNs is a classic example which for sure has shown safety and efficacy in a large number of clinical studies during the last 25 years but, regardless, is still being considered experimental or not evidence-based therapy, in the world without access to IFN.

Accordingly, promotion of rapidly accumulating evidence for the concept of MPNs as "A Human Inflammation Model for Cancer Development" into clinical practice with upfront treatment with IFN to inhibit clonal expansion ("stopping the fuel that feeds the fire") requires a global signature from the MPN scientific community, a fusion of the two worlds, and an urgent action from health authorities to accept that approval of a drug for orphan diseases—IFN

in MPNs—is applicable when safety and efficacy have been demonstrated in a large number of single-arm studies during the last 2 decades [102, 126].

Thirdly, the proof of concept that chronic inflammation may elicit MPNs needs to be further investigated in other mouse models than the ones already published, including the MPN-mouse model from Heike Pahl's group and the mouse model that has displayed formaldehyde (FA) by inhalation to be able to induce inflammation and ROS accumulation in the bone marrow with ensuing MPN-like blood and bone marrow features such as anemia, leukopenia and thrombocythemia, and megakaryocyte hyperplasia with myelofibrosis, respectively [13, 127, 128].

Fourthly, considering chronic inflammation as a potential trigger of MPNs evolution and the experimental proof that FA induces inflammation in the bone marrow with myelofibrosis, it is indeed intriguing to speculate if cigarette smoke that contains thousands of toxic inflammatory agents, including FA, may actually be a risk factor for development of MPNs [129]. Thus, smoking is associated with elevated hematocrit, leukocytosis, monocytosis, and occasionally thrombocytosis—all are hallmarks in patients with MPNs. To this end the JAK-STAT and NF-kB signalling pathways are activated in both smokers and in patients with MPNs. Additionally, both share elevated levels of several proinflammatory cytokines, in vivo activation of leukocytes and platelets, endothelial dysfunction, and increased systemic oxidative stress. Indeed, smoke as a chronic inflammation stimulus giving rise to a chronic myelomonocytic response and ultimately MPNs fits very well with the excellent inflammation model for MPNs development as recently described by Hermouet and coworkers [31]. Accordingly, there is reason to believe that smoking may be both a trigger for and a driver of clonal evolution in MPNs taking into account that both smoking and MPNs are associated with chronic inflammation and systemic oxidative stress. In this context smoking may augment chronic inflammation in MPNs, thereby magnifying the risk of thrombosis, clonal expansion, and second cancers. The role of smoking in MPNs pathogenesis is further supported by a most recent study showing that a high proportion of MPNs patients actually have a smoking history [130]. An association between smoking and MPNs evolution is also supported by the fact that the most frequent second cancers in patients with MPNs are lung and urinary tract cancers which are most prevalent in smokers [3].

Fifthly, chronic systemic inflammation in patients with MPNs may predispose to or aggravate existing inflammation-mediated diseases in MPNs patients. Thus, it might be anticipated that chronic inflammation associated with (other) chronic inflammatory diseases, for instance, inflammatory rheumatological or dermatological diseases (e.g., polymyalgia rheumatica, rheumatoid arthritis, psoriasis, hidradenitis, and systemic lupus erythematosus), chronic inflammatory bowel diseases (Crohn's disease, colitis ulcerosa), chronic obstructive pulmonary disease and cancers (e.g., lung cancer) might ultimately elicit MPNs in a subset of the patients consequent to the chronic inflammation-mediated myelomonocytic drive [31]. Importantly, in these patients, anemia, leukocytosis, and thrombocythemia are ascribed to their chronic inflammatory disease or cancer and, accordingly, they are not normally screened for JAK2V617F, CALR, or MPL mutations. In the context of MPNs as inflammatory diseases, being potentially triggered and driven by chronic inflammation, the time is ripe to consider if the above disease categories should be investigated more rigorously for MPNs than being clinical practice today. Indeed, such studies are urgently needed to elucidate and expand the role of chronic inflammation as a true trigger for and driver of clonal evolution in MPNs.

Sixthly, chronic inflammation and oxidative stress may have therapeutic implications. Thus, it might be anticipated that patients with systemic chronic inflammation due to concurrent inflammation-mediated comorbidities may exhibit an inferior response to cytoreductive therapy necessitating higher dosages of, for example, hydroxyurea to obtain normal leukocyte and platelet counts. Furthermore, the response to IFN might be impaired considering that IFN signalling is impaired by inflammation and oxidative stress [131].

Seventhly, in the context that "triple-negative" (negative for JAK2V617F, CALR, and MPL-mutations) ET patients have a much more favourable prognosis than mutation-positive ET patients, some triple-negative "ET" patients may actually not have a MPNs but instead polyclonal inflammation-driven thrombocythemia. If so, the subset of "triple-negative" "ET" patients may be associated with a heavy comorbidity burden of chronic inflammatory diseases, an issue which deserves to be investigated systematically.

Eighthly, by dampening chronic inflammation using potent anti-inflammatory agents such as JAK2 inhibitor treatment and statins, it is anticipated that the rate of thromboembolic events will likely decline, since chronic inflammation per se carries an increased risk of thrombosis due to several factors as outlined above (leukocytosis, thrombocytosis, and in vivo leukocyte-platelet and endothelial activation). This issue on inflammation-mediated thrombogenesis has been dealt with most recently [132].

Ninthly, chronic inflammation in MPNs, if left untreated with elevated platelet counts, may worsen the prognosis of second cancers, which MPNs patients are prone to develop, not only after the MPNs diagnosis but also prior to the diagnosis [3, 76]. This particular issue, the "Platelet-Cancer-Loop" in MPNs, and the perspectives for prognosis of second cancers when not treating elevated platelet counts in MPNs have most recently been reviewed and debated [78, 133]. Indeed, elevated platelet counts in MPNs may contribute to the inferior prognosis of second cancers in these patients, most recently being reported in a large Danish epidemiological study [134].

Tenthly, the notion of treating these diseases only when far advanced is antithetical to treating other forms of cancer. The model of clonal evolution, the occurrence of additional molecular abnormalities, and the development of metastatic sites of disease following extramedullary hematopoiesis of $CD34^+$ cells in the spleen and liver are just some of the compelling reasons to consider treating sooner rather than later, when the tumor burden is less rather than more and before disease progression occurs. The fact that both rIFN and JAK1/2 inhibition can cause molecular change in JAK2V617F allele burden and revert cytogenetic and other

clonal abnormalities adds impetus to this argument. From the perspective that chronic inflammation may drive clonal expansion in these neoplasms early treatment may induce a state of minimal disease in a substantial number of patients. This may alter the natural history of the MPNs and the otherwise inevitable path towards thrombosis, irreversible MF, and leukemic transformation [97–103].

Eleventh, statins have, in addition to a cholesterol-lowering effect, many so-called pleiotropic effects, including antiproliferative, proapoptotic, antiangiogenic, antithrombotic, and especially potent anti-inflammatory effects [135]. Most recently, it has been shown that statins also significantly inhibit the malignant MPNs cell growth, including a potent synergistic effect with JAK inhibition [136, 137]. Thus, the perspectives may be that statins will achieve an important role in the future MPNs treatment in combination with JAK1/2 inhibitors and IFN-alpha2, a combination therapy, which—if instituted already from the time of diagnosis by potent inhibition of clonal proliferation and hence blockage of chronic inflammation generated by the malignant clone itself—may envisage the hope of reverting MPNs disease progression by inhibiting inflammation-driven genomic instability, subclone formation, mutagenesis, and thereby the ultimate transformation to myelofibrosis and acute myeloid leukemia. In regard to the anti-inflammatory, antithrombotic, and cytoreductive potential of statins and most lately the epidemiological evidence that statins reduce cancer-related mortality the rationale for the use of statins in patients with MPNs—per se accompanied by an increased risk of second cancers with an inferior prognosis—is only further supported [134–138]. Taking into account that MPNs patients may be prone to develop inflammation-mediated osteopenia with an increased risk of fractures early diagnosis and treatment of osteopenia with bisphosphonates may be an option in the future. Indeed, bisphosphonates also possess potent anti-inflammatory, immunomodulatory anticancer properties and may have a synergistic effect with statins in targeting the bone marrow stroma niche, thereby inhibiting the egress of $CD34^+$ cells from stem cell niches [139]. To this end, several reports have documented beneficial effects of treatment with bisphosphonates in MPNs [140–145]. The rationales for the mevalonate pathway as a therapeutic target in the treatment of MPNs have been thoroughly described in recent reviews [135, 146].

5. Conclusion

The concept of chronic inflammation as a major driver of disease progression in MPNs opens the avenue for clinical trials in which the two most promising agents within MPNs—IFN and ruxolitinib—are combined and instituted in the early disease stage according to the early intervention concept. The proof of concept and the rationales for this combination therapy have most recently been published [147] and a Danish study on combination therapy with low-dose pegylated IFN and ruxolitinib is ongoing with very promising preliminary results. The ability of IFN to induce deep molecular responses with normalisation of the bone marrow, even years after cessation of IFN, and the role of inflammation in the initiation and progression of MPNs make the combination of IFN and ruxolitinib one of the most promising new treatment strategies for patients with MPNs [8, 9, 11–13].

Conflict of Interests

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Review Article

HSP90 and HSP70: Implication in Inflammation Processes and Therapeutic Approaches for Myeloproliferative Neoplasms

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Myeloproliferative neoplasms (MPN) are clonal stem cell disorders that lead to the excessive production of one or more blood cell lineages. It has been reported that, in most MPN, inflammatory cytokines are frequently increased, indicating that inflammation plays a crucial role in these disorders. Heat shock proteins (HSP) are induced in response to many stressful conditions from heat shock to hypoxia and inflammation. Besides their chaperone and cytoprotective functions, HSPs are key players during inflammation, hence the term "chaperokine." Through their chaperone activity, HSP90, a stabilizer of many oncogenes (e.g., JAK2), and HSP70, a powerful antiapoptotic chaperone, tightly regulate Nuclear Factor-kappa B signalling, a critical pathway in mediating inflammatory responses. In light of this potential, several HSP90 inhibitors have been generated as anticancer agents able to degrade oncogenes. As it turns out, however, these drugs are also potent inhibitors of the inflammatory response in various diseases. Given the chaperone potential of HSP70 and the fact that HSP90 inhibitors induce HSP70, interest in HSP70 inhibitors is also increasing. Here, we focus on the implication of HSP90 and HSP70 in inflammatory responses and on the emergence of new therapeutic approaches in MPN based on HSP inhibitors.

1. Introduction

1.1. Philadelphia Chromosome-Negative Myeloproliferative Neoplasms. Philadelphia chromosome-negative myeloproliferative neoplasms (MPNs) are acquired clonal disorders of haematopoietic stem cells (HSC) characterized by hyperplasia of one or several myeloid lineages. They include essential thrombocythaemia (ET), polycythaemia vera (PV), and myelofibrosis (PMF). The V617F mutation of the Janus kinase protein, JAK2, is the most prevalent genetic abnormality in these three types of MPN and is found in 95% of PV, and about 50% of ET and PMF [1–4]. This mutation, which usually affects only one of the JAK2 gene alleles in ET, frequently becomes homozygous in PV and MF. Subsequently, JAK2V617F induces the constitutive activation of downstream signalling pathways including PI3K (Phosphatidyl-Inositol-3 Kinase), MAPK (Mitogen Activating Protein Kinase), and STAT (Signal Transducers and Activators of Transcription) and thus cytokine independent growth and hypersensitivity [2]. Other abnormalities in the TPO receptor (MPL)/JAK2 axis, such as mutations in MPL, LNK, [5] or CBL epigenetic regulators (TET2 (Tet methylcytosine dioxygenase 2) [6] and DNMT3A (DNA Methyl Transferase 3b) [7]), have been identified. More recently, two groups have identified novel alterations of the calreticulin gene (CALR) in around 67% and 88% of JAK2-negative ET and PMF, respectively. These alterations were exceptionally found in PV patients. Inflammation seems to be independent from the identified mutations, and better understanding of the causes and molecular mechanisms that underlie chronic inflammation in MPNs seems necessary to improve the treatments currently proposed to MPN patients. Depending on the beneficial effects of JAK2 inhibitors on inflammatory conditions observed in myelofibrosis, one may

reasonably wonder whether other anti-inflammatory therapeutics could be useful. In this review, we focus on the key role of heat shock proteins in inflammatory responses and on the emergence of new therapeutic approaches based on HSP inhibitors.

1.2. Heat Shock Proteins (HSPs). Stress or heat shock proteins (HSPs), first discovered in 1962 by Ritossa [8], are a set of ubiquitous and highly conserved proteins. Mammalian HSPs have been classified into two groups according to their size: high molecular weight HSPs and small molecular weight HSPs. The first group includes four major families: HSP110, HSP90, HSP70, and HSP60. Some of these are expressed constitutively whereas expression of the others is induced by stressful conditions [9]. High molecular weight HSPs are ATP-dependent chaperones and require cochaperones to modulate their conformation and ATP binding. In contrast, small molecular weight HSPs, such as HSP27, are ATP-independent chaperones. HSPs are induced by a variety of physiological and environmental insults, from temperature stress to hypoxia, inflammation, infections, or anticancer chemotherapy [10]. Even in the absence of stress, HSPs play key roles in living systems by acting as chaperones. They assist in (i) the folding of newly synthesized polypeptides, (ii) the assembly of multiprotein complexes, and (iii) the transport of proteins across cellular membranes [11].

Stress proteins allow cells to survive in otherwise lethal conditions, and several mechanisms account for their cytoprotective effect: (i) as mentioned above, they are powerful chaperones; (ii) they participate in the proteasome-mediated degradation of proteins under stress conditions, thereby contributing to the so-called "protein triage"; (iii) they inhibit key effectors of the apoptotic machinery at the pre- and postmitochondrial level [12]. Among the different HSPs, HSP27 and HSP70 are the most strongly induced after stresses such as anticancer drugs, oxidative stress, radiation, and shock inflammatory stress. This need for HSPs increases not only after proteotoxic damage, but also during physiological conditions, such as differentiation processes, in a tissue and stage-specific manner. HSPs like HSP90 and HSP70 participate in the monomacrophagic differentiation of primary monocytes [13, 14]. In zebrafish, mutation of GRP75 (HSP70 family) specifically impairs the development of erythrocytes, granulocytes, and haematopoietic progenitors, thus giving rise to a human myelodysplastic-like syndrome (MDS) [15]. Moreover, HSP70 and HSP27 are required for erythroid differentiation of human primary erythroblasts [16, 17]. Apart from their cytoprotective functions, HSPs such as HSP90 and HSP70 have been shown to have additional cellular functions directly related to inflammation and the innate immune response. The term "chaperokine" was therefore attributed to these HSPs, which combine their unique function to act both as a chaperone and as cytokine (see Section 3).

In this review, we will focus mainly on the role of HSP90 and HSP70 in inflammation and on the therapeutic approaches based on their inhibition.

The Chaperone HSP90. The HSP90 family includes HSP90-alpha and HSP90beta, which functions as an ATP-dependent chaperone. HSP90 forms large protein complexes with other molecular chaperones (p23, cdc37, HSP70, and so on) known as multichaperone complexes. This chaperone complex is involved in the folding, activation, and assembly of numerous proteins, including key mediators of signal transduction and transcriptional regulation [18]. The alpha and beta isoforms of HSP90, which are essential for the viability of eukaryotic cells, represent 1-2% of total cytosolic proteins usually in a latent, uncomplexed form and can be further induced by stresses. Conversely, tumours often express high levels of catalytically active HSP90, which is found in complexes with oncogenic client proteins [19]. HSP90 is overexpressed in breast tumours, lung cancer, leukaemia (i.e., myelodysplastic syndromes and acute myeloid leukaemia), and Hodgkin lymphoma [20], and thus it contributes to tumorigenicity and cancer cell resistance. HSP90 acts through both its antiapoptotic role and its chaperone function of stabilizing many kinases involved in cancer-cell signalling, including tyrosine kinases (i.e., FLT3 [21], JAK2 [22], v-Src [23], and serine/threonine kinases (AKT, Raf-1)). HSP90 also interacts with and stabilizes the receptor interacting kinase (RIP). Upon stimulation by Tumour Necrosis Factor alpha (TNF-α), RIP is recruited to the TNF receptor, thereby allowing the activation of Nuclear Factor-kappa B (NF-κB), a key component of the inflammatory response. Following depletion of HSP90, RIP is degraded and activation of NF-κB is prevented [24]. In this context, HSP90, by its stabilizing effect on the conformations of mutant proteins during transformation, favours the emergence of polymorphisms and mutations that support the evolution of resistant clones. Therefore, a rationale exists for targeting HSP90-dependent pathways in cancers and inflammatory diseases.

The Chaperone HSP70. The HSP70 family constitutes the most conserved and well-known class of HSPs. Among them, the inducible HSP70 (also called HSP72/HSPA1) and the constitutively expressed HSC70 (HSP73/HSPA8) are mainly localized in the cytosol, while others are located in the mitochondria (mtHSP70) or in the endoplasmic reticulum (GRP78/Bip). HSP70 cochaperones (HSP40, HSP110, CHIP, HOP, HIP, BAG-1, and BAG-3) modulate the chaperone activity of the protein through their binding to functional domains of HSP70 either the NH2-terminal ATP-binding domain (ABD) or the COOH-terminal peptide-binding domain (PBD).

HSP70 is a powerful antiapoptotic protein that inhibits both caspase-dependent (extrinsic and intrinsic pathways) and independent cell death [25, 26]. The important role of HSP70 was further demonstrated using mouse embryonic cells that lacked inducible HSP70 (encoded by HSP70.1 and HSP70.3), which display hypersensitivity to a wide range of lethal stimuli [27].

The high expression of HSP70 is associated with a poor prognosis and resistance to chemotherapeutic drugs in many cancers such as breast, endometrial, or gastric cancer [28, 29]. In Bcr-abl leukaemia cells, the expression of the protein HSP70 is also elevated and requires the GATA-response element in the HSP70 promoter [30]. Recently, Gallardo and colleagues identified a role for HSP70 in the

proliferation and survival of the erythroid lineage in PV. In an *ex vivo* model, inhibition of HSP70 expression led to the dose-dependent inhibition of cell growth and burst formation unit erythroid (BFU-E) in PV and ET. This effect was associated with increased apoptosis of the erythroid lineage and decreased phospho-JAK2 signalling [31]. HSP70 might contribute to cell proliferation through the regulation of STAT signalling. Overexpression of HSP70 has been shown to upregulate STAT5 levels and activity, thus allowing the expression of the antiapoptotic protein Bcl-xl [32].

2. Focus on Emerging Drugs as HSP Inhibitors

2.1. HSP90 Inhibitors. The HSP90 chaperone family has a unique pocket in their N-terminal region, which binds ATP and ADP. This domain is crucial for the control of conformation and activity. Most competitive inhibitors of HSP90 bind to this "pocket" and block interaction with its client proteins.

There are two major classes of active molecules: natural HSP90 inhibitors and their derivatives, as well as synthetic inhibitors. In this review, we focus on HSP90 inhibitors, which have been proved to be useful for patients with haematological malignancies.

Most of the natural HSP90 inhibitor derivatives come from Geldanamycin and Radicicol [33]. Geldanamycin is an ansamycin-derivative benzoquinone that binds to the ATP binding "pocket" of HSP90 with higher affinity than natural nucleotides. This compound was discovered in 1970 [34]. It was first identified for its antibiotic and antitumoural potential in leukaemia (L1210) and nasopharynx KB cell lines [34]. A preclinical study subsequently revealed the hepatotoxicity of this inhibitor, thereby limiting its application [35]. Many derivatives have been reported to have less severe hepatotoxic effects and demonstrate potent anticancer activity at nontoxic doses, as is the case for 17-allylamino-17-demethoxygeldanamycin (tanespimycin, 17-AAG) and 17-[2-(dimethylamino) ethyl] amino-17-demethoxygeldanamycin (alvespimycin, 17-DMAG) [36]. 17-DMAG, which has better bioavailability and water solubility, went through a phase I evaluation [37] and 17-AAG with an improved formulation (DMSO-free) is in a phase III clinical trial.

Similarly to Geldanamycin, Radicicol is a macrocyclic antifungal antibiotic [38] that binds to the N-terminal domain of HSP90 [39] and destabilizes HSP90 client proteins. While *in vitro* studies have shown the efficacy of Radicicol, it was found unstable *in vivo*. Derivatives of Radicicol like VER-52296 (NVP-AUY922) were thus developed and entered in clinical trials in 2007 [40].

In parallel with natural HSP90 inhibitor derivatives, novel synthetic inhibitors have been developed, including purine-based compounds. The first class of such scaffolds was the PU series, such as PU-H71 and PU-DZ8, which share biological activity with Geldanamycin and Radicicol [33]. The PU series have a higher affinity for HSP90 than does ADP and are more water soluble and specific [41]. One of them, PU-H71, has been shown to induce tumour regression in a xenograft model of triple-negative breast cancers [42] and is currently in clinical trials. Another family of chemical compounds, not yet disclosed, has been produced by Serenex using a chemoproteomics technology platform. Among this family, SNX-7081, a small oral molecule, is more potent than 17-AAG in Chronic Lymphocytic Leukaemia [43] and SNX-5422 has entered a phase I trial [43] (Pfizer Inc., New York, NY, USA) [44, 45].

2.2. HSP70 Inhibitors. Members of the HSP70 family comprise three major domains: an N-terminal domain, which binds ATP, a substrate-binding domain, and a C-terminal domain, which also acts as a substrate-binding domain. Inhibitors of HSP70 could be classified in two groups according to the targeted domains, ABD or PBD.

Different inhibitors targeting the PBD have been developed. The first inhibitor was derived from the apoptosis inducing factor (AIF) protein, named ADD70 (AIF derived decoy for HSP70) [27]. It prevents caspase-independent cell death through its association with HSP70 [46]. Other small chemical inhibitors of HSP70, like 2-phenylethynesulfonamide (PES), which is also called pifithrin-μ, induce an apoptotic caspase-dependent cell death [47].

Other strategies to inhibit HSP70 are based on molecules that target the ABD. The first inhibitor tested was VER-155008. This is an adenosine-derived inhibitor that inhibits cell proliferation and induces apoptosis by targeting the ABD of both the inducible and constitutive (HSC70) form of HSP70 [48]. Furthermore, combined with small HSP90 inhibitors, VER-155008 displays a potentiator effect in colon cancer cells [48].

In order to protect the activity of constitutive HSC70, peptide inhibitors selectively directed against the inducible form of HSP70 have been designed. By screening, our group has selected peptide aptamers with (i) high affinity for the ABD of HSP70 and (ii) a strong inhibitory capacity on the chaperone activity of HSP70 *in vitro*. One of these aptamers, named A17, has also demonstrated a strong antitumoural potential in *vivo* [49].

Designing molecules that directly target HSP70 protein is not the only strategy. Other molecules, like KNK437 (N-formyl-3,4-methylenedioxy-benzylidene-gamma-butyrolactam), are able to inhibit the expression of inducible HSPs. In particular, KNK434 has been shown to prevent the induction of HSP70 following treatment with HSP90 inhibitors [50].

3. HSP90 and HSP70: Role in the Inflammatory Process

3.1. HSP90 and Inflammation. As previously mentioned, HSP90 functions as part of a multichaperone complex via its association with cochaperones (e.g., p23 and HSP70) and client proteins. Inhibition of HSP90 by inhibitors such as benzoquinone ansamycins leads to both upregulation of HSP expression (especially HSP70 and HSP27) and degradation of various client proteins via proteasomal degradation [51]. Several key regulators of signalling pathways that play critical roles in mediating inflammatory and immune responses are clients of HSP90. These include JAK/Signal Transducer

and Activator of Transcription (STATs) (JAK2), Toll Like-Receptor- (TLR-) 4 (e.g., TGF-β-activated kinase- (TAK-) 1 and RIP kinase), and NF-κB (Inhibitor of IκB Kinase (IKK)) signalling pathways [52].

Although the main application of HSP90 inhibitors is related to cancer therapy, these drugs are potent inhibitors of certain proinflammatory mediators in different cell types [50, 51]. The use of HSP90 inhibitors causes the dissociation of the IKK complex and thus prevents NF-κB activation [53]. In a number of *in vitro* models, Geldanamycin has inhibited TNF-α-mediated IKK and NF-κB activation [24, 50, 54]. In cultured human respiratory epithelium, Geldanamycin also inhibits TNF-alpha-mediated IL-8 gene expression [54]. SNX-7081 (by Serenex), a potent inhibitor of HSP90, increases HSP70 levels, and prevents NF-κB nuclear translocation, and cytokine and nitric oxide (NO) production following the stimulation of Jurkat cells by TNFalpha, Interleukin(IL)1-beta or LPS [55]. Data show that HSP90 immunostaining is increased in inflammatory regions of human atherosclerotic plaque. Atherosclerotic plaque of mice treated with either 17-AAG or 17-DMAG showed reduced activation of the transcription factors STATs and NF-κB and chemokine expression induced by proinflammatory cytokines [56]. 17-AAG was also shown to attenuate the inflammatory response in autoimmune encephalomyelitis and in severe sepsis [55, 57]. More recently, Yun et al. showed that a synthetic HSP90 inhibitor, EC144, is potent and selective and is efficacious in an inflammatory mouse model of endotoxic shock. EC144 is able to prevent LPS-mediated TLR4 signalling, thus decreasing proinflammatory cytokines, such as TNF-α and IL-6 [58].

3.2. HSP70 and Inflammation. Inflammation occurs in response to various cellular stresses including infection or heat shock. During infection, the level of HSP70 is increased and confers cytoprotection [59] via the inhibition of inflammatory signalling pathways, including the NF-κB pathway [60].

HSP70 proteins are implicated in the regulation of the immune responses and modulate inflammation through several mechanisms. Of note, extracellular HSP70 is released from intact cells via an active nonclassical secretory pathway. HSP70 could be secreted associated with exosomes or by an endolysosomal dependent pathway [61, 62]. Extracellular HSP70 was first shown to enhance the cross-presentation of the HSP-bound peptide by MHC-I on dendritic cells (DCs). HSP70s, by associating with peptides, form an HSP-peptide complex that binds to cell surface receptors, such as CD91 and Lox-1, and is further internalized by endocytosis [63]. Extracellular HSP70 functions as a "danger signal" [64], since, compared with serum from healthy individuals, serum from patients with autoimmune diseases, patients with trauma, and children with septic shock shows high concentrations of HSP70 [65–67]. As a mediator of inflammation, exogenous HSP70 is able to activate cell surface receptors on immune cells such as TLR2 and TLR4 [68], thus leading to NF-κB activation, and TNF-α, IL1-β, and IL-6 production [69]. As well as activating the NF-κB pathway, downstream signalling induced by HSP70 elicits a rapid calcium flux, and the production of inflammatory cytokines and chemokines by monocytes and DCs [69].

4. HSP90 as a Therapeutic Target in MPN and the Potential Implication of HSP70

Malignant cells have more unfolded proteins than do normal cells, which lead to higher chaperone expression. In transformed myeloid cells, oncogenic transducer molecules have been shown to depend more on HSP90 chaperoning than is the case in untransformed cells [70]. Among the client proteins that interact with and are stabilized by HSP90, there are several transducer molecules, such as kinases like BCR-ABL [71, 72], FLT-3 [21], and JAK2 [22]. In myeloproliferative neoplasms, one promising therapy is based on using HSP90 as a therapeutic target to destabilize its oncogenic partners and induce their proteolytic degradation. Different specific inhibitors of HSP90 (i.e., geldanamycin derivatives, resorcinol derivatives, purine analogues, and other synthetic inhibitors) are currently used as anticancer drugs and display potent activity in clinical trials in patients with solid tumours and haematological malignancies [42, 68].

It was first established that targeting HSP90 in JAK2-mutant cells lines by using 17-AAG reduced JAK2 levels and inhibited JAK2 signalling pathway activation [73]. This study showed a decrease in the expression of oncoprotein AKT and in the phosphorylated form of STAT5 and JAK2. In addition to this direct inhibition strategy, the authors combined JAK2 inhibitors with 17-AAG and observed no synergic effect. However, a more detailed study that examined the effects of PU-H71 [74] showed promising results in preclinical models of solid cancers [42] or haematological cancers [75]. In addition, the toxic side effects of PU-H71 on nonhaematopoietic cells were lower than those associated with geldanamycin derivatives, such as 17-AAG or 17-DMAG [74]. In this work, PU-H71 demonstrated interesting effects *in vitro* in JAK2-dependent cell lines, *in vivo* in murine models of PV and ET, and in samples from patients with primary MPN [74]. Moreover, PU-H71, which is able to induce degradation of client proteins of HSP90 including JAK2, inhibits proliferation and attenuates JAK-STAT pathways in JAK2V617F-positive or MPLW515L-positive cell lines. Following these results, the authors observed additive effects of a combined treatment using PU-H71 and a JAK2 inhibitor (TG101348), suggesting that using inhibitors of HSP90 alone or in combination might be an interesting therapeutic approach.

In light of these results, other inhibitors of HSP90 were tested. Among these molecules, a nongeldanamycin analogue (NVP-AUY922, AUY922), already known to inhibit the chaperone association of HSP90 with its client proteins [72, 75], was investigated in the context of a preclinical MPN study [70]. This study found a synergic effect of AUY922 and a JAK2 inhibitor (TG101209) in JAK2V617F cells lines. In addition, it has been shown that genetic resistance to enzymatic inhibitors of JAK2 is overcome by HSP90 inhibition [76].

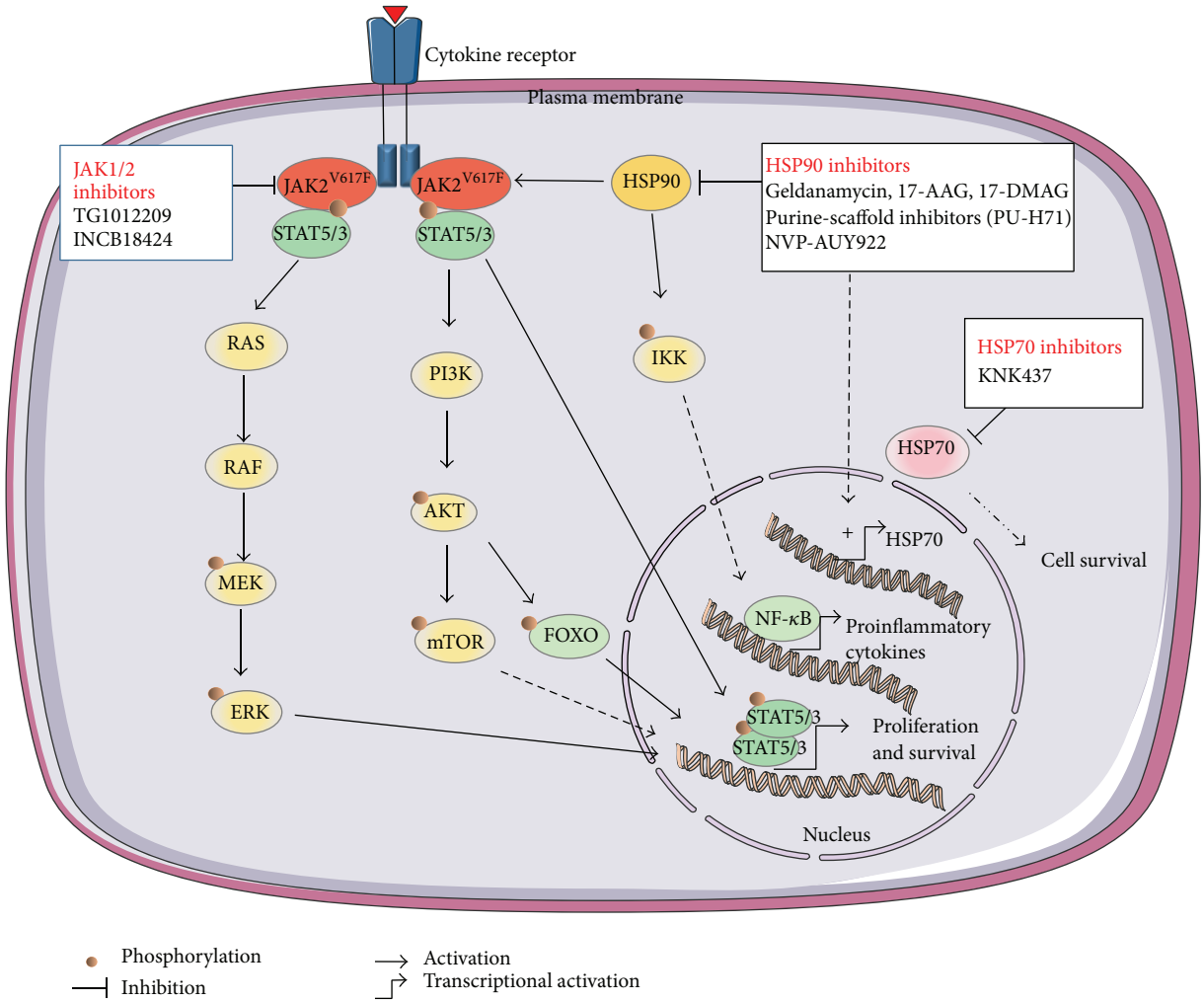

Figure 1: Schematic representation of the potential effects of HSP inhibitors on the main activated pathways in myeloproliferative neoplasms. PI3K (Phosphatidyl-Inositol-3 Kinase), STAT (Signal Transducers and Activators of Transcription), ERK (extracellular signal-regulated kinases), MEK (mitogen-activated protein kinase kinase), HSP (heat shock protein), mTOR (mammalian target of rapamycin), FOXO (Forkhead transcription factors), NF-κB (Nuclear Factor-kappa B), and IKK (Inhibitor of IκB Kinase).

This combination was also evaluated on CD34+ cells harvested from the peripheral blood of patients suffering from myelofibrosis. In these primary cells, the combined treatment was more effective than each agent alone and caused a greater decrease in JAK2 and AKT levels in addition to a decrease in the phosphorylated forms of STAT3 and STAT5 without affecting their expression. More recently, it was also observed that the effect of JAK inhibitors and especially the dual JAK1/JAK2 inhibitor INCB18424 (ruxolitinib, JAKAVI) was enhanced by PU-H71 in a murine model of myelofibrosis [77]. Nevertheless, HSP90 inhibitors have been reported to increase the expression of other HSPs including HSP70, which provides a selective survival advantage in tumour cells.

The implication of HSP70 in MPN has not been investigated; however, several in-depth studies have explored the implication of HSP70 in other types of haematological malignancies including chronic [78, 79] or acute myeloid leukaemia [80]. Recently, proteomic analysis of samples from PV and ET patients revealed that HSP70 is overexpressed in PV samples but not in ET samples. Additionally, JAK2V617F cells from patients with PV were sensitized by siRNA HSP70 interference assay or treatment with an inhibitor of HSP70 (KNK437). Their results supported the hypothesis that HSP70 is implicated in the pathogenesis of PV and suggested that HSP70 could constitute a new molecular target [31]. Furthermore, the association of ruxolitinib with the HSP70 inhibitor, KNK437, showed a synergic effect in both JAK2 V617F cells lines and in culture colonies of PV [81]. These data confirm that HSP70 must participate in the pathogenesis of PV and seems to be a promising therapeutic target in MPN.

5. Concluding Remarks

Inhibition of HSP90 as a therapeutic approach was first evaluated in the context of cancer treatments [19, 77]. However, growing evidence indicates that HSP90 inhibitors could be beneficial for both MPN and inflammatory reactions. Moreover, and given the substantial increase in HSP70 induced

by HSP90 inhibitors, a combination with HSP70 inhibitors might be an interesting alternative to optimise the treatment [74, 75] (Figure 1).

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

The Hen or the Egg: Inflammatory Aspects of Murine MPN Models

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It has been known for some time that solid tumors, especially gastrointestinal tumors, can arise on the basis of chronic inflammation. However, the role of inflammation in the genesis of hematological malignancies has not been extensively studied. Recent evidence clearly shows that changes in the bone marrow niche can suffice to induce myeloid diseases. Nonetheless, while it has been demonstrated that myeloproliferative neoplasms (MPN) are associated with a proinflammatory state, it is not clear whether inflammatory processes contribute to the induction or maintenance of MPN. More provocatively stated: which comes first, the hen or the egg, inflammation or MPN? In other words, can chronic inflammation itself trigger an MPN? In this review, we will describe the evidence supporting a role for inflammation in initiating and promoting MPN development. Furthermore, we will compare and contrast the data obtained in gastrointestinal tumors with observations in MPN patients and models, pointing out the opportunities provided by novel murine MPN models to address fundamental questions regarding the role of inflammatory stimuli in the molecular pathogenesis of MPN.

1. Introduction

"Dass Carcinome nicht selten auf einfach entzündliche Reize, wie Traumen, entstehen, ist bekannt" (that carcinomas arise, not seldom, at the site of inflammatory stimuli, such as traumas, is known) wrote Virchow in 1869 [1]. This far-sighted statement, worded as a fact rather than a hypothesis, was validated almost 150 years later when Hanahan and Weinberg named "inflammation" as an underlying principle that contributes to and fosters the newly named "hallmarks of cancer" [2].

2. Inflammatory Etiology of Solid Tumors

In the interval between these two pivotal publications, a large collection of data was accrued that supports the postulated role for inflammation in carcinogenesis. It is now known that solid tumors can arise on the basis of chronic inflammation, most notably Gastrointestinal Stromal Tumor (GIST) following *Helicobacter pylori* infection. Additional examples include enteropathy-associated T cell lymphoma and adenocarcinomas in patients with coeliac disease as well as the increased risk of colorectal carcinoma in patients with inflammatory bowel disease [3, 4].

The model for neoplastic transformation in these disorders implies a multistep process (Figure 1). Initially, chronic inflammation causes epithelial cells as well as stromal macrophages to release cytokines and other stimulatory molecules that promote proliferation of surrounding cells, for example, the interstitial cells of Cajal in the stomach during active *H. pylori* infection [5]. In a second series of steps, enhanced proliferation increases the chance of stochastic mutations, leading first to hyperplasia and subsequently, with the accumulation of additional aberrations, to neoplasia. While this model has been validated experimentally for several solid tumor entities, the role of inflammation in

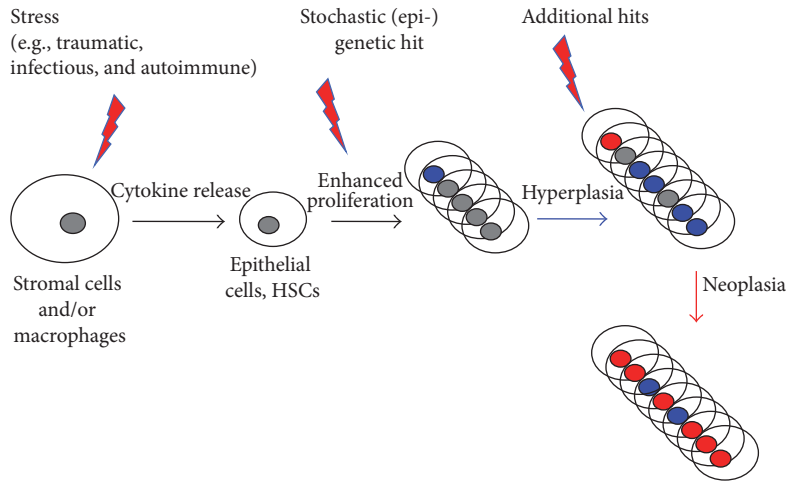

Figure 1: Multistep process for inflammatory driven neoplastic transformation. Stress, induced by various intrinsic and extrinsic factors, causes epithelial cells as well as stromal macrophages to release cytokines and other proliferation-promoting molecules, which lead to enhanced proliferation of surrounding cells. In a second step, enhanced proliferation increases the chance of stochastic mutations, leading first to hyperplasia and subsequently, with the accumulation of additional aberrations, to neoplasia.

the genesis of hematological malignancies has not been extensively studied.

3. Cell Extrinsic Influences on the Development of Myeloid Malignancies

The microenvironment and stromal tissue that surround solid tumors can be seen as analogous in function and in cell-cell interactions to the bone marrow niche cells that surround hematopoietic stem cells. During the past years, several observations have strengthened the hypothesis that the bone marrow niche can contribute to the development of myeloid malignancies. In one seminal study, Raaijmakers and colleagues demonstrated that altering gene expression by deletion of *Dicer1* specifically in osteoprogenitor cells, but not in the bone marrow, led first to the development of myelodysplasia and, subsequently, to the emergence of acute myeloid leukemia [6]. Leukemia arose in hematopoietic cells that expressed *Dicer1* but had acquired other genetic abnormalities. Importantly, transplantation of BM from anemic, thrombocytopenic mice, in which *Dicer1* has been deleted in the osteoprogenitors, into lethally irradiated wild-type recipient mice led to complete resolution of the cytopenias, demonstrating that they were niche-induced and not attributed to cell autonomous changes in hematopoietic stem cells themselves [6]. Conversely, transplanting wild-type bone marrow cells into mice which carried the *Dicer1* deletion in osteoprogenitors resulted in an MDS phenotype and induction of AML. These data clearly demonstrate that changes in the bone marrow niche can be sufficient to induce leukemia. Interestingly, deleting *Dicer1* in mature osteoblasts did not induce either MDS or leukemia, demonstrating that very specific alterations in the bone marrow are required for niche-induced oncogenesis. The precise nature of these changes is currently being investigated and it is not known whether inflammatory mechanisms contribute to leukemia induction in this model.

4. Association of MPN with Inflammatory and Autoimmune Diseases

While the data by Raaijmakers and colleagues thus constitute a proof of principle that leukemia can be induced by changes in the bone marrow microenvironment, the question remains whether inflammatory processes in particular contribute to the induction or maintenance of myeloid malignancies, specifically to myeloproliferative neoplasms (MPN). Several studies have recently suggested an inflammatory etiology for MDS, AML, and MPN [7–10], most notably a large epidemiological study in Sweden, which demonstrated a significantly increased risk of AML or MDS in patients with a history of any infectious disease [9]. Esplin et al. have shown that continuous TLR activation by chronic exposure to Lipopolysaccharides (LPS) alters the self-renewal capacity of HSCs in mice. Prolonged TLR activation occurs in various bacterial infections, for example, during oral infections such as Gram-negative periodontitis and during subacute bacterial endocarditis [11]. In their mice, Esplin and colleagues were able to show a myeloid bias and, conversely, a selective loss of lymphopoietic potential as well as an increased proportion of $CD150^{hi}CD48^{-}$ long-term HSCs [12]. The emergence of a myeloid bias has been witnessed during normal aging of HSC [13–15]. Signer et al. point out that the risk of developing myeloid and lymphoid leukemias increases with age [16]. It seems likely that HSCs acquire random genetic hits either under chronic TLR activation induced by LPS or during normal aging. These parallels strengthen the hypothesis of inflammatory driven myeloid malignancies, in some cases perhaps induced directly by an infectious cause.

While inflammatory processes involve various factors, including cytokines, reactive oxygen species, and immune

cells like macrophages, autoimmune phenomena are characterized by activation of T and B cells including the production of autoantibodies. Autoimmune diseases thus mainly involve changed T and B cell function but might share aspects of inflammatory processes resulting from altered cytokine release, such as increased IL-6 levels [17].

MPN patients with an antecedent autoimmune disorder carried a 1.7- and 2.1-fold increased risk to develop an AML or an MDS, respectively [9, 18]. In particular patients with MPN-associated myelofibrosis may show various autoimmune phenomena, including antibodies against red blood cells or anti-nuclear [9, 18] or anti-mitochondrial antibodies. To some extent, this might explain the pathogenesis of anemia and the accompanied compensatory reticulocytosis in this cohort of patients [19, 20]. The resulting increased malignant and nonmalignant myeloproliferation themselves thereby increase the risk for stochastic secondary (epi-)genetic hits and disease progression. However, neither the inflammatory nor the autoimmune hypotheses regarding MPN etiology have yet been directly confirmed by experimental studies.

5. The Inflammatory Hypothesis of MPN

MPN patients show elevated serum levels of various proinflammatory cytokines including IL-1, IL-6, IL-8, IL-11, IL-17, TNF-α, and TGF-β, as well as of the anti-inflammatory IL-10 [21–26]. Treatment with Ruxolitinib, JAK1 and JAK2 inhibitor, significantly decreased the level of circulating cytokines [27]. While these data demonstrate that MPN is accompanied by inflammatory changes, the causal order of events has not been determined. Does the malignant clone trigger an inflammatory response or—and this would constitute a change in perspective—can chronic inflammation itself trigger a MPN? In the latter model, sustained low-level, probably subclinical inflammation initially increases the proliferation of healthy, polyclonal hematopoietic stem and progenitor cells. Since each cell division carries the risk of acquiring a mutation, a malignant MPN clone arises and evolves on the basis of chronic, inflammation-induced proliferation.

Is there evidence supporting such a change in perspective or can it be procured using recently established, novel murine MPN models?

6. Murine Models to Test the Inflammatory Hypothesis of MPN

The field of gastrointestinal tumors has made use of sophisticated mouse models to detail the role of inflammation for the initiation and promotion of carcinomas. Multiple tissue specific knockout and transgenic lines have been generated to study the underlying molecular mechanisms and signal transduction pathways [34]. During the past five years, various mouse models with a myeloproliferative neoplasm-(MPN-) like phenotype have also been reported [32, 33, 45–52]. In this review, we will describe the evidence supporting a role for inflammation in initiating and promoting MPN development. Furthermore, we will compare and contrast the data from GI tumors with observations in MPN patients and models, pointing out the opportunities provided by the novel murine MPN models to address fundamental questions regarding the role of inflammatory stimuli in the molecular pathogenesis of MPN.

Various murine MPN models based on the most commonly occurring mutations have been developed. The alleles, which were introduced either in bone marrow transplant models, as transgenes, or as constitutively or inducibly active knock-ins, include $JAK2^{V617F}$, $JAK2^{Exon12}$, $cMpl^{W515L}$, TET2, ASXL1, and NFE2 (see Table 1) [32, 33, 45–52]. Of these, the NFE2 mice consistently show spontaneous transformation to acute leukemia, suggesting that elevated NFE2 activity promotes not only MPN development but also a sustained acquisition of additional aberrations leading to leukemic transformation [32, 33]. The transcription factor NFE2 is overexpressed in the majority of MPN patients, irrespective of the underlying driver mutation [53, 54]. NFE2 is central to the inflammatory process. On the one hand, it is induced by inflammatory cytokines, such as IL1β [55]. Elevated NFE2 activity in turn increases cell proliferation by increasing transcription of cell cycle regulators and promoting G1/S transition [33]. On the other hand, NFE2 itself promotes inflammation as it has been shown to directly regulate transcription of IL-8, a proinflammatory cytokine [56]. Interestingly, inhibition of NFE2, by shRNA, abrogates endogenous erythroid colonies (EEC) formation [57], a pathognomonic hallmark of PV, supporting a central role for this inflammatory axis in promoting growth of the neoplastic clone.

Two distinct groups of murine models are used to study the role of inflammation in GI cancers (reviewed in [34]). The first are genetically altered mice, either transgenic or knock-in strains, that carry mutations in the "adenomatous polyposis coli" (APC) gene or in genes affecting the Wnt signaling pathway. The APC gene is mutated in 80% of human colorectal cancers, while a further 10% carry mutations in beta-catenin, a central regulator of the Wnt-signaling pathway [58, 59]. In the second type of models, chemical carcinogens and promoters of inflammation, frequently azoxymethane (AOM) and dextran sodium sulfate (DSS), are used to induce the development of colitis associated colon cancer (CAC) [34].

7. The Role of the COX2/PGE2 Axis

By generating double or triple mutant mice, for example, strains that carry APC mutations in addition to tissue specific knockouts of critical signal transducing molecules, the role of various molecular pathways was investigated. The data reveal a critical role for the cyclooxygenase-2 (COX-2)/prostaglandin-E2 (PGE2) pathway even in mice that carry APC mutations [35–37, 43]. COX-2 is a central mediator of inflammation. It oxidizes arachidonic acid to prostaglandin H2, which is subsequently converted to PGE2. PGE2 promotes inflammation by affecting a variety of cellular

Table 1: Disease models involving inflammation.

Affected compartment	Cause	Intervention	Phenotype	Reference
	Genetic alteration			
Hematopoiesis	$JAK2^{V617F}$	TNF-α deletion	Attenuation of MPN development	[28]
Hematopoiesis	Gata-1^{lo}		Myelofibrosis	[29]
Hematopoiesis	Gata-1^{lo}	TGF-β inhibition	Restored hematopoiesis, reduced fibrosis	[30]
Hematopoiesis	TPO^{hi} with	TGF-β inhibition	Restored hematopoiesis	[31]
Hematopoiesis	NFE2 overexpression/mutations		MPN, sAML	[32, 33]
Gastrointestinal mucosa	APC mutations		Colorectal cancer	Reviewed in [34]
Gastrointestinal mucosa	$APC^{\Delta 716}$	COX-2 knockout	Suppression of intestinal polyposis	[35]
Gastrointestinal mucosa	$APC^{\Delta 716}$	PGE2-receptor-2 knockout	Suppression of intestinal polyposis	[36]
Gastrointestinal mucosa	$APC^{\Delta 716}$	Prostaglandin synthase knockout	Suppression of intestinal polyposis	[37]
Gastrointestinal mucosa	$APC^{\Delta 716}$	15-prostaglandin dehydrogenase (15-PDGH) knockout	Disease exacerbation	[38]
Gastrointestinal mucosa	$APC^{\Delta 716}$	Deletion of either IL-17, IL-6, CCR2, TNFR, or p55	Suppression of intestinal polyposis	[39–42]
	Infectious cause			
Hematopoiesis cell intrinsic and extrinsic	TLR activation by bacterial infection		HSC exhaustion	[12]
	Chemical cause			
Gastrointestinal mucosa	Azoxymethane (AOM) Dextran Sodium Sulfate (DSS)		Colitis associated colon cancer (CAC)	Reviewed in [34]
Gastrointestinal mucosa	Azoxymethane (AOM)	COX-2 transgene	Increased development of tumors	[43]
Gastrointestinal mucosa	Azoxymethane (AOM) Dextran Sodium Sulfate (DSS)	COX-2 deletion	Increased development of tumors	[44]
Gastrointestinal mucosa	AOM or DSS plus deletion of either IL-17, IL-6, CCR2, TNFR, or p55		Suppression of CAC	[39–42]

functions. In contrast to COX-1, which is constitutively expressed, COX-2 is specifically induced by proinflammatory stimuli and mitogens.

Knockout of COX-2 in mice carrying the $APC^{\Delta 716}$ mutation drastically suppressed the development of intestinal polyposis as did treatment of mice with COX-2 inhibitors [35]. Conversely, transgenic overexpression of COX-2 in colon epithelium increased the development of intestinal tumors [43]. A similar strategy could easily be used to test the importance of the COX-2/PGE2 axis in MPN models. The COX-2 knockout is not tissue specific, so that development of the MPN phenotype in the presence or absence of systemic COX-2 could be investigated. In this context, the use of inducible models appears especially interesting, as the role of inflammatory processes in disease initiation could be investigated [48, 50, 60].

The logic described above was applied to various other genes in the COX-2/PGE2 axis, and the results consistently underwrite an essential role for an inflammatory response in the development of APC-driven cancers. For example, knockout of the gene for either the PGE2-receptor-2 or the microsomal PGE synthase resulted in the suppression of intestinal polyp formation [37]. Conversely, deletion of the gene for 15-prostaglandin dehydrogenase (15-PDGH), an enzyme that catabolizes and inactivates prostaglandins, resulted in disease exacerbation, animals carrying mutant APC but lacking 15-PDGH developing significantly more polyps than their control littermates [38]. In addition, and perhaps less surprisingly, the COX-2/PGE2 axis was also shown to be essential in the AOM/DSS inflammation-associated colon tumor model, as deletion of COX-2 exacerbates CAC development [44, 61].

Equivalent mouse strains could be generated in the context of various MPN mutations to investigate the contribution of the COX-2/PGE2 inflammatory axis to MPN disease initiation or maintenance. Inducible expression of MPN alleles in the background of a constitutive COX-2/PGE2 knockout will test the role of inflammation in MPN initiation, whereas constitutive expression of MPN mutations and subsequent inducible deletion of a COX-2/PGE2 axis gene will test for the requirement of an inflammatory milieu in maintaining the MPN phenotype.

8. The Role of Specific Immune Cells

During the past decade, various mouse strains lacking specific immune cells have been developed. These mice can attest to the requirement of specific cell types for disease development. For example, crossing $APC^{\Delta 716}$ mice with op/op mice, which are devoid of functional macrophages, led to a suppression of polyp formation, as did the generation of APC-mutant, $kit^{W/W}$ mice, which lack mast cells [62]. Hence, both macrophages and mast cells are required to elaborate the microenvironment in which mutant APC can induce polyp formation. A recent paper by Ramos and colleagues provides compelling evidence that similar but distinct mechanisms operate in MPN [63]. In mice with an established $JAK2^{V617F}$ driven erythrocytosis, depletion of macrophages with clodronate normalized hematocrit and RBC counts as well as reducing reticulocytosis. Since these authors used a Vav-Cre/$JAK2^{V617F}$ BMT model, it is likely that the macrophages were also carrying the $JAK2^{V617F}$ mutation and were therefore part of the malignant clone. The molecular mechanism is thus slightly different from that in gastric cancer, where macrophages appear necessary for paracrine stimulation of the neoplastic epithelial cells. In MPN, macrophages that are part of the malignant clone would be perpetuating the neoplasia in an autocrine manner. However, if the op/op mice are used in models similar to those detailed above, a role for healthy macrophages in MPN initiation from healthy HSCs may be revealed.

9. The Role of Cytokines

The requirement for macrophages and mast cells points to a rather obvious role for cytokines in tumor formation. While the essential role for cytokines in various physiological processes makes the construction of knockout mice deficient in these signaling molecules challenging, several strains have been generated and examined for cytokine contribution to gastric cancer development. Deletion of IL-17, IL-6, CCR2, or TNF-receptor p55 [39–42] led to a suppression of intestinal polyp development or CAC development in both the APC-mutant and the AOM/DSS models.

One very similar study points to an important role for TNF-α in promoting $JAK2^{V617F}$ driven MPN [28]. Deletion of TNF-α limited expansion of $JAK2^{V617F}$ positive cells and attenuated disease development, pointing to a disease-promoting role for this cytokine. Analogous investigations for other inflammatory cytokines are required, especially addressing the question whether they are necessary for successful disease initiation. Candidates that should be investigated with priority include those factors for whom elevated levels have been documented in MPN patients and who have been shown to play a role in the genesis of other entities with an inflammatory component.

In this light, IL-11 stands out as its levels are elevated in PV patients and has been shown to induce healthy bone marrow to form endogenous erythroid colonies [22, 64]. EEC constitute a characteristic abnormality of PV, one that may be used diagnostically because of its high sensitivity and specificity. Antibodies to IL-11 inhibit EEC formation in PV cells [64]. IL-11 has been shown to promote gastric tumor development, while, conversely, deletion of the IL-11 coreceptor alpha ablated the development of gastric tumors [65].

IL-8 has likewise been shown to induce EEC formation from healthy bone marrow cells [64]. As detailed above, IL-8 is a direct target of NFE2 and both are overexpressed in MPN patients. Furthermore, Hermouet and colleagues have shown that IL-8 promotes hematopoietic progenitor survival [66]. Conversely, inactivation of the IL-8 pathway inhibited $CD34^{+}$ cell proliferation and colony formation [66]. As IL-8 levels constitute an independent predictor of survival in PMF patients, this cytokine is highly likely to contribute to MPN pathophysiology, perhaps as one of the pivotal inflammatory mediators that initiate hyperproliferation of healthy HSCs in the bone marrow [26].

The role of TGF-beta in the dysmegakaryopoiesis and fibrosis characteristic of PMF has been investigated in a murine model of myelofibrosis due to low Gata-1 expression (Gata-1^{lo}) [29, 30]. While the mutation decreasing Gata-1 levels in this model is not found in PMF patients, Gata-1 levels are specifically downregulated in a subset of PMF megakaryocytes [67]. In Gata-1^{lo} mice, inhibition of TGF-beta signaling restored hematopoiesis, normalized megakaryocyte development, and reduced fibrosis [30]. Similar results were obtained by Dr. Vainchenker's group in mice overexpressing thrombopoietin (TPO). Mice displaying high TPO levels develop an MPN phenotype with fibrosis. In the absence of TGF-beta, these mice still show a myeloproliferative syndrome, yet no fibrosis [31]. Interestingly, while they express normal TGF-beta levels, untreated Gata-1^{lo} mice nonetheless show specific TGF-beta signaling alterations in bone marrow and spleen, such as overexpression of EVI1. This signaling abnormality is comparable to the abnormal TGF-beta profile observed in PMF patients, which includes overexpression of STAT1 and IL-6, factors directly related to autoimmune fibrosis [68].

These data clearly indicate that TGF-beta plays a pivotal role in propagating the PMF phenotype and the development of fibrosis, which contributes to the cytopenias that constitute the leading cause of morbidity and mortality in this patient population. Targeted deletion or tissue specific overexpression of TGF-beta is now required to determine whether the cytokine is required or sufficient for disease initiation. Observations in other organs suggest that the latter is likely: liver specific overexpression of TGF-beta results in hepatic fibrosis [69].

Another novel, autocrine inflammatory pathway has recently been described. Dr. Hoffman's laboratory showed that MPN myeloid cells secrete elevated levels of lipocalin-2, an inflammatory cytokine, and that lipocalin-2 levels are elevated in PMF patients [70]. Lipocalin secretion is known to be stimulated by IL-1, IL-6, and IL-17, all of which are elevated in MPN [23, 24, 71–73]. Lipocalin induces reactive oxygen intermediates (ROS) formation with subsequent induction of double stranded DNA breaks leading to apoptosis of healthy HSCs but not PMF HCSs [70]. Hence, protection of PMF cells from lipocalin action, by a yet unknown mechanism, could constitute one way in which the microenvironment or the MPN clone itself uses inflammatory mediators to create an environment that provides a selective advantage to the MPN clone.

10. The Inflammatory Hypothesis of MPN: Awaiting Proof from Murine Models

While the evidence presented above supports a change in perspective, in which inflammation may induce and promote MPN, rather than simply being a consequence of it, several aspects of this hypothesis remain to be experimentally proven. A murine model, which does not carry a specific MPN mutation, but rather models a prolonged, chronic inflammation, would constitute a valuable tool. If in such a model, the inflammatory milieu alone was sufficient to induce malignant myeloproliferation or even leukemic transformation, this would constitute a proof of principle.

Proving the inflammatory hypothesis in MPN patients directly may, however, not be feasible. Diagnosing the underlying inflammatory process, postulated to be present even prior to the clinical MPN presentation, will not be possible in most cases. However, this will not be required. If the inflammatory hypothesis can be proven experimentally, this provides sufficient evidence for the initiation of clinical trials examining the effectiveness of early therapeutic intervention with the goal of suppressing chronic inflammation, thereby intersecting the vicious cycle that promotes MPN progression. Again, epidemiological data from the field of gastric cancers may point the way. Two landmark studies, published over 20 years ago, demonstrated that regular use of nonsteroidal anti-inflammatory drugs (NSAIDs) reduces the risk of colon cancer [74]. NSAIDs including aspirin are well known to function as a COX-1/2 inhibitor and therefore inhibit the production of PGE2. The Efficacy and Safety of Low-Dose Aspirin study (ECLAP), nomen est omen, proved both the safety and efficacy of aspirin in PV patients [75]. While overall survival was not increased during the observation period in patients treated with low-dose aspirin, longer followup is required to observe a beneficial effect if aspirin use prevents leukemic transformation by suppressing a chronic inflammatory stimulus. As mentioned above, mouse strains carrying MPN mutations in the context of COX-2 deficiency may reveal the impact of the COX-2/PGE2 inflammatory axis to MPN disease initiation and maintenance as well as leukemic progression.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Cytokine Regulation of Microenvironmental Cells in Myeloproliferative Neoplasms

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The term myeloproliferative neoplasms (MPN) refers to a heterogeneous group of diseases including not only polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF), but also chronic myeloid leukemia (CML), and systemic mastocytosis (SM). Despite the clinical and biological differences between these diseases, common pathophysiological mechanisms have been identified in MPN. First, aberrant tyrosine kinase signaling due to somatic mutations in certain driver genes is common to these MPN. Second, alterations of the bone marrow microenvironment are found in all MPN types and have been implicated in the pathogenesis of the diseases. Finally, elevated levels of proinflammatory and microenvironment-regulating cytokines are commonly found in all MPN-variants. In this paper, we review the effects of MPN-related oncogenes on cytokine expression and release and describe common as well as distinct pathogenetic mechanisms underlying microenvironmental changes in various MPN. Furthermore, targeting of the microenvironment in MPN is discussed. Such novel therapies may enhance the efficacy and may overcome resistance to established tyrosine kinase inhibitor treatment in these patients. Nevertheless, additional basic studies on the complex interplay of neoplastic and stromal cells are required in order to optimize targeting strategies and to translate these concepts into clinical application.

1. Myeloproliferative Neoplasms

Myeloproliferative neoplasms (MPN) are clonal hematopoietic stem cell disorders characterized by abnormal proliferation and expansion of one or more myeloid lineages [1, 2]. The WHO classification of MPN comprises four classic MPN and additional nonclassic MPN. The group of the common, classic MPN includes chronic myeloid leukemia (CML) defined by the Philadelphia chromosome (Ph) and the three Ph-negative entities' polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF). The group of nonclassic MPN includes systemic mastocytosis (SM), chronic neutrophilic leukemia (CNL), and chronic eosinophilic leukemia (CEL) [1, 3].

Aberrant tyrosine kinase (TK) signaling is a common hallmark in MPN and has been shown to represent a key driver of the disease. The *BCR-ABL1* fusion gene, which results in a constitutive activation of ABL1 kinase activity, characterizes CML [4–6]. In a majority of patients with PV, ET, and PMF, the activating V617F mutation in the receptor-associated TK *JAK2* is detected [7–10]. In addition, mutations in exon 12 of *JAK2* and mutations in the thrombopoietin receptor (*MPL* W515K/L) have been described in these entities [11, 12]. More recently, somatic mutations in *CALR* were found in *JAK2*- and *MPL*-negative patients with ET or PMF [13, 14]. The activating point mutation D816V in the *KIT* receptor TK is a diagnostic criterion for SM and is found in more than 80% of all patients with SM [15].

A constitutively activated *FIP1L1-PDGFRA* fusion TK has been identified in patients with CEL with or without an accompanying hypereosinophilic syndrome (HES) [16, 17]. More recently, *CSFR3* mutations have been described as a recurrent defect in patients with CNL [18].

Common pathogenic mechanisms are observed despite the variety of different oncogenic mutations underling specific MPN types. Aberrant expression of inflammatory cytokines has been associated with patients' symptoms and alterations of the bone marrow (BM) microenvironment as well as progression of the disease. Several different studies have suggested an important role for the BM microenvironment in the pathogenesis of hematologic malignancies including MPN. In fact, alterations in the BM microenvironment such as increased microvessel density (angiogenesis), fibrosis, and thickening of bone trabeculae are typical pathological findings in MPN and may contribute to disease phenotypes and disease progression. This review focuses on the cytokine regulation of microenvironmental cells with special emphasis on common as well as distinct pathogenetic mechanisms in various MPN. In particular, expression and functional relevance of interleukin-6 (IL-6), IL-8, vascular endothelial growth factor (VEGF), basic fibroblast growth factor (FGF-b), hepatocyte growth factor (HGF), platelet derived growth factor (PDGF), oncostatin M (OSM), tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), and stroma derived factor-1 (SDF-1, CXCL-12) are reviewed. Evidence for increased expression of these cytokines in various MPN is summarized in Table 1. Furthermore, the effect of JAK1/2 inhibitors on the cytokine storm in MPN and targeted drugs for VEGF/VEGFR, HGF/c-MET, and SDF-1/CXCR-4 are discussed.

Table 1: Increased expression of cytokines in myeloproliferative neoplasms. Evidence for increased expression of the cytokines fibroblast growth factor (FGF), hepatocyte growth factor (HGF), interleukin-6 (IL-6), IL-8, oncostatin M (OSM), platelet derived growth factor (PDGF), transforming growth factor-β (TGF-β), tumor necrosis factor-α (TNF-α), and vascular endothelial growth factor (VEGF) in the myeloproliferative neoplasms chronic myeloid leukemia (CML), polycythemia vera (PV), essential thrombocythemia (ET), primary myelofibrosis (PMF), and systemic mastocytosis (SM) is shown. The numbers indicate selected references for elevated expression of the cytokine in the given myeloproliferative neoplasm.

Disease	CML	PV, ET, PMF	SM
Oncogene	*BCR-ABL1*	*JAK2* V617F, *CALR*, *MPL*	*KIT* D816V
FGF	[31, 36, 37]	[36–46]	[47, 48]
HGF	[30, 31, 33]	[38, 39, 49, 50]	
IL-6	[51, 52]	[38, 49, 51, 53]	[54–56]
IL-8	[57]	[49, 53, 58]	
OSM		[59]	[60, 61]
PDGF	[35]	[22, 46, 62–65]	
TGF-β	[66, 67]	[40, 42, 46, 68–72]	[48]
TNF-α	[31, 52]	[38, 49, 73]	[74]
VEGF	[26, 27, 29, 31, 33, 36, 75–77]	[21, 36, 38, 45, 46, 49, 58, 75, 76, 78–87]	[54, 88–91]

2. Cytokine Expression in Classical MPN

2.1. Chronic Myeloid Leukemia. CML is characterized by the reciprocal chromosome translocation t(9;22) and the resulting *BCR-ABL1* fusion gene [5, 6]. The BCR-ABL1 oncoprotein exhibits constitutive TK activity and triggers key signaling pathways, including the RAS-RAF-MEK-ERK pathway, the phosphoinositide 3-kinase-AKT pathway, and STAT5 [19, 20]. Cytokines and other effector molecules downstream of these aberrant signaling cascades have been implicated in the pathogenesis of CML [21].

Aguayo et al. investigated BM vascularity and cytokine levels in CML and other hematologic neoplasms [22]. CML patients reportedly have increased BM vessel density and elevated serum levels of VEGF, HGF, FGF-b, and TNF-α compared to controls [23, 24]. Furthermore, high VEGF levels were found to correlate with a shorter survival of patients in chronic phase CML [25]. Immunohistochemical staining of BM sections showed that VEGF is expressed primarily in myeloid progenitor cells, megakaryocytes, and mature granulomonocytic cells in chronic phase CML as well as in myeloid differentiated blast cells in the blast phase of CML [26]. The BCR-ABL1 oncoprotein was found to upregulate expression of VEGF in CML cells, and analysis of signaling pathways downstream of BCR-ABL1 revealed that the mammalian target of rapamycin (mTOR) contributes to BCR-ABL1-dependent expression of VEGF [27]. Targeting of mTOR by rapamycin in CML cells inhibited not only VEGF expression but also the *in vitro* growth of leukemic cells [28]. CD34+ BM cells derived from CML patients secreted up to 10 times more VEGF, FGF-b, HGF, and IL-8 compared to normal donors' BM CD34+ cells. Furthermore, BM mononuclear cells isolated from CML patients induced vascularization of matrigel implants in mice [29]. A number of additional studies described expression of HGF in CML cells [30–33]. In particular, elevated HGF levels in BM and peripheral blood and a correlation of HGF expression with microvessel density in the BM were found. Zhelyazkova and colleagues reported evaluated plasma HGF, cellular HGF, and expression of the HGF receptor c-MET in CML patients. The plasma HGF level correlated with markers reflecting the tumor burden as well as with the phase of CML and overall survival in these patients. In contrast, no prognostic relevance for VEGF levels in chronic phase CML was observed in this study [33]. Also, contrary to VEGF, BCR-ABL1 did not induce synthesis of HGF *in vitro* and targeting of BCR-ABL1 with imatinib showed no effect on HGF expression [34]. Although various cell types may express and release HGF, immunostaining of BM sections revealed that basophils are a primary source of HGF in CML [32]. Expression of PDGF was reported to be associated with BM fibrosis in accelerated and blast phase CML [35].

IL-2 and IL-6 serum levels in patients with CML were found to be significantly elevated compared to controls.

Moreover, IL-6 levels in CML patients were found to correlate with BM angiogenesis and reportedly increase during disease progression [51, 52, 92]. The BCR-ABL1 targeting TK inhibitor (TKI) imatinib was found to downregulate IL-6 and IL-8 release in primary CML cells *in vitro* [93]. Hantschel et al. identified IL-8 as one of the strongest downregulated genes in CML upon treatment with the TKI dasatinib [57]. Expression of BCR-ABL1 resulted in a substantial upregulation of IL-8 which was inhibited by dasatinib or nilotinib [57]. TNF-α has recently been implicated in stem cell biology of MPN [94, 95]. A study investigated IL-1, IL-6, and TNF-α serum levels in CML patients and described no significant difference for TNF-α compared to controls [52]. However, CML stem/progenitor cells were found to produce TNF-α in a kinase-independent fashion, and at higher levels relative to their normal CD34+ counterparts. In addition, TNF-α concentrations were found to be elevated in BM supernatants derived from *BCR-ABL1* transgenic mice compared to wild type mice [95].

2.2. Polycythemia Vera, Essential Thrombocythemia, and Primary Myelofibrosis. Elevated levels of inflammatory cytokines have been reported in all entities of classical MPN [38, 49, 51, 53, 58, 96–99]. In particular in PMF, patients suffer from severe constitutional symptoms, and increasing evidence shows that several of these symptoms are mediated by proinflammatory cytokines. Tefferi et al. investigated the prognostic significance of cytokines in PMF by determining serum levels of a comprehensive cytokine panel. In this study, IL-1β, IL-1RA, IL-2R, IL-6, IL-8, IL-10, IL-12, IL-13, IL-15, TNF-α, granulocyte colony-stimulating factor (G-CSF), interferon α (IFN-α), macrophage inflammatory protein 1α (MIP-1α), MIP-1β, HGF, IFN-γ-inducible protein 10 (IP-10), monokine induced by IFN-γ (MIG), monocyte chemotactic protein 1 (MCP-1), and VEGF levels were found to be elevated in PMF patients. In addition, the authors identified IL-8, IL-2R, IL-12, and IL-15 levels as independent prognostic factors for survival of patients with PMF [49]. These findings are in line with other studies showing elevated cytokine level in PMF, ET, and PV [38, 51, 53, 58, 96–98]. However, the methods applied in these studies differed and the panels of elevated cytokines within different studies showed some inconsistences between these studies as reviewed by Hasselbalch [99]. Thus, better standardization is apparently needed to directly compare cytokine production in different MPN cohorts. Nevertheless, increasing evidence indicates that the disease burden of MPN is not only mediated by the primary neoplastic clone but also mediated by a secondary inflammation with an aberrant cytokine production and changes of the BM microenvironment. The concept of cytokines contributing to tissue fibrosis, angiogenesis, and osteosclerosis/osteopenia in MPN has been well established. In particular, FGF-b, IL-8, VEGF, HGF, PDGFR, TGF-β, TNF-α, and OSM have been implicated in BM microenvironment alterations in patients with MPN [21, 22, 39–41, 68, 69]. Evidence for expression of these cytokines in PV, ET, and PMF is discussed in the next paragraphs.

FGF-b was found to be elevated in the serum of MPN patients. While Musolino et al. reported increased FGF-b levels in PV, ET, and PMF [36], Vaidya et al. found FGF-b—together with IL-1β, IL-1RA, IL-2R, EGF, IL-10, FGF-b, IL-12, IFN-α, and RANTES—to be particularly elevated in PMF when compared to PV patients [38]. Moreover, high levels of IL-6 and FGF-b were observed in a coculture model of *JAK2* V617F positive hematopoietic cells and stroma cells [34]. Emadi et al. studied IL-8 production in PMF. IL-8 serum levels were significantly increased in patients with PMF, and IL-8 expression was observed in various hematopoietic cell types, including granulocytes, monocytes, megakaryocytic cells, CD34+ progenitor cells, and platelets [100]. Increased serum levels of IL-8 have also been described in patients with PV and ET [38, 58, 101], and IL-8 was found to enhance formation of erythroid colonies *in vitro* [102]. Within a PMF patient cohort, IL-8 serum level was an independent prognostic factor for survival [49].

A number of studies have described elevated VEGF serum levels in MPN [36, 49, 78]. Immunohistochemical studies performed on BM sections of ET, PV, and PMF patients revealed an increased expression of VEGF and its receptor in all MPN groups compared to controls [79, 80]. Megakaryocytes, macrophages, and immature myeloid precursors showed positive immunostaining while erythroid (precursor) cells stained negative for VEGF [80]. Boissinot et al. detected elevated levels of HGF in the serum and BM plasma obtained from PV patients compared to secondary erythrocytosis patients that were employed as controls. Furthermore, BM stem cells and clonal erythroblasts were identified as the major sources of HGF in patients with PV [50]. Further studies analyzing cytokine panels in plasma of MPN patients confirmed elevated HGF levels in PMF, PV, and ET [38, 49].

Wickenhauser et al. described production of TGF-β and PDGF in normal human megakaryocytes [103]. Subsequent studies found higher levels of TGF-β in megakaryocytes in the BM of patients with myelofibrosis compared to controls. In contrast, no increase in TGF-β was found in BM cells of patients with ET [70]. TNF-α was found to be elevated in a subset of patients with PMF. Tefferi et al. studied 127 PMF patients and observed significantly higher levels of TNF-α compared to controls. However, a substantial number of patients showed no detectable TNF-α in peripheral blood and no association with clinical parameters and disease progression was observed [49]. Another study identified TNF-α as one of two cytokines that were differentially expressed when stratifying ET and PV patients according to their *JAK2* V617F mutation status [58]. In line with this finding, a murine BM transplant model for *JAK2* V617F showed a marked increase of TNF-α serum levels. This increased TNF-α level was found to be accompanied by a decrease in erythropoietin and G-CSF, which the authors discussed as a possible suppressive effect of TNF-α on normal hematopoiesis [73]. We studied *JAK2* V617F-mediated gene expression and identified IL-6 and the IL-6 family members OSM and leukemia inhibitory factor (LIF) to be directly upregulated by V617F-mutated *JAK2*. Furthermore, oncogene-dependent upregulation of IL-8 and VEGF was observed [59]. Immunohistochemistry

staining of BM section from patients with PMF, ET, and PV showed that megakaryocytes, endothelial cells, and myeloid progenitors stain positive for OSM, whereas erythroid cells were OSM negative. This pattern correlates with expression of phosphorylated STAT5, which was identified as the major signaling pathway of oncogene-dependent OSM expression [59].

3. Cytokine Expression in Nonclassical MPN: Systemic Mastocytosis

SM is MPN characterized by an abnormal accumulation of mast cells in the BM and other organs [104]. In a substantial subset of patients, SM is accompanied by increased release of various mediators from mast cells and consecutive clinical symptoms [105–107]. The majority of SM patients harbor the somatic *KIT* point mutation D816V. KIT is a receptor TK, and activation of KIT signaling through its ligand stem cell factor (SCF) mediates cell proliferation and survival in immature progenitor cells and mast cell differentiation, as well as mast cells migration, activation, and adhesion [108]. KIT D816V shows constitutively active TK signaling and induces the recruitment of several downstream signaling pathways, including PI3-kinase/AKT [109], mTOR [110], and STAT5 [109, 111].

Brockow et al. measured levels of growth factors in plasma and skin blister fluid of patients with SM [54]. IL-3 and IL-4 were not detectable, and SCF as well as VEGF levels showed no significant difference between patient samples and controls. In contrast, IL-6 was significantly increased in plasma of SM patients and correlated with serum tryptase levels [54]. Subsequent studies confirmed increased IL-6 plasma levels in SM cohorts and suggested a correlation with the severity of symptoms and the presence of osteoporosis [55]. Moreover, IL-6 levels were found to correlate with disease category, severity of BM pathology, organomegaly, and extent of skin involvement. Thus, the authors suggested that IL-6 was a useful surrogate marker of severity of disease [56]. Moreover Rabenhorst et al. investigated cytokines potentially involved in the development of osteopenia or osteoporosis in SM. Again, elevated levels of the proinflammatory cytokine IL-6 were found in patients with SM. High levels of IL-6 were accompanied by increased levels of the osteoclast-regulating factors receptor activator of nuclear factor kappa-B ligand (RANKL) and osteoprotegerin. The authors argue that cytokines produced by mast cells might shift the balance of bone turnover towards increased bone resorption and decreased bone formation [112]. IL-31 has been implicated in the induction of chronic skin inflammation and was found to be increased in patients with SM and to correlate with disease severity [113]. Gene expression studies of purified BM mast cells in SM detected high expression of CCL-23 in indolent and aggressive SM, whereas IL-1β, IL-13, or OSM were particularly upregulated in aggressive SM [60].

A number of studies used mast cell lines, in particular the *KIT* D816V mutated human mast cell line HMC-1, to investigate cytokine expression in SM. Selvan and colleagues described expression of MCP-1, MIP-1α, MIP-1β, RANTES, and IL-8 in HMC-1 cells [114]. Subsequent studies showed expression of TNF-α [74], IL-1β [74], and OSM [61] in HMC-1 cells. FGF-b was found to be expressed in a number of murine mast cell lines and to be regulated by SCF, TGF-β, and TNF-α [47]. Immunohistochemical staining of BM sections derived from patients with SM showed expression of FGF-b and in some cases weak expression of TGF-β [48]. Furthermore, mast cell infiltrates expressed VEGF as determined by immunohistochemistry of BM sections [88]. Although no significant elevation of VEGF levels was found in plasma of SM patients [54], it is likely that VEGF is locally increased in the BM microenvironment and contributes to increased angiogenesis in SM. Comparative oncology studies in dogs showed expression of VEGF in neoplastic mast cells [89, 90]. Moreover, a correlation of VEGF plasma levels with tumor grade and microvascular density was observed in canine mastocytoma [91].

We studied the effect of *KIT* D816V on cytokine expression in various *in vitro* models. The cytokine profile induced by *KIT* D816V showed a marked overlap when compared to the profile induced by *JAK2* V617F and *FIP1L1-PDGFRA*. A number of cytokines, including OSM, were found to be regulated by all three oncogenes [59, 61, 115]. These studies suggest that the mutant TK in MPN activate common signaling pathways resulting in overlapping effects on cytokine production. Moreover, these and other data indicate that targeting of TK signaling or relevant downstream signaling molecules will reduce the aberrant inflammatory cytokine production not only in PMF, ET, and PV but also in other MPN. A comprehensive analysis of cytokine serum levels in a large cohort of SM patients would be useful to compare the expression of inflammatory cytokines in SM with the pattern observed in other MPN.

4. Cytokine Regulation of Microenvironmental Cells

4.1. Fibrosis in MPN. Fibrosis is considered to be a reactive process that is often associated with tissue remodeling and tissue repair. Tissue fibrosis may occur in various organs and involves fibroblasts and other connective tissue cells [116]. Concerning development and characteristics of MPN, fibrosis is one of the major pathological findings [117]. The process of fibrosis involves not only local fibroblasts and infiltrating leukocytes resulting in persistence of inflammation in the tissue, but also the proliferation of cells with a myofibroblast phenotype. The pathological mechanisms underlying the development of fibrosis in MPN patients are still not fully understood. Involved cells produce different growth factors, proteolytic enzymes, angiogenic factors, and fibrogenic cytokines, which results in enhancement of connective tissue elements' deposition. This leads to progressively remodeling and finally destruction of physiological tissue architecture [116].

PMF and CML have the highest potential of inducing myelofibrosis. In general, all MPN can develop BM fibrosis, although the likelihood for this varies considerably between the subtypes. The fibrotic potential of MPN with predominant thrombocytosis such as ET can be differentiated from PMF on the basis of morphology. In PMF, the stromal

reaction that accompanies clonal hematopoietic stem cell proliferation is characterized by a consistent myelofibrosis associated with osteosclerosis and neoangiogenesis. Thus, fibrosis is a disease-defining hallmark of PMF at diagnosis [118]. In addition, a higher fibrosis grade in patients with PMF correlated with worse prognosis [119]. In patients with PV or ET, reticulin fibrosis at the time of diagnosis is associated with an increased risk of transformation to post-PV or post-ET myelofibrosis [119]. In CML, BM fibrosis occurs in up to 40% of patients at diagnosis and is associated with a poor prognosis [120]. Recently, BM fibrosis in CML was proposed as an independent predictor of responses to TKI therapy [121]. Mastocytosis is also commonly associated with slight-to-moderate BM fibrosis [48, 117]. In the BM of SM patients, mast cell infiltration is often accompanied by fibrosis. In addition, mast cell infiltration with consecutive fibrosis may also occur in the liver, spleen, and lymph nodes [48, 117]. Mast cells produce fibrogenic cytokines including TGF-β and FGF-b. Immunohistochemical studies show a close correlation between the mast cell expression of FGF-b and the reticulin fibrosis of mastocytosis lesions [48].

Concerning PMF development, the megakaryocytic lineage seems to play an essential role in promoting myelofibrosis [68]. Megakaryocytic cells were found to produce a variety of growth factors and cytokines leading to proliferation of fibroblast and the development of fibrosis. PDGF is one of the first growth factors that has been implicated in the role of megakaryocytes in development of BM fibrosis [122]. Several studies described increased levels of PDGF in patients with PMF [62, 63], and immunohistochemical staining showed that megakaryocytes and erythroid precursors were highly positive for PDGF [64]. Patients with ET showed increased plasma levels of PDGF; in particular the subgroup of patients with reticulin fibrosis had higher PDGF plasma levels. In contrast, no alteration of intraplatelet PDGF levels was observed in this study [65]. PDGF not only enhances the replication, survival, and migration of myofibroblasts but also modulates the production and secretion of pro- and anti-inflammatory mediators in the pathogenesis of fibrotic diseases [123].

Further studies revealed that the expression and production of TGF-β were increased in patients suffering from MPN. Several groups have evaluated TGF-β expression in PMF, PV, and ET. These groups reported on quantitative alterations of TGF-β and its receptors in megakaryocytic, platelet, and CD34+ progenitor cells and concluded that TGF-β was involved in myelofibrosis and myeloproliferation [39, 40, 42, 69–72, 124, 125]. TGF-β is a growth factor displaying potent fibrogenic properties and is furthermore associated with not only BM fibrosis, but also clonal hematopoietic expansion and angiogenesis. Moreover, TGF-β has been described to negatively regulate progenitor cell growth [126, 127]. In addition, TGF-β reportedly promotes the deposition of extracellular matrix in different tissues [128, 129]. In PMF, the pathogenic relevance of TGF-β is based on the ability to induce production of types I, III, and IV collagens, fibronectin, tenascin, and proteoglycans. Furthermore, TGF-β blocks matrix degradation by reducing collagenase-like protease synthesis, while enhancing protease inhibitor expression [116]. Importantly, TGF-β downstream signaling, through SMAD2/3 phosphorylation, has been shown to be active in megakaryocytes extending proplatelets, indicating an autocrine stimulation in megakaryocyte development [125]. TGF-β induced PI3-kinase/AKT/NF-κB signaling in hemangioblasts, and activation of this pathway enhanced the production of matrix metalloproteinase-9 [66, 67].

Apart from TGF-β and PDGF, FGF-b is considered to be a cytokine with potent fibrogenic characteristics. Several groups analyzed expression of FGF-b in different MPN. The levels of circulating FGF-b were significantly higher in the serum of MPN patients when compared to healthy controls, the highest levels being measured in patients with marked BM fibrosis [37, 39–41, 43, 44, 124]. FGF-b was found to promote fibroblast proliferation in cortical kidney [130]. Furthermore FGF-b promotes cardiac hypertrophy and fibrosis by activating MAPK signaling [131]. Further studies are required to identify the importance of FGF-b in development and progression of MPN. Dalley et al. determined concentration of FGF-b and calmodulin in urine. They showed a significantly elevated calmodulin excretion in PMF patients when compared to PV, ET, and CML. Using a neutralizing antibody to calmodulin influenced the *in vitro* proliferation of normal human fibroblasts. Extracellular calmodulin should also be considered a potential mitogen involved in the stroma cell reaction in patients with PMF [43].

A special situation is *FIP1L1-PDGFRA*+ CEL. In these patients, fibrosis is usually detected in the endomyocardium, which is not the case in other MPN types. One hypothesis is that eosinophils, once entering cardiac tissues, can promote local fibrosis. Eosinophil-related tissue fibrosis has been attributed to infiltration of the tissues with eosinophils and deposition of eosinophil granule proteins [132]. Furthermore, eosinophils were shown to produce the cytokines IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, GM-CSF, TGF-α, TGF-β, TNF-α, MIP-1α, RANTES, eotaxin, and OSM [133]. Many of the eosinophil-derived cytokines have the potential to stimulate fibroblast proliferation and contribute to local inflammation as well as recruitment of other leukocytes [115, 132]. However, further *in vitro* and *in vivo* models are mandatory to deeply understand the role of cytokines for organ specific fibrosis in CEL.

4.2. Angiogenesis in MPN. Angiogenesis, the formation of new vessels from preexisting vessels, plays an important role in development and progression of different tumor types, and targeting of angiogenesis has been successfully translated into clinical practice in various solid tumor models [134]. The process of angiogenesis in hematological malignancies is comparable to the process observed in solid tumors. Endothelial cells from preexisting vessels are activated in the BM by an angiogenic stimulus (e.g., VEGF) and proliferate, migrate, and form new vessels. Initiation of leukemia-induced angiogenesis involves secretion of angiogenic cytokines by leukemic cells and their interaction with the BM stroma [135]. Apart from solid tumors, the importance of angiogenesis becomes increasingly evident in various MPN and other hematologic malignancies. Angiogenesis in the BM of MPN patients was described to correlate

with disease burden, progression, and prognosis [36, 79]. Among the classical *BCR-ABL1* negative MPN, increased BM microvessel density (MVD) has been observed in a number of studies in all MPN entities but is most abundant in PMF [45, 75, 80–83, 136]. Not only does PMF show the highest MVD among the classical MPN, but MVD was also described as an independent adverse prognostic factor in PMF [137]. Significantly higher MVD was also found in the BM of patients with post-PV or post-ET myelofibrosis compared to PV or ET [138]. The association of *JAK2* V617F mutation status with MVD showed no significant difference between *JAK2* wild type and mutant MPN patients in two out of three studies [79, 81, 138]. In contrast to the observation that the increase in BM vascularity seems to be generally independent of the *JAK2* V617F status, MVD correlated with *JAK2* V617F mutant allele burden within the *JAK2* V617F+ subgroup [79]. In chronic phase CML, the BM is hypercellular, with a prominent myeloid compartment and left shift in the granulomonocytic cell compartment [23]. Along with myeloid hyperplasia, augmented BM angiogenesis is a typical finding [24, 75]. In particular, the BM of patients with CML shows a significant increase in MVD, functionally associated with elevated levels of angiogenic cytokines [23]. Furthermore, the BCR-ABL1 targeting TKI imatinib was found to reduce the MVD in CML [137]. Alterations of the BM microenvironment are frequently noticed not only in classical MPN but also in SM. These alterations include angiogenesis, thickened bone trabeculae, and sometimes massive BM fibrosis [48, 88, 117, 139]. Our group studied MVD and expression of VEGF in SM. The median BM MVD was found to be significantly higher in SM compared to cutaneous mastocytosis or controls. Furthermore, MVD correlated with the grade of mast cell infiltration in the BM [88].

The process of angiogenesis is tightly controlled by a variety of angiogenic and antiangiogenic cytokines. Leukemic cells upregulate several angiogenic factors leading to increased BM vascularity. VEGF is the most important proangiogenic cytokine that is involved in tumor angiogenesis. VEGF is able to bind to three receptors: VEGF receptor-1 (VEGFR-1; fms-like tyrosine kinase-1, Flt-1), VEGFR-2 (human kinase domain region, KDR/murine fetal liver kinase-1, Flk-1), and VEGFR-3 (Flt-4). VEGFR-2 was found to be both necessary and sufficient to mediate effects of VEGF on endothelial cells, like induction of vascular permeability and angiogenesis [135]. In addition, VEGFR-1 is expressed on hematopoietic stem cells and frequently on leukemic cells [140], whereas megakaryocytes express VEGFR-2 [135], and VEGFR-3 is mainly involved in the regulation of lymphangiogenesis. VEGF not only promotes BM neovascularization but was also found to signal through VEGFRs expressed on the surface of neoplastic hematopoietic cells [141]. Thus, secreted VEGF has been considered to contribute to disease progression by an autocrine or paracrine mechanism [135]. Numerous studies reported increased levels of VEGF in the blood as well as expression in the BM of patients with PV, ET, and PMF [21, 36, 46, 49, 75, 76, 80, 83–87]. Increased expression of VEGF was also found in CML [23, 25, 26, 77] and in BM section of SM patients [88].

HGF and FGF-b are other cytokines with potent angiogenic potential. Endothelial cells express the HGF receptor c-MET and the role of HGF in angiogenesis is well established [142]. HGF enhances vascular matrix degradation and endothelial cell invasion and migration, as well as proliferation of vascular endothelial cells. Furthermore, HGF induces capillary tube formation in a matrigel assay and promotes angiogenesis *in vivo*. HGF acts synergistically with VEGF on endothelial growth but has also been shown to induce angiogenesis independent of VEGF [142]. Elevated levels of HGF have been described in patients with PV, ET, and PMF [38, 49, 50], as well as in CML [30–33]. FGF-b regulates proliferation and function of various mesenchymal cells. It induces growth of fibroblasts and endothelial cells *in vitro* [143] and stimulates angiogenesis and fibrosis *in vivo* [144]. Elevated levels of FGF-b have been described in PV and ET, but particular high levels were found in patients with PMF [36, 38]. In addition, CML patients showed also increased expression of FGF-b [24, 29].

IL-6 is a proinflammatory cytokine that has been implicated in the pathogenesis of various MPN. Among many other functions, IL-6 has been reported to stimulate angiogenesis in the tumor microenvironment and to enhance proliferation and migration of endothelial cells [145–147]. A recent study reported defective pericyte coverage of vessels after IL-6 stimulation compared to VEGF-stimulated vessels [148]. We identified the IL-6 family member OSM as an oncoprotein-dependent cytokine in neoplastic cells of *JAK2* V617F, *KIT* D816V, and *FIP1L1-PDGFRA* positive MPN [59, 61, 115]. OSM has been described to act as a growth factor for various mesenchymal cells, including fibroblasts, osteoblasts, and endothelial cells and to induce angiogenesis *in vitro* and *in vivo* [149–152]. Thus, OSM has been implicated in tissue remodeling, inflammation, and tissue fibrosis [151, 153–155]. Similarly, IL-8 is a multifunctional proinflammatory cytokine which is highly expressed in various MPN. It has been implicated in tumor growth and angiogenesis. In particular, IL-8 was shown to promote endothelial cell proliferation, capillary tube organization, and matrix metalloproteinase expression in endothelial cells [156]. In summary, a number of inflammatory cytokines, abundantly expressed in various MPN, have the potential to trigger angiogenesis in the BM and other organ systems. This pathogenetic process has therefore been proposed as a potential target in CML and other MPN and is best studied for targeted drugs against VEGF/VEGFR and HGF/c-MET.

4.3. Bone Marrow Niche Interactions. Apart from direct effects on endothelial cells and fibroblast, neoplastic cell-derived inflammatory cytokines are also involved in autocrine and paracrine loops between neoplastic (stem) cells and mesenchymal (stem) cells (Figure 1). Hematopoietic stem cells (HSC) rely on their interactions with the BM niche to maintain their quiescent state and to protect their integrity and functions but also to undergo asymmetrical cell division and differentiation in order to regulate and support blood cell production on demand. Similarly, disease-initiating leukemic stem cells (LSC) interact with the BM niche. However, the BM niche in hematopoietic malignancies is commonly altered

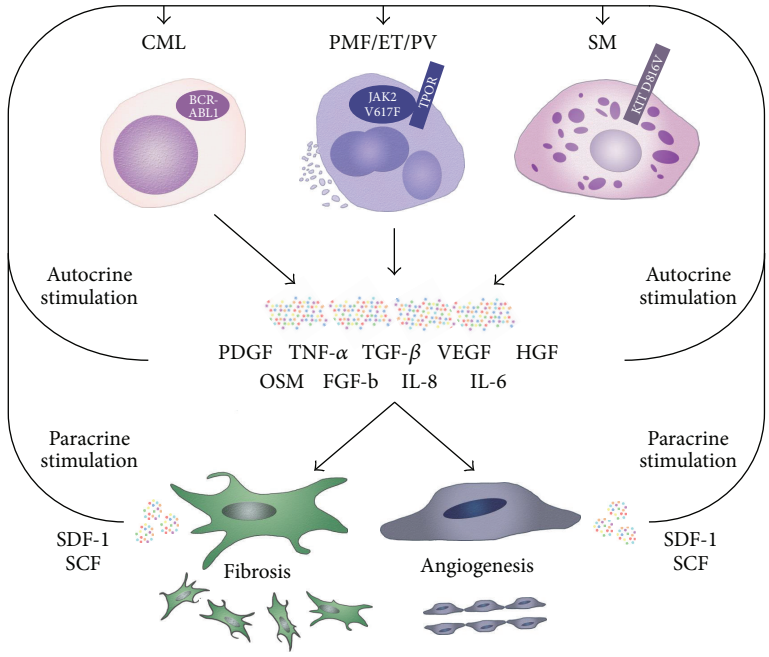

Figure 1: Inflammatory cytokines induce alterations of the bone marrow microenvironment and mediate autocrine and paracrine stimulation of neoplastic cells in myeloproliferative neoplasms. Neoplastic hematopoietic cells in chronic myeloid leukemia (CML), polycythemia vera (PV), essential thrombocythemia (ET), primary myelofibrosis (PMF), and systemic mastocytosis (SM) secrete various cytokines including fibroblast growth factor (FGF), hepatocyte growth factor (HGF), interleukin-6 (IL-6), IL-8, oncostatin M (OSM), platelet derived growth factor (PDGF), transforming growth factor-β (TGF-β), tumor necrosis factor-α (TNF-α), and vascular endothelial growth factor (VEGF). These cytokines bind to their receptors on the surface of fibroblast, endothelial cells, and other cells of the bone marrow stroma and induce fibrosis and angiogenesis. In turn, cytokine production in stromal cells (e.g., stroma derived factor-1, SDF-1, or stem cell factor, SCF) has been implicated in proliferation, migration, and clonal selection of hematopoietic cells as well as in resistance to therapy.

and the leukemia-induced remodeling of the niche may directly contribute to the aberrant function of LSC [157]. A number of these complex interactions have been described as potentially interesting targets in MPN, of which some are exemplified in this section.

Arranz et al. recently described the effect of nestin-positive mesenchymal stromal cells and sympathetic nerve fibers on the regulation of hematopoietic stem cells in *JAK2* V617F positive MPN. Sympathetic nerve fibers, supporting Schwann cells, and nestin-positive mesenchymal stromal cells were found to be reduced in the BM of MPN patients and murine MPN models, a process that may be triggered by IL-1β produced by mutated MPN cells. Depletion of nestin-positive cells or their production of stroma derived factor-1 (SDF-1, CXCL12) accelerated MPN progression. This elegant study demonstrates how inflammatory cytokines produced by neoplastic cells alter or even damage the niche-forming mesenchymal stromal cells in MPN. Furthermore, neuroprotective or sympathomimetic drugs were described as potential therapeutic agents to target this interaction [158].

Expanded myeloid CML cells were found to produce the proinflammatory cytokine IL-6 in inducible *BCR-ABL1* transgenic mouse model recapitulation features of human chronic phase CML. IL-6 served as a positive feedback loop to sustain CML development in this model and reprogrammed both normal and leukemic multipotent progenitor cells towards myeloid development at the expense of lymphoid differentiation. Interestingly, knockout of IL-6 signaling was observed to delay CML development. These results suggest that blocking of IL-6 or targeting the IL-6 signal transduction pathway could represent a valuable target in CML. Moreover, the authors suggested that such self-reinforcing loop—involving IL-6, or other secreted proinflammatory factors—might be relevant in a broad spectrum of MPN [92]. Traer and colleagues studied the effect of the BM microenvironment on imatinib resistance in CML. FGF released from stromal cells was found to promote growth of CML cells through the FGF receptor and the MAP-kinase pathway. In line with the *in vitro* data, CML patients resistant to imatinib without kinase domain mutation showed increased expression of FGF in the BM. Resistance could be overcome with ponatinib, a multikinase inhibitor that targets the FGF receptor in addition to BCR-ABL1 [159]. Another study focused on the effect of stromal cells on the resistance to JAK2 inhibitor treatment in *JAK2* V617F+ disease. Cytokines were found to contribute to this protective effects of stromal cells, and neutralizing antibodies against IL-6, FGF, or CXCL-10 restored the apoptosis induced by JAK2 inhibition [34].

We found that OSM secreted by neoplastic cells did not only stimulate growth of fibroblasts, osteoblasts and microvascular endothelial cells but also induced the production of the angiogenic and profibrogenic cytokines HGF and VEGF in human fibroblasts [59]. In addition, marked production of SDF-1 was induced by OSM in these cells. Thus,

specific tumor cell-stroma cell interactions may potentiate the cytokine storm observed in MPN, that is, by inducing the production and release of cytokines that modulate growth of stromal cells as well as their activation, with consecutive expression of additional cytokines and cytokine receptors [58]. Schwaller et al. showed that retroviral overexpression of OSM in BM cells was sufficient to induce a lethal MPN with marked BM fibrosis and polyclonal expansion of myeloid cells [160].

Fleischman and colleagues studied the effect of the proinflammatory cytokine TNF-α in MPN. *JAK2* V617F induced TNF-α expression in cell lines and primary MPN cells. TNF-α in turn was found to reduce colony formation in normal hematopoietic cells while *JAK2* V617F+ progenitor cells were resistant to TNF-α. Thus, oncogenic *JAK2* generates a TNF-α rich environment which facilitates clonal expansion of mutant cells in MPN [94]. Similarly, CML stem and progenitor cells were found to produce higher levels of TNF-α than their normal CD34+ counterparts. TNF-α promoted survival of CML stem cells in an autocrine manner by the nuclear factor κB/p65 pathway and expression of IL-3. Importantly, TNF-α inhibition induced apoptosis of CML cells and acted synergistically with nilotinib [95]. Together, these findings suggest TNF-α as new putative therapeutic target in MPN.

5. Targeting the Cytokine Storm and the Microenvironment in MPN: A Novel Concept

5.1. JAK Inhibitors and Cytokine Production in MPN. Increased cytokine production was described as a hallmark of classical MPN that contributes to symptom burden of the patients and was referred to as cytokine storm. Targeting of this increased overall cytokine production has been successfully implicated in PMF, PV, and ET. In particular, the identification of the *JAK2* V617F mutation led to the development of various JAK2 inhibitors. Ruxolitinib is the first JAK2 inhibitor approved for treatment of PMF. It targets wild type and mutant JAK2 as well as JAK1 and was found to induce marked and durable reductions in splenomegaly and symptoms in patients with PMF [161]. Despite having only limited effects on the *JAK2* V617F allele burden, significant improvements of fatigue, pain, night sweats, and pruritus were observed after ruxolitinib treatment. In addition, a reduction of cytokine serum levels—including IL-6, IL-8, TNF-α, VEGF, and FGF-b—was found. Changes in cytokine level correlated with reduction in spleen size and coincided with symptom improvement [161]. Thus, it is tempting to speculate that the cytokine storm observed in PMF significantly contributes to the symptom burden in PMF. Interestingly, these changes were not related to the patients' *JAK2* mutational status [161]. This is in line with the observation of similar activation patterns of downstream signaling pathways in *JAK2* mutant and wild type cases. The majority of *JAK2* wild type patients harbor *CALR* mutations. Initial observations suggest that mutant *CALR* also activates JAK-STAT signaling [13]. Therefore, targeting of JAK1/JAK2 is effective to reduce proinflammatory cytokines in PMF irrespective of the *JAK2*/*MPL*/*CALR* mutation status [162].

Autocrine GM-CSF stimulation was identified as mechanism of imatinib resistance in CML leading to *BCR-ABL1*-independent activation of JAK/STAT signaling. Wang et al. used the JAK2 inhibitor AG490 to target GM-CSF induced activation of JAK/STAT signaling and could thus overcome resistance to imatinib and nilotinib *in vitro* [163]. Furthermore, activated JAK2/STAT5 signaling has been described as a potential target in LSC in CML [164, 165]. BCR-ABL1 was shown to activate JAK2 and subsequently STAT5 [166]. In addition, BCR-ABL1 was also found to activate STAT5 directly and independently of JAK2, and high levels of STAT5 activation contributed to imatinib resistance [167]. Gallipoli et al. showed that the JAK1/2 inhibitor ruxolitinib synergized with nilotinib in inhibiting the proliferation of CD34+ cells in patients with CML [164]. These findings provide a rationale for the application of JAK2 inhibitors to eradicate residual disease in CML. Clinical trials combining these drugs are now warranted to test this concept in patients.

5.2. Targeting of the VEGF/VEGFR Axis. Targeting of VEGF and/or the VEGF receptors (VEGFRs) is a widely used concept of antiangiogenesis in oncology. Neutralizing antibodies and soluble receptors are used to inhibit the interaction between VEGF and its receptors (Figure 2(b)). In addition, small molecule inhibitors targeting the kinase activity of VEGFR are applied [168]. Targeting of VEGFR with kinase inhibitors resulted in a reduction in stromal fibroblasts, macrophages, and endothelial cells in *in vitro* cultures of human BM whereas hematopoietic colony formation was not impaired [169].

Bevacizumab is a humanized monoclonal antibody against VEGF approved for antiangiogenic treatment in solid tumors. Mesa and colleagues performed a phase II study enrolling 13 patients with myelofibrosis. None of the patients treated with bevacizumab had an objective response, but significant toxicity was observed. Therefore, this study was terminated early [170]. VEGF promotes angiogenesis mainly through VEGFR-1 and VEGFR-2. Small molecule inhibitors targeting VEGFR and other kinases, for example, sorafenib and sunitinib, have been approved for treatment of patients with renal cell and hepatocellular carcinoma [168]. Sunitinib was tested in a small cohort of patients with PMF. Only one out of 14 patients showed clinical improvement, whereas a high rate of adverse events was observed [171]. Vatalanib is a VEGFR kinase inhibitor with greater potency against VEGFR-2 than against VEGFR-1 or VEGFR-3. In addition, inhibitory effects on PDGF receptor and KIT are observed. A phase I study in PMF showed modest activity with clinical improvement in 20% of the patients examined [172].

mTOR was identified to mediate *BCR-ABL1*-dependent VEGF expression in CML [27]. Targeting of mTOR by rapamycin in CML cells inhibited not only VEGF expression but also the *in vitro* growth of leukemic cells [28]. A clinical pilot study to evaluate the antileukemic and antiangiogenic effects of rapamycin in patients with imatinib-resistant CML showed transient antileukemic effects in a subset of cases [173]. In summary, despite promising data in preclinical

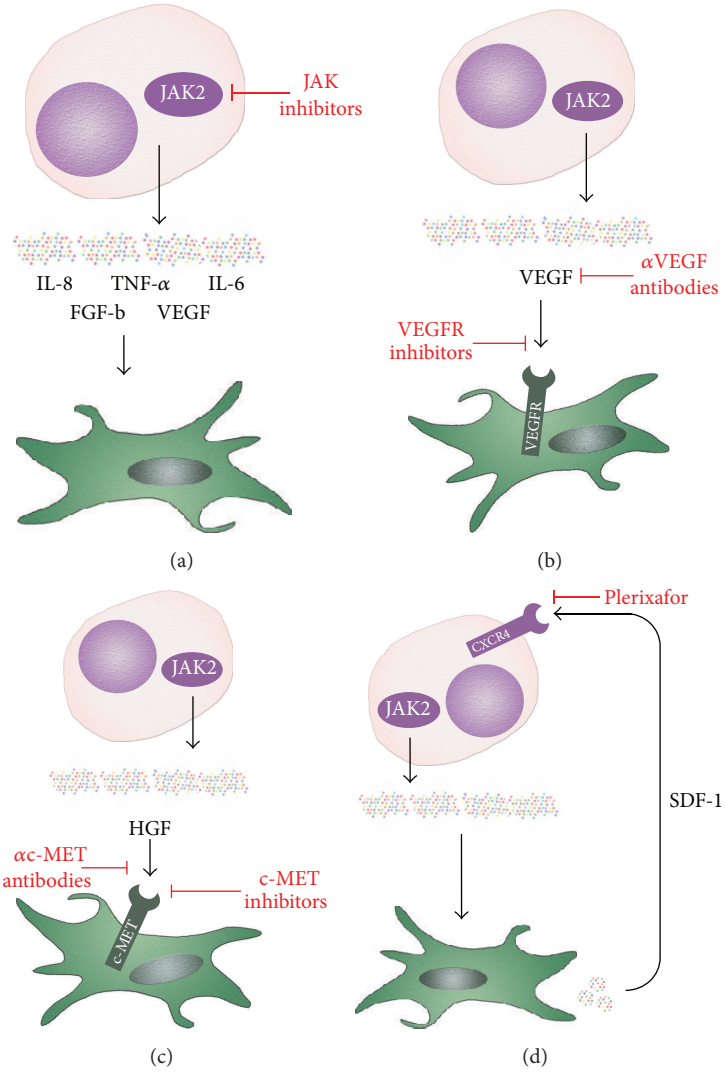

Figure 2: Targeting of cytokines and microenvironment interaction in myeloproliferative neoplasms. (a) JAK inhibitors reduce the expression of various cytokines in MPN. (b) Antibodies against vascular endothelial growth factor (VEGF) and kinase inhibitors targeting the VEGF receptors (VEGFRs) result in disruption of the VEGF/VEGFR axis. (c) Antibodies and kinase inhibitors targeting the hepatocyte growth factor (HGF) receptor c-MET attenuate the HGF/c-MET axis. (d) Plerixafor inhibits the interaction of stroma derived factor-1 (SDF-1) with its receptor CXCR4 which results in mobilization of leukemic stem cells.

models, direct targeting of VEGF resulted only in modest clinical effects on patients with MPN so far.

5.3. Targeting of the HGF/c-MET Axis. Aberrant activation of HGF and/or its receptor c-MET has been described in solid tumors as well as in acute myeloid leukemia (AML), myeloma, and MPN. Production of HGF was found to be independent of *BCR-ABL1* in CML and independent of *JAK2* V617F in other MPN [32]. Thus, blocking of the HGF/c-MET function was suggested as an independent therapeutic target which could synergize with TKI treatment in MPN [174].

c-MET neutralizing antibodies bind to the extracellular domain of the receptor and prevent binding of HGF to c-MET (Figure 2(c)) [160]. These antibodies have shown promising effects on solid tumors [174]. *In vitro* studies have shown that c-MET neutralizing antibodies can effectively suppress the growth of *JAK2* V617F-mutated cells, including PV erythroblasts and the HEL cell line, which expresses HGF at high levels [50]. In addition, small molecule inhibitors targeting c-MET and the c-MET-related RON receptor have been developed. The c-MET inhibitors SU-11274 and PHA-665752 decreased the survival of AML cells in a dose dependent manner [174]. SU-11274 was found to inhibit colony formation, to reduce viability, and to induce differentiation in A9M, U937, and OCI-AML cells [175]. Moreover, the c-MET inhibitors were found to block the response to HGF in a myeloma model [176]. Our group tested the effects of SU-11274 and PF-02341066 (crizotinib) on *BCR-ABL1* positive cells and found that both drugs induce a significant growth reduction in KU812 cells and K562 cells [34]. Furthermore, c-MET inhibitors were found to reduce proliferation of primary

CML cells *in vitro* [32]. Boissinot and colleagues tested the efficacy of combining c-MET and JAK inhibitors on the proliferation of the *JAK2* V167F positive HEL and UKE-1 cell lines. Only a weak inhibition was observed when molecules were tested separately, whereas the combination of the c-MET inhibitor PF-2341066 and the JAK inhibitor ruxolitinib inhibited growth of UKE-1 cells [174]. In summary, preclinical models show promising results for HGF/c-MET inhibition in MPN. The clinical efficacy of this targeting approach remains to be tested in clinical trials.

5.4. Targeting of the SDF-1/CXCR4 Axis. Increasing evidence suggests an important role of the BM microenvironment in the regulation of proliferation and survival of normal and leukemic hematopoietic stem cells. Thus, targeting of the specific BM niches and stem cell-niche interactions has been suggested as a promising therapeutic strategy [177]. SDF-1 (CXCL-12) is a chemokine produced by mesenchymal cells of the BM stroma (e.g., endothelial cells and osteoblasts) with particularly high expression in perivascular, niche-forming mesenchymal stromal cells [178]. Hematopoietic stem and progenitor cells express the SDF-1 receptor CXCR4 and migrate specifically towards SDF-1. Plerixafor (AMD3100) inhibits the SDF-1/CXCR4 interaction and is clinically used to mobilize hematopoietic stem and progenitor cells in stem cell transplant donors [179]. The SDF-1/CXCR4 axis is one potential target in the interplay of leukemic stem cells (LSC) and the BM microenvironment (Figure 2(d)).

CXCR4 is highly expressed on the surface of malignant cell in chronic lymphocytic leukemia (CLL), and SDF-1 was found to promote chemotaxis of CLL cells and their interaction with stromal cells, which was shown to induce resistance of CLL cells to cytotoxic agents, and was furthermore suggested to mediate persistence of minimal residual disease in the BM during therapy. In line with this concept, CXCR4 antagonists were successfully used to block interactions between CLL and stromal cells and to mobilize CLL cells from their protective microenvironments, becoming thus accessible to conventional drugs [180]. Similar targeting concepts were applied in preclinical models for AML and acute lymphoblastic leukemia (ALL). CXCR4 antagonist inhibited the proliferation of AML cells and reduced protection against chemotherapy by stromal cells *in vitro* and *in vivo* [181–183]. Leukemic cells in T-ALL were found to be in direct, stable contact with SDF-1-producing BM stroma. Furthermore, genetic targeting of CXCR4 in murine T-ALL led to rapid, sustained disease remission and CXCR4 antagonism suppressed human T-ALL in primary xenograft models [184].

Partly ambivalent results have been published for the role of SDF-1/CXCR4 in MPN, and although increased levels of SDF-1 have been reported, this may not necessarily result in a sustained activation of CXCR4 signaling in neoplastic cells [185, 186]. On the one hand, mobilization of CD34+ cells in patients with PMF has been attributed to reduced CXCR4 expression and hypermethylation of the CXCR4 promoter [187, 188]. Moreover, although elevated levels of immunoreactive forms of SDF-1 were found in the BM and peripheral blood of patients with PMF and PV, detailed studies using mass spectrometry have shown that SDF-1 was mainly truncated and thus expressed in an inactive form in these patients. The authors of this study concluded that reduced levels of intact SDF-1 due to proteolytic degradation would contribute to the mobilization of hematopoietic stem cells in PMF [186]. In line with these data, CD34+ cells in CML showed an impaired chemotactic response to SDF-1 although no decrease in CXCR4 expression was observed [189, 190]. Our group identified the cell surface enzyme dipeptidylpeptidase-IV (CD26) as a marker of CML LSC. CD26 was shown to disrupt the SDF-1/CXCR4 axis by cleaving SDF-1, and targeting of CD26 by gliptins suppressed the expansion of *BCR-ABL1*+ cells. CD26 expression may explain the mobilization of LSC and the observed extramedullary spread of hematopoietic stem and progenitor cells in CML, and inhibition of CD26 may revert abnormal LSC function [191].

On the other hand, the SDF-1/CXCR axis between stroma and leukemic cells contributes to resistance to TKI treatment in CML. Imatinib was found to enhance migration of CML cells towards stromal cell layers, which may in turn promote nonpharmacological resistance to imatinib [192, 193]. Mechanistically, this finding was linked to CXCR4 redistribution into the lipid raft fraction, in which CXCR4 colocalized with active LYN after TKI treatment [193]. The CXCR4 inhibitor plerixafor diminished migration of *BCR-ABL1* positive cells and reduced adhesion of these cells to extracellular-matrix components and to BM stromal cells *in vitro*. Moreover, plerixafor was also found to decrease the drug resistance of CML cells induced by coculture with BM stromal cells *in vitro*. Importantly, plerixafor was shown to mobilize leukemic cells *in vivo* and to act synergistically with nilotinib to reduce the leukemia burden in a mouse model. The authors of this study argue that the combination of CXCR4 inhibition with TKI treatment in CML might be a useful approach to override drug resistance and to achieve deeper responses in CML [194]. In contrast, another study tested the effects of plerixafor in combination with either imatinib or dasatinib in a murine CML BM transplant model. In this study, no beneficial effect of plerixafor over TKI monotherapy was observed. Moreover, an increase in CNS infiltration after plerixafor treatment was described [195]. The discrepancy of these date can partly be explained by difference in the CML mouse model (e.g., irradiation possibly contributing to CNS infiltration) and in the TKI administration. Weisberg et al. applied plerixafor after marked reduction of disease burden with nilotinib as a model of minimal residual disease and argued that the absence of significant disease burden was relevant for the beneficial effects of the combination therapy [194].

Thus, the SDF-1/CXCR4 axis is a promising but still controversial target in CML and other types of MPN. The effect of CXCR4 inhibitors in PV, ET, PMF, and SM remains to be addressed in further preclinical models.

6. Concluding Remarks and Future Perspectives

The complex interplay between neoplastic cells and microenvironmental cells in MPN has gained increasing interest in

recent years. The resulting research revealed new important insights into the pathogenesis of MPN. One important aspect is that oncogenic signaling promotes cytokine production in MPN cells and alters their interaction with the BM stroma. A number of pathogenetic mechanisms are found to be conserved between various MPN, and lessons learned from one disease can be exploited for the other MPN types. Thus, it will be important to compare systematically the various common as well as rare MPN-variants in terms of basic and clinical science.

More recently, the pathologically altered interactions between neoplastic cells and their microenvironment have been investigated with the aim of defining new potential targets of therapy and to develop novel therapeutic approaches. First, the increased angiogenesis and BM fibrosis may serve as novel targets of therapy in MPN. Indeed, several TKI used to treat MPN may also suppress angiogenesis and/or fibrosis through inhibition of vascular target kinases. Thus, the VEGF/VEGFR, HGF/c-MET, and SDF-1/CXCR4 axis are potential targets in MPN, and a number of other molecular targets are under investigation. Many open questions still have to be addressed in preclinical model, and so far only few of the many exciting approaches were successfully translated to the clinic. Best evidence for targeting of the inflammatory cytokine storm is derived from the clinical efficacy of JAK inhibitors in MPN, which show marked benefits in patients despite their lack of specificity for mutant JAK2. Other targeting approaches for inflammatory cytokines will most likely be combined with established or experimental inhibitors of the primary oncoprotein in the given MPN. Increasing knowledge of the LSC-niche interaction will help to optimize this combined targeting approach and to establish synergistic strategies for therapy or even cure of MPN.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

The Role of Reactive Oxygen Species in Myelofibrosis and Related Neoplasms

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Reactive oxygen species (ROS) have been implicated in a wide variety of disorders ranging between traumatic, infectious, inflammatory, and malignant diseases. ROS are involved in inflammation-induced oxidative damage to cellular components including regulatory proteins and DNA. Furthermore, ROS have a major role in carcinogenesis and disease progression in the myeloproliferative neoplasms (MPNs), where the malignant clone itself produces excess of ROS thereby creating a vicious self-perpetuating circle in which ROS activate proinflammatory pathways (NF-κB) which in turn create more ROS. Targeting ROS may be a therapeutic option, which could possibly prevent genomic instability and ultimately myelofibrotic and leukemic transformation. In regard to the potent efficacy of the ROS-scavenger N-acetyl-cysteine (NAC) in decreasing ROS levels, it is intriguing to consider if NAC treatment might benefit patients with MPN. The encouraging results from studies in cystic fibrosis, systemic lupus erythematosus, and chronic obstructive pulmonary disease warrant such studies. In addition, the antioxidative potential of the widely used agents, interferon-alpha2, statins, and JAK inhibitors, should be investigated as well. A combinatorial approach using old agents with anticancer properties together with novel JAK1/2 inhibitors may open a new era for patients with MPNs, the outlook not only being "minimal residual disease" and potential cure but also a marked improvement in inflammation-mediated comorbidities.

1. Introduction

The Philadelphia negative chronic myeloproliferative neoplasms (MPNs) encompass essential thrombocythemia (ET), polycythemia vera (PV), and myelofibrosis (MF). These neoplasms arise due to an acquired stem cell lesion with subsequent clonal evolution being driven by several mutations, including the highly prevalent JAK2V617F somatic mutation in PV (in >95%, and in about 50% of patients with ET and PMF, resp.) and the *CALR* and *MPL* somatic mutations [1–9]. These mutations are virtually mutually exclusive and are all considered "second hits" or "driving mutations" within the MPNs whereas the primary genetic hit or "founding mutation" remains unknown [4].

Common clinical denominators for the MPNs are high rates of thrombohemorrhagic complications, hypermetabolic symptoms, splenomegaly, uncontrolled myeloproliferation, low-grade chronic inflammation, a massive inflammation-mediated comorbidity burden, and immune-deregulation [10–16]. The MPNs have an inherent propensity to progress in *a biological continuum* from early cancer stages (ET/PV) to more advanced cancer stages (MF or acute myeloid leukemia (AML)) [17, 18]. The concept of such a *biological continuum* is being increasingly recognized and supported by clinical and molecular studies, the latter displaying increasing JAK2V617F allelic burden throughout the stages. The fact that a *JAK2* positive phenotype only persists in 20–50% of the cases when MPNs transform to AML (or even develops biphenotypic AML) also demonstrates the inherent risk of subclone formation which is a characteristic shared by many other cancers [19–23]. Consequently, the malignant clones are heterogeneous and thus difficult to target with chemotherapy, accounting for the inferior survival in MPN associated AML compared to *de novo* AML [24–26].

The MPNs have recently been described as "*A Human Inflammation Model,*" in which the fuel that feeds the fire is low-grade chronic inflammation [27]. The hypothesis is that the MPN—with uncontrolled myeloproliferation and uncontrolled cytokine secretion as a consequence of constitutively activated JAK-STAT signalling—by itself creates a proinflammatory milieu in the bone marrow and in the circulation. This proinflammatory milieu founds increasing genomic instability accounting for the propensity of the MPNs to acquire new mutations facilitating clonal evolution and ultimately progression to myelofibrosis and AML. It also links the MPNs with a heavy inflammation-mediated comorbidity burden, including premature atherosclerosis, other inflammatory diseases, and second cancers [22, 27–38]. In this context, it has been known for several years that chronic inflammation *per se* increases the risk of cancer development, solid as well as hematological, but the major questions in MPNs are, among others, how low-grade inflammation is eliciting genomic instability and clonal evolution and how the founding clone evades the immune system.

In MPNs, the *optimal therapeutic goals* are to normalize peripheral blood counts, minimize symptoms, prevent vascular complications, restore bone marrow architecture/morphology, and eliminate the risk of progression to MF or evolution to AML. It is crucial to acknowledge that the majority of ET and PV patients have long life-expectancies and therefore treatment related toxicities and long-term side effects influence treatment options [39–42]. The therapeutic agents display striking differences. Treatment with interferon-alpha2 (IFN) has been used successfully for decades, demonstrating its ability to normalize blood counts in the majority of patients, to reduce the JAK2V617F (and *CALR*) allelic burden, and to restore bone marrow morphology and induce major molecular remission in a subset of patients [43–55]. Because of the immune-enhancing properties, some patients experience autoimmune phenomena, primarily thyroiditis, during IFN treatment. A subset of patients also experiences symptoms similar to those arising in patients with systemic inflammation, including chronic fatigue, flue-like symptoms with low-grade fever, weight loss, and depression all symptoms being associated with chronic inflammation [56–58]. Despite undisputed hematological efficacy and safety being shown in a large number of single-arm IFN studies, similar results obtained from large randomized studies between IFN and the most widely used cytoreductive agent in MPNs, hydroxyurea, are still lacking. Most MPN experts agree that HU increases the risk of skin cancer and concern is increasing in regard to its potential of inducing AML after long-term use (>10 years) [59–64]. With the introduction of JAK inhibitors, the therapeutic landscape has expanded considerably. However, these novel agents potently suppress virtually all immune cells including NK-cells, CD4+ T-cells (Th1 and Th17), regulatory T-cells, macrophages, and dendritic cells (DCs) with ensuing impairment of immune regulation and consequently an increased risk of infections [65–71]. This risk is well documented and involves mainly urinary tract infections and herpes zoster but also more rare infections such as tuberculosis, toxoplasmosis, and progressive multifocal leukoencephalopathy [72–76]. Although patients are exposed to an increased risk of infections during treatment with JAK1/2 inhibitors, this novel treatment modality has definitely demonstrated its efficacy in terms of improvement of quality of life due to a rapid resolution of constitutional symptoms within days in concert with a marked reduction in symptomatic splenomegaly within the next weeks or months in the large majority of patients with myelofibrosis [77–80]. To this end, JAK1/2 inhibition in myelofibrosis is associated with an improved overall survival as well [81, 82]. The impact of JAK1/2 inhibition on symptom burden and splenomegaly in myelofibrosis is considered to be driven mainly by its pronounced anti-inflammatory efficacy as evidenced by a marked reduction in several proinflammatory cytokines during JAK-inhibition therapy [77, 83]. In this regard, the improved survival in ruxolitinib-treated MF patients is likely mainly explained by an improvement in inflammation-mediated comorbidities as well [84]. However, ruxolitinib has failed to demonstrate significant impact on the *JAK2* clone [85] which substantiates the need for combinatorial approaches when treating MPNs [28].

Taking into account that chronic inflammation with the production of reactive oxygen species (ROS) may have an important role for the development and progression of MPNs—likely being a very potent driver of clonal evolution and mutagenesis in a vicious self-perpetuating circle—we herein will discuss the role of ROS in MPN pathogenesis and its impact upon comorbidity burden, immune regulation, and disease progression [27, 29, 86–90].

2. Reactive Oxygen Species

Reactive oxygen species (ROS) are a group of oxygen-containing molecules involved in many biological processes including normal cellular signalling and immune defence. Consequently, lacking the ability to produce ROS results in organ dysfunction and disease as evidenced by, for example, chronic granulomatous disease in which the immune system is unable to combat invading bacteria and fungi due to impaired production of ROS by neutrophils [91–95]. However, the same ROS compounds are also involved in several inflammation-driven diseases where an excess of ROS production is thought to account for the tissue damage, dysfunction, and fibrosis associated with the diseases [96, 97]. In addition, elevated levels of ROS, often referred to as *oxidative stress*, have a major role in cancer development, both in solid tumors and in hematological malignancies [86–90, 97]. There is no clear cut-off that defines exactly which compounds are to be included in the ROS category, and often nonoxygen molecules buffering ROS levels are also included in the analysis of cellular oxidative status. The molecules superoxide (O_2^-) and hydrogen-peroxide (H_2O_2) are obvious ROS, but intracellular levels of glutathione and reduced glutathione are also crucial in the cellular redox interplay. Hydrogen-peroxide is of particular interest since it can freely diffuse across cellular membranes and interact with cells in close proximity to the H_2O_2 producing cells. This includes the endothelial cells within the intima of artery walls, and oxidative stress has already been linked to cardiovascular

diseases, especially the development of premature atherosclerosis in chronic inflammatory diseases [96, 98–100]. H_2O_2 has been shown to activate NF-κB pathway, thus creating self-perpetuating vicious circles in which inflammation creates ROS which in turn creates more inflammation [101–103]. To avoid such situations, the system has a fail-safe: *suppressors of cytokine signalling* (SOCS), a family of proteins dedicated to creating negative feedback loops. They are normally activated by inflammatory mediators such as IFN, IL-4, TNF-alpha, and H_2O_2 [104, 105]. Activated SOCS proteins bind to JAKs disrupting the JAK-STAT pathway, thereby ensuring that the inflammatory process is not being sustained. However, in MPNs, this pathway is constitutively activated and the much warranted SOCS brake is overruled. Furthermore, aberrant methylation of SOCS-coding DNA and consequent dysregulation of SOCS have also been reported in MPNs [106, 107].

3. Hepatitis C as a Model of Inflammation-Mediated Fibrosis and Cancer Development: Similarities to MPNs as "A Human Inflammation Model for Cancer Development"

The initiating event in hepatitis C is a viral infection. This results in chronic inflammation, increased production of ROS and consequently oxidative stress, inability of the immune system to clear the infected cells, an increased risk of progression to terminal cirrhosis, and ultimately an increased risk of developing hepatocellular carcinoma (HCC) or lymphoma [108–113]. In MPNs, the initiating event is unknown, but after acquisition of the *JAK2* mutation, MPNs (much like hepatitis patients) exhibit evidence of low-grade chronic inflammation with ensuing fibrosis and bone marrow failure in addition to an increased risk of developing AML. Another similarity is the inability of the host immune system to identify and clear the fundamental problem, for example, the malignant clone in regard to MPNs. Another striking similarity is the existence of a common effective treatment modality: the very potent immune-enhancing, antiviral agent IFN which has been used successfully for decades in hepatitis patients as well as in MPN patients. In this regard, it has recently been hypothesized that a virus infection (e.g., human retrovirus) might be implicated in MPN pathogenesis [27, 114]. It is also of particular interest to note that oxidative stress has been implicated in the therapeutic response. Thus, it was demonstrated, that increasing levels of ROS disrupt IFN signalling, thus counteracting therapy [115].

4. ROS and MPNs

The ROS molecules are produced mainly by neutrophils, macrophages, and monocytes. In the context of MPNs, this is crucial, since the MPN cells are clonal and autonomously dysregulated and have been shown to produce excessive ROS *in vitro* [87]. Furthermore, MPN patients demonstrate elevated levels of ROS *in vivo* [86, 116]. An increased ROS production has been observed in other cancers, and in some cases the cancer cells express catalase (the enzyme that metabolizes H_2O_2) in excess and in addition produce large amounts of H_2O_2. In this way, the malignant clone itself avoids the toxic effects of H_2O_2 and suppresses the neighbouring healthy cells (ROS induce apoptosis in healthy cells) thereby facilitating clonal expansion [117–126]. This mechanism has not yet been established in MPNs but certainly warrants further investigation, especially since the excessive ROS production in MPNs gives rise to a proliferative advantage to *JAK2* positive clones [28, 87, 127, 128] (Figure 1). In this regard, the model proposed by Marty et al. is in agreement with the MPN inflammation model and excessive ROS accumulation in a vicious self-perpetuating circle. In this context, considering the role of NF-E2 in MPN disease pathogenesis, it is intriguing to speculate if NF-E2 may contribute in driving the vicious inflammation wheel, including ROS accumulation as most recently discussed [29, 129–134]. On the other hand, it has also been demonstrated that the hematopoietic stem cell niche (HSC) in MPNs downregulates catalase activity resulting in an increase in oxidative DNA damage (8-oxo-G) and subsequent double-stranded- (ds-) DNA breaks, a widely accepted measure of ROS induced DNA damage, and perhaps in this way induces instability of the HSC niche [87].

In a mouse model, ds-DNA breaks were shown to be a consequence of ROS accumulation, and it was also shown that the CD34+ HSCs themselves produced this excess ROS, probably as a consequence of catalase downregulation [87, 127]. Furthermore, a functional lack of superoxide dismutase (SOD) activity could also be of importance. ROS negatively influence the AKT pathway, which in turn influences Forkhead O/FoxO which regulates the transcription of several antioxidative defence pathways, including GPx, catalase, and SOD [135]. These mice developed aggressive PV phenotype but when they were treated with the potent ROS-scavenger molecule n-acetyl-cysteine (NAC) they developed normal phenotype, demonstrating the direct role in MPN disease development and disease progression [87]. This was substantiated by the finding that NAC treatment of the PV phenotype mice delayed progression to MF phenotype when compared to nontreated mice.

The damaging effects of ROS (besides the proliferative advantage) are also attributed to the consequent oxidation of lipids, proteins, and, most importantly, the ds-DNA breaks due to oxidation. In healthy cells, this insult will be rapidly repaired but a hallmark of most cancers is a defective DNA repair (sometimes even induced by therapy, e.g., hydroxyurea). Furthermore, the response to DNA damage is also affected as demonstrated by the negatively regulated p53 pathway in MPNs [136]. This is also demonstrated in MPNs, where the CHEK2 germline mutations, which are associated with ET and PV, account for an increased risk of developing an MPN. Together with other proteins, the CHEK2 proteins are associated with DNA damage and binding of TP53 (p53) and CHEK2 are involved in many cancers [137–143]. Consequently, harbouring this CHEK2 mutation can result in inadequate DNA repair and consequently increased risk of developing (and sustaining) genetic hits in several cancer types. It is intriguing to consider if germline CHEK2 mutation accounts for the initial genetic

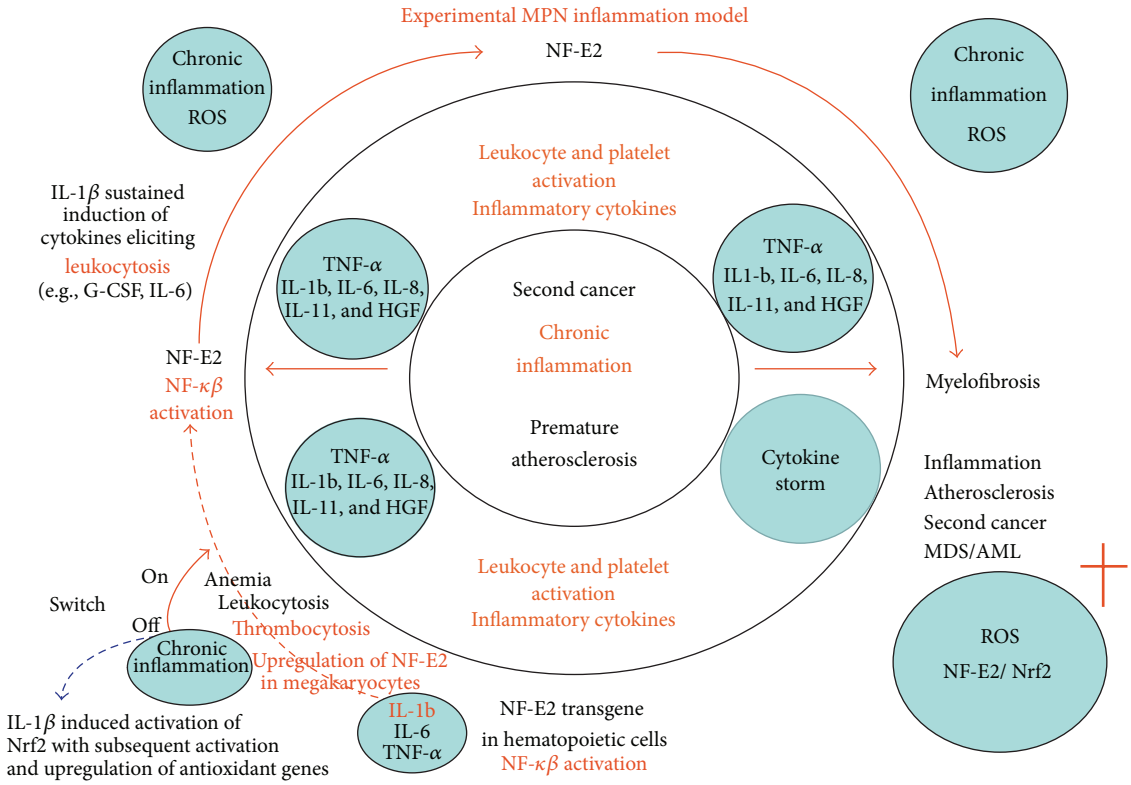

Figure 1: Sustained NF-E2 expression likely elicits a pronounced oxidative stress milieu with excessive ROS giving rise to myeloid expansion with leukocytosis and excessive thrombocytosis and inflammation-mediated *in vivo* activation of leukocytes and platelets, thereby further promoting a sustained, self-perpetuating release of inflammatory products. In this vicious circle, an oxidative stress burden with NF-E2 domination over Nrf2 promotes ROS accumulation and megakaryocytic differentiation. Increasing oxidative stress-induced DNA damage of hematopoietic stem cells (HSCs) elicits genomic instability and clonal MPN evolution with accumulation of mutations ultimately terminating in myelofibrotic and leukemic transformation. A relative deficiency of Nrf2 may also result in expansion of the HSC and progenitor cell compartment and ultimately migration of HSCs from their stem cell niches into the circulation ("leaving the burning nest") to seed in the spleen and liver (myelofibrosis with myeloid metaplasia). The vicious circle may be locked by early intervention with interferon-alpha2 (stopping the fuel to the fire) in combination with a JAK1-2 inhibitor (e.g., ruxolitinib) and a statin, the latter agents "cooling down the system" by their highly potent anti-inflammatory properties which may actually be enhanced (synergism) when being administered simultaneously. With permission from Leukemia Research [29].

instability in some MPN patients. In time and by "chance" this might result in a somatic *JAK2* mutation with ensuing increased production of ROS, clonal expansion, and increasing genomic instability due to ineffective DNA repair and an increase in ROS induced DNA damage, all of which further facilitate disease progression with subclone formation and inflammation-mediated bone marrow fibrosis. The role of chronic inflammation and ROS in MPN pathogenesis has most recently been substantiated in a mouse model, in which mice were exposed to the highly potent inflammatory compound, formaldehyde (FA), by inhalation [144]. This agent induced bone marrow toxicity with typical MPN-like alterations in the mice, including an increased number of megakaryocytes and myelofibrosis in concert with the development of anemia, leukopenia, and thrombocythemia. Highly interestingly, these changes were accompanied by evidence of oxidative stress and inflammation in the bone marrow as assessed by significant increases in ROS levels, increased NF-κB activity at both mRNA and protein levels, and significant increases in the inflammatory markers, TNF-alpha and IL-1beta, as well [144]. These observations are in accordance with studies demonstrating that oxidative stress in hematopoietic stem cells can lead to DNA damage, premature senescence, and loss of stem cell function [145]. Accordingly, all together these findings are supportive of the concept that chronic inflammation by induction of oxidative stress and an inflammatory bone marrow microenvironment may give rise to DNA damage and likely an impaired stem cell function with ensuing development of myelofibrosis.

In hepatitis, it has been demonstrated, that the excess of ROS and consequent *oxidative stress* inhibits IFN signalling, thus counteracting the normal immunosurveillance by NK-cells and CD8+ cytotoxic T-cells (CTLs) [115]. The reduced IFN signalling and ensuing reduction of MHC-I expression by virally infected cells provide an escape route from the innate and adaptive immunosurveillance. A prerequisite of this model is that the MHC-I expression is low enough to avoid CTL activation by antigen recognition, but also

high enough to avoid "missing self" activation of NK-cells. This model deserves to be tested in MPNs to elucidate if increased ROS levels might facilitate both clonal expansion and immune evasion, implying ROS-mediated inhibition of IFN signalling and the immune evasion to exhibit dual actions. In this regard, reduced MHC-I expression might keep the tumor below detection limit of CTLs, and even *if* a tumor cell is indeed detected, probably due to threshold expression of "self" by MHC-I, the consequent IFN signalling from the activated NK-cell will likely have only a limited impact since the pathway is functionally blocked by excess of ROS. By this mechanism, recruitment and activation of other immune cells, in particular macrophages, may be inhibited and the immune response remains unamplified and thus ineffective in combating the clone. This concept is partly supported by the finding of downregulation of HLA expression in ET, PV, and MF and further supported by efficacious treatment with IFN, which increases MHC-I expression of the clonal cells (thus making them "legitimate" targets for CTLs) but also increases the NK-cell compartment, thus inducing the much warranted amplification of the immune system. IFN also mobilizes dormant stem cells rendering them susceptible to targeted therapies [146–149].

Since ROS appear to play a crucial role in disease progression of MPNs, the targeting of ROS seems intuitive, especially since the increased ROS can interfere with both endogenous tumor surveillance and treatment response. Treatment with NAC has been used successfully in an *in vivo* mouse model after JAK2V617F bone marrow transplant, but never in human MPN subjects. The majority of experiences with human NAC treatment are based on the treatment of patients suffering from paracetamol poisoning. In this setting, the NAC treatment is intensive and of short duration. NAC treatment has also been investigated in spinal cord injuries but again the treatment duration is short [150–153]. However, longer exposure has been investigated in chronic pulmonary diseases: chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF). Both diseases have a significant inflammatory component, and both diseases showed positive responses to NAC treatment with fewer exacerbations (COPD) and more stable lung function (CF) in the NAC-treated groups [154–156]. Similar results were obtained in patients suffering from systemic lupus erythematosus (SLE) [157].

In order to target MPN related oxidative stress, it is important to acknowledge that the optimal level of ROS is not known. In the experimental MPN models, treatment with NAC almost totally removed any existing ROS, which in an experimental model might give satisfying results but in a human trial might result in a dismal outcome [87, 127]. *In vivo*, ROS are needed to some extent to ensure normal cellular signalling and to enable the immune system in combating invading bacteria and an obvious problem might be an increase in infectious diseases and (other) neoplastic diseases. However, this has not been identified so far with NAC treatment of COPD and CF, both diseases otherwise heavily burdened by (chronic) infections and the NAC treatment resulted in disease relevant improvements.

5. Discussion and Perspectives

The MPNs are clonal neoplasms intimately associated with a dysregulated immune system [16, 17, 148, 149, 158–160]. As in many other diseases, inflammation and excess generation of ROS are thought to play a major role, both in disease initiation and associated inflammation-mediated comorbidities [27, 28, 84, 135]. The initiation of disease is probably a consequence of defective DNA repair and/or increased acquisition of DNA damage. This could be caused by many factors, for example, germline CHEK2 mutation [142, 143]. Of note, it is also intriguing to consider that the initiation of the disease might be consequent to a "fertile ground" changing the fitness of the stem cell niche for a preexisting abnormal hematopoietic stem cell [4, 161–163]. By chance, the JAK2 mutation is acquired and consequent generation of ROS with clonal expansion and evolution due to genomic instability characterizes the further course of the disease. ROS are also involved in cardiovascular diseases which are major contributors to the comorbidity burden and mortality in MPN patients [11, 15, 96, 98, 99]. Accordingly, the targeting of ROS is an obvious therapeutic option, especially since one of the main goals is to prevent genomic instability, likely facilitated by increased ROS, and thereby ultimately fibrotic and leukemic transformation. In regard to the potent efficacy of NAC in decreasing ROS levels, it is intriguing to consider if NAC treatment might benefit patients with MPNs. The encouraging results from studies in CF, SLE, and COPD warrant such studies.

Furthermore, the antioxidative potential of the widely used agents, IFN, JAK inhibitors, and statins, both as monotherapies and in various combinations, should be investigated. Studies on combinations with IFN, the only agent with the potential to induce "minimal residual disease," as the *backbone* and "old antioxidative drugs" (statins, NAC) and the novel JAK1/2-inhibitors are urgently needed. Such studies may further enhance the potency of the novel combination therapy with IFN and ruxolitinib, a concept which already has been shown to be highly efficacious in patients with PV and hyperproliferative MF, implying an improvement in inflammation-mediated comorbidities as well [28, 84, 164]. Such a combinatorial approach using old agents (statins, NAC, and IFN) with anticancer properties (antiproliferative, proapoptotic, antiangiogenic, anti-inflammatory, and antioxidative properties) together with novel JAK1/2 inhibitors may open a new era for patients with MPNs, the outlook not only being "minimal residual disease" and potential cure but also a marked improvement in inflammation-mediated comorbidities. These goals will not only set new standards for treatment of MPNs in the future but may also likely be highly cost-effective when considering the potential of decreasing dosages of very expensive drugs (JAK1/2 inhibitors/ IFN) due to synergism between them and for example, statins, and therefore a reduction in side effects of single agents as well [27, 28, 165–168]. This novel treatment concept, targeting the oxidative stress mechanisms in MPNs, is foreseen to alleviate the heavy disease burden, which encompasses not only an increased risk of severe cardiovascular complications and second cancers but likely also an increased risk of premature

atherosclerosis (early ageing?) [28, 29, 135]. By eliminating the oxidative stress overload, improving the defective antioxidative stress defence, and improving "Tumor Immune Surveillance" according to the novel treatment concept as outlined above, we are convinced that the future will look bright for our patients and will enlighten new horizons.

Conflict of Interests

Mads Emil Bjørn has received partial funding for his Ph.D. study from Novartis Oncology. Hans Carl Hasselbalch has received research grants from Novartis Oncology.

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Review Article

Pathogenesis of Myeloproliferative Neoplasms: Role and Mechanisms of Chronic Inflammation

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Myeloproliferative neoplasms (MPNs) are a heterogeneous group of clonal diseases characterized by the excessive and chronic production of mature cells from one or several of the myeloid lineages. Recent advances in the biology of MPNs have greatly facilitated their molecular diagnosis since most patients present with mutation(s) in the *JAK2, MPL,* or *CALR* genes. Yet the roles played by these mutations in the pathogenesis and main complications of the different subtypes of MPNs are not fully elucidated. Importantly, chronic inflammation has long been associated with MPN disease and some of the symptoms and complications can be linked to inflammation. Moreover, the JAK inhibitor clinical trials showed that the reduction of symptoms linked to inflammation was beneficial to patients even in the absence of significant decrease in the *JAK2*-V617F mutant load. These observations suggested that part of the inflammation observed in patients with *JAK2*-mutated MPNs may not be the consequence of *JAK2* mutation. The aim of this paper is to review the different aspects of inflammation in MPNs, the molecular mechanisms involved, the role of specific genetic defects, and the evidence that increased production of certain cytokines depends or not on MPN-associated mutations, and to discuss possible nongenetic causes of inflammation.

1. Introduction

Chronic myeloproliferative neoplasms (MPNs) are rare hematologic diseases characterized by the clonal proliferation of mature blood elements from several myeloid lineages, associated in certain cases with bone marrow fibrosis, splenomegaly, and/or hepatomegaly. They include chronic myelogenous leukemia (CML), three related entities named polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF) (called Philadelphia chromosome-negative (Phi-negative) MPNs), chronic eosinophilic leukaemia, mastocytosis, and unclassifiable MPNs [1]. CML and other MPNs are classified based on the presence or the absence of the *BCR-ABL* fusion gene which is the hallmark of CML [2]. This review focuses solely on Phi-negative MPNs. Three types of molecular markers are associated with Phi-negative MPNs: activating mutations in the *JAK2* gene (*JAK2*-V617F being the most frequent mutation, present in all subtypes of MPNs); activating mutations in the *MPL* gene (*MPL*-W515L/K mostly); and alterations of *CALR*, the gene coding calreticulin (*CALR*), detected in ET and in PMF [3–11]. A small percentage of MPN patients (<15%) do not carry mutations in the *JAK2*, *MPL*, or *CALR* genes.

The exact roles played by *JAK2*, *MPL*, and *CALR* mutations in the pathogenesis, phenotype, and complications of the three MPN subtypes are not fully elucidated. None of the *JAK2*-V617F, *MPL*-W515L/K, or *CALR* mutations is specific of a particular MPN subtype. They are detected in patients with very different phenotype and disease evolution, and therefore their presence alone is not sufficient to explain the clinical presentation and complications observed in MPN patients.

Moreover, for subsets of patients, the *JAK2*-V617F mutation has been shown to be a rather late event, sometimes recurrent, which indicates that other genetic events are responsible for clonality in these patients [14–18]. Interestingly, some of the clinical symptoms and complications appear to be linked to the chronic inflammation which almost always accompanies MPN disease, and reduction of symptoms linked to inflammation is beneficial to patients [19, 20]. Presently it is unclear whether the inflammation-related biological markers and clinical symptoms observed in MPN patients are consecutive or reactive to, or perhaps even precede, the main mutations harbored by MPN clones. Obviously, a better understanding of the mechanisms that underlie inflammation in the different MPN subtypes should have a significant impact on the design of future protocols tested for the therapy of MPNs. To help address this issue, the present review describes the role played by somatic as well as germline genetic defects in the increased production of inflammatory cytokines and other inflammation markers in MPNs; potential nongenetic causes of chronic inflammation are also discussed.

2. Chronic Inflammation, including Inflammation Associated with Solid Cancer or MPNs

Inflammation is a pathological process typically triggered by an external aggression, which may be a physical or chemical injury, irradiation, or infection. In addition, chronic hypoxia (e.g., when cells accumulate in a solid tumor or in the bone marrow in the context of blood malignancy or in any type of tissue in case of venous or arterial thrombosis) can also lead to inflammation [21–23]. Chronic inflammation is characterized by the prolonged stimulation of the production of immune blood cells from the lymphoid and myeloid lineages and the release of various mediators, notably inflammatory cytokines, in blood vessels and in tissues. Myelopoiesis is stimulated during inflammation so as to produce sufficient quantities of polyclonal granulocytes, monocytes, and macrophages to ensure the destruction of damaged cells, tissues, or infectious pathogens, adequate phagocytosis, and presentation of antigens to lymphocytes. The production of polyclonal megakaryocytes and platelets is frequently increased, to ensure thrombus formation and hemostasis in case of damaged blood vessels in inflamed tissues. Chronic inflammation may lead to hypoxia of variable severity in the damaged tissues and, accordingly, to increased production of polyclonal erythroid progenitors and red blood cells in an effort to improve cell and tissue oxygenation. Conversely, hypoxia can lead to increased production of inflammatory cytokines: individuals with mountain sickness present with elevated levels of inflammatory cytokines in peripheral blood, and healthy volunteers exposed to a hypoxic environment (three nights in high altitude above 3400 meters) presented with a high level of interleukin- (IL-) 6 [24, 25]. Patients with Chuvash polycythemia associated with homozygous germline mutation in the Von Hippel-Lindau (*VHL*) gene, a major actor of the hypoxia sensing pathway, present with elevated levels of tumor necrosis factor- (TNF-) α and interferon- (IFN-) γ [26]. Inflammatory diseases such as inflammatory bowel disease and rheumatoid arthritis also provide evidence of cross talk between hypoxia and inflammation [27]. In rheumatoid arthritis, hypoxia-inducible factor- (HIF-) 2α is the HIF isoform that plays a major role in inflammation, notably by inducing expression of IL-6 and TNF-α [28]. Importantly, HIF-1α plays an essential role in survival and function of myeloid cells during inflammation [29].

If the initial "injury" persists, the inflammation response and associated chronic stimulation of hematopoiesis are prolonged, and the risk of DNA alteration increases in cells from the damaged tissues or/and in overstimulated hematopoietic progenitors. Over time the acquisition of genetic defects in the inflamed tissues or/and hematopoietic progenitors may eventually lead to the development of solid cancer or/and clonal hematopoiesis and hematological malignancy (Figure 1). In fact, all types of solid and blood cancers, including MPNs, are accompanied by some degree of chronic inflammation [21, 22]. The mechanisms of inflammation in the context of cancer are complex and multiple. Chronic inflammation is an early event in many types of cancers and in certain lymphoma but in MPNs, the possibility that chronic inflammation precedes the acquisition of the main MPN mutations is a new subject of research. Whatever its chronology, chronic inflammation facilitates further DNA alteration in cancer and adjacent cells, and targeting inflammation and its causes should offer new opportunities of cancer treatment and also help reduce complications [21–23].

In the context of solid cancer, chronic inflammation may be reactive to a persistent tissue injury (exposure to toxics or to infectious agents) or/and to the tumor itself; it may also be a consequence of tumor-associated mutations or of treatment (radiotherapy or chemotherapy) (Figure 2). Thus inflammation may precede or/and accompany malignancy, and polyclonal hematopoietic cells of the myeloid and lymphoid lineages participate in the inflammation process. Whatever the cause(s) of inflammation, sustained stimulation of the proliferation of lymphoid or myeloid cells to maintain inflammation over months or years increases the risk of DNA alteration in these cells and the subsequent emergence of a mutated clone (initiation of malignancy) or of additional mutated clones (during or after radio- or chemotherapy). Figure 2 represents progression from chronic inflammation and stimulation of polyclonal myelopoiesis to clonal myelopoiesis, expansion of a mutated myeloid clone, and myeloid malignancy.

In MPNs, cells from all myeloid lineages may belong to the malignant clone: erythroid cells, megakaryocytes, neutrophils, and monocytes; B-lymphocytes or/and T-lymphocytes may be mutated too, but only rarely and usually in PMF [30]. In contrast to patients with solid cancer, for whom myelopoiesis is normal and polyclonal, the immune response in patients with MPNs includes the mobilization and activity of mutated (clonal) myeloid cells as well as of healthy myeloid and lymphoid cells. Depending on the small or large size of the MPN clone, the myeloid part of immune and inflammatory responses may be partially or mostly clonal and subsequently mildly or severely defective. This side of

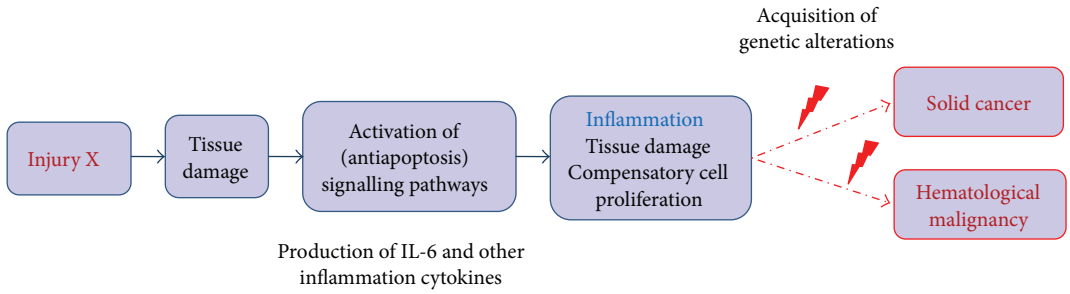

Figure 1: Progression from chronic inflammation to solid and blood cancers. A physical, chemical, or infectious injury leads to tissue and cell damage and activation of antiapoptosis signaling pathways in affected cells, which results in the autocrine and paracrine production and consumption of prosurvival, inflammatory cytokines, as well as chemokines, to attract immune cells of the lymphoid and myeloid lineages to the site of injury. Over time, established inflammation (chronic inflammation) constantly overstimulates the production of hematopoietic cells and induces more tissue and cell damage, hereby increasing the rate of DNA duplication and risk of defective DNA reparation and mutation, both in cells from affected tissues (increased risk of solid cancer) and in lymphoid and myeloid cells participating in the immune/inflammatory response (increased risk of hematological malignancy).

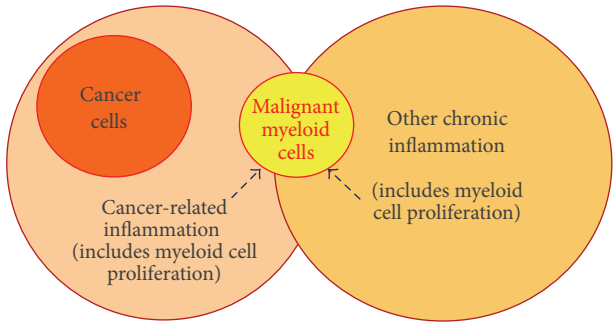

Figure 2: Increased risk of myeloid malignancy in case of chronic inflammation. Chronic inflammation may be related to solid cancer or to other causes (infectious, toxic, and physical). In all cases the immune response includes an increased stimulation of the production of myeloid cells, with the associated increased risk of DNA alteration in dividing progenitor cells. Over the years, a myeloid progenitor may acquire a defect in a gene critical for survival or proliferation (*MPL*, *JAK2*, and *CALR*?) and a *MPL*-, *JAK2*-, or *CALR*-mutated malignant clone may expand and lead to a MPN. Other mutations providing a mild growth advantage (*TET2*, *IDH1/2*?) may occur before or after the *MPL*, *JAK2*, or *CALR* mutations. In the case of inflammation related to solid cancer, cancer cells and the inflammatory cytokines they produce likely affect immune cells.

myeloid malignancy is often neglected but likely important in the pathogenesis and complications of MPNs.

One cause of chronic inflammation recognized as increasing the risk of malignant transformation of affected cells and tissues is chronic infection. Indeed it is now well established that latent infection can be associated with various types of solid cancer or/and with lymphoid malignancy [31–37]. In blood malignancies, two main transforming mechanisms may be at play: direct cell infection and transformation by oncogenic molecules or indirect transformation via chronic antigen stimulation and cell proliferation resulting in increased risk of acquisition of genetic defects.

2.1. Molecular Pathways Activated in Chronic Inflammation. During inflammation, cytokines are released which signal cells such as T-lymphocytes and monocytes-macrophages to travel to the site of injury. In turn, activated immune cells increase their production of inflammatory cytokines, chemokines, hematopoietic cytokines, and other growth factors, hereby stimulating numerous cell types from their environment (fibroblasts and endothelial cells), which further increases the production of inflammatory cytokines. In this context, the nuclear factor kappa-B (NF-κB) and JAK1/STAT1 pathways are the two main molecular pathways activated to enhance the production of inflammation cytokines (Figures 3 and 4) [12, 21, 38]. In case of inflammation linked to hypoxia, which may occur after thrombosis or because of cell accumulation, the production of inflammatory cytokines and growth factors by the cells exposed to hypoxia is upregulated via the HIF-1α pathway [39, 40]. As shown in Figure 3, the NF-κB, HIF-1α, and JAK/STAT pathways interact closely. They act in synergy, NF-κB activating the HIF-1α pathway, which in turn leads to increased activation of several signaling pathways, including JAK2/STAT5 (via the production of erythropoietin (EPO)), STAT3 (via inflammation cytokines from the IL-6 family or via EPO, hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF)), and STAT1 (via type I and type II inflammatory cytokines) (Figure 4). Moreover, the level of JAK activity affects the expression of transcription factors HIF-1 and HIF-3 [13, 41]. In the context of malignancy, the genetic mutations associated with the tumor may or may not induce the production of inflammation cytokines in mutated cells. This aspect is particularly important in the context of blood cancers since the mutated cells are involved in the immune response or/and are major sources of production of inflammatory cytokines.

Situations where chronic inflammation results from more than one cause are not rare: physical injury and infection, thrombosis and hypoxia, solid cancer and infection, *JAK2*-mutated MPN and thrombosis, and so forth. The degree of activation and overall synergistic action of the three

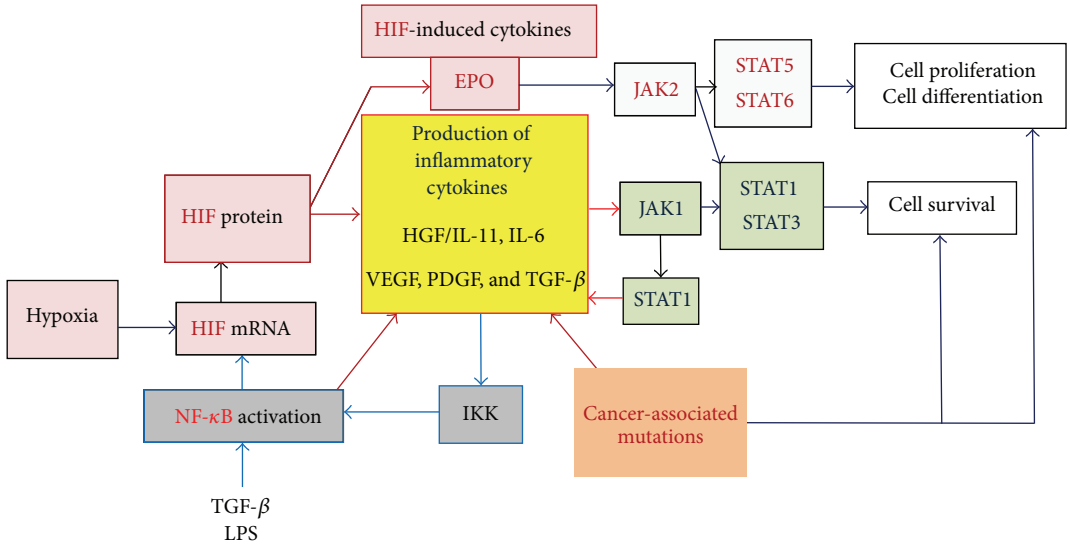

Figure 3: Main molecular pathways activated for the production of inflammatory cytokines. Three main transcription factors control the production of inflammatory cytokines and subsequently cell survival and proliferation: (i) HIF-1α, activated in hypoxic tissues, regulates the transcription of multiple genes including numerous inflammatory cytokines and growth factors that promote cell survival, fibrosis, and neoangiogenesis [12, 13]; (ii) NF-κB induces the expression of many inflammation cytokines and growth factors, as well as HIF-1α mRNA; (iii) STAT1, like NF-κB, induces the expression of several inflammation cytokines. To a lesser degree, STAT3 also regulates cytokine transcription, notably of IL-6. STAT1 and STAT3 are activated by JAK kinases, essentially JAK1, but other kinases also activate STAT transcription factors (e.g., MET, the HGF receptor). In addition, cancer-associated mutations may affect the expression (*TET2* and *IDH1/2* mutations) or signaling (*JAK2*-V617F, *CBL*, or *LNK* mutations) of cytokines or cytokine receptors. Certain growth factors (TGF-β) and other molecules such as liposaccharide (LPS), a component of Gram-negative bacteria, can also activate the NF-κB pathway and subsequently the HIF and JAK/STAT pathways. Red arrows represent pathways that directly lead to increased production of inflammatory cytokines.

main pathways which control the production of inflammatory cytokines may vary widely, which allows for infinite qualitative and quantitative differences (Figure 4). Thus the cytokine profile and degree of overproduction of inflammatory cytokines and other mediators of inflammation are expected to vary from patient to patient, according to the cause(s) of inflammation, the cell types being stimulated, and the molecular pathways involved.

2.2. Main Inflammatory Cytokines, Cellular Sources, and Role in Expansion of the MPN Clone. Cytokines may be divided into four groups on the basis of their biological functions: (i) natural immunity, for TNF-α, IL-1, IL-6, IL-5, IL-8, and chemokines; (ii) lymphocyte activation, growth, and differentiation, for IL-2, IL-4, and transforming growth factor-β (TGF-β); (iii) regulation of inflammation, also for IL-4, TGF-β, and IL-1, IL-10, IFN-γ, and granulocyte macrophage-colony stimulating factor (GM-CSF); and (iv) stimulation of leucocyte growth and differentiation, for IL-1, IL-3, IL-5, IL-6, granulocyte-CSF (G-CSF), macrophage-CSF (M-CSF), and GM-CSF. Cytokines are also classified as Th1 (proinflammatory) cytokines (IL-1, IL-2, IL-12, TNF-α, and IFN-γ) and Th2 (anti-inflammatory) cytokines (IL-4 and IL-10, notably). Th1 cytokines cause stimulation of CD8-positive cytolytic T-lymphocytes, leading, for instance, to viral clearance. Hence the cytokines produced during chronic inflammation vary according to the cause of inflammation and the cell types involved.

The cytokines produced in large quantities during inflammation may also vary according to the molecular pathways that are being activated (due to the acquisition of mutation(s), infection, hypoxia, etc.). The cytokines produced following activation of the NF-κB and JAK1/STAT1 pathways include IL-1β, IL-6, IL-8, IL-10, IL-11, IL-12, IL-13, IL-15, IL-22, vascular endothelial growth factor (VEGF), TNF-α, TGF-β, platelet-derived growth factor- (PDGF-) BB, b-fibroblast growth factor (FGF), G-CSF, GM-CSF, IFN-α, macrophage inflammatory protein- (MIP-) 1α, MIP-1β, MIP-3α, HGF, IFN-γ-inducible protein 10 (IP-10), monocyte chemotactic protein- (MCP-) 1, monokine induced by IFN-γ (MIG), and regulated on activation, normal T-cell expressed and secreted (RANTES) [13, 40, 41]. In case of hypoxia, increased HIF-1α expression leads to the upregulation of the production of EPO, VEGF, insulin growth factor 2 (IGF-2), TNF-α, TGF-β, PDGF, fibroblast growth factor (FGF) 2, IL-6, HGF, and its receptor MET (list not exclusive) [42]. Most inflammation cytokines activate the JAK1/STAT3 pathway, thus ensuring enhanced survival of many cell types, including fibroblasts, endothelial cells, and hematopoietic progenitors. Certain cytokines and growth factors activate other molecular pathways, such as the Smad proteins for TGF-β, JAK1/STAT1 for IFN, or the JAK2/STAT5 pathways for EPO and G-CSF, which stimulate the production of red blood cells and granulocytes, respectively [43–46].

In MPNs, several inflammatory cytokines and growth factors (IL-6, IL-8, GM-CSF, HGF, VEGF, b-FGF, and TGF-β) are found to be significantly overproduced in all subtypes,

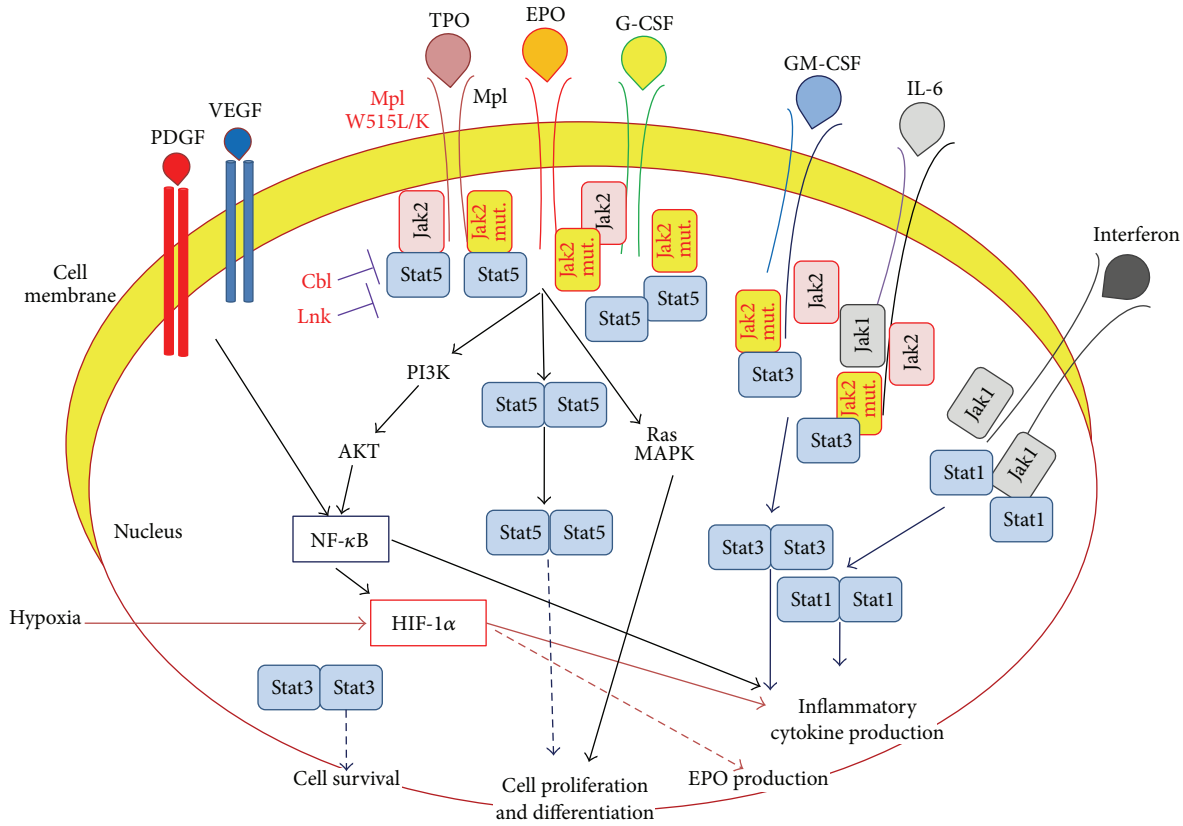

Figure 4: Molecular pathways activated by inflammatory cytokines and growth factors and affected by MPN-associated mutations. Most cytokine and growth factor receptors can activate the HIF-1α, NF-κB, and/or one or more of the different JAK/STAT pathways, either directly or indirectly. Among the JAK kinases, myeloid cells express essentially JAK2 and to a lesser degree JAK1 and TYK2 (not represented). JAK2 activates STAT5 and STAT3. JAK1 activates mainly STAT1 and to a lesser degree STAT3. The different STAT transcription factors form homodimers as well as heterodimers, which allows for a differential regulation of the expression of inflammatory cytokines. In MPN clonal cells, the JAK2-coupled receptors of EPO, TPO, and G-CSF may form complexes with and activate wild type JAK2 only, V617F-mutated JAK2 only, or wild type and V617F-mutated JAK2, which likely result in different levels of activation of the JAK2/STAT pathways concerned. Moreover, EPO, TPO, and G-CSF activate other molecular pathways besides the JAK/STAT pathways, such as the antiapoptosis, prosurvival PI3K/AKT pathway and the proproliferation RAS/MAPK pathway. Of note, activation of HIF-1α leads to an increased production of inflammatory cytokines in all cell types, but HIF-1α induces EPO expression only in the rare EPO-producing cell types (renal cells, neuronal cells, and certain tumors). LNK loss-of-function mutants result in enhanced activation of JAK2/STAT5. The *CBL* mutants detected in MPNs also enhance JAK/STAT signaling. Blue arrows represent JAK/STAT pathways, and red arrows represent HIF pathways.

yet with a large variability in quantity (Table 1). Of note, TGF-β1 inhibits normal hematopoiesis in humans via its receptor II (TGF-βRII). In cancer cells, a reduced expression of TGF-βRII is frequent, which suggests that malignant MPN progenitors may also acquire resistance to TGF-β1 by downregulating TGF-βRII expression [47, 48]. For certain cytokines, qualitative and quantitative differences in production can be related to the MPN phenotype. Excess production of IL-4, IL-10, and TNF-α has been reported in ET; elevation of IL-11 levels has been described only in PV; and in PMF, many cytokines, growth factors, and chemokines are produced at high levels but IFN-γ levels are usually low (Table 1) [45, 49–54].

The cellular sources of production of inflammation cytokines, chemokines, and growth factors are many and of course vary depending on the MPN subtype and associated complications (thrombosis and bone marrow fibrosis). However, they usually include most of the cell types which constitute the bone marrow microenvironment or hematopoietic niche, fibroblasts, macrophages, T-lymphocytes, and endothelial cells, as well as healthy or mutated (clonal) hematopoietic progenitors and mature blood elements, platelets, neutrophils, monocytes, and macrophages [55–59]. Macrophages may present with a M1 phenotype, where they produce large amounts of TNF-α and IL-12 (both elevated in PV and PMF) as well as IL-23. Macrophages of the M2 phenotype secrete IL-4 or IL-10 (both elevated in ET).

In MPNs the EPO level is low and typically undetectable in PV [60]. The presence of the activating *JAK2*-V617F mutation in >95% of PV cases likely compensates for the low EPO production by rendering erythroid progenitors highly responsive to low doses of EPO, to result in polycythemia.

Table 1: Qualitative differences in inflammatory cytokine expression in ET, PV, and PMF.

MPN subtype	Cytokines produced in excess	Main cellular sources
All MPNs	IL-6, IL-8, IL-2, soluble IL-2R, HGF, TNF-α, TGF-β, GM-CSF, VEGF, and bFGF	Bone marrow fibroblasts, endothelial cells, monocytes, macrophages, T-lymphocytes, hematopoietic progenitors, and hepatocytes
ET	IL-4, IL-10, IFN-γ, MCP-1, PDGF-BB, and soluble IL-6R (gp80)	M2 macrophages, platelets, and T-lymphocytes
PV	IL-11, IL-12, IL-13, IL-5, and IL-7	Bone marrow fibroblasts, T-lymphocytes, M1 macrophages, and hematopoietic progenitors
PMF	IL-1β, IL-10, IL-12, IL-13, IL-15, IL-33, G-CSF, IFN-α, MIP-1α, MIP-1β, IP-10, MIG, MCP-1, and PDGF-BB	Bone marrow fibroblasts, activated T-lymphocytes, neutrophils, macrophages, hematopoietic progenitors, megakaryocytes, and platelets

This explanation is likely also valid for the 50–60% of ET and PMF which are *JAK2*-V617F-mutated. Intriguingly, blood levels of other cytokines which also activate the JAK2/STAT5 pathways (TPO and G-CSF) or the JAK2/STAT3 pathways (GM-CSF, IL-12, IL-33, and cytokines of the IL-6 family: IL-6, IL-11, and oncostatin M (OSM)) are often normal or elevated in MPNs (Table 2). Several of these cytokines are produced by nonhematopoietic cells and also by myeloid progenitors, and they promote the survival and proliferation of both clonal and nonclonal myeloid progenitor cells. This is the case notably for TPO, IL-6, IL-8, IL-11, IL-33, GM-CSF, HGF, and TNF-α and several of these cytokines have been proven to contribute to the expansion of the *JAK2*-V617F-mutated cells [51–53, 59, 61]. Regarding TPO, it is important to note that the low surface expression of Mpl (the TPO receptor) observed in MPN progenitors and platelets likely limits the effect of high circulating levels of TPO. The reasons for the low expression of Mpl in MPN patients are not fully understood. On one hand, *JAK2*-V617F is thought to be less efficient than wild type JAK2 to bring Mpl receptors to the cell surface and possibly to increase Mpl destruction. On the other hand, a high TPO level and activating *JAK2*-V617F or *MPL*-W515L/K mutations may be ways of counteracting Mpl repression in progenitor cells.

Another intriguing observation is the elevated production of IL-33. IL-33 is an alarmin known to help fight viral infection that is implicated in autoimmunity, and an increased risk of autoimmune disease has been reported in MPN patients [61, 62]. Chronic stimulation by the above cytokines also facilitates the survival and expansion of fibroblasts and fibrosis (IL-6 and b-FGF), monocytes-macrophages (IL-6 and GM-GSF), and platelet production (IL-6) and neoangiogenesis (VEGF), whereas IL-12 and IL-33 activate T-lymphocytes and natural killer (NK) cells. In addition, MPN cells accumulate in the bone marrow, which leads to some degree of hypoxia and subsequent activation of the HIF-1α pathway, with upregulation of STAT3 expression and production of cytokines which further promote cell survival (IGF-2, HGF, and IL-6), fibrosis (PDGF, FGF2, and IL-6), and neoangiogenesis (VEGF) [42, 63].

Altogether, the qualitative and quantitative differences found in cytokine production in the three MPNs subtypes hint that the causes and mechanisms of chronic inflammation likely differ in ET, PV, and PMF. The *JAK2*-V617F, *MPL*-W515L/K, and *CALR* mutations likely influence clinical symptoms but do not explain differences in inflammation. For instance, *JAK2*-V617F, *MPL*, and *CALR* mutations are detected at similar levels of expression in ET (associated with mild or very mild inflammation) and in PMF (characterized by severe inflammation). Thus it is important to investigate and understand the mechanisms of inflammation at play in each MPN subtype, including those independent of *JAK2*, *MPL*, or *CALR* mutations.

2.3. Main Clinical and Biological Symptoms. The main clinical symptoms observed in MPNs which are linked to an increased production of inflammation cytokines are fatigue, fever, itching, night sweats, weight loss, and, to some extent, splenomegaly. These symptoms are frequent in PMF; they occur in PV but are mostly absent in ET, which is a good reflection of the degree of production of inflammation cytokines characteristic of PMF (high or very high), PV (moderate to high), and ET (mild).

The main biological parameters routinely assessed which are affected in case of inflammation include blood cell counts (in particular leukocyte, neutrophil, and platelet counts), iron levels, and several proteins: the C-reactive protein (CRP), haptoglobin, alpha-1 acid glycoprotein (orosomucoid), ferritin and fibrinogen (increased), and albumin and transferrin (decreased). The major stimulus of increased synthesis by liver (hepatocytes) is IL-6. These inflammatory proteins present with different kinetics: inflammatory positive markers which are increased early include CRP, haptoglobin, and alpha-1 acid glycoprotein, whereas fibrinogen, ferritin, and transferrin are late-acting inflammatory proteins.

Elevation of the leukocyte and platelet counts is typical of MPNs and thus does not allow distinguishing between inflammation and MPN. CRP is elevated in MPNs, particularly in PMF, and pentraxin 3 has been reported to decrease in MPNs [64]. A high CRP and low pentraxin 3 were linked to a high risk of thrombotic events in PV and in ET, and a high CRP was associated with shortened leukemia-free survival in MPN patients with myelofibrosis [64, 65]. The level of IL-6 in serum is almost constantly increased in case of MPN but IL-6

Table 2: Infectious pathogens and toxic compounds known to be associated with chronic inflammation and solid and blood cancers.

Exposure to infectious pathogens	Affected cells	Associated solid cancer	Associated hematological malignancy
Human T-cell leukemia virus 1 (HTLV-1)	T-lymphocytes		T-cell leukemia T-cell lymphoma
Hepatitis C virus (*Flavivirus*)	Hepatocytes B-lymphocytes	Hepatocarcinoma	B-cell lymphoma, plasma cell leukemia, MGUS, and myeloma
Hepatitis B virus (Hepadnavirus)	Hepatocytes	Hepatocarcinoma	
Epstein-Barr virus (human herpes virus 4)	B-lymphocytes	Nasopharynx	B-cell lymphoma MGUS, and myeloma
Human herpes virus 8 (herpes virus)	Fibroblasts B-lymphocytes	Sarcoma	B-cell lymphoma, myeloma, and plasma cell leukemia
Human herpes virus 2 (herpes virus)	Epithelial cells	Genital cancer	
Human papilloma (papilloma virus)	Epithelial cells	Genital cancer	
Helicobacter pylori (bacterium)	Epithelial cells	Gastric carcinoma	MALT lymphoma, MGUS, and myeloma
Borrelia burgdorferi (bacterium)	Keratinocytes, fibroblasts, dendritic cells, T-lymphocytes, and monocytes/macrophages		Skin lymphoma
Campylobacter jejuni (bacterium)	Intestinal cells		Immunoproliferative small intestine disease (IPSID)
Chlamydia psittaci (bacterium)	Nasal and pulmonary cells		Ocular adnexal lymphoma
Toxoplasma gondii (protozoon)	Macrophages		Intraocular B-cell lymphoma
Schistosoma haematobium (Platyhelminthe, Trematoda)		Bladder carcinoma	
Opisthorchis viverrini (Platyhelminthe, Trematoda)		Bile duct carcinoma	
Porphyromonas gingivalis (bacterium)		Gingival/mouth cancer, pancreatic cancer?	
Exposure to toxics	**Affected cells**	**Associated solid cancer**	**Associated hematological disorder or malignancy**
Tobacco	Epithelial cells	Lung cancer Bladder cancer Others	Chronic stimulation of myelopoiesis
Asbestos fibers (results in asbestosis, an inflammatory condition)	Mesothelial cells	Mesothelioma	
Pesticides	All		Lymphoma
Benzene	All	Bladder cancer	Chronic myelogenous leukemia and other myeloid and lymphoid malignancies

levels (and other inflammatory cytokines) are not measured in routine laboratory practice.

3. Activation of the Molecular Pathways of Inflammation by MPN-Associated Mutations

MPNs are characterized by the activating *JAK2*-V617F and *MPL*-W515L/K mutations, the *CALR* mutations, and also high levels of total Jak2 (wild type and V617F-mutated) in neutrophils and platelets [3–10]. The effect of *JAK2*-V617F mutation is to activate primarily the STAT5 pathways but the STAT3 pathways are also activated (Figure 4) [66]. The *MPL*-W515L/K mutations presumably stabilize Mpl, the Jak2-coupled dimeric receptor for TPO [67]. TPO is known to stimulate the JAK/STAT pathways and also PI3K/AKT, ERK, p38, NF-κB, and HIF [67–69]. Accordingly, in transfected cells expression of *MPL*-W515L/K mutants resulted in increased activation of ERK (extracellular signal-regulated

kinases) 1 and ERK 2 (ERK1/2) and AKT (protein kinase B) in absence and in presence of TPO [5, unpublished observations]. To our knowledge, the effect of *MPL*-W515L/K mutations on NF-κB and HIF has not been studied. In any case, the *JAK2*-V617F mutation activates STAT3 and the *MPL*-W515L/K mutations activate STAT1 and STAT3, which implies that they may stimulate the production of inflammatory cytokines (Figure 4). However, in MPN progenitor cells and platelets, the expression of Mpl receptors at the membrane surface is often very low, which likely attenuates the effect of TPO stimulation and *MPL*-W515L/K mutation.

The molecular pathways possibly activated by *CALR* mutations remain unclear. Calreticulin is a calcium-binding protein chaperone normally located in the endoplasmic reticulum (ER); the *CALR* mutations associated with MPNs all result in C-terminal truncated forms of calreticulin located in the cytosol. Thus it is presumed but not formally demonstrated that *CALR* mutants may affect intracellular calcium flux as well as the trafficking and secretion of glycoproteins, which could potentially lead to altered expression and activation of cytokines, receptors, Jak2, and other signaling molecules. Consistently, the initial papers reported an activation of the JAK2/STAT5 pathway in transfected cells which expressed *CALR* exon 9 mutants [8, 9]. However, the precise molecular mechanisms linking *CALR* mutants and the JAK2/STAT5 pathway have not been identified.

More rarely, in ET or PMF the "driving" mutation may be a loss-of-function mutation in the *LNK* gene or in the *CBL* gene [70–74]. LNK is an adaptor protein which acts as a negative regulator of TPO/Mpl-mediated activation of JAK2. Expression of LNK loss-of-function mutants also results in enhanced activation of the JAK2/STAT5 pathway. *CBL* codes for an E3-ubiquitin ligase which promotes the ubiquitination of signaling molecules, including tyrosine kinases. The *CBL* mutations detected in MPNs cause the loss of E3-ubiquitin ligase activity, thus resulting in increased signaling and cell proliferation. So far there is no evidence that *LNK* or *CBL* mutations induce the production of inflammatory cytokines, but they may alter their signaling. Figure 4 summarizes the pathways activated by the main MPN-driving mutations.

Mutations in the *TET2*, *IDH1*, *IDH2*, *EZH2*, *ASLX1*, or *DNMT3A* genes may also be found in MPNs. They are not specific of MPNs: they are found also in other blood and solid malignancies. Their main action is to alter the regulation of gene expression [75–83]. *TET2* and *IDH1/2* mutants impair the hydroxylation of methylcytosine and thus affect DNA methylation. More precisely, *TET* gene products catalyze the conversion of 5-methylcytosine to 5-hydroxy-methylcytosine (5-OH-MeC), a reaction that depends in part on iron and oxygen [80, 81]. *EZH2* (enhancer of zeste homolog 2) gene codes for a histone methyl transferase, and *ASXL1* (additional sex combs like transcriptional regulator 1) gene product belongs to the Polycomb group of proteins and thus is thought to disrupt chromatin and alter gene transcription. *DNMT3A* codes for a DNA methyltransferase and mutations presumably alter the epigenetic regulation of gene expression [82]. Thus one cannot exclude that these mutations may alter the expression of genes coding for inflammatory cytokines or receptors. Interestingly, some of these mutations have been shown to precede *JAK2*-V617F [15].

4. Inflammatory Cytokines Produced as a Consequence of MPN-Associated Mutations

Not surprisingly, *JAK2*-V617F has received most of the attention. Several groups have studied the production of inflammation cytokines in *JAK2*-V617F-mutated cells or in murine *JAK2*-V617F-driven MPN models. So far published reports concluded that, *in vitro*, *JAK2*-V617F can increase the production of IL-6, IL-8, IL-9, OSM, CCL3, CCL4, and TNF-α [53, 59, 84, 85]. However, in MPN patients there is no correlation between the *JAK2*-V617F burden and the blood or serum levels of these cytokines. In fact, it is highly probable that only a fraction of these cytokines is under the control of *JAK2*-V617F. Firstly, IL-6, IL-8, and OSM are abundantly produced by nonhematopoietic (nonclonal and nonmutated) cells [51–53]. Secondly, certain molecules produced under the control of *JAK2*-V617F, such as OSM, in turn stimulate the production of other inflammatory cytokines in a *JAK2*-V617F-independent manner [85]. Thirdly, in the *JAK2*-V617F$^{+/+}$ HEL cell line, anti-JAK2 miRNA experiments had only a partial inhibiting effect on IL-6 mRNA expression; in these experiments, anti-JAK2 miRNA experiments had no effect on the expression of IL-11 and HGF [53]. Thus in *JAK2*-V617F-mutated cells, major inflammatory cytokines may be controlled partially (IL-6) or totally (IL-11 and HGF) by molecular pathways not regulated by *JAK2*-V617F.

Regarding *MPL*-W515 mutations, only one group reported the analysis of inflammation cytokines produced in *MPL*-W515L-mutated cells, in a murine bone marrow transplantation model: expression of *MPL*-W515L was associated with a significant increase in the production of IL-6, IL-10, IL-12 (p40), TNF-α, CSF3, and chemokines CCL2, CXCL9, and CXCL10 [84]. Again, *MPL*-W515L-mutated cells were not the sole source of production of these cytokines.

Regarding the *CALR* exon 9 mutations associated with MPNs, their effect on cytokine expression is not known. It is interesting to note that soluble calreticulin has been reported associated with increased production of IL-6 and TNF-α [86].

Regarding *TET2*, *IDH1/2*, and *ASXL1* mutations, it was reported that mutated forms of IDH1/2 were associated with specific DNA hypermethylation profiles, and the list of genes found to be differentially methylated includes several genes linked to inflammation, particularly the IL11-Rα and TGF-βRI receptors [79]. Interestingly, IL-11 and TGF-β are secreted at high levels in case of inflammation and both alter myelopoiesis. IL-11Rα is also differentially methylated in *TET2*-mutated cells [79]. Hypermethylation of the genes encoding IL11-Rα and TGF-βRI receptors would presumably lower their expression and subsequently make clonal progenitor cells less sensitive to the inhibiting action of TGF-β and anti-inflammatory action of IL-11. Since *TET2* and *IDH1/2* mutations are mostly found in PMF, it is possible that these mutants play a role in the aggravation of inflammation observed in severe forms of PMF [87, 88]. In myelodysplastic syndromes, *ASXL1* mutations combined with *SETBP1* mutations were reported to repress the TGF-β pathways [89]. However no study of cytokine or receptor protein expression in relation to *ASXL1* mutation in MPNs has been published.

5. Inflammatory Cytokines Produced as a Consequence of Germline Genetic Defects

Germline defects, variants, or haplotypes can affect, directly or indirectly, the expression or signaling of inflammatory cytokines and receptors, thus potentially attenuating or aggravating chronic inflammation. The 46/1 (*JAK2* GGCC) haplotype and single-nucleotide polymorphisms (SNP) in *JAK2*, in the telomerase reverse transcriptase (*TERT*), in the MDS1 and EVI1 complex locus (*MECOM*), or in *HBS1L-MYB* have been reported to be associated with a predisposition to mutation in the *JAK2* gene on the same allele (*JAK2* GGCC haplotype) or a predisposition to the development of a MPN (*MECOM*, *TERT*, *JAK2*, and *HBS1L-MYB* variants) [90–94]. To this day it remains unclear how these hereditary genetic variants act to facilitate the development of MPNs, but alteration of the transcription of the concerned genes is possible. Regarding germline *JAK2* variation, inappropriate expression of *JAK2* would clearly disturb myelopoiesis and alter the contribution of myeloid cells to inflammation responses. Consistently, the *JAK2* GGCC haplotype was reported to be associated with a defective response to cytokine stimulation, increased risk of inflammation, and impaired defense against infection [95, 96]. In CML, cells with short telomere length were found to express a specific "telomere-associated" cytokine and chemokine secretory phenotype [97]. Little is known on the functional effects of MECOM variants on cytokine production but Yasui et al. recently reported that the EVI1 oncoprotein could alter TGF-β signaling and TGF-β-mediated growth inhibition [98, 99].

It is established that variations due to SNPs in the promoter region of genes coding for inflammatory cytokines and receptors potentially affect their production. Many groups have published SNPs associated with an altered production of a cytokine or a cytokine receptor, and such SNPs concern all the main cytokines involved in inflammation: IL-1, IL-1Rα, IL-2R, IL-6, IL-8, IL-10, IL-12, IL-33, TNF-α, HGF, and MCP1/CCL2 [100–115]. SNPs have been shown to control the expression of these cytokines *in vitro* and individuals who carry the SNP are described as high or low producers [116–118]. Cytokine polymorphisms have been studied in association with specific diseases, response to infectious agents, or immune response to inflammation. To our knowledge, this type of analyses has never been performed in MPNs.

6. Clonal and Nonclonal Chronic Inflammation in MPNs

Chronic inflammation associated with MPN may have several causes, and their recognition should allow offering improved and individualized treatment to MPN patients.

6.1. MPN-Related Chronic Inflammation

6.1.1. Clonal Inflammation. As described above, part of the inflammation is clonal since MPN clonal cells produce inflammatory cytokines (IL-6, IL-8, IL-9, IL-11, OSM, TNF-α, CCL3 (MIP-1α), and CCL4 (MIP-1β)); the eventual acquisition of *IDH1/2* or *TET2* mutations may aggravate "clonal inflammation" by altering the expression of certain receptors (IL-11Rα, TGF-βR1). The consequences are multiple: (i) enhanced survival and growth of clonal cells (IL-6, IL-8, IL-11, and TNF-α); (ii) increased production of inflammation cytokines that target bone marrow stromal cells as well as hematopoietic progenitors, via the action of OSM, IL-11, and IL-6; (iii) resistance of clonal cells to growth inhibitors, via a reduced expression of IL-11Rα or TGF-βR1 [53, 59, 61, 63, 119, 120]. In addition, clonal cells can recruit and activate neutrophils, monocytes, and natural killer cells via the production of CCL3 and CCL4; the neutrophils and monocytes potentially recruited may be clonal or non-clonal.

6.1.2. Nonclonal, Reactive Inflammation. Any malignant process induces nonclonal immune responses which aim to restrict and eventually destroy the malignant cells. In case of advanced disease, clonal cells accumulate and nonclonal, hypoxia-induced inflammation can develop. Nonclonal inflammation may also be reactive to treatment. In MPNs, the mature myeloid cells which participate in the antitumoral or hypoxia-induced or therapy-related "reactive" inflammation response may be clonal or nonclonal. Depending on the MPN subtype, the size of the clonal population is likely to be large (PV and PMF) or moderate or small (ET), implying that the clonal part of reactive inflammation is probably more significant in PMF and PV than in ET. This should not be overlooked because clonal cells likely mount a less efficient immune response than healthy cells, meaning that the inflammation/immune response could be rather inefficient in PV and PMF. Consistently, an increased risk of a second cancer was reported in MPN patients [121].

6.2. Chronic Inflammation and Myeloid Stimulation as Predisposition to MPNs. The observation that major inflammatory cytokines are produced independently of MPN-associated mutations and the demonstration that *JAK2*-V617F can be a late event in MPN development are consistent with the hypothesis that chronic stimulation of myelopoiesis (via inflammation) may precede the acquisition of mutation in the *JAK2* (*MPL* and *CALR*?) gene(s) in subsets of MPN patients. A frequent objection is the lack of evidence of inflammation or myeloid stimulation prior to the diagnosis of a MPN. However, it is not rare that routine blood tests of patients, especially older patients, reveal a slight elevation of leukocyte or platelet counts, or hematocrit, with or without biological evidence of mild inflammation. There are dozens of reasons for mild alterations of blood counts, ranging from smoking, stress, obesity, and diverse latent infections to mild forms of chronic inflammatory diseases (intestinal, rheumatoid, skin, type 2 diabetes, atherosclerosis, etc.). Such patients are simply observed; investigation begins when blood counts rise significantly (reach at least one of the WHO criteria of MPN) or when patients present clinical symptoms related to MPN or to the underlying cause of chronic myeloid stimulation or inflammation. Also, it is not rare to detect lymphoid infiltrates in the bone marrow of MPN patients and monocytosis or lymphopenia in peripheral blood, sometimes prior to the diagnosis of MPN; these observations may be considered as

evidence of a disturbed immune response. Thorough investigation of the stages preceding the diagnosis of overt MPN, similar, for instance, to the studies that established monoclonal gammopathy of undetermined significance (MGUS) as the precancerous stage of multiple myeloma, is needed in MPNs to validate the hypothesis of chronic (antigen-mediated or not) stimulation of myelopoiesis preceding the acquisition of *JAK2*, *MPL*, or *CALR* mutation.

The chronic inflammation and myeloid stimulation hypothesis is attractive, because it can explain several if not all of the mysteries that persist in MPNs. For instance, chronic myeloid stimulation allows the recurrent acquisition of *JAK2*-V617F, multiple *JAK2* mutations, and combinations of *JAK2*, *MPL*, or *CALR* mutations regularly reported in MPNs. Early chronic inflammation and myeloid stimulation would explain that *JAK2*-V617F burden and clinical symptoms and disease severity are not correlated. The recent reports that patients under treatment with JAK inhibitors may develop or reactivate viral infection, possibly due to impaired NK cell function, are also consistent with chronic infection contributing to the inflammation associated with MPNs [122]. Importantly, the chronic stimulation hypothesis allows for multiple causes of inflammation, infectious or not, some mild (as observed in ET) and some severe (as typical of PMF). Last but not least, the chronic myeloid stimulation hypothesis allows for many different initial causes and thus would explain why the *JAK2*-V617F mutation and to a lesser degree the *MPL* exon 10 and *CALR* exon 9 mutations are associated with very different diseases (ET, PV, PMF, RARS-T, and splanchnic thrombosis for *JAK2*-V617F; ET, PMF, and RARS-T for *MPL* and *CALR* mutations). For all these reasons, chronic myeloid stimulation and inflammation, and notably latent infection, deserve investigation as initial, early, or complicating events of MPNs.

Indeed chronic exposure to various toxic compounds or to infectious pathogens and subsequent chronic inflammation frequently precedes malignant cell transformation in the context of solid cancers; importantly, the same toxics or infectious agents associated with solid cancer may also lead to lymphoid malignancy (Table 2) [31–37]. Normal immune responses following infection include the stimulation of myelopoiesis (granulocytes and monocytes). The production of B-lymphocytes and plasma cells is stimulated to produce polyclonal Ig. If infection becomes chronic, a focusing of the Ig response from polyclonal to monoclonal (mc) immunoglobulins (Ig) may occur, and that will persist as long as the infection. Epstein-Barr virus (EBV), Hepatitis C virus (HCV), Hepatitis B virus (HBV), or *Helicobacter pylori* (*H. pylori*) stimulate polyclonal B-cell proliferation, and these pathogens are implicated in several B-cell malignancies (Burkitt, Hodgkin, non-Hodgkin lymphoma, and chronic lymphocytic leukemia) via cell infection and direct transformation (EBV and HCV), via antigen-driven stimulation (*H. pylori*), or both (EBV and HCV) [32–37]. Moreover, EBV, cytomegalovirus (CMV), HHV-8, and HHV-6 can induce a chronic monoclonal Ig response [123–126].

In support of the hypothesis that infection may predispose to chronic hematological malignancy, we showed that, for about 25% of patients with multiple myeloma, the purified mc Ig specifically recognizes an antigen from HCV, EBV, or *H. pylori* [124–127]. These important findings suggest that infectious agents may also initiate multiple myeloma, not just certain types of lymphoma, which opens new possibilities of curative treatment, as demonstrated recently by the regression of one case of HCV-associated myeloma following treatment by IFN-γ [128]. Antigen-driven proliferation as a facilitator of DNA mutation acquisition and cell transformation is rarely investigated in the context of myeloid malignancies but since chronic antigen stimulation also concerns myeloid cells, latent infection as a cause of inflammation in chronic myeloid disorders should not be excluded. Thus a promising research approach for chronic myeloid disorders is to propose that, for subsets of patients, malignancy may result from chronic, polyclonal abnormal immune response by myeloid cells, eventually facilitating excessive myeloid proliferation, acquisition of genetic alterations in genes that are critical for myelopoiesis (*JAK2* and *MPL*; *CALR*?), and transformation of progenitor cells from the most stimulated lineage(s) and then expansion of a malignant clone.

7. Consequences for the Treatment of MPNs

Logically, the *JAK2*-V617F mutation rapidly became the main target of treatment in MPNs after its discovery in 2005. In contrast, chronic inflammation has so far been neglected in the treatment of these diseases.

Recognition of the importance of inflammation in the pathogenesis of MPNs offers great opportunities to improve therapy. The JAK inhibitor trials showed that blocking JAK2 function significantly reduced inflammatory cytokine levels and other markers of inflammation in PMF patients, resulting in improved clinical symptoms. Patients benefited from JAK inhibitors even when the *JAK2*-V617F mutant burden was not reduced or when their disease was not associated with *JAK2* mutation. Although the comprehension of the causes and mechanisms of inflammation in MPNs is still very incomplete, accumulated knowledge indicates that NF-κB and JAK1 are major pathways for the production or/and signaling of inflammatory cytokines. In certain cases, the HIF-1α pathways may also be activated. The three pathways are closely linked (Figures 3 and 4), and used alone, inhibitors of the JAK/STAT pathways (or inhibitors of NF-κB) cannot be expected to completely block cytokine production and signalling in MPNs. In a murine model of *JAK2*-V617F-mutated MPN, selective STAT blocking resulted in increased inflammation and thrombocytosis [129]. In fact, alteration of STAT3 function (deletion or hyperactivation) is known to lead to altered myelopoiesis and increased expression of STAT1 and inflammatory cytokines, notably IL-6, a strong stimulant of platelet production, fibroblast proliferation, and inflammatory acute phase protein production [130–132]. In support of this mechanism, Grisouard et al. reported increased expression of STAT1 and STAT1 target genes in *JAK2*-V617F mice after STAT3 deletion; IL-6 and other inflammatory cytokines were not measured in this study [129].

Ideally to cure a MPN, one should aim to reduce the effects of the *JAK2*, *MPL*, or *CALR* mutant carried by the

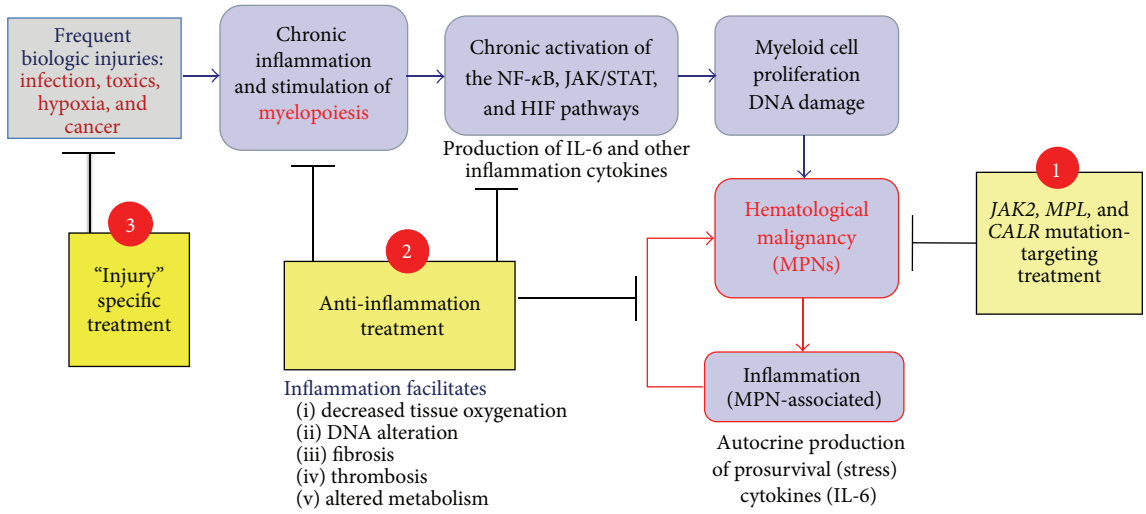

Figure 5: Chronic inflammation in myeloid neoplasms and new therapeutic options. In MPN patients, chronic inflammation includes the participation of *JAK2*-V617F-, *MPL*-W515 L/K-, or *CALR*-mutated cells and the production of inflammation cytokines under the control of these mutations. Chronic inflammation may also be reactive to the MPN clone or to other coexisting causes of inflammation (hypoxia due to cell accumulation in the bone marrow; thrombosis; infection; others). Healthy and mutated (clonal) myeloid cells participate in MPN-associated reactive inflammation, and the NF-κB, JAK/STAT, and HIF pathways are chronically activated in the MPN clone and in cells from the bone marrow environment. Ideally treatment should combine the following: (1) inhibition of the *JAK2*-V617F, *MPL*-W515L/K, or *CALR* mutations, possible with JAK inhibitors; (2) inhibition of chronic inflammation, via the neutralization or inhibition of inflammation cytokines or receptors, and/or the inhibition of the NF-κB and HIF pathways; (3) in cases where chronic inflammation precedes mutation, and a cause is identified, adequate treatment of the initial cause of inflammation could be added (e.g., antibiotics or antiviral treatment in case of latent infection).

MPN clone, as well as the production and signaling of the main inflammation cytokines produced by the patient. This can be achieved by blocking the three main pathways responsible for cytokine production (these include the JAK1/JAK2 pathways) and by suppressing the cause(s) of MPN mutation, when identified. Used alone, JAK1/2 inhibitors have the capacity to block the *JAK2*-V617F and *MPL*-W515L/K mutations and a large fraction of the production and signaling of inflammatory cytokines. But for complete treatment of inflammation and mutations in MPNs, the addition of NF-κB and HIF-1α inhibitor drugs should benefit patients [133–138]. This contrasts with myeloma, a disease not driven by the activation of the JAK2/STAT pathways where NF-κB and HIF-1α inhibitors used alone can reduce both disease and inflammatory cytokines [139]. Another advantage of such combination therapies would allow lowering the dose of each drug and hopefully reduce toxicity. Of note, one reason why IFN-α is able to induce a complete clinical, biological, and molecular remission (*JAK2*-V617F-negativation) in PV and in ET patients is that IFN-α acts on several JAK/STAT pathways as well as on other pathways critical for the production of inflammatory cytokines [140, 141]. In short, as represented in Figure 5, the ideal MPN therapy may combine the following: (1) inhibition of the JAK1 pathway and *JAK2*-V617F, *MPL*-W515L/K, or *CALR* mutations with a JAK1/2 inhibitor and (2) NF-κB and HIF inhibitors (note that (1) and (2) may be achieved with IFN-α). Whenever an early cause of chronic inflammation is identified, adequate treatment should be added: for instance, antibiotics or antiviral treatment in case of latent infection.

The complexity of inflammation in MPNs should not discourage attempts to define it biologically at the time of diagnosis, prior to therapeutic decisions, and during treatment monitoring. Knowing the precise inflammation status of each MPN patient would greatly help improve his/her treatment. As described earlier, the inflammation status and cytokine profile of a MPN patient are expected to vary according to the MPN subtype, presence of *JAK2*, *MPL*, or *CALR* mutation, eventual cause of inflammation preceding MPN-driving mutation, and personal genetic background. Yet what matters for therapy is the resulting cytokine profile of the patient, and nowadays establishing the inflammation cytokine profile of an individual is technically simple and not overly expansive and requires only a blood sample. Knowing that a patient is a strong producer of IL-6, HGF, or TNF-α, for instance, would allow focusing treatment on the target cytokine(s), perhaps by adding to the patient's combination therapy one of the existing antagonist drugs or neutralizing antibodies that specifically block these cytokines or receptors [119, 142–144].

Last but not least, extensive genetic studies and murine models have not succeeded to fully explain most of the chronic hematological malignancies, including MPNs. This suggests that genetic aberrations, although crucial, are probably not sufficient for a lymphoid or myeloid malignancy to develop, and more attention is now given to the hematopoietic niche and cytokine production, the human microbiome and oncogenic infectious pathogens, and the host's immune response [145, 146]. There is no reason to limit these important pathogenic mechanisms to lymphoid malignancies and

solid cancer, and perhaps the next major research effort in the MPN field should be to investigate the validity of the hypothesis of chronic inflammation/myeloid stimulation preceding mutation acquisition. More specifically, a systematic search for latent infection in MPN patients is feasible and simple, thanks to various tests based on the multiplexed antigen or peptide microarray technology; these assays require only a small blood sample [127, 147]. Obviously the identification of an infectious cause of inflammation in subsets of MPN patients would offer additional possibilities of combined treatment (with antibiotics or antiviral therapy) (Figure 5). Regarding research and animal models of MPNs, it is possible to develop new murine models of chronic myeloid stimulation, antigen-mediated or not [148].

In conclusion, inflammation is very complex yet there are relatively simple laboratory tools to diagnose and characterize inflammation in patients. Several predictive inflammation markers are already identified in MPNs, and potent drugs that target the molecular pathways of inflammation or the inflammatory cytokines detected in excess in patients already exist. Designing new, more complete, and individualized combination treatments that include drugs that block MPN mutations as well as the main inflammation pathways is possible, and such protocols should benefit MPN patients.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Impact of Inflammation on Myeloproliferative Neoplasm Symptom Development

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Myeloproliferative neoplasms (essential thrombocythemia, ET; polycythemia vera, PV; myelofibrosis, MF) are monoclonal malignancies associated with genomic instability, dysregulated signaling pathways, and subsequent overproduction of inflammatory markers. Acknowledged for their debilitating symptom profiles, recent investigations have aimed to determine the identity of these markers, the upstream sources stimulating their development, their prevalence within the MPN population, and the role they play in symptom development. Creation of dedicated Patient Reported Outcome (PRO) tools, in combination with expanded access to cytokine analysis technology, has resulted in a surge of investigations evaluating the potential associations between symptoms and inflammation. Emerging data demonstrates clear relationships between individual MPN symptoms (fatigue, abdominal complaints, microvascular symptoms, and constitutional symptoms) and cytokines, particularly IL-1, IL-6, IL-8, and TNF-α. Information is also compiling on the role symptoms paradoxically play in the development of cytokines, as in the case of fatigue-driven sedentary lifestyles. In this paper, we explore the symptoms inherent to the MPN disorders and the potential role inflammation plays in their development.

1. Introduction

In 1951, Sir William Dameshek postulated the concept of "myeloproliferative disorders" and furthermore ascribed their development to "a hitherto of undiscovered stimulus." The past half-century has since brought light to the cryptic "stimulus" believed to drive these disabling neoplasms. Developing from a host of myelostimulatory mutations, myeloproliferative neoplasms (MPNs) including polycythemia vera (PV), essential thrombocythemia (ET), and myelofibrosis (MF) propagate through an evolving cascade of inflammatory conduits well documented to inflict dramatic symptomatology and impair quality of life. Great gains have been made in our understanding of how these disrupted signaling pathways coalesce into dysregulated synthesis of cytokines, chemokines, and reactive species that ultimately induce symptoms. In this paper, we discuss the role inflammation plays in MPN pathobiology, disease advancement, and symptom development.

2. Characterization of MPN Symptoms

The burdensome symptom profile is arguably the most recognizable feature of the MPN disease process and in itself may contribute to reduced life expectancy, as observed in myelofibrosis risk scoring [1, 2]. Prominent symptoms include fatigue (92.7%), early satiety (61.9%), abdominal pain (45.9%), abdominal discomfort (53.2%), inactivity (60.5%), headache (48.3%), concentration problems (61.7%), dizziness (55.2%), numbness (61.3%), insomnia (65.4%), sad mood (62.7%), sexuality problems (57.9%), cough (46.4%), night sweats (56.4%), itching (52.6%), bone pain (48.5%), fever (20.2%), weight loss (34.2%), and impaired quality of life (84.2%) [3]. The MPN symptom burden has been closely examined for its impact on patient daily living through the MPN LANDMARK survey. This study systematically surveyed 813 MPN patients and discovered that MPN symptoms negatively impacted work hours, number of sick days taken, the need for medical disability and/or early retirement,

and overall activities of daily living. Patients additionally described feeling anxious and worried about their conditions (MF, 91%; PV, 78%; ET, 74%) which in turn compromised overall quality of life (MF, 81%; PV, 66%; ET, 57%) [4]. Adding to the complexity is the recent revelation that MPN symptoms indeed promote the development of other symptoms. An investigation of the symptom of insomnia revealed that the complaint correlates closely with most other MPN related symptoms and functional domains [5]. A similar study investigating correlations with MPN-related sexuality complaints found that this symptom also correlated with other MPN symptoms (insomnia, depression/sad mood, night sweats, and QOL), as well as emotional, cognitive, and social domains of functioning [6].

It has been well recognized that the prevalence and severity of symptoms differ by MPN subtype. However, more recent studies have demonstrated that significant heterogeneity exists even within MPN subtypes. A prospective evaluation of 1470 MPN patients discovered the presence of five clusters in PV and ET, respectively, and four clusters in MF [7]. Symptom clusters in ET and PV differed by clinical variables including age, language, gender, the presence of laboratory abnormalities, spleen size, history of hemorrhage, and Myeloproliferative Neoplasm Symptom Assessment Form Total Symptom Score (MPN-SAF TSS) value. Notably, neither PV nor ET clusters differed by risk scores suggesting symptomatology likely presents independent of disease stage and risk scoring tools should not be applied as surrogate measurements of disease severity. In MF, clusters differed by a variety of clinical variables as well as risk scores (DIPSS) with increasing degrees of symptomatology correlating with higher risk score categories.

Recent efforts have aimed to analyze the scope and extent of MPN symptoms in a systematic format. The first investigation was completed in 2007 as a self-reported internet survey of 1179 MPN patients [8]. This revealing study showed that fatigue, pruritus, bone pain, fevers, and weight loss led to restricted participation in physical and social functions and furthermore that available treatment regimens including androgens, steroids, hydroxyurea, and erythropoiesis-stimulating agents not only failed to improve the symptom burden but paradoxically contributed to its development. This survey served as a benchmark for the development of three MPN-specific PRO tools: MF-SAF, MPN-SAF, and MPN-SAF TSS/MPN-10. From these instruments, we have gathered key information on both the spectrum and severity of MPN symptoms. Below we discuss these tools individually (Table 1).

Table 1: MPN symptom assessment forms.

PRO tool	Year	Total number of questions	Question composition	Available languages
MF-SAF [9]	2009	20	Fatigue Inactivity Bone pain Cough Pruritus Night sweats Fever Weight loss Abdominal pain/discomfort Early satiety Quality of life	English
MPN-SAF [3]	2011	27	Fatigue Inactivity Headache Dizziness Concentration problems Numbness Insomnia Sad mood Sexuality problems Bone pain Cough Pruritus Night sweats Fever Weight loss Abdominal pain/discomfort Early satiety Quality of life	English Italian German French Mandarin Arabic Spanish Dutch Swedish Portuguese Japanese Hebrew Czech
MPN-SAF TSS [14]	2013	10	Fatigue Inactivity Concentration problems Bone pain Pruritus Night sweats Fever Weight loss Abdominal discomfort Early satiety	English Italian German French Mandarin Arabic Spanish Dutch Swedish Portuguese Japanese Hebrew Czech

MF-SAF: Myelofibrosis Symptom Assessment Form; MPN-SAF: Myeloproliferative Neoplasm Symptom Assessment Form; MPN-SAF TSS: Myelofibrosis Symptom Assessment Form Total Symptom Score; PRO: Patient Reported Outcome; QOL: quality of life.

2.1. MF-SAF. The Myelofibrosis Symptom Assessment Form (MF-SAF) was created in 2009 and served as the first validated MPN Patient Reported Outcome (PRO) tool to be made available for clinical and trial settings [9]. A 20-item instrument, the survey attempted to capture the most common symptoms within myelofibrosis and content included issues related to catabolic/proliferative symptoms, quality of life, fatigue, and splenomegaly-associated issues. Questions were constructed in a "yes," "no," or 0 (absent) to 10 (worst imaginable) scale. The tool proved useful in the open label phase II trial of the *JAK2* inhibitor, ruxolitinib [10].

2.2. MPN-SAF. The Myeloproliferative Neoplasm Symptom Assessment Form (MPN-SAF) was developed two years later in efforts to capture the symptoms within PV and ET as well as MF [3]. This survey included the items present within

the MF-SAF, along with questions related to microvasculature complications such as headaches, concentration problems, lightheadedness, dizziness, vertigo, numbness/tingling, and sexual dysfunction. This expanded version was structured in a similar format to the MF-SAF and proved beneficial in evaluation of a variety of novel targeted compounds including ruxolitinib, LY2784544, SAR302503, Vorinostat, and pegylated interferon [11–13].

2.3. MPN-SAF TSS (MPN-10). The Myeloproliferative Neoplasm Symptom Assessment Form Total Symptom Score (MPN-SAF TSS; MPN-10) is an abbreviated version of the MPN-SAF containing the 10 most symptomatic and pertinent items [14]. This tool allows for rapid administration in clinical and trial formats and has replaced the MPN-SAF in most settings. The survey has been successfully cross-validated against the EORTC QLQ-C30 and is available in a variety of languages including English, Italian, German, French, Mandarin, Arabic, Spanish, Dutch, Swedish, Portuguese, Japanese, Hebrew, and Czech.

3. Origins of Inflammation in MPN Patients

In healthy individuals, the inflammatory cascade is driven by a delicate interplay between cellular responses and neurohormonal stimulatory factors/cytokines. Dysregulation of this system is a hallmark feature of the MPNs. Although the initial inciting event has yet to be clearly elucidated, all MPN disorders arise from genetic defects within pluripotent stem cell populations that accumulate over the disease course. JAK2V617F, a member of the Janus kinase signal transduction pathway, was the first recognized mutation inherent to the MPN population (PV 96%, ET 50%, and MF 50%) [15]. The role the Janus kinase cascade (including *JAK2*, *JAK2*, *JAK3*, and TYK2) plays in the signaling of inflammatory cytokines is well documented and profound. As JAKs are essential to the signaling of surface growth factor receptors and cytokines bereft of intrinsic kinase activity, constitutive activation, as observed in JAK2V617F, induces unregulated signaling of STAT transcription factors with resultant cellular growth and propagation [16]. STAT3, in particular, is linked closely with cancer development via activation of immunomodulatory cytokines (IL-6, IL-10, and IL-17), growth factors (FGF, VEGF), and matrix metalloproteinases [17]. These products further induce positive autofeedback through the JAK/STAT pathway, perpetuating cellular malignant potential. In general, cytokines (interleukins, interferons, and soluble growth factors; definitions in Table 2) are important regulators of cellular processes, particularly those involving immunomodulatory activities, cellular growth angiogenesis, and migration [18]. Cytokine dysregulation is believed to be associated with other mutations observed within MPNs (IDH1/2, TET2) but requires additional investigation [19].

Chronic inflammation has been hypothesized to play a supportive role in oncogenesis given its promotion of genomic instability through DNA mutations and epigenetic changes, prevention of tumor immune surveillance, and encouragement of clonal evolution [17, 20, 21]. MPN cells (leukocytes, platelets) with inherent hypersensitivity to cytokines and or growth factors respond in a proliferative fashion with resultant production of more stimulatory factors. As chronic inflammatory conditions, MPN disorders revolve around a perpetual cycle of DNA damage, cellular remodeling, and subsequent fibrosis [22]. This process has been a topic of great interest, especially as it relates to the heterozygous clinical presentation of MPN patients.

Table 2: Cytokine descriptions.

Acronym	Description
B2MICG	Beta-2 microglobulin
BMP6	Bone morphogenetic protein 6
CRP	C-reactive protein
FGF	Fibroblast growth-factor
GCSF	Growth colony stimulating factor
HGF	Hepatocyte growth factor
Hs-CRP	High-sensitivity C-reactive protein
IFN-α	Interferon-alpha
IFN-γ	Interferon-gamma
IFN-γ-IP	Interferon-gamma inducible protein
IP-10	Interferon-gamma inducible protein 10
IL-1B	Interleukin-1B
IL-1RA	Interleukin-1RA
IL-2R	Interleukin-2R
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-13	Interleukin-13
IL-15	Interleukin-15
IL-17	Interleukin-17
MIG	Monokine-induced by gamma
MIP-1β	Macrophage inflammatory protein-1β
NF-KB	Nuclear factor-KB
PAI1	Plasminogen activator inhibitor-1
PTX3	Pentraxin-3
TIMP1	Tissue inhibitor of metalloproteinase-1
TNF-1	Tumor necrosis factor-1
TNF-α	Tumor necrosis-factor-α
TNF-RII	Tumor necrosis-factor-RII
VCAM1	Vascular adhesion molecule
VEGF	Vascular endothelial growth factor
EGF	Epidermal growth factor

4. Inflammation and MPN Symptom Development

The relationship between chronic inflammation and MPN symptom development has also been a topic of recent interest. An evaluation of abnormal cytokine expression within myelofibrosis determined that primary myelofibrosis (PMF) patients had significantly increased levels of IL-1B, IL-1RA,

IL-2R, IL-6, IL-8, IL-10, IL-12, IL-13, and IL-15 and TNF-1, G-CSF, IFN-α, MIP-1α, MIP-1β, HGF, INF-γ-IP, and VEGF in addition to reduced IFN-γ levels [23]. IL-2R, IL-12, IL-15, and IP-10 were independently predictive of inferior survival. IL-2R and IL-12 were associated with transfusion needs and HGF, MIG, and IL-1RA were associated with marked splenomegaly.

Evaluation of the association between cytokines and MF symptoms was undertaken in 2013 through an ad hoc analysis of 309 MF patients during the blinded phase of the COMFORT-1 trial evaluating ruxolitinib against placebo [24]. Changing levels of five cytokines was significantly associated with change in the MPN-SAF TSS when controlled for arm, visit-by-arm interaction, age, sex, and body mass index (BMI). Cytokines included VCAM1, LEPTIN, TNFRII, TIMP1, and B2MICG. IL-8 appears to play a uniquely important role within MPNs. As a potent chemokine, it has previously been shown within other malignancies to promote angiogenesis, induce leukocyte chemotaxis/activation, and stimulate cellular reproduction. A recent study determined IL-8 to be associated with elevated levels of circulating blasts and the presence of constitutional symptoms [23]. In polycythemia vera, patients demonstrate increased levels of IL-1RA, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IFN-γ, GM-CSF, MCP-1, MIP-1α, MIP-1β, HGF, IP-10, MIG, MCP-1, PDGF-BB, TNF-α, IFN-γ, and VEGF [25, 26]. Conversely, PV patients also demonstrate lower levels of EGF and RANTES. PV patients also had significantly elevated levels of IL-7, GM-CSF, MIP-1α, IP-10, MIG, eotaxin, IFN-γ, and VEGF in comparison to primary MF patients. On multivariate analysis, MIP-1β was shown to be associated with inferior survival. In addition, hemoglobin count correlated with IL-4 and MCP-1, hematocrit count correlated with TNF-α and MCP-1, lymphocyte count correlated with IL-6 and TNF-1, and JAK2V617F mutation status correlated with TNF-1 and PDGF-BB [26]. An analysis of ET patients determined this population to have elevated levels of IL-1B, IL-4, IL-6, IL-8, IL-10, IL-12, HGF, GM-CSF, IFN-γ, MCP-1, PDGF-BB, TNF-α, and VEGF. Interestingly, IL-4, IL-8, GM-CSF, IFN-γ, MCP-1, PDGF-BB, and VEGF appeared to be significantly higher in ET patients when compared to PV populations and may serve as useful markers to distinguish the two disorders [26]. Also within ET patients, polynuclear cell counts were found to correlate with HGF, IL-6, IL-12, MG-CSF, and VEGF whereas red cell counts correlated with PDGF-BB levels. JAK2V617F positive status also correlated with PDGF-BB and TNF-α [26]. In comparing PV and ET patients with vascular complications versus those without complications, no significant differences in cytokine levels were noted. However, in comparing PV and ET patients with a history of vascular events, ET patients have significantly increased levels of IL-4, IL-8, GM-CSF, IFN-γ, MCP-1, and VEGF [26].

Interestingly, the specific combination of inflammatory markers appears to be as important as the type of factor present. In MF, the combination of TNF-α and TIMP-1 has been shown to promote survival of CD34+ stem cells whereas the combination of ATP and TNF-α has been shown to reduce proliferation [27]. Specific inflammatory markers are also associated with disease severity and complications. For example, pentraxin and CRP are well established to play a role in thrombosis and atherogenesis. These biomarkers have been associated with the development of major thrombotic events in PV and ET [28]. A recent study also identified low levels of IL-12 in MPN patients with vascular complications [26]. Altered levels of PDGF, FGF, and VEGFb have also been noted in stromal cells of patients with PV, ET, and PMF suggesting proinflammatory cytokines promote bone marrow fibrosis which is well established to contribute to anemia and subsequently fatigue [29, 30]. Cytokines (BMP-1, BMP-R2, BMP-6, and BMP-7) may have a role in promoting the advancement of MPNs from early to later stages [31]. Of special interest, the presence of specific gene mutations impacts the type and degree of cytokine expression. For instance, JAK2V617F positive patients have significantly higher levels of IL1B, IL-8, IL-17A, and IFN versus triple-negative (*JAK2*, *MPL* negative) patients [32]. Much has yet to be learned about the role cytokines play in MPN symptom development. The growing availability of cost-sensitive cytokine profile testing has offered us what can best be recognized as preliminary data on this complex topic. Below we discuss the available literature on specific MPN symptoms and their relationships to inflammation (Table 3).

4.1. Fatigue. Fatigue is a common complaint among cancer patients, present within 30–60% of the cancer population [33]. The symptom is particularly prominent within MPNs (PV 85%, ET 72%, and MF 84%), representing the most common symptom voiced regardless of subtype. MPN-fatigue has been shown to correlate closely with functional capacities. In a novel evaluation of MPN patient functionality, participants were found to perform an average of 25.1 metabolic equivalents (METS), akin to scores observed in Parkinson disease patients and dramatically lower than healthy controls (45.8 METS) [9].

Cytokines have been documented to induce fatigue in both malignant and nonmalignant states. A recent study identified positive associations between TNF-α and postchemotherapy fatigue in women with breast cancer [34]. Anemia is a recognized contributor to this symptom and recent studies have demonstrated that cytokines play a critical role in the development and perpetuation of this comorbidity. IL-1, IL-6, and TNF were recently shown to promote deregulation of erythropoietin with resultant anemia in acute myelogenous leukemia (AML) and myelodysplastic syndromes (MDS) [35, 36]. Cytokine-induced hypocortisolism is also a potential source of fatigue. Cytokines have been shown to induce dysregulation of the HPA axis and promote a blunted stress response due to subtherapeutic cortisol production [37]. In cancer, fatigue has been closely associated with depressed mood and increased levels of IL-6, a cytokine observed within MPNs [38, 39]. A survey of 1788 MPN patients confirmed that 32% have been seen or diagnosed with depression and 22.2% had received active treatment of mood disorder within the prior six months suggesting potential association in this population [40].

Table 3: Associations between MPNs and cytokines.

Inflammatory marker*	Impact	Disorder
B2MICG	Symptoms	MF
BMP1	Disease advancement	PMF
BMP6	Disease advancement	PMF
BMP7	Disease advancement	PMF
BMP-Rcp2	Disease advancement	PMF
CD40L	Loss of appetite	MF
CRP	Thrombosis; atherogenesis	PV, ET
Ferritin	Pruritus	MF
FGF	Marrow fibrosis	PV, ET, PMF
HGF	Splenomegaly	PMF
IFN	Associated with JAK2V617F	MF
IL-12	Inferior survival; transfusion requirements, vascular complications	MF
IL-15	Inferior survival	MF
IL-17A	Associated with JAK2V617F	MF
IL-1B	Associated with JAK2V617F	MF
IL-1RA	Splenomegaly	PMF
IL-2R	Inferior survival; transfusion requirements	MF
IL-8	Elevated blasts; constitutional symptoms	MF
IL-8	Associated with JAK2V617F	MF
IP-10	Inferior survival	MF
LEPTIN	Symptoms; weight loss	MF
MIG	Splenomegaly	PMF
PAL1	Insomnia	MF
PTX	Thrombosis; atherogenesis	PV, ET
RANTES	Insomnia	MF
TIMP1	Symptoms	MF
TNF-1	Clonal expansion	JAK2V617F+ MPNs
TNFRII	Symptoms	MF
VCAM1	Symptoms	MF
VEGFb	Marrow fibrosis	PV, ET, PMF

ET: essential thrombocythemia; MF: myelofibrosis; MPN: myeloproliferative neoplasm; PMF: primary myelofibrosis; PV: primary myelofibrosis.
*Refer to Table 2 for definition.

The combination of cancer-related depression and fatigue also contributes to a sedentary lifestyle which further encourages a proinflammatory state that propagates symptoms. Multiple randomized controlled trials have demonstrated that cancer-related fatigue may be reduced through aerobic physical activity, potentially through modulation of cytokine production [37]. The mechanism has yet to be elucidated as intense physical activity has been shown to increase circulating levels of IL-6 which subsequently stimulates the production of other anti-inflammatory cytokines including IL-1RA and IL-10, and inhibit proinflammatory cytokines such as TNF-α. The MPN Fatigue Project is an international effort performed in collaboration with the *MPN Forum* aiming at evaluating the breadth and efficacy of current strategies targeting MPN fatigue [41]. The study remains ongoing and includes evaluation of treatments such as sleep deprivation, dietary supplements, and exercise.

4.2. Splenomegaly. Abdominal-related complaints are common among MPN patients, largely attributable to splenomegaly, portal hypertension, mechanical obstruction, and splenic infarcts. An independent source of morbidity and mortality complaints related to the abdomen has included early satiety (76%), abdominal pain (63%), abdominal discomfort (72%), and weight loss (48%) [3]. However, cytokines may also play an important role in the development of this symptom. Splenomegaly has been associated with expansion of the malignant clone from the bone marrow microenvironment to extramedullary sites including the spleen. TNF-α, in particular, promotes clonal expansion in JAK2V617F positive MPNs [42, 43]. The development of splenomegaly has also been associated with specific cytokines including MIG, HGF, and IL-1RA [23]. However, the mechanism involved in stimulation has yet to be established. Interestingly, JAK2/STAT3 signaling has been shown to promote fibrosis, angiogenesis, and inflammation in the setting of portal hypertension, independent of the presence of malignancy suggesting that regional inflammation also plays a role in abdominal pain, whether or not cancer is present [44]. Thrombosis may result in a variety of abdominal complaints, particularly with ET and PV. An evaluation of 244 consecutive PV and ET patients demonstrated that patients within the highest CRP protein tertile had the highest rate of major thrombotic events [28]. Similarly patients demonstrating the lowest pentraxin 3 levels had higher risks for major thrombotic events. Of interest, values of hs-CRP and PTX3 also correlated with JAK2V617F allele burden.

Abdominal pain may also be exacerbated by cytokine-induced nerve hyperstimulation, both peripherally and centrally. Animal studies have shown increased expression of TNF-α, IL-1, and IL-6 after nerve injury, cytokines all disproportionately high in the MPN population. In addition, inflammation and trauma have been shown to induce peripheral nerve cell release of inflammatory cytokines within the central nervous system via glial stimulation [45]. The effects of cytokine-mediated pain were demonstrated in a recent study of patients with painful neuropathies where it was observed that this population had twofold higher levels of IL-2 mRNA and TNF-α mRNA in comparison to healthy controls [46]. The direct impact these cytokines have on nerves within MPN populations has yet to be investigated.

4.3. Microvascular, Cognitive Symptoms and Pruritus. Microvascular complaints typically refer to those symptoms that result from disease activity occurring at a capillary level. In the MPNs, these symptoms may include headaches,

concentration problems, lightheadedness, dizziness, vertigo, numbness/tingling, and sexual dysfunction. Historically, neurocognitive disturbances have been attributed to cellular stasis and microthrombosis. Proinflammatory cytokine production is believed to be a contributor to cognitive impairment in cancer patients via the disruption of neurohormonal signaling and impaired creation of neurotransmitters. These neurotransmitters include serotonin, dopamine, and norepinephrine, all of which are critical to functions involving homeostasis of sleep, mood, and memory [47–49]. A recent analysis of patients treated with ruxolitinib in the COMFORT-II trials identified RANTES and Pal1 levels to correlate with the complaints of insomnia [50].

The role of inflammation in cognitive impairment has been intensely studied in both animal and human models. In an evaluation of IL-6 deficient animals, injection of lipopolysaccharide (LPS, shown to inhibit memory and learning in animals) failed to induce cognitive impairments suggesting IL-6 plays a key role in interrupting the process of memory and learning [49]. In human studies, elevated levels of IL-1, TNF-α, IL-6, and CRP were also linked to impaired memory and neurodegenerative disorders in the elderly [51–53]. Within hematological disorders, patients with elevated levels of IL-6 were found to have worsened executive function [36]. Interestingly, AML and MDS patients with higher levels of IL-8 were found to have improved memory. Pruritus has also been linked to the inflammatory cascade. A recent study of JAK2V617F transgenic mice demonstrated increased number of mast cells in those with the PV phenotype [54]. These mast cells represent a key source of prostaglandin, leukotriene, histamine, and tryptase, mediators of the inflammatory response with recognized ties to pruritus. A study evaluating symptoms of ruxolitinib treated patients within the COMFORT-II trial determined that baseline pruritus was associated with lower ferritin levels, a surrogate marker of inflammation [50]. Pruritus, most prominent in PV patients (65%), may be tied to an inflammatory cellular response as well. Basophils have been instigated as a primary mediator for symptom development and studies have demonstrated that the number of constitutively activated and hypersensitive circulating basophils is increased in PV, correlating with the degree of pruritus [55]. In addition, mast cells may play a role. Recent studies using infrared thermography have documented mast cell degranulation due to temperature shifts with the release of pyrogenic factors such as interleukins, histamine, and leukotrienes [56, 57].

4.4. Constitutional Symptoms. Fevers, night sweats, and weight loss are foundational symptoms in MPNs. Both fevers and night sweats are recognized to be partially cytokine driven, typically by IL-1, IL-2, IL-6, TNF-α, and IFN. Within other malignancies, IL-6 has been found to correlate with the presence of B symptoms which serve as a prognostic factor in Chronic Lymphocytic Leukemia (CLL) and Hodgkin's lymphoma [58–60].

The development of MPN-associated weight loss is complex and relates to a variety of factors including splenomegaly, portal hypertension, and cancer-cachexia. The role of a cytokine-driven proinflammatory state as the nidus for cancer-cachexia is well supported by literature [61, 62]. Defined as a process of dysregulated carbohydrate and fat metabolism with ongoing skeletal muscle breakdown, the presence of cancer-cachexia is linked to a dismal survival rate in comparison to cancer patients lacking this feature [63]. In cancer patients, TNF-α has been shown to induce proteolysis of skeletal muscle and furthermore enhance the expression of genes related to enzymes in the ubiquitin-dependent proteolytic pathway [64]. In a review of cytokine levels present in patients from the COMFORT-II trial, weight loss was associated with lower leptin levels and high CD40L was associated with loss of appetite. Whether these aberrant cytokines function to support cancer-cachexia or involve an alternative mechanism of action has yet to be investigated.

5. Improving Symptoms by Targeting Cytokines

Recognizing the substantial impact chronic inflammation has on the MPN symptom burden, attentions have turned to therapies demonstrating efficacy in reducing cytokines. As in other malignancies, improving physical activity and reducing fat intake may reduce inflammatory cytokines and improve survival [65]. In noncancer patients, increased physical activity has been shown to reduce TLR4 signaling and truncate release of inflammatory cytokines [66]. In obese subjects, proinflammatory cytokines have also been shown to be released by white fat which may be subsequently removed through physical activity. In addition to evaluating the effects of sedentary living on the MPN symptom burden, the final phases of the MPN Fatigue Project involve the development of comprehensive patient activity programs which may have subsequent impact on cytokine-induced symptomatology [41].

Recognizing the role constitutive Janus kinase signaling plays in inflammation, *JAK2* inhibition has become a rational target for preventing cytokine dysregulation. A recent study evaluated meaningful changes in cytokine expression following 24 months of ruxolitinib therapy in 63 high-risk MF patients [67]. Ruxolitinib was able to induce profound reductions in the expression of TNF-α and MIP-1α at both 4 weeks and 24 months. The expression of IgE was also strongly reduced in almost all patients with direct impact on the amount of activated anti-inflammatory macrophages. A similar study utilized the Functional Assessment of Cancer Therapy-Lymphoma (FACT-Lym) to evaluate symptoms of ruxolitinib treated patients within the COMFORT II trial and compare them to changes in cytokine levels [50]. Ten symptoms including fever, weight loss, fatigue, loss of appetite, pain, itching, sleeping well, lack of energy, night sweats, and trouble sleeping were assessed at baseline and weeks 8, 24, and 48. Treatment with ruxolitinib led to improved items of itching, night sweats, and weight loss with subsequent reduction in numerous cytokines. Loss of appetite improved over time and negatively correlated with decreases in IL-1RA levels in ruxolitinib treated patients.

The impact emerging *JAK2* inhibitors such as momelotinib and pacritinib will have on cytokines is of high interest

as they progress through clinical trials. Importantly, their limited hematological toxicities may be reflective of their selective inhibition of kinases. A recent investigation of pacritinib demonstrated that it selectively spares *JAK1* while inhibiting *JAK2*, JAK2V617F, FLT3, and IRAK1, an IL-1 receptor kinase associated with the inflammatory response and suppression of normal hematopoiesis [68]. Whether inhibition of IRAK1 is of clinical significance from a symptomatic standpoint has yet to be investigated. Other cellular signaling networks such as the PI3K-Akt-mTOR pathway impact cytokine development and represent novel targets for intervention. Similarly, as antifibrosing agents, hedgehog inhibitors, hypomethylating agents, histone deacetylation inhibitors, and HSP90 inhibitors enter the treatment landscape, knowledge of their impact on inflammation is of great interest.

6. Conclusion

Clear relationships exist between MPN symptoms and markers of inflammation. Though most data remains in early stages of investigation, knowledge gleaned from other malignancies has offered us potential mechanisms that explain these observed cytokine-symptom associations. A variety of MPN symptoms correlate with the presence of specific markers of inflammation. However, how these markers differ between MPN subtypes, change with disease progression, and relate to transformation remain unknown. The expanded access to targeted agents has provided a platform by which cytokine signals may be inhibited early within the cascade, limiting their potential for toxicity. It is with great anticipation that we venture into this uncharted territory of cytokine-symptom associations and explore novel therapies hosting high potential for symptomatic benefit.

Conflict of Interests

Geyer, Scherber, and Dueck have no conflict of interests to declare. Mesa works in consultancy for Novartis and in research for Incyte, Gilead, CTI, Genentech, Promedior, and NS Pharma.

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Review Article

Inflammation as a Driver of Clonal Evolution in Myeloproliferative Neoplasm

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Our understanding of inflammation's role in the pathogenesis of myeloproliferative neoplasm (MPN) is evolving. The impact of chronic inflammation, a characteristic feature of MPN, likely goes far beyond its role as a driver of constitutional symptoms. An inflammatory response to the neoplastic clone may be responsible for some pathologic aspects of MPN. Moreover, *JAK2V617F* mutated hematopoietic stem and progenitor cells are resistant to inflammation, and this gives the neoplastic clone a selective advantage allowing for its clonal expansion. Because inflammation plays a central role in MPN inflammation is a logical therapeutic target in MPN.

1. Introduction

Inflammation has a central role in myeloproliferative neoplasm (MPN). Excessive inflammation not only causes many of the debilitating symptoms associated with the disease but also drives the selective expansion of the neoplastic clone [1–3]. Inflammation may also be directly responsible for some pathologic features of the disease such as bone marrow fibrosis and anemia [4–9]. Below, I will provide evidence for increased inflammatory cytokine production in MPN, describe the effect of inflammation on both normal and neoplastic hematopoietic stem and progenitor cells, and propose a model whereby inflammatory insult upon a vulnerable hematopoietic stem cell pool drives the emergence of the MPN neoplastic clone.

2. What Causes Inflammation in MPN?

A deranged inflammatory cytokine profile is a shared characteristic of both humans with MPN as well as mouse MPN models. This suggests that the *JAK2V617F* clone is responsible for inflammation but exactly how the *JAK2V617F* clone induces inflammation is unclear. Excessive inflammatory cytokine production is not exclusive to the neoplastic cells in MPN. For example, the inflammatory cytokine tumor necrosis factor-alpha (TNF) is equally overproduced in both the *JAK2V617F* and the *JAK2WT* cells from MPN patients and in MPN mice (AF unpublished results). Moreover, mice injected with Ba/F3 cells expressing *JAK2V617F* have elevated production of inflammatory cytokines including TNF and IL-6, but the Ba/F3 *JAK2V617F* cells are not the source of these cytokines [10]. Ross Levine's group used single cell cytokine analysis to measure cytokine production in a mouse MPN model [11] and found that both neoplastic and nonneoplastic cells produce excessive inflammatory cytokines. They also used STAT3 knockout mice to investigate the role of STAT3 mediated cytokine production in the pathogenesis of MPN. In mice with pan-hematopoietic deletion of STAT3, the MPN phenotype was attenuated in a *MPLW515L* MPN model, but if STAT3 was deleted in the neoplastic cells but remained intact in the nonneoplastic cells, the disease phenotype remained robust [11]. Taken together, these data suggest that the MPN neoplastic clone may induce an inflammatory reaction by nonneoplastic host cells and that the inflammation mediated by these nonneoplastic cells plays a key role in MPN pathogenesis.

Inflammatory cells of both the innate and adaptive immune systems contribute to MPN pathology in mouse models, supporting the notion that some pathologic features

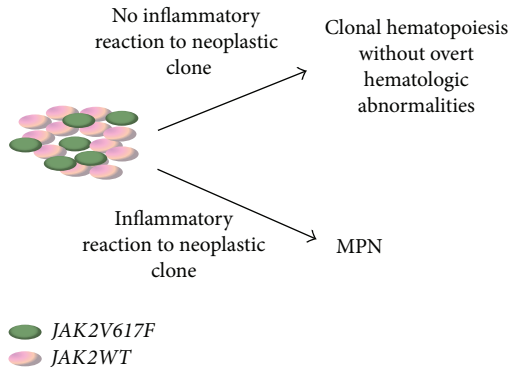

Figure 1: Putative role of inflammation in shaping MPN disease phenotype.

of MPN are caused by the host's reaction to the neoplastic clone rather than a direct consequence of the neoplastic clone itself. For example, erythrocytosis was attenuated in MPN mouse models deficient in macrophages [12, 13]. We have found that in a background devoid of T and B cells (RAG2$^{-/-}$) a *JAK2V617F* transduction-transplantation MPN model had no fibrosis, attenuated splenomegaly, and reduced leukocytosis yet erythrocytosis and excessive megakaryopoiesis were preserved (AF, unpublished results). These data demonstrate that each cellular subset of the immune system affects distinct aspects of MPN pathophysiology.

The nature or extent of the host's inflammatory response to a *JAK2V617F* clone could modulate the MPN phenotype (Figure 1). It is possible that an inflammatory response to the *JAK2V617F* clone is required to develop a clinically relevant MPN, and without this inflammatory response *JAK2V617F* results in clonal hematopoiesis without overt hematologic abnormalities. This would explain the observation of normal elderly individuals with a detectable *JAK2V617F* clone [14–16]. It is also possible that the nature of the host's inflammatory response to the clone shapes the resulting MPN phenotype that may help explain why this one mutation can lead to three distinct disease entities.

3. Inflammation as a Therapeutic Target in MPN

Targeting inflammation is a logical therapeutic approach in MPN. JAK inhibitors are not only used in MPN but are also utilized in autoimmune and inflammatory diseases [17] because JAK/STAT signalling is involved in the production of many inflammatory cytokines. Treatment of MPN patients with the JAK1/2 inhibitor ruxolitinib results in prompt resolution of constitutional symptoms concurrent with reduction in the inflammatory cytokines profile in plasma [18]. Ruxolitinib does not reduce *JAK2V617F* allele burden immediately, suggesting that its effect is not by direct targeting of the *JAK2V617F* neoplastic cells. Over time, however, there appears to be a decrease in the *JAK2V617F* allele burden [19] in patients treated with ruxolitinib.

It is possible that ruxolitinib's effect in MPN is via its anti-inflammatory properties and not necessarily through its inhibition of the mutant JAK2. Decreasing inflammation may reduce the selective advantage of the *JAK2V617F* clone and lead to its contraction, albeit slow. Inhibitors with primarily JAK1 inhibitory activity and little JAK2 inhibitory activity have been investigated in myelofibrosis (MF) to test this hypothesis [20]. Treatment of MF patients with a JAK1 inhibitor resulted in improvements in MF-related symptoms and a modest decrease in spleen size (abstract 714 ASH 2014) demonstrating that agents that selectively target JAK1 while sparing JAK2 do have activity in MF.

Perhaps chronic inflammation produces an environment that is highly selective for hematopoietic stem cell clones with MPN associated mutations such as *JAK2V617F*. How exactly MPN associated mutations may give hematopoietic stem cells a selective advantage in the setting of chronic inflammation is unknown, but the known consequences of chronic inflammation on hematopoietic stem cells provide us with some clues.

4. Impact of Chronic Inflammation on Hematopoietic Stem Cells

Chronic inflammation causes age-related hematopoietic stem cell (HSC) functional decline. The ability of hematopoietic stem cells to be quickly called into cycle is crucial for the rapid hematopoiesis required during times of stress, but HSC bombarded by chronic inflammation eventually exhausts. This HSC exhaustion is mediated by chronic cycling of HSC. In mouse model systems repeated exposures to inflammatory stimuli such as lipopolysaccharide (LPS) [21] negatively affected HSC. HSC function was also compromised in mice with a deletion of miR-146a, a micro-RNA whose role is to suppress inflammatory cytokine production. Aged miR-146a knock-out mice developed hematopoietic stem cell exhaustion, myeloproliferation, marrow fibrosis, and extramedullary hematopoiesis. This stem cell exhaustion and myeloproliferative phenotype could be induced in young miR-146a$^{-/-}$ mice by chronic bacterial exposure. This suggests that exposure to pathogens and the ensuing inflammatory response catalyzes HSC exhaustion. To further illustrate the role of inflammation in HSC exhaustion, a cross of the miR146a$^{-/-}$ mice onto a RAG1$^{-/-}$ background (which lacks T and B cells) rescued HSC from exhaustion. Together these data demonstrate that chronic inflammation leads to HSC exhaustion and promotes the development of myeloproliferation, marrow fibrosis, and extramedullary hematopoiesis.

5. Thrombopoietin/MPL Signaling Pathway in HSC Quiescence and Its Relevance to MPN

The thrombopoietin (TPO) signaling axis plays a central role in hematopoietic stem cell cycling [22]. The hematopoietic stem cell phenotype in mice deficient in TPO and its receptor MPL highlight the importance of this pathway for HSC maintenance. MPL signaling maintains HSC quiescence.

HSC from $TPO^{-/-}$ mice display accelerated cell-cycle kinetics and reduced transcriptional expression of negative cell-cycle regulators. This chronic cycling leads to HSC attrition, resulting in a 150-fold reduction of HSC in $TPO^{-/-}$ mice. Mutations which enhance MPL signaling result in superior hematopoietic stem cell reconstitution ability and self-renewal in mouse models. This includes mice deficient in negative regulators of MPL signaling such as Lnk [23] and MERIT-40 [24].

Activation of the MPL pathway is the common theme among mutations associated with MPN including *MPL*, *JAK2*, and *LNK*. It is currently unknown whether calreticulin mutations affect the MPL signaling pathway, but clues such as high expression of mutated calreticulin in MPN megakaryocytes [25] implicate a potential role for calreticulin in megakaryopoiesis. The observation that thrombopoietin mimetics induce reversible marrow fibrosis [26] demonstrates that excessive signaling through MPL is in itself capable of mediating bone marrow fibrosis.

Intact MPL signaling is required for development of *JAK2V617F* induced MPN in mouse models [27]. Hitchcock's group crossed *JAK2V617F* transgenic mice onto a $MPL^{-/-}$ background which resulted in thrombocytopenia and lessened neutrophilia compared to *JAK2V617F* transgenic mice on a *MPL* wild-type background. Crossing of *JAK2V617F* transgenic mice onto a thrombopoietin deficient ($TPO^{-/-}$) background however had a different effect. $TPO^{-/-}$ mice are thrombocytopenic, and expression of *JAK2V617F* restored platelets to a normal level. *JAK2V617F* mice on a $TPO^{-/-}$ background had larger spleens and increased numbers of megakaryocytes in the spleen compared to *JAK2V617F* transgenics on a wild-type background.

Spivak's group also found that a functional thrombopoietin receptor is required for development of polycythemia vera in a mouse transgenic model of PV (abstract 427, ASH 2012). In their hands, abrogation of TPO production ($TPO^{-/-}$) delayed erythrocytosis, decreased spleen size, and reversed fibrosis. They found that loss of functional MPL ameliorated PV, with a decreased hematocrit, decreased leukocytosis, decreased platelet count, and reversal of fibrosis. Taken together, these data demonstrate that activation of the Tpo/MPL signaling pathway is crucial for the MPN disease phenotype. It is also attempting to speculate that activation of the MPL signaling pathway by *JAK2V617F* may also affect HSC cycling.

6. *JAK2V617F* Downregulates MPL

Decreased expression of MPL on the cell surface of platelets, megakaryocytes, and $CD34^+$ cells is an established feature of polycythemia vera and myelofibrosis [28–30]. *JAK2V617F* directly downmodulates MPL [31]. Wild-type *JAK2* but not *JAK2V617F* renders MPL resistant to endoglycosidase H-mediated degradation which allows MPL to be recycled back to the cell surface after ligand binding. JAK2 wild-type cells will have high levels of MPL on their cell surface when exposed to high concentrations of TPO, leading to quiescence and apoptosis. In the context of *JAK2V617F*, MPL is internalized and ubiquitinated and undergoes proteasomal degradation. As a result, cell surface expression of MPL is much lower in cells with *JAK2V617F*, facilitating resistance to the quiescence/apoptosis that would normally be encountered after exposure to high levels of TPO and allowing these cells to continue to proliferate.

7. Reactive Oxygen Species as Mediators of DNA Damage and HSC Aging

HSC cycling induces ROS which results in DNA damage, and this accumulation of DNA damage leads to HSC aging [32]. Genetic defects that cause increased production of ROS result in premature HSC aging and are associated with bone marrow failure syndromes such as Fanconi Anemia (FA). In a mouse model of FA the bone marrow failure phenotype only becomes evident when mice are exposed to chronic inflammation [32]. This inflammation induced HSC exhaustion can be prevented in FA mice with the ROS scavenger N-acetyl cysteine (NAC) [33]. This demonstrates that chronic inflammation promotes HSC attrition by increasing ROS production and more importantly that protection of HSC from inflammatory stress may be of value therapeutically for those individuals susceptible to accelerated HSC aging.

Increased ROS is a feature of MPN patients and mouse models. *JAK2V617F* cells confer paracrine DNA damage to neighboring normal cells as well as to themselves through increased ROS. The increased ROS was found to be mediated by lipocalin-2 (Lcn2) which is overexpressed in *JAK2V617F* cells [34, 35]. Normal bystander hematopoietic cells exposed to Lcn2 demonstrated p53 pathway activation, increased apoptosis, and decreased cellular proliferation. *JAK2V617F* cells were resistant to Lcn2-induced growth suppression which conferred a relative growth advantage to *JAK2V617F* clones. Treatment of a mouse knock-in *JAK2V617F* model with NAC reduced erythrocytosis and splenomegaly [36] suggesting that accumulation of ROS may play a direct role in the pathogenesis of MPN and that therapeutics that reduce ROS could be of value in MPN.

8. *JAK2V617F* Cells Have a Selective Advantage in Inflammatory Environments

Chronic inflammation may promote the emergence of the *JAK2V617F* neoplastic clone because *JAK2V617F* mutated hematopoietic progenitor cells are resistant to inflammatory cues which suppress normal hematopoietic progenitors (Figure 2). MPN hematopoietic cells overproduce inflammatory cytokines including tumor necrosis factor-alpha (TNF). We have found that the *JAK2V617F* mutation confers upon hematopoietic progenitor cells high-level resistance to the suppressive effects of TNF [1]. Consequently, because the overproduction of TNF suppresses the replicative activity of the nonmutated progenitors while enhancing the replication of the *JAK2V617F* mutant progenitors, the overall influence of the mutation *in vivo* is to alter the coefficient of selection in favor of the *JAK2V617F* clone.

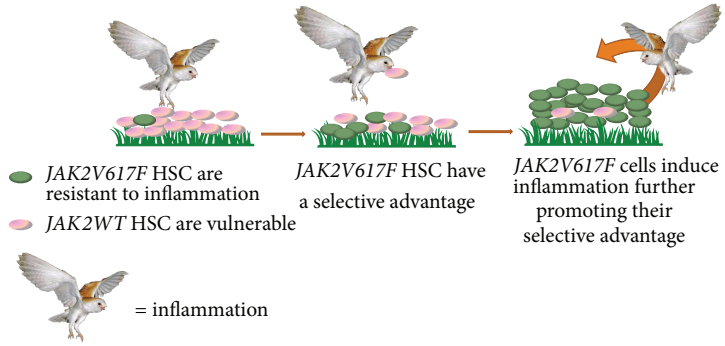

Figure 2: Model of inflammation as a driver of expansion of the *JAK2V617F* clone in MPN. *JAK2V617F*-mutated hematopoietic stem cells (HSC) are resistant to inflammation giving them a selective advantage in environments with high inflammation.

Neoplastic cells in MPN have been found to be resistant to other specific inflammatory cytokines. This includes IL-33, a member of the IL-1 family of cytokines, which has been implicated in many autoimmune diseases [37]. Transplantation of bone marrow from *JAK2V617F* transgenic mice into lethally irradiated IL-33$^{-/-}$ mice delayed the onset of MPN, demonstrating that IL-33 production by stromal cells promotes development of MPN. Increased IL-33-expressing cells were detected in bone marrow biopsies from MPN patients. Exogenous IL-33 promoted colony formation by primary CD34^{+} MPN stem/progenitor cells from patients and also improved the survival of *JAK2V617F* positive cell lines [38]. Together, these data demonstrate that IL-33 promotes the growth of *JAK2V617F* mutated cells.

9. Does Inflammation Precede the *JAK2V617F* Clone?

It is clear that concomitant inflammation supports MPN pathogenesis, but it is also possible that inflammation precedes the development of MPN and may be a predisposing factor to acquire MPN. A prior history of any autoimmune disease has been found to be associated with a significantly increased risk of MPN. Specifically, there is an increased risk of MPN with prior immune thrombocytopenia (OR = 2.9), Crohn's disease (1.8), polymyalgia rheumatic (1.7), giant cell arteritis (5.9), Reiter's syndrome (15.9), and aplastic anemia (7.8) [39]. Each of these autoimmune diseases is associated with substantial overproduction of inflammatory cytokines, including TNF [40, 41].

We are working to determine whether exaggerated production of inflammatory cytokines in response to immunogenic stimuli may be a predisposing factor to acquire MPN. We find that monocytes from MPN patients produce excessive amounts of TNF after stimulation through Toll-like receptors (TLR) and crucial pattern recognition receptors for microbial products (Abstract 4584, ASH 2014 annual meeting). This excessive production of TNF is due to a defect in the negative regulatory feedback loop which normally serves to dampen TNF production. Both mutant and nonmutant monocytes from MPN patients overproduce TNF, demonstrating the excessive cytokine production is not directly mediated by *JAK2V617F*.

10. Conclusion

MPN is a unique hematologic malignancy with a multifaceted and complex pathobiology. The unrestrained expansion of mature myeloid cells can be directly attributed to the constitutive activation of JAK2 by *JAK2V617F* but many of the other pathologic features of the disease such as marrow fibrosis, constitutional symptoms, and cytopenias could be due to the host's inflammatory reaction to the neoplastic clone. It is clear that inflammation plays a critical role in the pathogenesis of MPN but the details are still evolving. A more in-depth understanding of why inflammation is high in MPN and how the host's immune system responds to the neoplastic clone will likely reveal fundamental insights into MPN disease pathogenesis and will also identify novel therapeutic targets in this disease.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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Review Article

Circulating Cytokine Levels as Markers of Inflammation in Philadelphia Negative Myeloproliferative Neoplasms: Diagnostic and Prognostic Interest

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Cytokines are well known mediators of numerous physiological and pathological processes. They contribute to the regulation of normal hematopoiesis but increasing data suggest that they also have a clinical impact in some hematopoietic malignancies. In particular, there is evidence that cytokines are implicated in the functional symptoms of Philadelphia negative myeloproliferative neoplasms (Ph− MPNs), suggesting that evaluation of circulating levels of cytokines could be of clinical interest for the characterization of patients at the time of diagnosis and for disease prognosis. In this review, we present the current knowledge on alteration of circulating cytokine profiles in MPNs and their role in myelofibrosis pathogenesis. Phenotypic correlation, prognostic value of cytokines, and impact of JAK inhibitors are also discussed.

1. Cytokine Networks in Myeloproliferative Neoplasms

Cytokines are known to play essential roles in hematopoiesis such as the regulation of differentiation and production of progenitor cells and mature blood cells [1]. The knowledge of cytokine function has not only contributed to the development of supportive therapies (i.e., Erythropoietin (EPO)), but dysregulation of cytokines also argues in the diagnosis of some hematopoietic disorders. For example, one of the minor criteria of polycythemia vera (PV) according to WHO 2008 classification is the subnormal serum EPO level [2]. Recently, clinical trials with Janus kinase (JAK) inhibitors have confirmed the presence of aberrant production of inflammatory cytokines and highlighted their role in the pathophysiology of Philadelphia negative myeloproliferative neoplasms (Ph− MPNs). Indeed, clinical impact of JAK inhibitors on the functional symptoms and splenomegaly in patients were concomitant with a significant effect on the plasma levels of many cytokines [3, 4].

The first experimental data that showed elevations of serum and/or plasma cytokines in Ph− MPN date back to more than 15 years. In the 90s, changes in serum levels of interleukin (IL) such as IL6 [5, 6], IL2 and its soluble receptor [7], and of tumor necrosis factor (TNFα) [8] were already reported associated with disturbances of blood cell counts. The study of Hermouet et al. [9] in 2002 has expanded this panel, showing elevated serum levels of IL8 and IL11 in patients with PV, compared to healthy subjects. Elevated serum concentrations of IL11 and IL8 were observed in 30% and 100%, respectively, of the PV but not in controls. This high concentration of these two cytokines was also observed in the bone marrow plasma in 48% and 100% of PV patients, respectively, concerning IL11 and IL8. The authors have also shown that the stimulation of stromal cells with IL1β induced an increase in the production of these

two cytokines, suggesting that bone marrow stromal cells regulate IL11 and IL8 production. This study also described an elevation of IL8 both in sera and in bone marrow plasma among patients classified as idiopathic erythrocytosis (in the absence of endogenous erythroid colonies).

In 2005, Panteli et al. measured the serum levels of IL1α, IL1β, IL2, IL6, soluble IL2 receptor alpha (sIL2-Ra), and Thrombopoietin (TPO) in 25 primary myelofibrosis (PMF), 40 Essential Thrombocytemia (ET), and 8 PV in comparison with a group of 27 healthy subjects and a subgroup of 10 chronic myeloid leukemia (CML) patients [6]. The interest issue of this study was to show that all Ph− MPNs MPNs (PMF, ET, and PV) had significant increased serum levels of IL2 and its soluble receptor, compared to healthy subjects. The CML patients showed the same increases compared to the healthy subjects, but with significantly lower values than PMF. Similarly, PV and ET patients had significantly lower levels of IL2 compared to PMF ones. Overall, PMF patients displayed a gain of all cytokines measured in this study, with the exception of IL1α and IL1β, compared to healthy subjects and CML, PV, and ET patients. The profiles of ET and PV patients were relatively similar with no significant difference reported between these 2 subgroups, although the rate of IL2 and its receptor were higher in PV (but not significant). Concerning all the patients evaluated (Ph− MPN and CML), the authors did not find any significant increase of IL1α nor IL1β compared to healthy subjects.

Regarding TPO results, the authors found a significant increase in TPO serum compared to controls only for patients with PMF. ET and PV patients, despite moderately higher median levels, had no significant overexpression of TPO (versus controls), although high levels of TPO in ET have previously been reported [10, 11]. No difference between PV and ET could be demonstrated in this study. The moderate increase of TPO levels must be interpreted in view of the decreasing rates of EPO reported in several studies, in correlation with EPO independent growth of hematopoietic progenitors in MPNs. In particular, a multicenter study on a cohort of 116 PV reported a significant reduction in rates of EPO in 85% of patients compared to secondary polycythemia, confirming the interest of the diagnostic assessment of serum EPO in PV [12]. In the study of Panteli et al. [6], the observed changes do not suggest that the assay of TPO can serve as a diagnostic marker of ET. Indeed, increasing levels of TPO were not correlated to platelet count or bone marrow megakaryocyte to clumping.

In 2011, Tefferi et al. [13], using a multiplex assay using magnetic nanobeads coupled with flow cytometry, have assessed plasma levels of 30 cytokines including several growth factors such as granulocyte colony-stimulating factor (G-CSF), vascular endothelial growth factor (VEGF), and hepatocyte growth factor (HGF), in a cohort of 127 patients with PMF. The assay was compared to a control population comprising 35 healthy subjects. Firstly, this study confirmed the wide deregulation of cytokine expression described in PMF patients. In fact, 20 of the 30 cytokines tested in plasma showed significant differences, compared to healthy subjects. The authors approved the previously described increases of IL2, IL6, and IL8 but also found significant increases in IL10, IL12, IL13, IL15, TNFα, and interferon alpha (INFα). Contrary to previous results [6], the authors found elevated levels of IL1α and IL1β. This difference is probably due to the different techniques/antibodies used (conventional ELISA versus multiplex assay). In this inflammatory profile, additional deregulations of hematopoietic growth factors such as G-CSF, HGF, and VEGF were observed. Of the 127 patients included in this study, 90 patients had a blood sample taken at diagnosis before any treatment, showing that inflammatory conditions characterized by a cytokine overproduction play an integral part in the disease.

Using the same technology, Vaidya et al. were able to study the cytokine profiles of another cohort of 65 PV compared to the results obtained in their cohort of 127 PMF and 35 controls [14]. In this study, they showed that several plasma cytokines were abnormally expressed in PV compared to normal controls, but PV patients presented a different pattern to PMF patients. Compared to normal controls, PV patients demonstrated significantly higher levels of IL1RA, IL5, IL6, IL7, IL8, IL12, IL13, IFNγ, granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage inflammatory protein 1α and 1β (MIP-1α and MIP-1β), HGF, IFNγ inducible protein 10 (IP-10), monokine induced by IFNγ (MIG), monocyte chemotactic protein-1 (MCP-1), and VEGF. Conversely, levels of epidermal growth factor (EGF) and regulated on activation normal T-cell expressed and secreted (RANTES) were lower in PV compared to normal controls. Differences between PV and PMF were numerous. Levels of the following cytokines were significantly higher in PMF compared to PV: IL-1β, IL1RA, IL-2R, EGF, IL10, basic fibroblast growth factor (b-FGF), IL12, IFNα, and RANTES. In contrast, levels of IL7, IFNγ, GM-CSF, MIP-1α, IP-10, MIG, and VEGF were significantly higher in PV compared to PMF.

Using the same multiplex assay technology, these results were improved by Pourcelot et al., who studied the plasma concentrations of 13 cytokines in the plasma of 17 PV and added data on 21 ET [15]. This study firstly permitted to highlight a significant elevation of these 13 cytokines in PV and in ET. As in the study of Vaidya et al. [14], the authors found previously reported increases in IL6, IL8, IL12, IFNγ, GM-CSF, HGF, and VEGF in PV. Moreover, the study of Pourcelot et al. found a significant increase in plasma levels of IL4, IL10, MCP-1, TNFα (not significant in the study of Vaidya et al.), and platelet derived growth factor (PDGF-BB) (not determined in the study of Vaidya et al.). Interestingly, the authors showed that PV and ET patients differ by their plasma cytokine profiles. ET patients had higher levels of IL4, IL8, GM-CSF, IFNγ, MCP-1, PDGF, and VEGF compared to PV. It is interesting to note that cytokines evaluated in this study were higher in ET patients than in PV.

All these studies confirmed the existence of an inflammatory context associated with MPNs; Gangemi et al. [16] have focused on the evaluation of IL22, IL23, and IL10 circulating cytokines which are considered as markers for the activation of T helper lymphocytes. This study showed a significant elevation of IL23 in PV patients compared to controls. However, ET patients did not show any changes in these 3 cytokines compared with controls and no difference between PV and ET could be demonstrated.

Table 1: Circulating cytokine expression in myeloproliferative neoplasms compared to healthy controls.

Types of cytokines	All MPNs	Essential thrombocythemia	Polycythemia vera	Primary myelofibrosis
Hematopoietic growth factors	↑ TPO [6, 10] NS IL6 [6, 8]	NS TPO [6] NS IL6 [6]	NS TPO [6] ↑ or NS IL6 [6, 14, 45] NS G-CSF [14] ↑ GM-CSF [14] ↑ IL5 [13, 14] ↑ IL7 [14] ↑ IL11 [9, 45]	↑ TPO [6] ↑ IL6 [6, 13] ↑ G-CSF [13]
Chemokines	ND	ND	↑ IL8 [9, 14, 45] ↑ IP-10 [14] ↑ MCP1 [14, 45] ↑ MIP1α [14] ↑ MIP1β [14] ↑ MIG [14] ↑ RANTES [14] NS EOTAXIN [14]	↑ IL8 [13] ↑ IP-10 [13] ↑ MCP1 [13] ↑ MIP1α [13] ↑ MIP1β [13] ↑ MIG [13]
Anti-inflammatory cytokines	↑ IL2 [6, 8] NS IL10 [8, 16]	↑ IL2 [6, 8, 14]	↑ or NS IL2 [6, 8, 14] ↑ IL1-Ra [14] NS IL10 [14] ↑ IL13 [14] ↑ HGF [14, 45] NS IL4 [14]	↑ IL2 [6, 8] ↑ IL1-Ra [13] ↑ IL10 [13] ↑ IL13 [13] ↑ HGF [13]
Proinflammatory cytokines	↑ sIL2-Ra [6, 8] ↑ TNFα [8] ↑ IL23 [16]	↑ sIL2-Ra [6, 8] NS IL23	↑ or NS sIL2-Ra [6, 8, 14] NS TNFα [14] ↑ IL23 [16] NS IL1β [14] ↑ IL12 [14] ↑ IL15 [14] ↑ IFNγ [14]	↑ sIL2-Ra [6, 8, 13] ↑ TNFα [13] ↑ IL1β [13] ↑ IL12 [13] ↑ IL15 [13] ↓ IFNγ [13]
Angiogenesis	ND	ND	↑ VEGF [14, 39] ↓ EGF [14] NS b-FGF [14]	↑ VEGF [13, 39]
Others	NS IL22 [16]	ND	NS INFα [14] ↑ Leptin [45]	↑ INFα [13] ND

This table summarizes cytokine expression possibly used as biomarkers in all myeloproliferative neoplasms (MPNs), essential thrombocythemia, polycythemia vera, and primary myelofibrosis compared to healthy donors. Because of the pleiotropic function of cytokines, they were arbitrarily classified according to proinflammatory, anti-inflammatory, hematopoietic growth factors, angiogenesis factors, chemokines, and others. References are reported in brackets. ↑ or ↓ means, respectively, increase and decrease of cytokine levels compared to healthy donors; NS means nonsignificant; ND means nondetermined.

From all of these studies, several comments emerge: (i) Ph– MPNs (PV, ET, and PMF) are all characterized by a significant change in the cytokine production objectified by increased plasma levels of many inflammatory cytokines (i.e., IL1, IL2, IL6, IL8, IL12, TNFα, and IFNγ), several growth factors (e.g., GM-CSF, G-CSF, HGF, PDGF, and EGF), and angiogenic factors (i.e., VEGF) (Table 1); (ii) deregulations also concern anti-inflammatory cytokines such as IL4 and IL10; (iii) there are differences in levels and cytokine profiles among MPNs but no particular continuum between these diseases could be objectified (Figure 1). Some cytokines are overexpressed in PMF versus PV (i.e., IL1β, IL1RA, IL2-Ra, EGF, and IL10). Conversely, some are overexpressed in PV versus PMF (i.e., IL7, IFNγ, GM-CSF, MIP-1α, IP-10, and MIG) and, finally, ET also have higher rates than PV concerning IL6, IL8, IL12, IFNγ, GM-CSF, and HGF; (iv) on a technical level, in the absence of standardization of methods between different studies, it is difficult to compare these results to each other; (v) the investigation of a large cohort of MFP, PV, and ET using the same technology would clarify these differences and allows to better define the existence of specific profile of each disease; (vi) interpretation of cytokine levels should take into account other factors that may limit the ability to use cytokines in everyday practice. For example, modifications of the immune system occur with age. Consequently, in healthy patients, age was shown to increase the measurement of IL6 and interferon-gamma inducible chemokines (MIG and IP-10) and conversely to decrease IL2, EGF, and EGFR measurements [17, 18].

2. Megakaryocytic and Granulocytic Cytokine Production in Myelofibrosis

In the normal bone marrow, the stroma cells comprise fibrocytes/fibroblasts, endothelial cells, osteocytes/osteoblasts as well as osteoclasts. Fibrosis is the result of collagen production by fibroblasts and its deposition in the extracellular

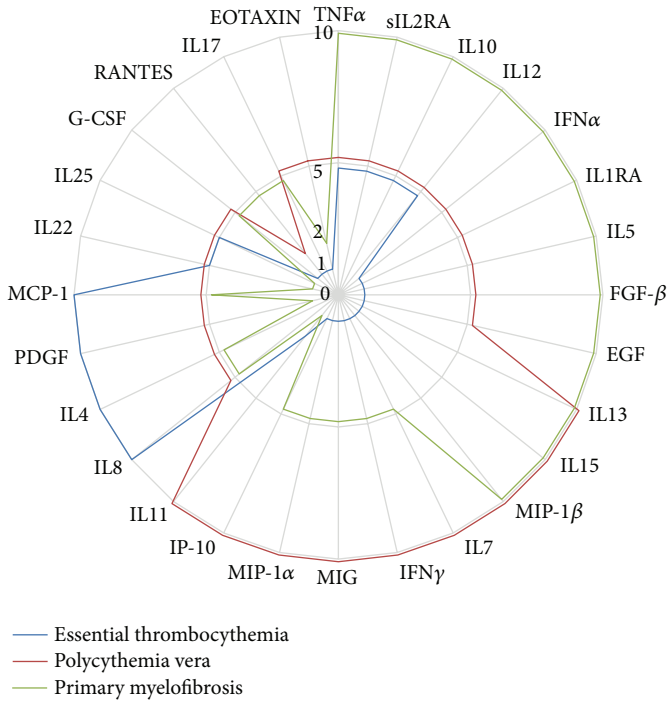

Figure 1: Radar graph of relative cytokine expression profiles according to MPN subtypes. Data were analysed from 6 studies [6, 13–16, 45]. For each myeloproliferative neoplasm, arbitrary scores were attributed to different cytokines according to their relative variations: 10 corresponds to an overexpression of cytokines compared to one or both MPNs; 2 corresponds to underexpression compared to one or both MPNs; 1 was attributed where no data was found in literature; 5 represents intermediary cytokine level. In cases of discordances between several studies, data are not added in this graph.

space and in parallel scar tissue formation [19]. In general, megakaryocytic clustering can be regarded as surrogate for increased megakaryocytic proliferation. In MPNs, clonal (mutated) neutrophilic or erythroid cells are morphologically indistinguishable from their polyclonal counterparts but the increase in bone marrow granulocytic progenitors and neutrophils is the morphological surrogate for aberrantly increased proliferation. Erythropoiesis is usually not increased in PMF and patients can have normal hemoglobin values or anemia [20]. The shape of the megakaryocytic nuclei and the emphasis of granulocytic proliferation are matters of dogmatic debate, in particular cloud-like (PMF) versus staghorn-like (ET) megakaryocytic nuclei [21–23].

The fundamental question is, why do fibroblasts start to produce more fibers and why are these fibers not degraded? All bone marrow cells (hematopoietic and nonhematopoietic) communicate with each other via direct cell-cell contact and via cytokine-receptor signaling. In his editorial article "Some Speculations on the Myeloproliferative Syndromes," William Dameshek raised the hypothesis that hormonal signaling factors ("myelostimulatory factors") may lead to these myeloid diseases [24]. Nowadays, although we have found clonal markers, aberrant expression of cell signaling molecules, and regulatory microRNAs [25–27], we still speculate on the causes of these diseases and in particular why progressive and prognostic adverse myelofibrosis develops in these patients.

The fact that prefibrotic PMF has similar megakaryocytic atypia as fibrotic-stage PMF and that megakaryoblastic acute myeloid leukemia (AML) is frequently associated with fibrosis make it likely that megakaryocytes could be the neoplastic cell subtype which predominantly forces fibroblasts to produce fibers. In contrast to ET megakaryocytes, PMF megakaryocytes form a more dense net of proplatelets within the bone marrow [28]. Therefore it is possible that increased proplatelet depositions lead to increased intramedullar cytokine release. Two central fibrosis-related cytokine/receptor pairs in MPNs are PDGF and its receptor (PDGFRA) and transforming growth factor beta 1 (TGFβ1) and the TGF type II receptor (TGFBR2) [29–31]. However, the link between the production of PDGF and TGFβ1 and MPN-associated mutations remains unclear. The *JAK2V617F* mutation does not directly result in fibrosis although it was observed that, in PMF, the mutant allele frequency is high [32]. In fact, *JAK2V617F* is mutated in 50–60% of PMF (including MF0-3) as well as 50–60% of ET, but in almost all cases of PV [25, 32]. *JAK2V617F*, through its association with PDGFRA and TGFBR2, may contribute to enhance their signaling, mimicking the action of their ligands. Currently, there is no known mutation which directly leads to myelofibrosis. It is more likely a progressive and long-lasting shift of the cytokine microenvironment towards fibrosis rather than one single genetic trigger. Several matrix modulating factors are increased, in particular thrombospondins (THBS) 1 and

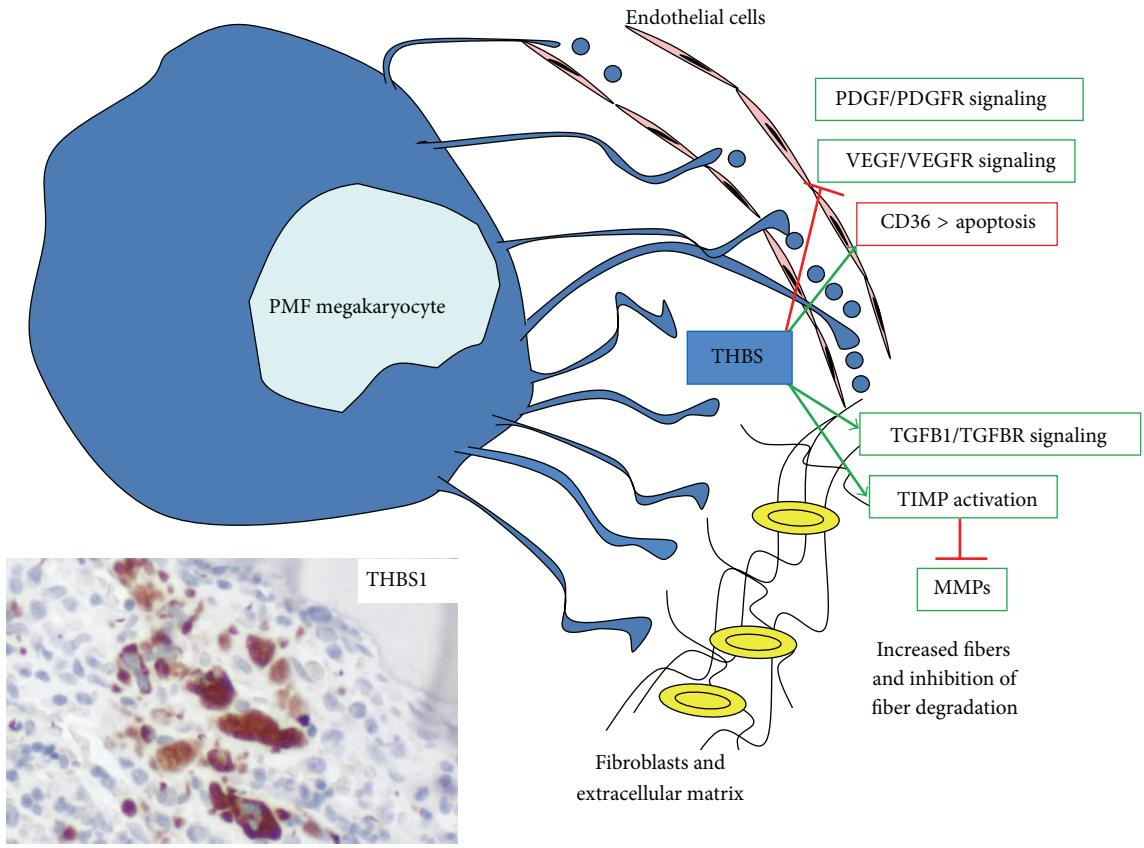

Figure 2: Role of THBS secreted by PMF megakaryocytes in angiogenesis and myelofibrosis development. Megakaryocytic THBS can lead to fibrosis via activation of TGFβ1 signaling and TIMPs but simultaneously inhibits angiogenesis via its receptor CD36 and inhibition of VEGF signaling. Nevertheless, myelofibrosis is associated with increased vascularization which could be mediated by PDGF signaling. The immunohistochemical image shows strong THBS expression in clustered PMF megakaryocytes and in small proplatelet depositions *in situ*.

2 and matrix metalloproteinases (MMPs) such as MMP14 which are produced by megakaryocytes (Figure 2) [33, 34]. MMPs degrade fibers while tissue inhibitors of metalloproteinase (TIMP) contribute to fiber accumulation but, in the most advanced stage PMF, TIMPs are not increased [34]. Therefore, increased expression of MMP14 could reflect a higher turnover of the extracellular matrix. Profibrogenic and simultaneously antiangiogenic THBS are matricellular factors which are not involved in structuring the extracellular matrix, but which regulate other factors of the extracellular matrix [35].

Fibrosis is not an isolated change but is usually accompanied by increased vascularization. It is thought that angiogenesis plays a critical role in the pathogenesis of PMF. Increased THBS acts ineffectively against exaggerated angiogenesis (e.g., mediated by increased PDGFRA levels) [29, 36]. Data from bone marrow histopathology suggest an increase in the microvessel density (MVD) and VEGF. Boveri et al. reported that PMF patients were characterized by a significant increase of MVD, particularly microvessels assessed by CD105 expression, compared to PV, ET, and controls [37]. Interestingly, they showed that prefibrotic PMF could be differentiated from ET by MVD. Similarly, post-PV and post-ET myelofibrosis harbored significantly higher numbers of microvessels compared to PV and ET, respectively. In parallel to the increasing density of microvessels, a significant increase expression of VEGF has been observed in PMF patients compared to ET, PV, and MDS/MPN [38]. Gianelli et al. confirmed high levels of VEGF expression in PMF compared to ET or controls but failed to objectify differences between PMF and PV [39]. Expression of VEGF receptor (VEGFR-1 and VEGFR-2) seems less specific. Both receptors were weakly expressed, mainly in megakaryopoietic and erythropoietic progenitors, with heterogeneous intensity [38]. MVD have been shown to correlate with a high *JAK2V617F* allele burden (≥55%) in mutated patients suggesting that angiogenesis may be influenced by allele burden in these patients, keeping in mind that about half of PMF are JAK2 wild type which clearly indicate that other factors (yet unknown mutations or aberrant cytokine expression) mediate microvessel proliferation.

3. Phenotypic Correlation and Prognostic Value of Circulating Levels of Cytokines

3.1. Correlation with Blood Cell Counts. One of the first issues is the existence of a correlation between the level of circulating cytokines and the intensity of hematopoietic production and/or the existence of specific cytokine overproduction

correlated to a cell type overproduction. The study of Tefferi et al. [13] showed, in PMF, the existence of a correlation between the rate of sIL2-Ra, HGF and IP-10 (only sIL2-Ra in multivariate analysis), and leukocytosis. In PV, correlation of IL-1β, IL2, IL7, b-FGF, and HGF with leukocytosis has been described [14]. More precisely, Pourcelot et al. reported a correlation between IL6, TNFα, and the number of lymphocytes in PV, and a correlation between HGF, IL6, IL12, GM-CSF, and VEGF with the numbers of neutrophils in ET [15]. Regarding erythrocyte production, significant correlations were reported in PV between IL12 and hematocrit (Ht) [14], TNFα, and Ht [15] and between IL4 or MCP-1 and hemoglobin (Hb) [15]. Moreover, Pourcelot et al. found in ET a correlation between PDGF-BB and red cell counts. These results suggest a relative specificity of the plasma levels of some cytokines with the deregulation of the red cell mass. Regarding the platelet count, no cytokine plasma levels were correlated with platelet count in ET patients in the literature. However, Vaidya et al. in 2012 reported a significant reduction of INFα and γ in patients with a platelet number greater than 450, and a significant increase of IL6, capable of regulating the platelet count, has been reported for ET patients in several studies.

The difficulty in interpretation of these data is related to the fact that the mechanisms of cytokine production can be multiple (bone marrow stroma cells, tumor cells, and extra-hematopoietic cells) and the plasma cytokine changes may also be linked to chronic inflammation associated with MPNs and therefore not only reflect myeloproliferation.

3.2. Correlation with JAK2V617F Status. Surprisingly, there are few data on cytokine plasma levels correlated to *JAK2V617F* status. In their study on PMF, Tefferi et al. found a correlation in multivariate analysis between the presence of the mutation and IL1RA, IP-10, and IL2-Ra rates [13]. The study of Pourcelot et al. showed a correlation between the presence of the mutation and the plasma concentration of TNFα and PDGF-BB in PV and ET, respectively [15].

The impact of the *JAK2V617F* mutation on cytokine secretion seems to be restricted, suggesting that the regulation of cytokine production is done by both phenomena *JAK2V617F*-driven and not driven. On the other hand, cytokines which appear *JAK2V617F*-driven differ between PMF, PV, and ET. This suggests that there is a difference in the cytokine impact of the mutation according to the pathology or conversely that these differences of cytokine profiles could contribute to the phenotypic differences within the JAK2 mutated MPN. Indeed, when comparing *JAK2V617F* mutated ET and PV patients, significant differences in the expression of some cytokines such as IL4, IL8, IFNγ, and PDGF-BB have been demonstrated [15]. To summarize, IL4 rates were also correlated with Ht in this study. Differences could be related to the intensity of the allelic load. For example, TNFα levels were reported previously to be correlated with *JAK2V67F* allelic burden. This may explain why in PV patients who express the highest levels of JAK2 mutated, a correlation between TNFα and the presence of the mutation was observed. In this study, TNFα levels were also correlated with Ht. This suggests in PV a direct link between plasma levels of TNFα, *JAK2V617F* allelic burden, and increased red cell mass.

The increase of PDGF in ET patients and its correlation with the presence of *JAK2V617F* mutation probably reflect the impact of the mutation on the regulation of megakaryopoiesis via TPO and the deregulation of this pathway in mutated patients. This deregulation could induce an upregulation of the synthesis of PDGF by megakaryocytes. This suggests that, in ET patients, PDGF assay may be a functional marker of *JAK2V617F* allelic load and indirectly a marker of JAK2 activation level. Thus, the plasma level of PDGF could identify ET patients for whom a JAK2 inhibitor therapy would be the most fruitful. To our knowledge, no studies have until now described any correlation between *CALR* or *MPL* mutations and circulating cytokines.

3.3. Correlation with the Clinical Course of Patients (Table 2). All MPNs are capable of inducing myelofibrosis or transforming into acute leukemia. Fibrosis progression during the chronic phase of myeloid neoplasms is regarded as an indirect surrogate of the aggressiveness of the clonal disease [20]. The mechanisms which lead to myelofibrosis, primary or secondary, are still an enigma. It is likely that in bone marrow cytokines secreted by MPN cells, in particular megakaryocytes, could induce activation of fibroblasts and endothelial cells. Moreover, circulating cytokines (not originating from bone marrow) could by themselves influence the bone marrow microenvironment and thereby contribute to the development of myelofibrosis. Hence, circulating cytokines represent an interesting opportunity of simple, accessible, and easily measurable biomarkers for the evaluation of the disease at diagnosis (in addition to usual genetic and clinical markers), but also the determination of prognosis.

In Tefferi et al. [13], the follow-up of patients naive to treatment assessed the prognostic value of some of these biomarkers. In particular, the rate of IL8, IL10, IL12, and IL15 and sIL2-Ra levels were independent predictors of low survival, correlated with DIPSS categories. The prognostic value of these biomarkers was confirmed retrospectively on 127 patients, including those who received a therapeutic treatment, proving their clinical interests. Plasma levels of IL8 and sIL2-Ra allowed a prognostic classification of patients as they showed an increase of one or two of these cytokines. Patients with elevation of at least one of these markers displayed a significantly decreased survival among both treatment-naive patients and those who had already received therapy at the time of cytokine explorations. Moreover, the study of distribution of patients according to their prognosis has shown a concomitant increase in the frequency of patients with elevation of one or two cytokines and the severity of the pathology. Thus, there were more frequently patients with an increase in one of these two markers within intermediate risk groups 1 and 2 (classified according to DIPSS plus). In addition, patients with 2 elevated markers were only found in risk group 2 patients.

In PV and ET, the study of Gangemi et al. found a link between increased levels of IL2 and its soluble receptor

Table 2: Cytokines with prognostic implications.

	Cytokines involved		
	Primary myelofibrosis	Essential thrombocythemia	Polycythemia vera
Low survival High DIPSS categories	↑ IL8 and/or sIL2Ra [13] ↑ IL10 [13] ↑ IL12 [13] ↑ IL15 [13]	ND	MIP-1β [13]
Progression to myelofibrosis	—	↑ IL2 [16] ↑ IL2ra [16]	↑ IL2 [16] ↑ IL2ra [16]
Leukemic transformation	↑ IL8 and/or sIL2Ra [13] ↑ IL2 and sIL2Ra [6, 8, 16] ↑ IL6 [6]	ND	ND
Vascular events	ND	ND	↑ IL12 [15] ↑ GM-CSF [15]

This table represents prognostic values of cytokines described in PV, ET, and PMF. ↑ means increase in cytokine level; ND means not described to our knowledge.

with progression to myelofibrosis [16]. Furthermore, the prognostic value of high levels of 13 cytokines significantly associated with a lower survival in PV patients has also been reported [13]. In univariate analysis, fibrotic transformation was significantly associated with high levels of the following cytokines: IL-1β, IL5, IL6, IL10, IL12, IL15, IL17, and IP-10. However, in multivariate analysis, only MIP-1β remained significant even when age and leukocytosis were added as covariates.

The risk of transformation in acute leukemia remains very difficult to predict. The ability to predict the evolution towards this serious complication by a circulating marker would allow early therapeutic management of these patients with a very poor prognosis. Tefferi et al. showed that PMF patients who evolved into acute leukemia had elevated IL8 and sIL2-Ra levels [13]. In particular, the elevation of IL8 levels was significantly correlated with decrease in leukemia-free survival and an increase in the incidence of transformation in acute leukemia. The predictive value of IL2 rate and its soluble receptor was also highlighted in the study of Gangemi in PMF patients progressing into acute leukemia [16]. The prognostic value of plasma levels of IL8 and sIL2-Ra has been reported in other hematological tumors [40, 41] or solid cancer (head, neck, and esophagus) [42]. To our knowledge, there is no data in the literature on predictive cytokine markers of leukemic evolution concerning PV or ET.

3.4. Correlation with Vascular Events. Assessment of vascular risk, particularly thrombosis, is a key element in the therapeutic management of patients for prescription of cytoreductive and antithrombotic treatments. This assessment of vascular risk is important because it could cause inappropriate exposure of patients to potentially leukemogenic drugs. This assessment is still based on indirect criteria such as age, a history of stroke, or the presence of vascular risk factors. The existence of an inflammatory context in thrombotic events has long been demonstrated, suggesting the importance of the evaluation of inflammatory cytokines in the evaluation of thrombotic risk. However, little is known about the existence of cytokine dysregulation associated with thromboembolic events in MPN and their potential predictive values.

In the study of Pourcelot et al., data on vascular events were available for 32 patients. They could therefore compare cytokine profiles in patients with or without vascular complications. Comparison of both subgroups did not show significant statistical difference for age, JAK2 mutational status, and biological parameters (leukocytes, platelets, numbers of neutrophils and lymphocytes, red cells, Hb, and Ht). Except for IL12 (p70) which was increased in patients with vascular complications, there were no significant differences in other cytokine levels between patients with or without vascular complications [15]. Comparison of vascular complications within PV and ET revealed a significant difference of IL12 and GM-CSF in the PV subgroup. Both parameters were increased in PV patients without complications. No significant difference was observed concerning ET patients.

The decrease of IL12 has been previously reported in patients presenting a thrombotic event without any MPN diagnosis [43] suggesting that this cytokine is a specific marker of the occurrence of thromboembolic events independent of MPN pathogenesis. However, these results must be interpreted with caution since some thrombotic events occurred before diagnosis, and therefore the cytokine evaluation was done after the accident. This may explain the difficulty in highlighting cytokine alterations predictive of thrombotic risk. It would be necessary to assess changes in cytokine levels at multiple times, before and after thrombosis, to identify more specifically predictive biomarkers of vascular complications.

A study of Barbui et al. has focused on the interest of C-reactive protein (CRP) and pentraxin 3 (PTX3) as markers of thrombotic risk [44]. In this study of 244 ET and PV patients, a difference in prognostic value was observed between these 2 markers. High thrombosis risk patients were characterized by a significant increase (>3rd percentile) of CRP. Conversely, these patients had significantly decreased rates of PTX3. Prognostic stratification based on serum levels of these two inflammation markers has shown that patients with high CRP and low PTX3 levels had a significantly higher risk of

thrombotic stroke (OR = 2.66, $P = 0.045$). In addition, the levels of these two markers were correlated with the mutational status of patients and with an allelic load greater than 50%.

4. Impact of JAK Inhibitors on Levels of Cytokines

In this review, we highlight the usefulness of cytokines as potent markers of prognosis. But in which way do treatments especially JAK inhibitors modify cytokines and cytokine levels? Treatment with ruxolitinib, the first JAK inhibitors approved in myelofibrosis, leads to a rapid and sustained downregulation of cytokine levels in myelofibrosis patients [3]. Another proof of the action of ruxolitinib on cytokine levels is the withdrawal syndrome consistent with cytokine storm observed after its discontinuation [46]. Independently of JAK status or of MPN subtype (myelofibrosis or PV), several cytokines were reduced after ruxolitinib treatment such as IL1Ra, IL6, IL8, TNFα, and bFGF [3, 4]. Only EPO and leptin were increased after ruxolitinib treatment. Reduction of IL16, IL18, VEGF, and MIP-1β was also reported more specifically in myelofibrosis and reduction of sIL2RA, sIL6R in PV. Moreover, a correlation between symptomatic reduction of the spleen size in myelofibrosis and reductions of IL-1ra, MIP-1β, IL6, and TNFα was observed [3]. Data reported on mouse models and on supernatants of *in vitro* cultures of mononuclear cells confirmed reductions of IL6 and TNFα after JAK1/2 inhibitors [47, 48]. Kleppe et al. showed that ruxolitinib treatment normalizes cytokine levels in mice transplanted with *JAK2V617F*-mutant as well as those transplanted with MPLW515L-mutant cells [49]. Beyond MPN, there is a rising interest in JAK inhibitors for other disorders such as autoimmune diseases, solid cancers, or other hematopoietic malignancies [50–52]. Reduction of cytokines was also observed in experimental models of those disorders [53, 54].

Nowadays, the only JAK inhibitor approved for the treatment of primary and secondary myelofibrosis is ruxolitinib, which inhibits not only JAK2 but also JAK1. Nevertheless, more selective JAK2 inhibitors (i.e., fedratinib, lestaurtinib, pacritinib) are in clinical development and differences between selective JAK2 inhibitors and JAK1/JAK2 inhibitors could be observed. On one hand, more selective JAK2 inhibitors appear to have a less pronounced anticytokine effect and, on the other hand, they induce a more pronounced antierythropoiesis effect [55]. For example, no consistent changes in levels of proinflammatory cytokines (IL6, IL2, IL8, and TNFα) relative to baseline were observed during the course of fedratinib treatment; however a rapid and durable improvement of symptoms concomitantly with an impact on *JAK2V617F* allele burden was induced [56]. Similarly, *Santos et al.* studied effects of lestaurtinib (CEP701), a selective JAK2 inhibitor, on the levels of 19 cytokines (IL-1β, IL-1Ra, IL2, IL6, IL8, IL9, IL10, IL12, IL13, IL15, bFGF, GM-CSF, IFNγ, IP-10, MIP-1α, MIP-1β, RANTES, TNF-α, and VEGF) [57]. In the same way, no significant change between baseline and treatment was noticed even in responders. In contrast to selective JAK2 inhibitors, momelotinib (CYT387), a JAK1/JAK2 inhibitor, normalized inflammatory cytokines in *JAK2V617F*-transduced mice [58].

Even if JAK inhibitors affect cytokine levels, recent studies suggest that cytokine regulation by JAK inhibitors is not enough by itself to fully abort this aberrant inflammatory cytokine production. Keohane et al. showed in myelofibrosis, PV, or ET patients receiving either ruxolitinib or fedratinib a significant decrease of cytokines after the first month (IFNα, IFNγ, IL10, IL2R, IL4, and IL17) but a weak rise in cytokine levels after six months [48]. This fact argues a possible therapeutic failure but also supports the interest for drug associations in MPN treatment targeting other molecular pathways implicated in inflammatory response. Moreover, the role of tumor microenvironment in hematopoietic neoplasms development is essential; not only malignant cells but also nonmalignant cells induce cytokine dysregulation [59]. Altogether, those studies suggest the interest of synergistic associations of JAK inhibitors with others drugs to normalize aberrant cytokine production in MPN.

5. Conclusion

The cytokine profiles of MPN patients involving deregulation of proinflammatory and anti-inflammatory cytokines as well as growth factors suggest that the impact of these deregulations is involved in hematological but also extra-hematological manifestations of these pathologies. These deregulations confirm the existence of an inflammatory reaction in MPNs that may contribute to the initiation and progression of the disease. In this context, circulating cytokine levels could be useful markers of MPNs for their characterization at diagnosis but could also be interesting in prognostic evaluation of these patients. Moreover, the impact of JAK2 inhibitors on plasma concentrations of inflammatory cytokines suggests that circulating cytokine assays could be useful to monitor therapeutic efficacy of these molecules. Long-term treatment with JAK2 inhibitors may also raise the question of patient compliance with their treatment. As a result, the significant reduction of cytokines by mechanisms of inhibition of JAK2, and most likely JAK1, could serve as an indirect marker for evaluation of therapeutic compliance in case of absence or inadequate therapeutic response.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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