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The Microenvironment in Myeloproliferative Neoplasms

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Introduction

Myeloproliferative neoplasms (MPN) are a classic example of a group of diseases in which inflammation and the neoplastic clone are so intimately entwined it is difficult to ascertain which is the “chicken” and which is the “egg”. Myeloproliferative neoplasms are typified by a chronic inflammatory milieu that provides a permissive microenvironment for disease progression and severity. Inflammatory signaling involving the malignant and non-malignant cells contribute to the MPN symptom burden, thrombotic risk and disease evolution and transformation to acute myeloid leukemia. Chronic inflammation is characterized by elevated levels of circulating inflammatory cytokines and chemokines while inflammatory cells and soluble mediators of inflammation constitute the inflammatory microenvironment in the MPNs. In addition to the presence of MPN driver mutations, disease heterogeneity suggests that host factors likely shape the pathologic consequences of the presence of the MPN neoplastic clone.

Plasma/serum measurements of various cytokines and other soluble proteins reflect inflammatory processes and could serve as noninvasive diagnostic or prognostic tools for predicting disease evolution in patients with MPNS [1]. Considerable progress has been made on better understanding the genetic basis of the MPNs since the discovery of major driver somatic mutations in Janus kinase 2 (*JAK2*)[2–5], calreticulin (*CALR*)[6, 7] and myeloproliferative leukemia virus oncogene (*MPL*)[8–10]. Despite this, mutation targeted, and selective MPN therapies have been slow to exploit this knowledge and remain challenging. One reason for this is significant disease heterogeneity due to the effect of an altered

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microenvironment on disease pathogenesis. Thus, applying broader approaches towards identifying novel biomarkers for disease monitoring and combined therapies will hopefully lead to better outcomes.

This review will discuss the role of an inflammatory microenvironment as a driver of clonal evolution in the MPNs, cytokine production in the MPNs, use of inflammatory cytokines as diagnostic and prognostic tools, and the use of the inflammatory microenvironment as a therapeutic targets.

Chronic inflammation as a contributor to the development of MPNs

There is evidence supporting the notion that chronic inflammation precedes the development of MPNs, thus creating a permissive environment for the expansion of the mutant MPN driver clone. A prior history of an autoimmune disease is associated with an increased risk of developing an MPN (Odds Ratio (OR)=1.2)[11]. A history of inflammation mediated by an infection has also been associated with an increased risk of myeloid malignancies, however only a history of cellulitis was associated with a significantly increased risk of an MPN (OR 1.34)[12]. Modifiable lifestyle factors that lead to chronic inflammation may also play a role in the development of MPNs. For example, smoking increases the risk of MPNs[13, 14]. Obesity has been associated with an increased risk for multiple malignancies, including MPNs [14–16]. This suggests that aggressive treatment of autoimmune and inflammatory conditions and lifestyle modifications aimed at reducing inflammation may be impactful to reduce one's risk of developing an MPN.

Non-modifiable factors such as the patient's germline predisposition also likely contribute to the development of an MPN. Although MPN driver mutations are clearly not inheritable, the predisposition to develop a somatic MPN driver mutation is. Interestingly, first-degree relatives of MPN patients have a 4–5-fold higher risk of developing an MPN[17]. Among the Single Nucleotide Polymorphisms (SNPs) identified as being associated with MPN include inflammasome-related genes[18] and monocyte chemoattractant protein-1 (MCP-1)[19], suggesting that the host's immune milieu may contribute to the genetic predisposition to acquire an MPN.

MPN subtype specific cytokine signatures

Although MPN subtypes can share identical driver mutations such as $JAK2^{V617F}$ the resulting phenotype is variable. Just like other clinical MPN subtype specific clinical features, there appears to be subtype specific cytokine signatures. A recent longitudinal study of more than 400 patients[20] identified specific inflammatory cytokine signatures according to disease subtypes. Ten cytokines including interferon gamma (INF-γ), interleukin-1 receptor antagonist (IL-1RA), IL-6, IL-8, IFN-γ–inducible protein 10 (IP-10), epidermal growth factor (EGF), eotaxin (CCL11), tumor necrosis factor-alpha (TNF-α), transforming growth factor-alpha (TGF-α), and growth-regulated oncogene (GRO-α or CXCL1) were significantly altered and showed strong disease subtype specificity. (Figure 1) Specifically, primary myelofibrosis (PMF) was associated with increased levels of TNF-

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α, IP-10, and IL-8 while TGF-α was unique to polycythemia vera (PV) and essential thrombocythemia (ET) presented with higher eotaxin, EGF and GRO-α levels[20].

Another study measured plasma cytokine levels in patients with MPN, younger than 35 years, to identify possible subtype-specific biomarkers. Dickkopf-related protein 1 (Dkk-1) was found to be the most significantly increased protein in patients with MPN compared with healthy donors.[21] Plasma Dkk-1 levels normalized to platelet counts were not significantly different between controls and ET but could discriminate ET from pre-PMF, in both $JAK2^{V617F}$ and CALR mutant patients (Fig. 2).

The patients with PV exhibit an altered cytokine milieu with significantly higher levels of IL-1RA, IL-5, IL-6, IL-7, IL-8, IL-12, IL-13, IFN-γ, granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage inflammatory protein-1alpha (MIP-1α), MIP-1β,, hepatocyte growth factor (HGF), IP-10, monokine induced by IFN-gamma (MIG), MCP-1, and vascular endothelial growth factor (VEGF) compared with normal controls.[22] A comparative study of plasma cytokine profiles in PV and ET MPN sub-types showed differentially elevated levels of IL-4, IL-8, GM-CSF, IFN-γ, MCP-1, platelet-derived growth factor (PDGF), and VEGF in ET as compared with PV.[23]

High levels of serum IL-8, IL-11, leptin, HGF and MCP-1 has been reported in PV [24, 25] and IL-11 and HGF levels were correlated with neutrophil counts and hematocrit levels in PV patients [25]. Also, while IL-10 and IL-22 were increased in both PV and ET patients compared to controls, IL-23 was selectively elevated only in PV [26].

Global cytokine analyses utilizing human cytokine arrays showed increased TIMP-1, MIP-1β and insulin-like growth factor binding factor-2 (IGFBP-2) in PMF patients but not ET or PV [27]. Serum IL-17 was also exclusively elevated in PMF patients compared to healthy controls but not in PV or ET patients [28]. IL-17 is a marker of angiogenic activity and is thought to enhance angiogenesis in the pre-fibrotic stage of PMF [28].

In treatment naïve patients, elevated cytokine profiles of GM-CSF, IL-1β, IL-4, IL-5, IL-6, IL-10, IFN-α2, MIP-1α, IL-12, and TNF-α were observed in all three MPN categories as compared to age-matched control subjects [29]. MPN subset analysis also revealed intra-disease variations with PMF displaying additional cytokine modulations such as increased IL-17A compared to controls, higher levels of IFN-γ, IL-12, IL-17A and IP-10 in comparison to ET patients and elevated plasma levels of IL-12, IL-4 and GM-CSF compared to PV patients [29]. $JAK2^{V617F}$ mutational status was also associated with higher IP-10 levels in myelofibrosis. Subsequently, although all MPN patients displayed an inflammatory status, PMF emerged as the highest producer of cytokines and chemokines [29].

Association of cytokines with specific disease outcomes

Specific cytokines are associated specific disease outcomes, suggesting that cytokine profiling could be useful clinically as predictive tools. A study involving 127 PMF patients showed increased 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), IFN-α, MIP-1α, MIP-1β, HGF, IP-10, MIG, MCP-1 and VEGF levels as well as decreased IFN-γ levels compared to normal

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controls. Treatment naïve PMF subjects displayed increased levels of IL-8, IL-2R, IL-12, IL-15, and IP-10 which predicted inferior survival. Association of phenotypic clinical features with cytokines included IL-8 with constitutional symptoms, leukocytosis and leukemia free survival, IL-2R and IL-12 with transfusion need, IP-10 correlated with thrombocytopenia while HGF, MIG and IL-1RA corresponded with marked splenomegaly [30].

Vaidya et al assessed the disease phenotypic and prognostic relevance of cytokine levels in PV and found that IL-12 levels correlated with hematocrit levels, IL-1β with leukocytosis and IFNα/IFNγ with thrombocytosis. MIP-1β, was significantly associated with an inferior overall survival [22]. A recent study found that high levels of GRO-α was associated with an increased risk of transformation of ET to MF. Additionally, longitudinal sampling indicated decreasing EGF levels in ET patients strongly correlated with disease transformation risk [20].

Bourantas et al., demonstrated increased serum beta-2-microglobulin, IL-2 and soluble IL-2 receptor alpha (sIL-2RA) in with MPNs patient progressing to advanced clinical stages [31]. Panteli et al., observed that serum levels of IL-2, sIL-2RA and IL-6 were increased when PMF progressed to MPN-blast phase (MPN-BP) and positively correlated with bone marrow angiogenesis, hence indicating that disease progression is coupled with amplified inflammation and that cytokine levels can be useful biomarkers to predict disease progression such as bone marrow angiogenesis [32].

Thus, measuring cytokines could potentially be leveraged as a tool for disease monitoring and to provide parallel information in addition to genomic and clinical data to predict disease progression/transformation.

Cellular components of inflammation in MPN

Emerging evidence indicates that inflammation in the bone marrow (BM) microenvironment and systemic inflammation contribute to the development and progression of MPNs. Different cell types act as mediators of inflammation in MPNs including mutant and normal hematopoietic stem and progenitor cells, mesenchymal stromal cells, megakaryocytes, monocytes, platelets, and endothelial cells. These cells producenumerous inflammatory cytokines that act in an autocrine and paracrine fashion to provide a self-perpetuating and permissive microenvironment for disease evolution ultimately resulting in bone marrow fibrosis and transformation to MPN-BP.

Bone marrow hematopoietic cells

The effect of MPN driver mutations on inflammatory transcriptional programs and cytokine secretion in hematopoietic stem and progenitor cells (HSPCs) can lead to an inflammatory bone marrow niche that supports the proliferation of mutant cells. We observed increased circulating TNF-α in MPN patients in comparison to healthy controls and also demonstrated that mononuclear cells and CD34+ cells from *JAK2*^{V617F} MPN patients unlike normal contols are resistant to the growth suppressive effects of TNF-α while colony formation.[33] Thus, the presence of *JAK2*^{V617F} not only increases TNF-α secretion but also creates a favorable environment for MPN mutant cell expansion[33]. Similarly, Lipocalin-2 is another

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molecule that has been shown to be increased in the serum and conditioned media of bone marrow mononuclear cells from MPN patients compared to controls [34]. The presence of lipocalin-2 also reduced the proliferation and colony-forming capacity of bone marrow $CD34^+$ cells from non-MPN patients or normal controls but not MPN patients thus providing a relative growth advantage to MPN clones [34, 35]. Lipocalin was expressed by myelofibrosis marrow myeloid cells and not erythroid or megakaryocytic cells [35]. Bone marrow neural death has been associated with IL-1β released from mutant HSCs reduced mesenchymal stromal cells and allowed the uncontrolled expansion of mutant HSCs and disease progression [36].

Using single-cell technology to understand disease pathology in myelofibrosis, Psaila et al., identified a megakaryocyte differentiation bias in early human multipotent stem cells and strong expression of fibrotic mediators in megakaryocyte progenitors [37]. Furthermore, cell surface expression of G6B was specific to mutant HSPCs from myelofibrosis patients thus identifying a potential selective target for MF HSPCs [37]. $CD34^+$ cells from patients with *CALR*-mutations were profiled by integrating target genotyping with single-cell RNA sequencing. This technology revealed that the frequency of *CALR*-mutated cells was higher in committed myeloid progenitors and megakaryocyte progenitors indicating increased fitness of the *CALR* mutation with myeloid differentiation. Compared to wild-type HSPCs, an upregulation of NF-κB pathway genes in undifferentiated mutant HSCs supports a cell-intrinsic role for *CALR* mutation in NF-κB activation[38].

Megakaryocytes in PMF possess an inflammatory and pro-fibrotic secretome that is a major driver of BM fibrosis [39]. The role of megakaryocytes in promoting inflammation has been reviewed separately in this edition and will not be discussed here.

Mesenchymal stromal cells

BM mesenchymal stromal cells (also mesenchymal stem cells or multipotent stromal cells (MSCs)) contribute to the maintenance of HSCs and normal hematopoiesis. Leukemic myeloid cells remodel the bone marrow niche into a “self-reinforcing leukemic niche” that favorably supports leukemic stem cells but not healthy stem cells [40]. Nestin-positive MSC reduction was observed in the bone marrow of MPN patients and MPN mouse models carrying the human $JAK2^{V617F}$ mutation due to IL-1β released by mutant HSCs resulting in a favorable environment for mutant HSC expansion [36].

MSCs from PMF patients are characterized by an increased secretion of TGFB, BMP and glycosaminoglycans and specific impairment of osteogenic abilities. Transcriptome analysis identified a TGF-β signature in primary MF MSCs [41]. Differentiation of glioma associated oncogene positive ($Gli1^+$) MSCs towards fibrosis driving myofibroblasts was shown in mouse models of myelofibrosis. Similarly, BM samples from MPN patients also showed an increased frequency of Gli1+ cells and corresponded to the severity of fibrosis by reticulin staining irrespective of $JAK2^{V617F}$ or *CALR* mutation status [42]. Leptin receptor (Lepr) expressing MSCs were also found to be expanded and fibrogenic in a mouse model of myelofibrosis [43].

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Monocytes

Mature hematopoietic cells in the peripheral blood are pivotal sources of increased systemic cytokines in MPN. Classical CD14+CD16- monocytes are a strongest producers of cytokines including TNF- α, IL-6, IL-8 and IL-10 in MF [44]. TNF-α is consistently increased in all MPN subtypes and has an integral role in the clonal expansion of *JAK2*^{V617F} cells [33]. We recently showed that primary monocytes from MPN patients have extensive TNF- α production compared to normal controls in response to stimulation due to a dampened response to the anti-inflammatory cytokine, IL-10 [45]. IL-10 receptor (IL-10R) signaling via suppressor of cytokine signaling-3 (SOCS3) was found to be downregulated in MPN patients. Interestingly, persistent TNF- α production was observed in both unmutated and *JAK2*^{V617F} monocytes indicating a non-cell autonomous role for monocytes in MPN inflammation [45]. Very recently, CD56+CD14+ pro-inflammatory monocytes have been identified as a pivotal source of GRO-α in ET patients thus creating an environment suitable for MPN disease evolution [20].

Granulocytes

Single cell-cytokine profiling of circulating granulocytes from PMF patients showed that several cytokines including IL-6, IL-8, IL-10, IL-12, TNF-α, CCL2, CCL3 and CCL5, were significantly increased compared to healthy controls [46]. This was the result of an increased fraction of cytokine secreting cells and the level of individual cytokines per cell. The proportion of cytokine secreting myeloid cells was higher than the *JAK2*^{V617F} -mutant allele burden, which suggested that non-malignant cells also contribute to cytokine production. Thus, aberrant inflammatory signaling in MPN is not restricted to cell-intrinsic effects but also non-cell autonomous processes [46].

The formation of neutrophil extracellular traps by neutrophils not only contributes to innate immunity and host defense but also promotes thrombosis [47]. Increased neutrophil activation has been reported in ET and PV with increased cell surface CD11b expression and circulating myeloperoxidase levels [48]. Recently, Wolach et al., demonstrated that neutrophils from MPN patients show increased NET formation with a pro-thrombotic phenotype which can be blocked by ruxolitinib [49]. Increased NET formation was associated with PAD4 overexpression in *JAK2*^{V617F} PV patient samples [49]. Thus, premature neutrophil activation in MPN is a cell-intrinsic effect of the *JAK2*^{V617F} mutation and is associated with thrombotic events. We also demonstrated that N-acetylcysteine (NAC) reduces NET formation in MPN patients and could be used a potential anti-thrombotic in MPN [50].

Platelets

Platelets play a role in innate immunity and inflammation in addition to their hemostatic function and contribute to thrombo-inflammation in MPN [51]. Thrombosis is a major cause of mortality and morbidity in MPN patients with several underlying mechanisms including membrane alterations on red blood cells, activated platelets, activated leukocytes, platelet-leukocyte aggregates, and dysfunctional endothelium. Systemic inflammation also plays a critical role in the development of vascular events as elevated high sensitivity C-reactive protein (hsCRP) is significantly associated with thrombosis risk in PV and ET patients [52].

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Platelet interactions with neutrophils and monocytes in MPN triggers activation of both cell types and stimulates inflammatory and thrombotic processes. We and others have reported increased platelet-leukocyte aggregates in MPN patients [50, 53]. We also observed that MPN platelets can induce NET formation with normal and MPN neutrophils without an external stimulus indicating that MPN platelets generate a prothrombotic microenvironment [50]. Platelet crosstalk with monocytes can also increase cytokine synthesis and release since monocytes are already known to play an important role in MPN inflammation [44, 45]. Inflammatory cytokines and reactive oxygen species result in an activated and pro-thrombotic endothelium as observed by increased von Willebrand factor (vWF) and E-selectin levels [54]. Recruitement of platelets and leukocytes to an activated endothelium results in a prothrombotic phenotype in MPN patients. Activated platelets themselves can act as immune cells by releasing pro-inflammatory cytokines such as CCL5 and platelet factor 4 (PF4 or CXCL4) stored in α-granules [55]. Elevated cytokine levels lead to reciprocal activation of platelets thus driving the thrombo-inflammatory loop in MPN.

Looking ahead – how could we incorporate knowledge of the microenvironment to aid in the clinical care of MPN patients?

Although our knowledge of the role of inflammation in MPN patients is rapidly expanding, we have yet to fully harness this knowledge toward improvements in diagnosis, prognostication, monitoring of disease progression or treatment, and use as a therapeutic target.

Cytokine profiling remains relatively untapped as a clinical tool. MPN subtypes appear to have unique cytokine profile signatures (see above), and it is conceivable that sometime in the future cytokine profiling could be incorporated as a diagnostic tool to aid in delineating the MPN subtype. Moreover, cytokine profiling could also possibly increase the accuracy of our prognostic scoring tools in MPN. Not only could cytokines be leveraged to help predict general prognosis but may be most helpful to identify patients at highest risk of specific outcomes such as thrombosis. Moreover, cytokines could also possibly be utilized to help aid in selection of drugs, for example those who are most likely to benefit from JAK 1/2 inhibitors.

Targeting of specific microenvironmental offender cell types could be applied therapeutically in MPNs. An example of targeting specific microenvironmental cell subtypes involved in fibrosis is the drug PRM-151, which targets the differentiation of fibrocytes, a cell type important for fibrosis[56]. There are a multitude of other potential targets, including Gli1 proteins on MSCs by GANT61[42], G6B on mutant HSCs[37], PDGFRA signaling pathway in fibrosis[43], and reducing NETs and platelet-leukocyte aggregates[50]. A greater understanding of the intricate relationshisp between inflammation and MPN disease pathogenesis will allow for more accurate therapeutic targeting to achieve the much-desired goal of disease modifying therapy in MPN.

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Synopsis:

Chronic inflammation is a hallmark of myeloproliferative neoplasms with elevated levels of pro-inflammatory cytokines being commonly found in all three subtypes. Systemic inflammation is responsible for the constitutional symptoms, thrombosis risk, premature atherosclerosis and disease evolution in MPN. Although the neoplastic clone and their differentiated progeny drive the inflammatory process, they also induce ancillary cytokine secretion from non-malignant cells. Here, we describe the inflammatory milieu in MPN based on soluble factors and cellular mediators. We also discuss the prognostic value of cytokine measurements in MPN patients and potential therapeutic strategies that target the cellular players in inflammation.

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Key points:

1. The inflammatory microenvironment in MPN encompasses soluble cytokines and associated cellular players.
2. A self-sustained inflammatory loop results in a milieu that supports the clonal expansion of the neoplastic clone.
3. Cytokine profiles in MPN can be leveraged for diagnosis, disease monitoring and prognosistication which will help to obtain more favorable patient outcomes.

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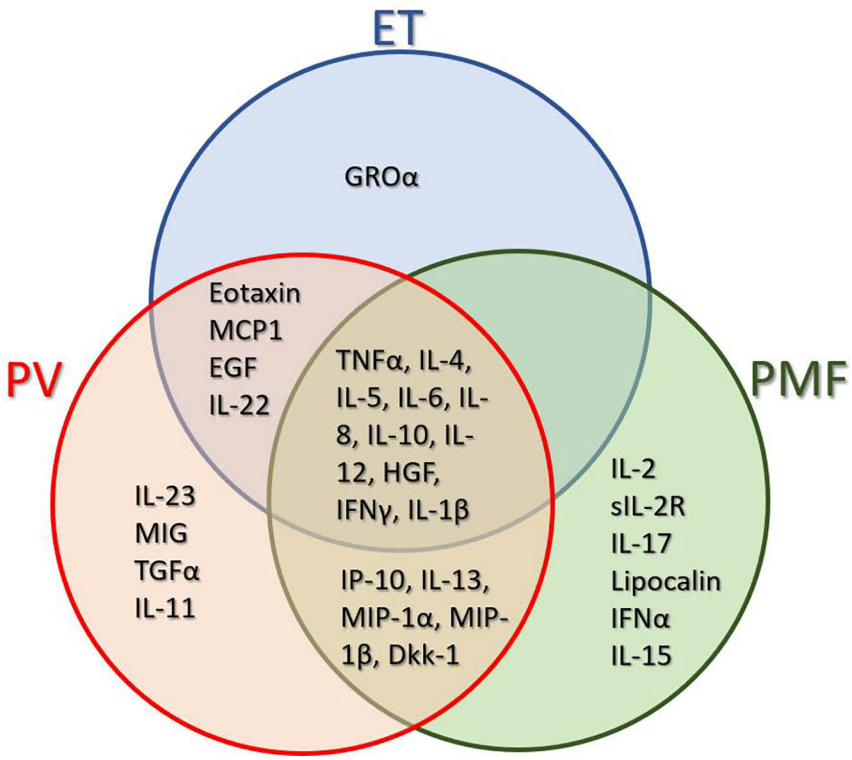

Figure 1.

MPN-associated cytokines and chemokines according to subtype. Dkk-1, Dickkopf-related protein 1; EGF, epidermal growth factor; GRO-a, growth-regulated oncogene; HGF, hepatocyte growth factor; IFNa, interferon alpha; IFNg, interferon gamma; IL-1RA, interleukin-1 receptor antagonist; IP-10, IFN-g–inducible protein 10; MIG, monokine induced by IFN-gamma; MIP, macrophage inflammatory protein-1; TGF-a, transforming growth factor alpha; TNFa, tumor necrosis factor alpha.

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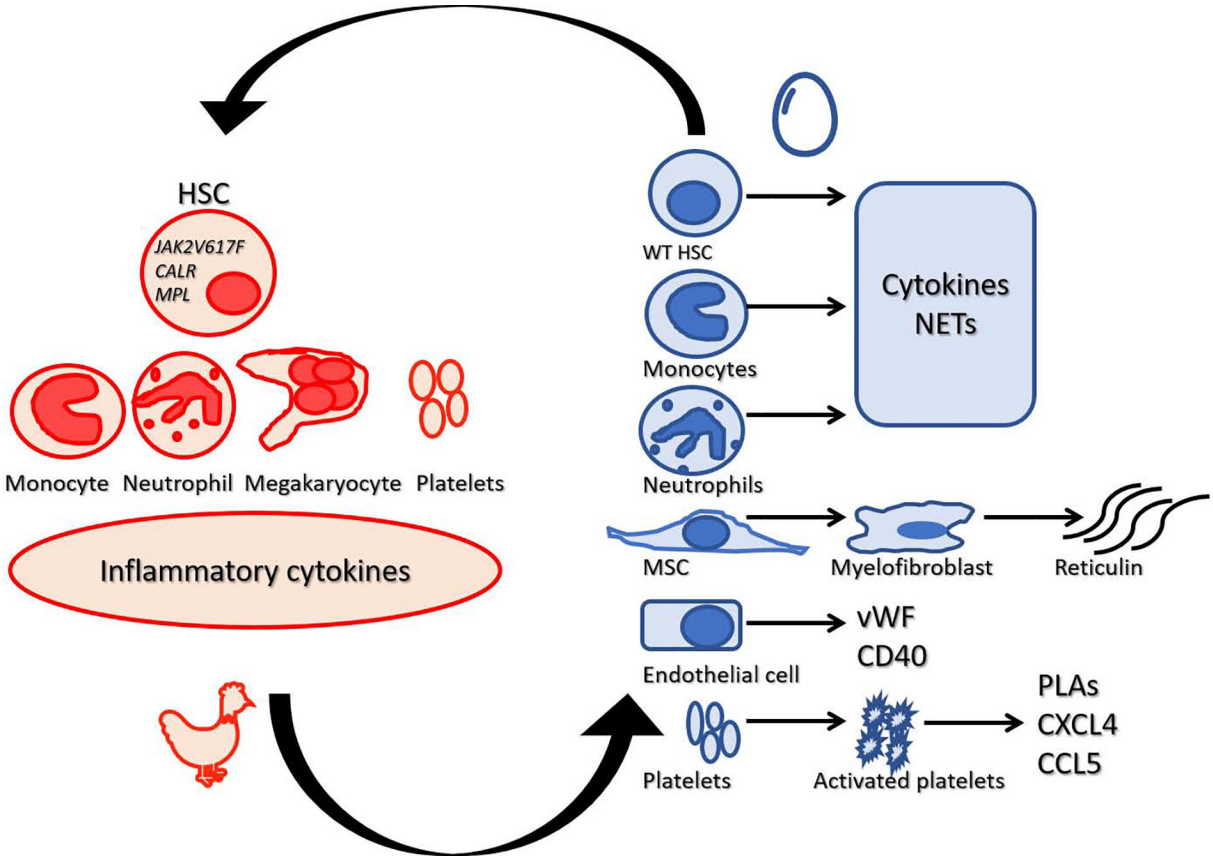

Figure 2.

Overview of the inflammatory loop in MPNs. The neoplastic hematopoietic stem cell (HSC) clone carrying the *JAK2*^{V617F}, *CALR*, or *MPL*, mutation secretes cytokines involved in inflammation and differentiate into malignant cells of the myeloid lineage such as megakaryocytes, monocytes, and granulocytes. Together, these cells produce a host of cytokines creating an inflammatory microenvironment which in turn results in aberrant activation and function of non-malignant cells in the bone marrow and peripheral blood. NETs, neutrophil extracellular traps; MSC, mesenchymal stromal cell; vWF, von Willebrand factor; PLAs, platelet-leukocyte aggregates.