Cytokine control of megakaryopoiesis

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Abstract

Platelets are anuclear blood cells required for hemostasis and are implicated in other processes including inflammation and metastasis. Platelets are produced by megakaryocytes, specialised cells that are themselves generated by a process of controlled differentiation and maturation of bone marrow stem and progenitor cells. This process of megakaryopoiesis involves the coordinated interplay of transcription factor-controlled cellular programming with extra-cellular cues produced locally in supporting niches or as circulating factors. This review focuses on these external cues, the cytokines and chemokines, that drive production of megakaryocytes and support the terminal process of platelet release. Emphasis is given to thrombopoietin (Tpo), the major cytokine regulator of steady-state megakaryopoiesis, and its specific cell surface receptor, the Mpl protein, including normal and pathological roles as well as clinical application. The potential for alternative or supplementary regulatory mechanisms for platelet production, particularly in times of acute need, or in states of infection or inflammation are also discussed.

Keywords: *megakaryopoiesis, cytokine, platelet, thrombopoietin, Mpl receptor*

Megakaryopoiesis

Megakaryocytes (Mk) are large, polyploid cells that originate in the bone marrow. Their biological function is to produce platelets, small disc-shaped cellular fragments with no nucleus, that circulate in the blood and play a critical role in hemostasis. Like other blood cells, Mk derive from hematopoietic stem cells (HSC) in a process termed megakaryopoiesis. According to the classical model of hematopoiesis, HSC sequentially transition through multipotent progenitors (MPP), common myeloid progenitors (CMP), bipotential Mk-erythroid progenitors (MEP) and unipotent Mk progenitors (MkP), which then mature into megakaryocytes (Nimmo et al. 2015). This model is under ongoing revision, with refined identification of progenitor cells and improved understanding of lineage commitment and hierarchical relationships (Adolfsson et al. 2005; Pronk et al. 2007; Iwasaki & Akashi 2007; Ng et al. 2012; Paul et al. 2015). Similarities between HSC and megakaryocytes have been noted, including shared expression of cell surface markers, transcription factors and signalling pathways (Månsson et al. 2007; Huang & Cantor 2009). Subsequently, murine thrombopoietin (Tpo)-dependent von Willebrand factor-expressing cells that exhibit platelet-biased long-term reconstitution capacity, were identified within the HSC compartment (platelet-biased LT-HSC) (Sanjuan-Pla et al. 2013). Concurrently, Yamamoto and colleagues demonstrated the existence of a Mk lineage-restricted cell with high self-renewal capacity residing within the phenotypic HSC compartment (Mk repopulating progenitor cells; MkRPs) and provided evidence that such cells do not pass through the MEP stage for production of platelets (Yamamoto et al. 2013). Soon after, a similar unipotent Mk progenitor cell was identified within the human HSC compartment (Notta et al. 2016). In mice, both platelet-biased LT-HSC and MkRPs contribute to megakaryocyte production during steady state. In contrast, a sophisticated combination of *in vivo* studies and proteomics has identified stem-like megakaryocyte-committed progenitors (SL-MkPs), that remain quiescent during steady state but become active, initiate translation of Mk transcripts and differentiate directly into MkPs, in situations of acute platelet need (Haas et al. 2015). These findings suggest that direct pathways exist to mediate rapid and efficient platelet production at times

of stress, such as inflammation-induced or other states of thrombocytopenia, that may also contribute at steady state. The precise contribution and conditions under which these alternative pathways drive production of megakaryocytes and platelets remains under study, as do the interrelationships, if any, between these cells and the traditionally defined megakaryopoietic cellular hierarchy (Figure 1).

As Mk progenitor cells mature they lose their proliferative ability and become polyploid through a process called endomitosis, a variation of the cell cycle during which the nucleus fails to segregate after S phase, resulting in diploid promegakaryoblasts that become tetraploid megakaryoblasts and successively larger and more polyploid promegakaryocytes and megakaryocytes (Geddis et al. 2007; Bluteau et al. 2009). In addition, Mk maturation involves the development of an extensive membrane system, known as the demarcation membrane system (DMS), which forms the plasma membranes of future platelets. A prevalent model of thrombopoiesis, the terminal stage of platelet release from Mks, is the proplatelet model. In this model, once mature Mk are generated they migrate to bone marrow sinusoids, into which they extrude dynamic transendothelial pseudopods, termed proplatelets. These proplatelets continue to elongate and segregate into multiple platelet-size beads connected to each other, and to their maternal Mks, by thin cytoplasmic bridges (Italiano et al. 1999). The final release of platelets is proposed to occur within the circulation, where shear forces support the shedding of new platelets from the tips of intravascular proplatelets (Junt et al. 2007).

Interestingly, a mounting body of evidence suggests the lung is a major site of platelet biogenesis. After the initial identification of megakaryocytes in the lung (Aschoff 1893), a variety of studies confirmed that blood leaving the lungs contains more platelets and fewer megakaryocytes than blood entering the lungs, suggesting pulmonary platelet production (Howell & Donahue 1937; Levine et al. 1993), and recent studies have confirmed the presence of megakaryocytes and their progenitors, of bone marrow origin, in the lungs, that generate significant numbers of platelets via

a distinct mechanism of proplatelet production in the microcirculation (Fuentes et al. 2010; Lefrançais et al. 2017).

Alternative models for platelet release have also been proposed, for example the platelet territory model, which proposes that, during Mk maturation, platelet territories or vesicles, representing nascent platelets, form from the developing DMS, and are released as platelets into the circulation via explosive fragmentation (Kosaki 2005). Similarly, as discussed further below, it has also been proposed that in response to acute platelet need, Mk rupture, characterized by rapid cytoplasmic fragmentation, results in greater numbers of platelets produced per Mk and allows more rapid replenishment of depleted platelet numbers (Nishimura et al. 2015).

Like all hematopoietic lineages, megakaryopoiesis is controlled by the interplay of extrinsic stimuli, including cytokines, chemokines and cell adhesion interactions, combined with intrinsic transcription factor regulation (Tijssen & Ghevaert 2013). In this review we will focus on extrinsic regulation of megakaryopoiesis by cytokines and chemokines.

Thrombopoietin, the major cytokine regulator of steady-state megakaryopoiesis

Thrombopoietin (Tpo) is encoded by a single gene located on human chromosome 3q26.3-3q27 (Chang et al. 1995), is composed of 332 amino acids, is heavily glycosylated (de Sauvage et al. 1994; Lok et al. 1994) and displays a four helical bundle fold (Feese et al. 2004). The biological activity of Tpo is mediated via binding to the Mpl receptor, expressed on HSC, Mks and platelets. Mpl is a member of the type I family of cytokine receptors, a group of transmembrane receptors that lacks intrinsic kinase activity but associate with the cytoplasmic Janus tyrosine kinases (JAK). In the unliganded state, Mpl exists as an inactive monomer or homodimer on the cell surface. Upon Tpo binding, Mpl dimers become active via conformational changes, resulting in transphosphorylation and activation of associated JAK2 proteins, further resulting in phosphorylation of intracellular receptor tyrosine residues (Figure 2). This active receptor scaffold supports subsequent recruitment and activation of multiple downstream biochemical cascades,

including the signal transducers and activators of transcription 3 and 5 (Stat3, Stat5), phosphoinositol-3-kinase (PI3K) and the mitogen-activated protein kinase (MAPK) pathways (Kaushansky 2009; Hitchcock & Kaushansky 2014).

Multiple mechanisms have been implicated in attenuation of Mpl signalling. Tpo stimulation results in Mpl internalisation, targeting to the lysosome and degradation (Hitchcock et al. 2008). Mpl is also poly-ubiquitinated in Tpo-stimulated cells, resulting in proteasome-mediated degradation, at least in part mediated by the c-Cbl E3 ubiquitin ligase (Saur et al. 2010). Specific negative regulators implicated in Tpo signalling include the Src-kinase family member Lyn, the Lnk adaptor protein and suppressors of cytokine signaling (SOCS) (Wang et al. 2000; Lannutti & Drachman 2004; Tong & Lodish 2004; Seita et al. 2007; Bersenev et al. 2008).

Tpo is constitutively produced by the liver (Decker et al. 2018). The concentration of circulating Tpo is controlled by Mpl-mediated internalisation and destruction, resulting in a typical inverse *in vivo* correlation between plasma Tpo levels and megakaryocyte and platelet mass (Fielder et al. 1996). This elegant feedback control reduces Tpo availability when platelets are replete and allows for increased Tpo concentration and promotion of megakaryocyte development and platelet production in times of need. More recently, binding of ageing, desialylated platelets to the Ashwell-Morell receptor on liver cells has been reported to stimulate Tpo expression (Grozovsky et al. 2015) and this may also contribute to feedback control of Tpo levels.

The indispensable role of Tpo is evident in mice or humans lacking Tpo or Mpl in the germline, where circulating platelet numbers are 5-10-fold lower than normal (de Sauvage et al. 1996; Alexander et al. 1996; Ihara et al. 1999). Tpo-independent platelet production, the mechanisms driving which are not fully understood, is nevertheless sufficient for basic hemostasis and capable of very significant megakaryopoiesis and platelet production following cytotoxic stress (Levin et al. 2001). The Tpo/Mpl-deficient thrombocytopenia is accompanied by deficiencies of a similar magnitude in megakaryocytes and their progenitor cells. Intriguingly, recent studies in genetically engineered mice with reduced or no Mpl expression - specifically on megakaryocytes

and platelets - revealed unexpected supra-physiological platelet numbers accompanied by significantly expanded numbers of high ploidy megakaryocytes and progenitor cells (Tiedt et al. 2009; Ng et al. 2014). This result suggests that Mpl expression on megakaryocytes themselves is dispensable for effective platelet production and that the primary mechanism by which Tpo signaling maintains platelet numbers is via stimulation of Mpl-expressing progenitor cells. A corollary of this conclusion is that the expression of Mpl on megakaryocytes and platelets is largely, if not entirely, for the regulation of circulating Tpo levels. This role is itself a crucial one to prevent platelet excess and associated myeloproliferation. Developmental differences are likely also to exist in the precise roles of Tpo and Mpl signaling in fetal, newborn and adult life (Potts et al. 2015; Lorenz et al. 2017; Sparger et al. 2018). Finally, it should be noted that Mpl signaling pathways are intact in platelets (Hitchcock & Kaushansky 2014), and a potential role for Tpo in regulation of platelet function can not be fully excluded.

In addition to cells within the megakaryocyte lineage, the Mpl receptor is also expressed on pluripotent HSC. Mice lacking Tpo or Mpl display reduced numbers of HSC that show defective hematopoietic reconstitution potential (Kimura et al. 1998). Similarly, in humans lacking Mpl, HSC defects also ensue, with such patients typically succumbing to bone marrow failure during childhood (Ballmaier et al. 2003; King et al. 2005; Walne et al. 2012). Tpo is required to maintain HSC quiescence (Qian et al. 2007; Yoshihara et al. 2007) and, at least in part, a deregulated balance between quiescence/self-renewal and HSC activation appears to underlie these phenotypes.

Other cytokines in steady-state megakaryopoiesis

The interleukin (IL)-6 family of cytokines is characterised by the shared use of the gp130 receptor signalling chain, which forms part of a hetero-oligomeric receptor complex that also typically includes a ligand-specific alpha-chain. Several members of the IL-6 family of cytokines, including IL-6, IL-11 and leukemia inhibitory factor (LIF) have little or no effect on megakaryocyte progenitor proliferation when acting alone, but can augment the *in vitro* megakaryopoietic actions

of IL-3 (Gordon & Hoffman 1992). Their major role appears to be in megakaryocyte maturation: IL-6, IL-11 and LIF each induce megakaryocytes to enlarge and increase DNA ploidy *in vitro* (Ishibashi et al. 1989; Teramura et al. 1992; Burstein et al. 1992). Injection of IL-6 (Hoffman 1989; Nagler et al. 1995), IL-11 (Neben et al. 1993; Gordon et al. 1996; Tepler et al. 1996) or LIF (Metcalf et al. 1990) increased platelet count and/or accelerated recovery from chemotherapy- or transplant-induced thrombocytopenia in mice and humans. However, genetic ablation of these cytokines or their specific receptor α-chains had no effect on megakaryocyte development or platelet counts, and did not abolish residual megakaryopoiesis in *Mpl*$^{-/-}$ mice (Escary et al. 1993; Bernad et al. 1994; Yoshida et al. 1996; Nandurkar et al. 1997; Gainsford et al. 2000). In contrast, inactivation of gp130-induced Stat3 signalling induced thrombocytopenia (Jenkins et al. 2002), while mice with constitutive activation of this pathway due to mutation of gp130 (gp130$^{Y757F/Y757F}$) exhibited excessive platelet production (Jenkins et al. 2005).

The primary roles of the colony-stimulating factors: granulocyte (G)-colony-stimulating factor (CSF), granulocyte-macrophage (GM)-CSF, macrophage (M)-CSF and IL-3 (or multi-CSF), are the coordination of granulocyte and macrophage development and function. In megakaryopoiesis, while G-CSF and M-CSF have no marked effects, GM-CSF and in particularly IL-3 promote Mk progenitor cell proliferation *in vitro* (Metcalf, Burgess, et al. 1986; Ishibashi et al. 1990), particularly in combination with other cytokines, such as members of the IL-6 family, Stem cell factor (SCF) and Erythropietin (Epo). However, while IL-3 has modest thrombopoietic activity when administered *in vivo* (Metcalf, Begley, et al. 1986), GM-CSF administration does not elevate platelet counts (Ishibashi et al. 1990), and platelet counts are normal in mice lacking these cytokines or their receptors (Stanley et al. 1994; Mach et al. 1998). In addition, the actions of IL-3 and GM-CSF do not contribute to basal platelet levels in the absence of thrombopoietin signalling (Chen et al. 1998; Scott et al. 2000).

Similarly, IL-9 has been shown to potentiate human megakaryopoiesis *in vitro,* in combination with Epo and SCF, and via a Tpo-independent mechanism (Fujiki et al. 2002). When

administered to mice, IL-9 stimulated megakaryopoiesis, which was inhibited by neutralizing endogenous IL-9 or IL-9R receptor blockade (Xiao et al. 2017).

As discussed above, recent studies suggest that megakaryocyte progenitors can diverge directly from HSC. Therefore, it is perhaps not surprising that a range of cytokines implicated in HSC-regulation also act on Mk progenitors. SCF and Flt3-ligand (FL) are produced by bone marrow stromal cells and serve as the ligands for the c-Kit and Flt3 receptor tyrosine kinase receptors, respectively. *In vitro*, both factors synergize with Tpo to stimulate megakaryocyte progenitor proliferation (Broudy et al. 1995; Metcalf et al. 2002; Sigurjónsson et al. 2002). FL and Tpo are thought to synergize to upregulate Bcl2 and delay apoptosis of megakaryocyte progenitors (Sigurjónsson et al. 2004), while SCF is proposed to augment Tpo-induced STAT5 signalling through JAK2 and Src kinase (Drayer et al. 2005). In addition, expression of membrane-bound SCF by bone marrow osteoblasts facilitates close cell-to-cell-interaction between megakaryocytic progenitors and stromal cells, which may also support megakaryopoiesis (Avraham et al. 1992).

Vascular endothelial growth factors (VEGFs) are secreted by various hematopoietic and non-hematopoietic cell types and act through a family of cognate receptor tyrosine kinases, namely VEGFR1, VEGFR 2 and VEGFR 3, all of which are expressed on megakaryocytic cells at various differentiation stages. While VEGFR1 and VEGFR3 are expressed by MEPs and MkPs, VEGFR2 expression is restricted to the late stages of MK maturation (Casella et al. 2003; Thiele et al. 2012). Activation of VEGFR1 by VEGFA has been shown to enhance MkP maturation and upregulate CXCR4 expression resulting in redistribution of Mks to the vascular niche (see below), ultimately leading to enhanced platelet production *in vivo* (Casella et al. 2003; Pitchford et al. 2012). Similarly, VEGFA-induced VEGFR2 activation facilitated increased Mk proliferation, survival and differentiation in a engineered cell line model (Coppola et al. 2006). In contrast, the VEGFC-VEGFR3 axis facilities negative regulation of Mk development: activation of VEGFR3 elevated the numbers of early Mk progenitors at the expense of more mature MkPs (Thiele et al. 2012). Of note, it has been shown that Mks express and secrete VEGFA (Katoh et al. 1995; Möhle et al. 1997) and

that Tpo promotes VEGFA production (Casella et al. 2003), indicating that autocrine and/or paracrine VEGF loops might contribute to megakaryopoiesis.

Many, if not all, the non-Tpo cytokines discussed, while capable, at least to some extent, of stimulating megakaryopoiesis *in vitro* or *in vivo*, appear dispensable when genetically ablated. These observations, combined with the fact that many of these cytokines are produced in high levels by immune cells in response to infection or inflammation, imply that significant roles of these cytokines in megakaryopoiesis may be in platelet production associated with stress conditions, rather than any significant contribution to steady state production, a theme discussed in further detail below.

Control of megakaryocyte migration and platelet release

The endosteal niche facilitates early megakaryocytic development, while terminal maturation occurs in the vascular niche. Thus, during differentiation megakaryocytic cells are required to migrate within the bone marrow. Stromal cell-derived factor 1 α (SDF1α) and fibroblast growth factor 4 (FGF4), two factors produced by bone marrow stromal cells, play pivotal roles in this process. SDF1α is a member of the CXC-family of chemokines. It specifically binds the CXC-chemokine receptor 4 (CXCR4), which is expressed throughout the megakaryocytic lineage (Wang et al. 1998). In humans, deregulation of the SDF1α-CXCR4 axis has been associated with thrombocytopenia (Salim et al. 2017) and, consistent with this role, injection of adenoviral vectors expressing SDF-1α increased platelet counts in normal mice and elevated thrombopoiesis in *Thpo*$^{-/-}$ and *Mpl*$^{-/-}$ mice, indicative of a TPO-independent mechanism (Avecilla et al. 2004). At the molecular level, SDF1α stimulates megakaryocyte CXCR4 surface polarisation and secretion of matrix metalloprotease 9 (Lane et al. 2000), enabling Mk migration towards an SDF1α gradient and facilitating localisation to the vasculature for platelet release (Niswander et al. 2014). FGF4 binds and activates FGF receptors, of which FGF receptor type 1 and type 2 are expressed on cells committed to the megakaryocytic lineage (Bikfalvi et al. 1992). Similar to SDF1α, administration

of FGF4 is capable of increasing platelet counts *in vivo* and partially rescues thrombopoiesis in *Thpo*$^{-/-}$ and *Mpl*$^{-/-}$ mice by directly augmenting cellular adhesion of megakaryocytes to endothelial cells. SDF1α- and FGF4-mediated vascular niche positioning relies on the vascular cell adhesion molecule 1 (VCAM1) - very late antigen 4 (VLA4)-mediated interaction between endothelial cells and megakaryocytes (Avecilla et al. 2004).

Once positioned in the vascular niche, the terminal events of thrombopoiesis are triggered by the bioactive sphingolipid sphingosine-1-phopsphate (S1P), a concentration gradient of which exists between vascular and nonvascular bone marrow niches, and a receptor for which, S1pr1, is expressed on megakaryocytes (Zhang et al. 2012). While germline deletion of S1pr1 in mouse models is embryonically lethal, hematopoietic-specific inactivation via generation of bone marrow chimaeras induced severe thrombocytopenia, with loss of a single *S1pr1* allele sufficient to result in more than 50% reduction in platelet counts. Furthermore S1pr1 activation with a synthetic agonist caused a rapid increase in platelet numbers (Zhang et al. 2012). These studies imply a requirement for S1P-S1pr1 at two stages during thrombocytopoiesis. Firstly, S1P acts as a chemo-attractant that initiates the entry of proplatelets into marrow sinusoids. Secondly, the few *S1pr1*$^{-/-}$ proplatelets that reach bone marrow sinusoids remained firmly attached to their parent Mk, suggesting that high vascular S1P concentration triggers proplatelet shedding into the blood stream (Zhang et al. 2012). S1P is released by activated platelets (Yatomi, Ruan, Hakomori, et al. 1995; Yatomi, Ruan, Ohta, et al. 1995), raising the possibility that a paracrine feedback loop might exist to augment platelet release during high platelet consumption.

Cytokines in emergency megakaryopoiesis

In addition to their well-established function in thrombus formation, platelets are also rapidly deployed to sites of inflammation. Recent studies reveal that modulation of inflammatory processes by platelets can occur via interaction with neutrophils, internalization of pathogens and secretion of cytokines, platelet microbicidal proteins (PMPs) and other inflammatory regulators

(Yeaman 2014). Thus, inflammation or infection can result in a transient high demand for platelets and thrombocytopenia is often reported in these situations. Recent studies suggest that emergency mechanisms exist to facilitate both the rapid and efficient production of megakaryocytes and the release of platelets to counteract platelet depletion.

Nishimura and colleagues have suggested that, under conditions of acute demand, platelet production via proplatelet formation is insufficient, and propose that megakaryocytes can undergo rapid cytoplasmic fragmentation, leading to the release of large numbers of platelets in a process termed megakaryocyte rupture (Nishimura et al. 2015). This process is triggered by the inflammatory cytokine IL1α. Administration of IL1α *in vivo* induced this Mk rupture, followed by a rapid and immediate increase in circulating platelet counts. Interestingly, *in vitro,* IL1α stimulation could also promote megakaryocyte differentiation and maturation. It is noteworthy that Mk rupture-mediated thrombopoiesis was also observed during steady state, although to a relatively minor extent, implying that alternative processes are more dominant for platelet biogenesis under normal homoestasis. A modest reduction in steady-state platelet count is observed in mice lacking IL-1α or its receptor, consistent with this activity (Nishimura et al. 2015).

Microbial pathogen-associated molecular patterns (PAMPs) interact with toll-like-receptors (TLR) and other pattern recognition receptors on the surface of innate immune cells, inducing production of a broad range of cytokines implicated in Mk and platelet production. For example, IFNα produced by monocytes and fibroblasts upon viral infections, stimulates HSC-derived SL-MkPs to initiate translation of pre-existing megakaryocytic transcripts, inducing rapid SL-MkP maturation and ultimately facilitating increased platelet production. Similarly, administration of lipopolysaccharide (LPS), mimicking a bacterial infection, or recombinant tumor necrosis factor alpha (TNFα), a cytokine involved in mediating acute inflammation, also triggered increased Mk-associated protein expression within the HSCs compartment (Haas et al. 2015). In addition, the pro-inflammatory cytokine IL-6 has been shown to enhance hepatic Tpo expression, increasing circulating Tpo levels during inflammation (Kaser et al. 2001) or in ovarian cancer patients (Stone

et al. 2012). Recent studies have also linked the inflammation-associated cytokines IL-21 and angiopoietin-like 4 with pro-megakaryopoietic activity (Schumacher et al. 2015; Benbarche et al. 2017).

Finally, several other cytokines discussed above in the context of steady state megakaryopoiesis are produced during infection or inflammation, including GM-CSF, SCF, IL-3, IL-11 and LIF (Müller-Newen et al. 2017). While having little or no apparent role in maintenance of steady state megakaryopoiesis, the contributions and potential synergies of these cytokines in emergency contexts remains a fertile area of study.

Pathobiology of thrombopoietin and the Mpl receptor

Consistent with the key regulatory role of Tpo-Mpl signalling in megakaryopoiesis, mutations at the *THPO* and *MPL* loci are associated with thrombocytopenia, thrombocythemia and related diseases. Congenital amegakaryocytic thrombocytopenia (CAMT) is a rare autosomal recessive bone marrow failure syndrome that presents with reduced numbers of megakaryocytes and severe thrombocytopenia. In the majority of cases, the underlying molecular mechanism for the disorder is biallelic (homozygous or compound heterozygous) loss-of-function mutations at the *MPL* locus (Ihara et al. 1999; Ballmaier et al. 2003). Because Tpo signalling is vital for the maintenance of HSC, CAMT patients nearly always progress to aplastic anemia (Ballmaier et al. 2003; King et al. 2005; Walne et al. 2012). Interestingly, the type of *MPL* mutation is predictive for the disease course. While nonsense *MPL* mutations, causing the complete loss of the receptor, induce the severe Type I-CAMT with chronically low platelet counts and an early onset of pancytopenia, missense *MPL* mutations that allow residual Mpl activity are associated with Type II-CAMT, a milder form of the disease in which thrombocytopenia is transiently ameliorated and pancytopenia delayed (Figure 2 C) (Ballmaier et al. 2003; Gandhi et al. 2005; Germeshausen et al. 2006).

Recently, a homozygous *THPO* mutation, *THPO R119C,* was described in a family with three children affected with CAMT, a lesion which is proposed to both reduce the capacity of Tpo

to stimulate Mpl, as well as reduce the efficiency of Tpo secretion (Pecci et al. 2018). Similarly, an inactivating mutation (*THPO R17C*) was reported in patients with mild asymptomatic thrombocytopenia (Mandrile et al. 2013), homozygosity of which induced aplastic anemia (Dasouki et al. 2013) and *THPO R99W* and *R157X* mutations have also been associated with inherited bone marrow failure (Seo et al. 2017). Finally, the minimal deleted chromosomal region in the rare 3q26.33-3q27.2 microdeletion syndrome, which is associated with diverse, multi-organ symptoms, including thrombocytopenia, includes the *THPO* gene, haploinsufficiency of which likely accounts for the latter symptom (Mandrile et al. 2013; Dasouki et al. 2014).

Just as loss of function mutations of *THPO* and *MPL* cause thrombocytopenia or aplastic anaemia, gain of function mutations in these genes are associated with leukemia, thrombocytosis, and the myeloproliferative neoplasms. Acute megakaryocytic leukaemia (AMKL), although extremely rare in adults, comprises up to 15% of pediatric acute myeloid leukemia (AML) and has two major forms: AMKL associated with Down syndrome (DS-AMKL) and non-DS-AMKL. Mutations in *GATA1* are universally associated with DS-AMKL and this lesion cooperates with secondary mutations to establish disease. These latter mutations often involve genes encoding cytokine signalling proteins, including activating mutations in *MPL* (Gruber & Downing 2015).

Inherited mutations that result in increased Tpo signalling have also been described in families with thrombocytosis (Figure 2 C) (Teofili & Larocca 2011; Hong & Gotlib 2014). The *MPL S505N* mutation within the transmembrane domain-encoding exon has been described in multiple families with elevated platelet counts, often accompanied by bone marrow fibrosis with ageing. This mutation causes constitutive activation of the receptor by inducing ligand-independent dimerisation and also results in low circulating Tpo levels, due to increased cytokine internalisation and degradation by the increased MPL mass associated with high platelet numbers (Ding et al. 2004; Hong & Gotlib 2014). Although more commonly associated with myeloproliferative neoplasia (see below), a mutation at MPL amino acid 515 (*MPL W515R*) has also been observed in a family with isolated thrombocytosis (Vilaine et al. 2012) and a germline *MPL R321W* mutation was identified

in a patient with essential thrombocytosis (Milosevic Feenstra et al. 2016). Polymorphisms in the *MPL* gene associated with elevated platelet counts have also been found, including the Baltimore mutation (*MPL K39N*) (Hong & Gotlib 2014). Interestingly, this incompletely penetrant autosomal dominant allele results in reduced cell surface receptor expression. A likely mechanism for the associated thrombocytosis is that sufficient receptor is produced to allow robust signalling, but reduced Tpo clearance results in increased available cytokine. An analogous mechanism has been proposed for the thrombocytosis associated with heterozygosity of *MPL R102P*, a loss of function mutation that causes CAMT when homozygous (Bellanné-Chantelot et al. 2017). Similarly, the *MPL P106L* polymorphism results in severe thrombocytosis when homozygous and milder symptoms when heterozygous (El-Harith et al. 2009). Lastly, mutations in splice sites or the 5'-untranslated region of the *THPO* gene have also been identified in families with thrombocytosis (Wiestner et al. 1998; Hong & Gotlib 2014). These mutations result in more efficiently translated transcripts and patients exhibit a sustained elevation in circulating Tpo levels despite the high platelet count.

The myeloproliferative neoplasms (MPN) are three distinct disorders, essential thrombocythemia (ET), polycythemia vera (PV), and primary myelofibrosis (PMF), that are characterised by activated cytokine receptor signalling pathways caused by three major classes of mutation. Activating mutations in *JAK2*, a key mediator of Tpo signalling, as well as that of Epo and other cytokines, are the most prevalent, particularly the *JAK2 V617F* mutation, and account for around two-thirds of MPN, including almost all PV. Several *in vitro* and *in vivo* studies have demonstrated that expression of a type I cytokine receptor, such as Mpl or the Epo receptor, is crucial for the development of *JAK2* mutant-induced disease (Lu et al. 2005; Sangkhae et al. 2014). Mutations in the calreticulin (*CALR*) gene are also common in ET and PMF. These mutations induce a frameshift to a specific alternative reading frame, resulting in a new C-terminal sequence with a positively charged tail that binds exclusively to, and constitutively activates, the Mpl receptor, explaining the megakaryocytic phenotype of the diseases (Klampfl et al. 2013; Araki et al. 2016;

Chachoua et al. 2016). Activating mutations in *MPL* itself are also observed in MPN, most frequently substitution of the W515 residue in the transmembrane domain, a key residue in regulating dimerisation, although rare mutations in the extracellular or cytoplasmic regions can also be found (Pikman et al. 2006; Defour et al. 2013; Milosevic Feenstra et al. 2016).

Clinical use of megakaryopoietic cytokines

The identification and cloning of Tpo in 1994 immediately stimulated efforts to evaluate this cytokine as a potential therapy for thrombocytopenia. Recombinant human (rh) Tpo and pegylated rh-megakaryocyte growth and development factor (PEG-rhMGDF), a truncated, non-glycosylated Tpo-protein coupled to polyethyleneglycol, were developed, and although highly efficient in stimulating platelet production in non-human primates and humans (Tomer & Harker 1996; Harker et al. 1997; Kuter et al. 2001), development was halted when thrombocytopenia occurred in several healthy volunteers receiving PEG-rhMGDF due to the development of antibodies that cross-reacted with endogenous Tpo (Li et al. 2001). Subsequently, an intensive search for non-immunogenic thrombopoietin mimetics capable of binding and activating the Mpl receptor began.

In 1997, Cwirla and colleagues identified a 14-amino acid peptide that bound to Mpl with high affinity but did not share homology with endogenous Tpo (Cwirla et al. 1997). To augment the activity of this Tpo mimetic, four such peptides were covalently fused to the antibody Fc fragment, creating a peptibody (Wang et al. 2004; Bussel et al. 2006). This agent, called Romiplostim, was shown to stimulate the growth of megakaryocyte progenitor cells, to increase the number, size and ploidy of megakaryocytes (Broudy & Lin 2004) and to enhance the production of platelets *in vivo* (Wang et al. 2004). Romiplostim activates the same downstream signalling pathways as rhTpo albeit with one fourth the affinity (Stasi et al. 2010), and competes with endogenous Tpo (Broudy & Lin 2004). Romiplostim proved effective in raising platelet counts in 80% of patients with idiopathic thrombocytopenia (ITP), an autoimmune disease characterized by autoantibody-mediated

destruction of platelets and 5-year follow-up documented that the drug was well tolerated and produced no antigenic response (Kuter 2007). In 2008 Romiplostim was approved for use in ITP.

High throughput screening for non-peptide Mpl agonists resulted in the identification of Eltrombopag. Stimulation of human $CD34^+$ cells with this agent increased megakaryocytic proliferation and differentiation and, upon administration *in vivo*, stimulated increased platelet counts (Erickson-Miller et al. 2005; Jenkins et al. 2007). Unlike Romiplostim, this small molecule does not compete with Tpo for Mpl-receptor binding sites, but instead interacts with the transmembrane domain of the receptor activating downstream JAK/STAT signalling, via Stat5, mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinases 1 and 2 (ERK1/2) (Erickson-Miller et al. 2005; Kim et al. 2007). Interestingly, unlike Romiplostim and Tpo itself, Eltrombopag does not stimulate Stat3 and AKT signalling, suggesting that Eltrombopag and Tpo may potentially act additively to increase platelet production. Eltrombopag proved effective in clinical trials in ITP (Stasi et al. 2010) and was approved as a second-line treatment for ITP as well as for hepatitis-C-related thrombocytopenia in patients undergoing antiviral treatment. Clinical trials have further proved Eltrombopag capable of restoring tri-lineage hematopoiesis in aplastic anemia (Desmond et al. 2014; Townsley et al. 2017).

Thrombocytopenia associated with chemotherapy or stem cell transplantation creates a significant demand for platelet transfusion. Limited data from early studies with rhTpo or PEG-rhMGDF suggested little or no benefit following myeloablative regimens in acute leukaemia or for thrombocytopenia associated with autologous stem cell transplants (Archimbaud et al. 1999; Nash et al. 2000; Schuster et al. 2002). These disappointing effects probably reflect the role of TPO in controlling platelet numbers – via stimulation of stem and progenitor cells, which are themselves ablated with aggressive chemotherapy. This contrasts with ITP, for example, where despite short circulating platelet half-life, sufficient stem and progenitor cell reserve exists for a beneficial effect of TPO. Nevertheless, future re-evaluation of newer TPO receptor agonists may be worthwhile in thrombocytopenia associated with myeloablative therapy, perhaps particularly Eltrombopag, which

appears to have a distinct mechanism of action to TPO. Finally, second generation oral thrombopoietin receptor agonists, such as Avatrombopag and Lusutrombopag are in clinical studies for the treatment of patients with ITP and chronic liver disease undergoing invasive procedures (Bussel et al. 2014; Terrault et al. 2014; Kim 2016), and ongoing studies continue to explore the potential use of Tpo agonists in other thrombocytopenic settings (Kuter 2013).

Summary and Perspectives

Cytokines and chemokines make an indispensable contribution to the controlled generation of megakaryocytes and the release of platelets, ensuring stable numbers of circulating platelets at steady state that suffices to maintain hemostasis without excess thrombosis (Table1). At steady state, Tpo acting through its receptor Mpl, is the major cytokine regulator of megakaryocyte number, controlling cellular production via stimulation of stem and progenitor cells. Nevertheless, residual megakaryopoiesis in Tpo-deficient mice clearly demonstrates the existence of a Tpo-independent mechanism of Mk generation, yet to be fully elucidated. Platelet release from megakaryocytes appears largely independent of Tpo; other factors such as SDF1α, FGF4 and S1P having prominent roles in the final stages of megakaryocyte migration and thrombopoiesis. However, regulation of terminal megakaryocyte maturation and platelet release remain to be fully explained. Further studies of the relative contribution of proplatelet versus other methods of platelet formation - at steady state and in pathological situations, as well as improved understanding of cytokine/chemokine contribution to these processes, are still required.

Consistent with its major role in megakaryopoiesis, mutations in the genes encoding Tpo and Mpl are associated with thrombocytopenia, when the pathway is compromised, and thrombocytosis or myeloproliferative disease when inappropriately activated. The specific nature of these mutations provides valuable information on the likely course of disease and can inform treatment options. While forms of Tpo or Mpl receptor agonists have found clinical utility,

particularly in ITP, considerable interest remains in exploring novel Tpo mimetics and additional clinical contexts in which such agents can be effectively applied.

Other cytokines, while often active in megakaryopoiesis when added to cultures or administered *in vivo*, appear dispensable for maintenance of steady state megakaryocyte numbers. However, many such cytokines, including members of the IL-6 family, interferons and others, are produced during infections or inflammation and a current area of fertile study is the role of these agents in generation of megakaryocytes and platelets at times of acute need. Similarly, the identification in recent years of specialized cells within the stem cell compartment that by-pass transit through the classically defined haematopoietic differentiation hierarchy and more directly differentiate to megakaryocytes, has opened new areas of investigation: the contexts in which these by-pass pathways are used, the relationship between these specialized megakaryocyte progenitor cells and their classically defined cousins, the roles of Tpo and other cytokines, and the potential for manipulating by-pass processes clinically, remain questions of great interest.

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Table 1. Cytokine control of megakaryopoiesis

Steady-state megakaryopoiesis			
Cytokine	**Receptor**	**Major function in megakaryopoiesis**	**References**
Tpo	Mpl	induces Mk progenitor cell proliferation and differentiation	de Sauvage et al., 1996; Alexander et al., 1996; Ihara et al., 1999
IL-6	IL6R	induces Mk maturation in combination with IL-3 *in vitro*	Ishibashi et al., 1989; Hoffman et al., 1989; Nagler et al., 1995
IL-11	IL11RA	induces Mk maturation in combination with IL-3 *in vitro*	Teramura et al., 1992; Neben et al., 1993; Gordon et al., 1996
LIF	LIFR	induces Mk maturation in combination with IL-3 *in vitro*	Burstein et al., 1992; Melcalf et al., 1990
GM-CSF	GM-CSFR	promotes Mk proliferation *in vitro*	Metcalf, Burgess et al., 1986; Ishibashi et al., 1990
IL-3	IL3R	promotes Mk proliferation *in vitro*	Metcalf, Begley et al., 1986
IL-9	IL9R	promotes Mk differentiation in combination with Epo and SCF *in vitro*	Fujiki et al., 2002; Xiao et al., 2017
SCF	c-KIT	promotes Mk proliferation in combination with Tpo *in vitro*	Broudy et al., 1995
FL	FLT3	promotes Mk proliferation in combination with Tpo *in vitro*	Sigurjonsson et al., 2002
VEGFA	VEGFR1/2	VEGFR1 activation increases Mk maturation, endosteal localisation and platelet counts; VEGFR2 activation promotes Mk proliferation, survival and differentiation *in vitro*	Casella et al., 2003; Pitchford et al., 2012; Coppola et al., 2006
VEGFC	VEGFR3	VEGFR3 activation attenuates Mk maturation	Thiele et al., 2012
Megakaryocyte migration			
SDF1-α	CXCR4	facilitates Mk migration to vasculature	Avecilla et al., 2004
FGF4	FGFR	augments cellular adhesion of Mks to endosteal niche	Avecilla et al., 2004
Platelet release			
S1P	S1pr1	initiates entry of proplatelets into BM sinusoids and triggers platelet shedding into the blood	Zhang et al., 2012
Emergency megakaryopoiesis			
IL1α	IL1R1	promotes Mk differentiation and maturation *in vitro*; induces Mk rupture *in vivo*	Nichimura et al., 2015
IFNα	IFNAR	stimulates rapid SL-MkP maturation	Haas et al., 2017
IL6	IL6R	promotes Tpo expression	Kaser et al., 2001; Stone et al., 2012
IL21	IL21R	increases Mk and platelet generation and platelet clearance	Benbarch et al., 2017
Angptl4	Integrins	promotes Mk reconstitution after myelosuppression	Schumacher et al., 2015

Figure 1

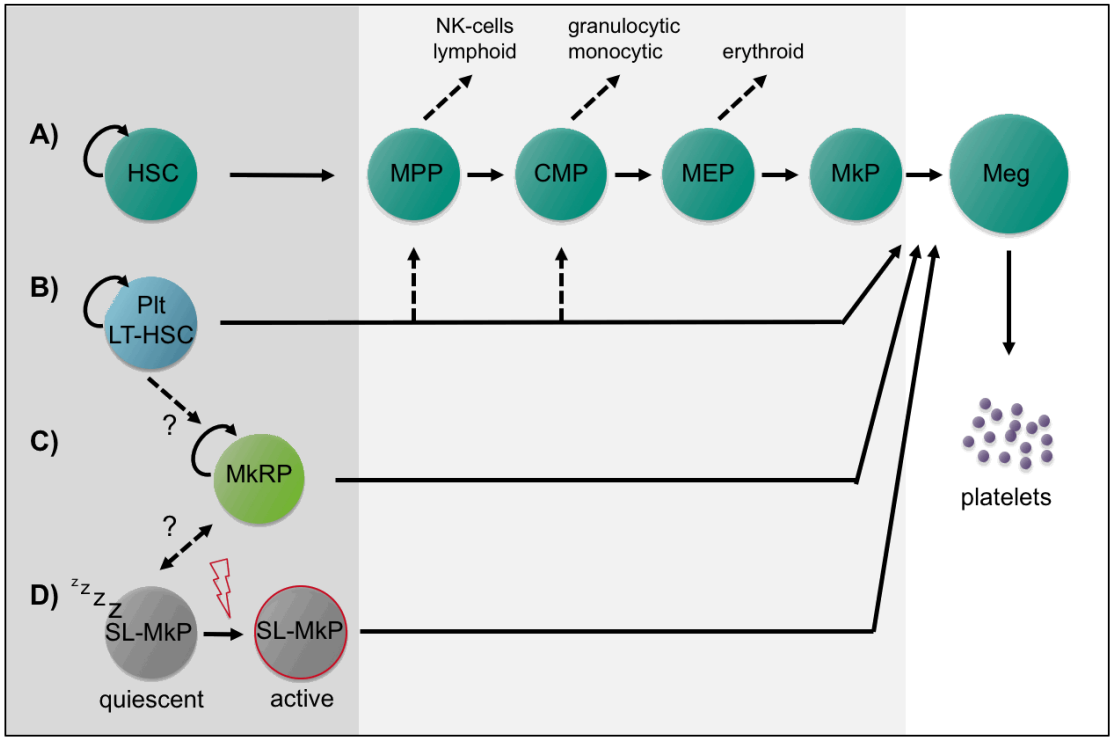

Figure 2