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## Novel mechanisms of platelet clearance and thrombopoietin regulation

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### Abstract

**Purpose of review**—The human body produces and removes  $10^{11}$  platelets daily to maintain a normal steady-state platelet count. Platelet production must be tightly regulated to avoid spontaneous bleeding or arterial occlusion and organ damage. Multifaceted and complex mechanisms control platelet removal and production in physiological and pathological conditions. This review will focus on different mechanisms of platelet clearance, with focus on the biological significance of platelet glycans.

**Recent findings**—The Ashwell-Morrell receptor (AMR) recognizes senescent, desialylated platelets under steady state conditions. Desialylated platelets and the AMR are the physiological ligand-receptor pair regulating hepatic thrombopoietin (TPO) mRNA production, resolving the longstanding mystery of steady state TPO regulation. The AMR-mediated removal of desialylated platelets regulates TPO synthesis in the liver by recruiting JAK2 and STAT3 to increase thrombopoiesis.

**Summary**—Inhibition of TPO production downstream of the hepatic AMR-JAK2 signaling cascade could additionally contribute to the thrombocytopenia associated with JAK1/2 treatment, which is clinically used in myeloproliferative neoplasms.

### Keywords

Ashwell-Morrell receptor; desialylated platelets; JAK2; STAT3; thrombopoietin

### Introduction

While the primary function of platelets is hemostasis, platelets also participate in antimicrobial host defense, secrete cytokines that can induce inflammation and growth factors that aid tissue repair. Chronic inflammation is often associated with reactive high platelet counts, and responses to acute infections may be accompanied by sudden reduction or increase of platelets (thrombocytopenia or thrombocytosis, respectively), placing platelets as reporters of disease progression or healing. A steady platelet supply is ensured by a

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### Conflicts of interest

The authors declare no conflicts of interest.

continuous platelet clearance and production of  $\sim 10^{11}$  platelets daily, and the rate of production rises sharply under conditions of platelet destruction to maintain levels of 150,000–400,000 platelets per microliter of blood. Platelet clearance and production must be therefore regulated to avoid spontaneous bleeding or arterial occlusion and organ damage, however both processes remain poorly understood. This review will focus on the current knowledge of platelet clearance and briefly address mechanisms of production.

## Thrombopoiesis

Platelet production is a complex process that requires differentiation of hematopoietic stem cells (HSCs) into specialized progenitors and their organized interplay with the bone marrow microenvironment and hematopoietic cytokines. Data support the existence of two anatomical and functional marrow microenvironmental “niches”: the osteoblastic niche and the vascular niche [1, 2]. Megakaryocyte (MK) maturation and platelet formation are dependent on cellular migration from the osteoblastic to the vascular niche, where once adequately mature, MKs extend proplatelet processes through or between cells of the sinusoidal endothelial layer and shed platelets into the bloodstream [3]. Marrow stromal cells are an integral part of these local microenvironments through expression of soluble and surface-bound cytokines, counter-receptors for integrins and other adhesion molecules on the surface of hematopoietic cells, and the secretion of extracellular macromolecules [4]. In reference to MK development, marrow stromal cells have been shown to secrete thrombopoietin (TPO), the primary regulator of thrombopoiesis [5], and CXCL12, a primary chemokine that attracts MKs and other hematopoietic cells to the marrow microenvironment [6, 7]. Additionally, CXCL12 acts to stimulate MKs to express cell surface SCF [8], which synergistically promotes MK growth with TPO [9], and to express VCAM-1 and fibronectin, which promote cell growth through their binding to the MK integrin  $\alpha 4\beta 1$  [10, 11]. The interaction of microenvironmental Von Willebrand Factor (VWF) and its MK receptor glycoprotein (GP) Ib-IX appears important for platelet formation and release, whereas in contrast, type I collagen, which localizes to the osteoblastic niche, prevents platelet formation [12]. Recent studies point to the fact that glycans, specifically glycan synthesis by the  $\beta 1,4$ -galactosyltransferase 1, are key elements in hematopoiesis regulating HSC function and MK migration (S.G., K.M.H. et al, unpublished data).

A major milestone in understanding the molecular mechanisms of thrombopoiesis was the discovery of TPO in 1994. TPO is the primary regulator of platelet production, supporting the survival, proliferation and differentiation of the platelet precursors, bone marrow MKs [13–15]. Since the discovery of TPO many molecular mechanisms of thrombopoiesis have been identified, including the development of polyploidy and proplatelet formation, the final fragmentation of the MK cytoplasm to yield blood platelets, and the regulation of this process [14, 16–19]. Although much progress has been made towards the understanding of thrombopoiesis, multiple unanswered questions remain. One unanswered question is the regulation of TPO production under steady state and under pathologic conditions.

Multiple organs display RNA transcripts, with hepatocytes having the highest levels and being the primary cells responsible for the production and secretion of TPO into the bloodstream. In one model hepatic TPO production has been identified as constitutive,

where TPO serum levels are maintained solely by its uptake and metabolism by platelets and MKs [20–24]. Thus, the removal and destruction of TPO released into the bloodstream is mediated by expression of its receptor, Mpl. In patients with thrombocytopenia little of the hepatocyte produced TPO is presumed to be removed by platelets and TPO blood levels rise; in contrast, thrombocytosis should be accompanied by low steady state levels of blood TPO, because platelet mediated TPO destruction surpasses TPO production [20–24]. Additional support for this model comes from *Thpo*<sup>+/-</sup> mice [25], where loss of one *Thpo* allele leads to a 40% reduction of platelet counts, and from a rabbit model of busulfan-induced thrombocytopenia [20].

A growing body of evidence lends credence to the assertion that platelet TPO metabolism is not the sole determinant of plasma TPO levels, implying that TPO levels are regulated in a more complex fashion. A number of inflammatory states are associated with thrombocytosis and increased blood TPO levels (e.g., ulcerative colitis, rheumatoid arthritis, ovarian cancer) [5, 26–34]. The inflammation-induced increase in TPO expression is mediated by IL-6, which stimulates hepatocyte TPO production both *in vitro* and *in vivo* [31, 32, 35, 36]. Selective liver irradiation also stimulates hepatocyte TPO production in humans [37]. In contrast to the “autoregulation” model of blood TPO levels, serum TPO levels are lower than expected in patients with Immune Thrombocytopenia (ITP) [38, 39], and high in patients with Essential Thrombocythemia (ET) [34, 40]. The notion that TPO production is regulated, rather than autonomous, is further supported by data showing that marrow stromal cells produce TPO in response to thrombocytopenia both in mice and in humans [5, 41].

In addition to its effects on MK progenitors and mature cells, TPO affects HSCs *in vitro*, especially when used in combination with IL-3 or SCF [42, 43]. HSCs express Mpl on their surface, indicating the stem cell effects of TPO are direct [44, 45]. Recently, mice lacking Mpl expression on MKs and platelets but expressing Mpl normally on stem/progenitor cells (*Mpl<sup>fl/fl</sup> Pf4-Cre* mice) were generated. Despite lacking Mpl on MKs and platelets, *Mpl<sup>fl/fl</sup> Pf4-Cre* mice displayed profound megakaryocytosis and thrombocytosis with a remarkable expansion of MK-committed and multipotential progenitor cells, the latter displaying biological responses and a gene expression signature indicative of chronic TPO overstimulation as the underlying causative mechanism. Even more surprising was the normal circulating TPO levels in these mice. Thus, the authors concluded that TPO signaling in MKs is dispensable for platelet production. The key role of TPO signaling is in controlling platelet numbers via generation and stimulation of the bipotential MK precursors. On the other hand, Mpl expression in MKs and platelets is essential to prevent megakaryocytosis and myeloproliferation by restricting the amount of TPO available to stimulate the production of MKs from the progenitor cell pool [46]. This is an intriguing finding as *Mpl<sup>fl/fl</sup> Pf4-Cre* mice were obviously able to “bypass” the lack of Mpl on the MK lineage, presenting more evidence that circulating TPO levels are regulated in a complicated manner.

A new model (detailed below) furthers our understanding of the regulation of blood TPO levels and thrombopoiesis: desialylated, senescent platelet clearance via the hepatic Ashwell-Morrell Receptor (AMR) enhances hepatic TPO production. In support of this

notion, injection of desialylated platelets in rabbits stimulates platelet production [47], presumably by stimulating liver TPO secretion.

## Platelet clearance

Several mechanisms mediate platelet clearance. One mechanism appears to function via aging (senescence) induced signals, *via* glycan degradation and apoptotic mechanisms. Platelets are also removed by immune (antibody) responses. Whether these mechanisms converge at a certain points during platelet-life time is unclear.

## The aging (senescence) induced platelet clearance

Studies have shown that platelet surface glycans mediate platelet clearance [48, 49]. Recently, loss of sialic acid has been identified as a determinant of senescent platelet removal [50]. Platelets lose sialic acid during circulation and are cleared via the hepatic AMR, a transmembrane heterooligomeric glycoprotein complex composed of ASGPR1 (CLEC4H1, HL-1) and ASGPR2 (CLEC4H2, HL-2) subunits. This highly conserved receptor has been largely regarded as an endocytic receptor [51], and since its discovery four decades ago the regulatory role of the hepatic AMR has remained largely unclear. Specifically, mice lacking either ASGPR1 or ASGPR2 subunit do not accumulate plasma proteins or lipids lacking sialic acid, which has been the predicted outcome of eliminating one of the AMR subunits [51]. It has therefore been a surprising discovery that platelets with reduced  $\alpha$ 2,3-linked sialic acid during sepsis, after cold storage (*in vitro* aging), or in mice lacking the sialyltransferase ST3GalIV are cleared by the hepatic endocytic AMR [52–55].

These findings led subsequently to the discovery that removal of senescent, sialic acid deprived platelets drives hepatic TPO mRNA expression *in vivo* and *in vitro* via JAK2 and STAT3 to increase MK numbers and *de novo* platelet production. The notion that loss of sialic acid determines platelet life span is not entirely novel [52, 53, 55–58], however, the recent study elucidates that aged, desialylated platelets regulate hepatic TPO mRNA production *in vivo* via the AMR. This feedback mechanism presents the AMR-desialylated platelet pair as the critical control point for TPO homeostasis and shows that TPO expression in hepatocytes is regulated and not constitutive (Figure 1). Importantly, disruption of AMR-desialylated platelet signaling by the JAK1/2 inhibitors AZD1480, TG101348 and BMS911543 adversely affect hepatic TPO mRNA expression and secretion in hepatocytes *in vitro* and *in vivo* [50]. Thrombocytopenia is a common adverse event of JAK1/2 inhibitor treatment, which is clinically used in myeloproliferative neoplasms (MPNs) [59, 60]. JAK1/2 inhibitors target hematopoietic stem and precursor cell mutant JAK2-V617F as well as wild-type JAK2, activation of which is essential for red blood cell and platelet production [61, 62]. This new study indicates that inhibition of TPO production downstream of the hepatic AMR-JAK2 signaling cascade could additionally contribute to the thrombocytopenia associated with JAK1/2 treatment. Clinical studies are necessary to investigate this notion.

## The AMR-IL-6R connection

Interestingly, the AMR signaling cascade, which involves JAK2 phosphorylation and STAT3 phosphorylation and translocation to the nucleus shares similarities with that of IL-6 [63]. IL-6 stimulates TPO mRNA expression in hepatocytes *in vivo* and in HepG2 and Hep3B cells *in vitro* [31, 32, 35, 64]. These data suggest that both desialylated platelets and IL-6 lead to STAT3-mediated hepatic TPO mRNA expression downstream of the AMR-JAK2 and IL6 receptor (IL-6R)-JAK1 signaling cascades, respectively.

Is there a cross-talk between the AMR and IL-6R? Binding of IL-6 to its hepatic receptor engages the signal transducing subunit gp130, leading to STAT3 tyrosine phosphorylation and activation by gp130-associated JAK1. Whether JAK2 and STAT3 directly associate with the AMR or require gp130 remains to be determined. A tyrosine kinase of 127 kDa (JAK2?) constitutively associates with the AMR ASGPR1 subunit in HepG2 cells [65]. It is therefore possible that both IL-6 and desialylated platelets lead to STAT3-mediated hepatic TPO mRNA expression downstream of JAK1 and JAK2, respectively. Hepatic STAT3 controls the transcription of mRNA for acute phase plasma proteins [66]. Since both the AMR and IL-6R share signaling through STAT3, it is therefore tempting to speculate that acute phase proteins are produced in response to AMR ligation, which would establish clearance of desialylated platelets as a component of the acute phase response. Consistent with this hypothesis, the AMR-mediated removal of desialylated platelets improves the probability of host survival during sepsis [54, 55]. Separate studies have shown that liver regeneration following injury is promoted by platelets [67] and requires AMR and hepatic STAT3 function [68, 69]. Thus, the platelet-AMR-STAT3 signaling cascade may connect desialylated platelets to inflammatory responses.

## Apoptosis as a platelet clearance mechanism

Platelet survival also depends on the interplay between prosurvival and proapoptotic members of the Bcl-2 family, which are critical regulators of the intrinsic apoptotic pathway. Platelet survival is extended in mice lacking the proapoptotic proteins Bak and Bax, whereas platelet clearance is accelerated in mice lacking the prosurvival proteins Bcl-2, Bcl-x<sub>L</sub> and Mcl-1, and in mice treated with the BH3 mimetic ABT-737 (inhibitor of prosurvival Bcl-2, Bcl-x<sub>L</sub> and Bcl-w) [70–72]. Whether members of the Bcl-2 family alter platelet surface sialic acid content is unclear. Interestingly, the primary platelet clearance site following administration of ABT-737 is the liver in dogs presumably *via* scavenger receptors [73], whereas the spleen does not appear to regulate the platelet life span in mice [72]. More data are needed to establish if glycan degradation *in vivo*, i.e. sialic acid loss, triggers the intrinsic apoptotic machinery in platelets, linking glycan degradation and intrinsic apoptotic machinery in the clearance mechanisms regulating platelet survival. Interestingly, data show that newborn and adult mice have similar platelet production rates, but neonatal platelets survived 1 day longer in circulation [74]. A study of pro-apoptotic and anti-apoptotic Bcl-2 family proteins shows that neonatal platelets have higher levels of the anti-apoptotic protein Bcl-2 and are more resistant to apoptosis induced by the Bcl-2/Bcl-x<sub>L</sub> inhibitor ABT-737 than adult platelets. However, genetic ablation or pharmacologic inhibition of Bcl-2 alone does not shorten neonatal platelet survival or reduce platelet counts in newborn mice,

indicating the existence of redundant or alternative mechanisms mediating the prolonged lifespan of neonatal platelets [74]. Whether glycans (increase in platelet surface sialic acid) play a role in the prolonged survival of neonatal platelets remains to be established.

## Antibody mediated platelet clearance

ITP is a common bleeding disorder caused primarily by autoantibodies directed against platelet integrin  $\alpha$ IIB $\beta$ 3 (GPIIb-IIIa) and/or the GPIb-IX complex. The prevailing model posits that antibody-mediated platelet destruction occurs in the spleen [75], where the interaction between the Fc portion of platelet-associated autoantibodies and Fc $\gamma$  receptors (Fc $\gamma$ R) on macrophages initiates phagocytosis. However, data show that, in contrast to anti- $\alpha$ IIB $\beta$ 3-mediated ITP, anti-GPIb $\alpha$ -mediated ITP is often refractory to therapies targeting Fc $\gamma$ R pathways or splenectomy. Recent findings show that certain anti-GPIb $\alpha$ -antibodies trigger platelet desialylation, a process that deviates platelet clearance from splenic macrophage Fc-receptors to the liver, likely via the AMR [76], showing that Fc $\gamma$ R-independent mechanisms of ITP exist [77]. In this regard, an adult chronic ITP patient with an anti-GPIb-IX autoantibody, who was resistant to corticosteroids, IVIG, recombinant human TPO, rituximab, danazol and vindesine (Eldisine®), has been successfully treated with oseltamivir phosphate, a sialidase inhibitor used to treat influenza [78]. The mechanism how anti-GPIb $\alpha$  antibody binding leads to desialylation remains to be established. It is likely that platelets secrete active sialidases (i.e. Neu1 and Neu3) upon platelet antibody binding and/or activation [79]. The notion that the AMR plays a significant role in the clearance of anti-GPIb $\alpha$ -opsonized and desialylated platelets provides a potential explanation for refractoriness to splenectomy, as well as to steroid and IVIG therapies. Recent data also show that platelet destruction in ITP patients is mediated by CD8<sup>+</sup> cytotoxic T lymphocytes (CTLs) [80].

## Conclusion

Platelet counts are controlled in a multifaceted, complex manner. Recent evidence shows that the AMR recognizes senescent, desialylated platelets under steady state conditions. Desialylated platelets and the AMR are the physiological ligand-receptor pair regulating hepatic TPO mRNA production, resolving the longstanding mystery of steady state TPO regulation. The AMR-mediated removal of desialylated platelets regulates TPO synthesis in the liver by recruiting JAK2 and STAT3 to increase thrombopoiesis. Senescent platelets are also removed from the circulation by apoptotic signals. Platelets are cleared by antibody binding to platelets via macrophage Fc $\gamma$ R during pathologic conditions. Recent findings suggest that antibodies can induce platelet desialylation, thereby converging signals for platelet removal with immune mediated platelet removal. Many questions remain concerning the mechanisms governing platelet numbers. How do the above processes work together to maintain platelet numbers? Do clearance systems communicate with the bone marrow environment to ensure adequate thrombopoiesis? Research as well as clinical trials will continue to elucidate the mechanisms that regulate billions of circulating platelets.

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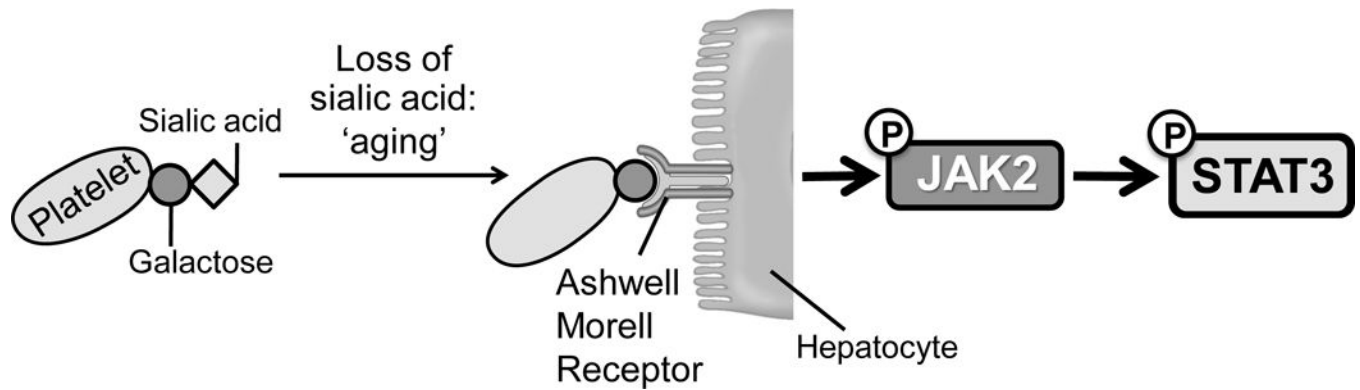


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**Key points**

- The AMR recognizes senescent, desialylated platelets under steady state conditions.
- Desialylated platelets and the AMR are the physiological ligand-receptor pair regulating hepatic TPO mRNA production.
- The AMR-mediated removal of desialylated platelets regulates TPO synthesis in the liver by recruiting JAK2 and STAT3 to increase thrombopoiesis.



**Figure 1. Scheme of hepatic TPO production via JAK2-STAT3 signaling after desialylated platelet uptake by the AMR**

Desialylated, senescent platelets are recognized by the hepatic AMR to regulate hepatic TPO production, bone marrow homeostasis and thrombopoiesis. Bone marrow MKs produce and release young sialic acid (purple ring) containing platelets into the blood stream. Young platelets maximally internalize TPO through Mpl receptors. Circulating platelets become desialylated by active blood-borne sialidases as they age (dashed purple ring), thereby becoming ligands for the AMR and being ingested by hepatocytes. Desialylated platelet ingestion positively stimulates hepatic TPO mRNA expression via JAK2-STAT3 signaling and TPO release in the circulation, thereby regulating bone marrow homeostasis and thrombopoiesis.