

Nuevos mecanismos en la depuración de plaquetas  
y control de la trombopoyetina

New mechanisms of platelet clearance  
and thrombopoietin regulation

## Mecanismos dependientes de glicanos reguladores de la sobrevivencia plaquetaria

Glycan-dependent regulatory mechanisms of platelet life

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### Summary

Platelets are important participants in hemostasis to repair vascular damage and in inflammatory events through secretion of cytokines and growth factors. Thus, chronic inflammation is often associated with reactive high platelet numbers, 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 or health. Platelet numbers are therefore intricately regulated to avoid spontaneous bleeding or arterial occlusion and organ damage. The growth factor thrombopoietin (TPO) drives platelet biogenesis by inducing megakaryocyte differentiation. A recent study in mice identified a feedback mechanism by which clearance of aged, desialylated platelets stimulates TPO synthesis by hepatocytes. This new finding generated renewed interest in platelet clearance mechanisms. Here, multiple established and emerging glycan dependent mechanisms of platelet senescence and clearance will be summarized.

### Thrombopoiesis

Thrombopoietin (TPO), the primary regulator of thrombopoiesis<sup>(1)</sup>, was discovered in 1994. TPO supports the survival, proliferation and differentiation of the platelet precursors, bone marrow megakaryocytes (MKs)<sup>(2-4)</sup>. Since the discovery of TPO many molecular mechanisms of thrombopoiesis have been identified and studied, including polyploidy and proplatelet formation, the final fragmentation of the MK cytoplasm to yield blood platelets<sup>(3,5-7)</sup>. Data suggest that MK maturation and platelet formation are dependent on migration of progenitor cells from the bone marrow osteoblastic niche to the vascular niche, where once adequately mature MKs extend pseudopodial projections, termed proplatelets, and shed platelets into the bloodstream<sup>(8)</sup>. Bone marrow stromal cells have been shown to support MK development through secretion of TPO and CXCL12, a primary chemokine that attracts MKs and other hematopoietic cells to the bone marrow microenvironment<sup>(9,10)</sup>. Additionally, CXCL12 induces MKs to express surface stem cell factor (SCF)

<sup>(11)</sup>, which synergistically promotes MK growth with TPO<sup>(12)</sup>, and VCAM-1 and fibronectin, which promote cell growth through their binding to the MK integrin  $\alpha 4\beta 1$ <sup>(13,14)</sup>. 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<sup>(15)</sup>.

### Regulation of TPO production

The regulation of TPO production under steady state and under pathologic conditions has been debated for decades. 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. TPO production has long been thought to be constitutive, with TPO serum levels maintained solely by its uptake and metabolism by platelets and MKs<sup>(16-20)</sup>. Circulating TPO levels are elevated in patients with congenital amegakaryocytic thrombocytopenia (CAMT), caused by germline *Mpl* mutations<sup>(21,22)</sup>, thrombocytopenia-absent radius (TAR) syndrome<sup>(23)</sup>, or acquired aplastic anemia<sup>(24,25)</sup>. In these cases, circulating TPO levels are inversely correlated to platelet counts. Under these conditions the removal and destruction of TPO released into the bloodstream depends on the platelet and MK mass and on expression of *Mpl* on the platelet and MK surface.

Support for this model also comes from mice, MKs and platelets of which specifically lack in *Mpl* or *Mpl*-regulatory proteins, i.e. the *Mpl*-associated tyrosine kinase JAK2 and the large GTPase dynamin 2 (DNM2), which regulates receptor-mediated endocytosis, e.g. *Mpl*<sup>(6,26,27)</sup>. *Mpl*<sup>fl/fl</sup> *Pf4-Cre* mice and *Jak2*<sup>fl/fl</sup> *Pf4-Cre* mice have 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<sup>(26,27)</sup>. These mice express *Mpl* and JAK2 normally in stem/progenitor cells. These findings are intriguing as *Mpl*<sup>fl/fl</sup> *Pf4-Cre* mice and *Jak2*<sup>fl/fl</sup> *Pf4-Cre* mice were able to “bypass” the lack of *Mpl* and JAK2 in the MK lineage, respectively, to produce platelets. The studies conclude that TPO signaling

in MKs is dispensable for platelet production, and that the key role of TPO signaling is in controlling platelet numbers via generation and stimulation of the bipotential MK precursors. Surprisingly, *Jak2*<sup>fl/fl</sup> *Pf4-Cre* and *Mpl*<sup>fl/fl</sup> *Pf4-Cre* mice have normal circulating TPO levels, showing that circulating TPO levels are regulated in a complicated manner. *Dnm2*<sup>fl/fl</sup> *Pf4-Cre* mice have impaired *Mpl*-mediated endocytosis, resulting in elevated plasma TPO levels and constitutive phosphorylation of JAK2, although JAK2 expression is reduced in platelets lacking DNM2<sup>(6)</sup>. *Dnm2*<sup>fl/fl</sup> *Pf4-Cre* mice develop MK hyperplasia, myelofibrosis, extramedullary hematopoiesis and severe splenomegaly. However, *Dnm2*<sup>fl/fl</sup> *Pf4-Cre* mice develop macrothrombocytopenia, not thrombocytosis, as DNM2-dependent receptor-mediated endocytosis plays an additional critical role in the formation of the MK demarcation membrane system required for platelet formation. The low blood platelet numbers of *Dnm2*<sup>fl/fl</sup> *Pf4-Cre* mice and their inability to clear circulating TPO likely exacerbate their rapid and severe myelofibrosis. Together the data show that MK and platelet mass are not the sole regulators of circulating TPO. In contrast to the “autoregulation” model of blood TPO levels, serum TPO levels are lower than expected in patients with immune thrombocytopenia (ITP)<sup>(28,29)</sup>, and high in patients with essential thrombocythemia (ET)<sup>(24,30)</sup>. In patients with ITP, the little TPO produced from hepatocytes 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 its production<sup>(16,20)</sup>. However, *Mpl* expression levels on the membrane surface of platelets are strongly decreased in patients with ET presenting the somatic JAK2 mutation V617F<sup>(31,32)</sup>, which can explain decreased TPO uptake and high circulation TPO levels.

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<sup>(1,33)</sup>. Selective liver irradiation in mice stimulates hepatic TPO production<sup>(34)</sup>. Further, in addition to marrow stromal cell TPO production, a number of inflammatory states (e.g. ulcerative colitis, rheumatoid arthritis, ovarian cancer) are associated with increased blood TPO levels and thrombocyto-

sis<sup>(1,30,33,35-41)</sup>. This inflammation-induced increase in TPO expression is mediated by interleukin 6 (IL-6), which stimulates hepatic TPO mRNA expression and production both in hepatocytes *in vivo* and in hepatoma HepG2 and Hep3B cells *in vitro*<sup>(38,39,41-43)</sup>. If hepatic TPO regulation by IL-6 is now well characterized, the ligand-receptor pair regulating hepatic TPO production at steady-state has remained elusive. 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 summary, circulating TPO levels are regulated in a complicated manner by platelet and MK Mpl-mediated endocytosis and destruction, and hepatic TPO production, regulated by IL-6 and desialylated, senescent platelets as further elucidated below.

#### Senescence-induced platelet sialic acid loss and clearance by the Ashwell-Morell receptor

Loss of sialic acid has recently been identified as a determinant of senescent platelet removal<sup>(44)</sup>. Platelets lose sialic acid during circulation and are cleared via the hepatic Ashwell Morell receptor (AMR), a transmembrane heterologous glycoprotein complex composed of ASGPR1 (HL-1) and ASGPR2 (HL-2) subunits. This highly conserved receptor has been largely regarded as an endocytic receptor<sup>(45)</sup>, and since its discovery four decades ago its regulatory role has remained largely unclear. Specifically, mice lacking either the 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<sup>(45)</sup>. 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 AMR<sup>(44,46-50)</sup>.

These findings led to the discovery that removal of senescent, sialic acid deprived platelets drives hepatic TPO mRNA expression *in vivo* and *in vitro* via JAK2 and signal transducer and activator of transcription 3 (STAT3) to increase MK numbers and *de novo* platelet production. The notion that loss of sialic acid determines platelet lifespan is not entirely novel<sup>(51-53)</sup>, however, the recent study elucidates that old (longer circulating), 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 not constitutive but is instead regulated. Importantly, disruption of AMR-desialylated platelet signaling using JAK1/2 inhibitors AZD1480, TG101348 and BMS911543 adversely affects hepatic TPO mRNA expression and secretion in hepatocytes<sup>(44)</sup>.

Polycythemia vera (PV), ET and myelofibrosis (MF) are clonal myeloproliferative neoplasms (MPNs) caused by mutations in the hematopoietic stem cells (HSCs) compartment. Most Philadelphia chromosome negative MPN patients with PV, ET and MF have an acquired mutation in Janus kinase 2 (*JAK2 V617F*), thrombopoietin receptor (*TpoR/MPL*) or calreticulin (*CALR*) gene in HSCs that results in constitutive JAK/STAT activity, leading to uncontrolled expansion of HSCs and erythroid, megakaryocytic and myeloid progenitors. Thrombocytopenia is a common adverse event of JAK1/2 inhibitor treatment, which is clinically used in MPNs<sup>(54,55)</sup>. 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<sup>(56,57)</sup>. The finding that JAK1/2 inhibitors disrupt AMR-desialylated platelet signaling and TPO expression in hepatocytes 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.

Sialylated derivatives of the glycan structure  $\beta$ 4-N-acetyllactosamine (Gal $\beta$ 4-GlcNAc) (type-2 LacNAc), a fundamental component of complex N-linked glycans, regulate thrombopoiesis and platelet lifespan. Unpublished data show that the major enzyme glycosyltransferase  $\beta$ 1,4Galactosyltransferase 1 ( $\beta$ 4GalT1) that synthesizes LacNAc is regulated via JAK/STAT signaling in MKs. Lack of  $\beta$ 4GalT1 results in severe thrombocytopenia in mice lending credence to the notion that JAK1/2 inhibitors may target  $\beta$ 4GalT1 in MKs leading to thrombocytopenia. Clinical studies are necessary to investigate this notion. In conclusion, these findings show that the regulatory mechanisms of TPO production, its effects and JAK2 regulation are complex and not

completely understood. Further investigation is necessary to determine the exact relationship between the TPO receptor Mpl, TPO and JAK2.

### Sequential functions of multiple glycosidases and endocytic lectin receptors in platelet clearance

Unmasking of N-acetylglucosamine (GlcNAc) and mannose (Man) linkages on derivatives of type-2 LacNAc, subsequent to neuraminidase (Neu) function implies the possibility that additional endocytic lectin receptors with different glycan binding specificities may also control platelet and secreted protein half-life and abundance. Multiple lectins capable of binding multivalent GlcNAc and Man linkages have been identified. For example, integrin  $\alpha$ M encoded by the *Itgam* gene can bind and endocytose proteins bearing exposed GlcNAc linkages that occur at the surface of chilled platelets<sup>(58,59)</sup>. Mice lacking the  $\alpha$ M subunit have increased platelet counts<sup>(60)</sup>, indicating that platelets are removed by this receptor under steady state conditions. The lectin domain also recognizes circulating cold-platelets and transfused cold-stored platelets via exposure of GlcNAc on platelet surfaces<sup>(61)</sup>. Cold-induced changes in GPIIb $\alpha$  glycan composition play a role in platelet binding to macrophages<sup>(62)</sup>. Interestingly, binding of VWF to platelets increases upon prolonged cooling<sup>(47)</sup>, indicating that VWF could facilitate clearance via the AMR and integrin  $\alpha$ M. Accumulation of secreted proteins with unmasked Gal and GlcNAc by lectin chromatography in plasma of mice lacking AMR and integrin  $\alpha$ M has been reported<sup>(63)</sup>, further implying the presence of glycosidases (Neu and  $\beta$ -galactosidase ( $\beta$ Gal)) activity in plasma.

### Immune-regulatory mechanisms regulating circulating platelet number

ITP is a common bleeding disorder caused primarily by platelet autoantibodies that accelerate platelet destruction, alter platelet function, and/or inhibit platelet production<sup>(64)</sup>. These autoantibodies are mainly directed against the two most abundant platelet GP complexes, GPIIb-IIIa (integrin  $\alpha$ IIB $\beta$ 3) and GPIb-IX. The prevailing model posits that antibody-mediated platelet destruction occurs in the spleen<sup>(65-67)</sup>, where the interaction between the Fc portion of platelet-associated immunoglobulin G antibodies and Fc $\gamma$  receptors (Fc $\gamma$ Rs) on macrophages initiates phagocytosis. However, in contrast

to anti- $\alpha$ IIB $\beta$ 3-mediated ITP, anti-GPIIb $\alpha$ -mediated ITP is often refractory to therapies targeting Fc $\gamma$ R pathways or splenectomy. Recent findings show that certain anti-GPIIb $\alpha$  antibodies trigger platelet desialylation, a process that deviates platelet clearance from splenic macrophage Fc receptors to the hepatic AMR, showing that Fc $\gamma$ R-independent platelet clearance mechanisms in ITP exist<sup>(66,68,69)</sup>. The mechanism of how anti-GPIIb $\alpha$  antibody binding leads to desialylation remains to be established. It is noteworthy that many antibodies targeting GPIIb $\beta$  and GPIIX subunits in the GPIIb-IX complex do not cause platelet clearance<sup>(70-72)</sup>. It is likely that platelets secrete neuraminidase upon antibody binding and/or platelet activation<sup>(73)</sup>. The notion that the AMR plays a significant role in the clearance of anti-GPIIb $\alpha$ -opsonized and desialylated platelets provides a potential explanation for refractoriness to splenectomy, as well as to steroid and intravenous immunoglobulin therapies.

The role of macrophages residing in peripheral organs in regulating platelet number at steady and the receptors involved *in situ* remains elusive. Transfusion experiments using fresh platelets show that platelets are cleared in the spleen and liver, presumably by macrophages<sup>(60)</sup>, however elimination of macrophages from wild type mice increases platelet count by only 5-10%<sup>(47)</sup>. Bone marrow macrophages regulate red blood cell number<sup>(74,75)</sup>. The role of bone marrow macrophages in thrombopoiesis has not been elucidated. Unpublished data show a novel role for the bone marrow macrophage in maintaining platelet count, by phagocytosing megakaryocytes that express the cancer-associated Thomsen-Friedenreich (TF) antigen, a disaccharide presented on O-glycans in cryptic form covered by a sialic acid moiety under healthy conditions.

### Role of intrinsic glycosidases

Removal of sialic acid from the cell surface glycoconjugates affects cell-cell interactions, binding to soluble molecules, viruses, bacteria and protozoa and modulates cell activity<sup>(76)</sup>. The significance of sialidase activity in the normal function of eukaryotic cells has been inferred from the heterogeneous clinical manifestations of individuals with genetic sialidase deficiencies<sup>(77-79)</sup>. At least four mammalian sialidases have been described in the human genome: sialidases 1 to 4 (Neu1-4)<sup>(80)</sup>. Neu1 is a mam-

malian lysosomal neuraminidase enzyme, while Neu3 is expressed on the cell surface where it may play a role in modulating the ganglioside content of the lipid bilayer.

Sialic acid loss from the platelet surface may be mediated by upregulation of platelet sialidases, i.e., Neu1 and Neu3, expressed in granular compartments and on the plasma membrane, respectively<sup>(73)</sup>. Platelet incubation with the sialidase inhibitor, 2-deoxy-2,3-dehydro-N-acetylneuraminic acid (DANA), enhances the recovery and survival of platelets stored at 4°C in mice<sup>(73)</sup>. *In vitro* studies demonstrated that addition of DANA during platelet storage at 4°C in the presence of plasma additive solutions preserves surface sialic acid<sup>(81)</sup>. The sialidase inhibitor oseltamivir phosphate (Tamiflu®), which is clinically used to treat influenza, has also been shown to increase platelet counts in 2 patients with immune thrombocytopenia (ITP), as well as in 77 patients from the Erasmus Medical Center, Rotterdam, independently of influenza diagnosis<sup>(82)</sup>. Desialylation is associated with apoptosis and phagocytosis of platelets in patients with prolonged isolated thrombocytopenia after allo-hematopoietic stem cell transplantation<sup>(83)</sup>. The sialidase inhibitor oseltamivir phosphate reduced platelet clearance in these patients, indicating that sialidases remove sialic acid from circulating platelets. Investigators also reported successful treatment with oseltamivir phosphate in a patient with chronic ITP positive for anti-GPIIb-IX autoantibody<sup>(84)</sup>. Hence, platelet sialic acid content dictates platelet interaction with lectins to induce clearance.

Platelets also express βGal in granular compartments and on their surface<sup>(73)</sup>. Since βGal is highly expressed and accumulates in lysosomes in senescent cells, it is used as a senescence biomarker both *in vivo* and *in vitro* in qualitative and quantitative assays, despite its limitations<sup>(85,86)</sup>. βGal expression and activity increases on platelet surfaces upon storage (K.M.H., unpublished data), presumably mediating surface terminal galactose cleavage, which may explain the exposure of GlcNAc on platelet surface glycoconjugates and subsequent clearance via macrophage integrin αM. Recent studies show that plasma Neu and βGal contribute to the accumulation of desialylated and de-galactosylated plasma glycoproteins.

The term “lysosome” was coined to convey the idea

of a membrane-bound lytic organelle that contains hydrolases active at acid pH within cells. Genetic defects causing secretory lysosome defects are associated with albinism, immunodeficiency and platelet disorders<sup>(87)</sup>. The fact that platelet Neu and βGal activities increase upon cold storage<sup>(87)</sup> and upon activation (K.M.H., unpublished data) suggests the presence of these enzymes in secretory lysosomes. Our unpublished results together with previous reported studies support the notion that platelets and likely megakaryocytes contain secretory lysosomes that can release Neu and βGal activities. However this notion and the cellular mechanisms triggering their secretion during *in vivo* aging remain to be investigated.

### Summary and conclusion

In summary, glycan-lectin interactions are part of the intricate regulatory apparatus that regulate thrombopoiesis, platelet production and their removal to maintain a balance of platelet numbers. Emerging data support the notion that modifications of LacNAc regulate hematopoiesis. Under steady state conditions, sialic acid loss is sensed by hepatic AMR receptors to induce TPO secretion and *de novo* platelet production, while loss of galactose severely impairs thrombopoiesis. Novel mechanisms are emerging to define how platelets lose sialic acid and galactose to regulate platelet count via multiple lectin receptors in the liver and the bone marrow.

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Los autores declaran que no poseen conflictos de interés.

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