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The Ashwell-Morell receptor regulates hepatic thrombopoietin production via JAK2-STAT3 signaling

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Abstract

The hepatic Ashwell-Morell receptor (AMR) can bind and remove desialylated platelets. We demonstrate that platelets become desialylated as they circulate and age in blood. Binding of desialylated platelets to the AMR induces hepatic thrombopoietin (TPO) gene transcription and translation, thereby regulating platelet production. The highly conserved endocytic AMR signals through Janus kinase 2 (JAK2) and the acute phase response signal transducer and activator of transcription 3 (STAT3) *in vivo* and *in vitro*. Recognition of this novel physiological feedback mechanism illuminates the pathophysiology of platelet diseases, such as Essential Thrombocythemia and Immune Thrombocytopenia, and contributes to our understanding of the mechanisms of thrombocytopenia observed with JAK1/2 inhibition.

Introduction

An adequate supply of platelets is essential to repair both continuously occurring vascular damage and to initiate thrombus formation following vascular injury. While platelets function primarily in hemostasis, they 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 (thrombocytosis), and responses to acute infections with sudden reduction of platelets (thrombocytopenia), placing platelets as reporters of disease progression or healing. To ensure a steady platelet supply, humans produce and remove ~10^{11} platelets daily, and the rate of production can rise sharply under conditions of platelet destruction. Platelet production must be tightly regulated to avoid spontaneous bleeding if counts are low or arterial occlusion and organ damage if counts are high.

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Thrombopoietin (TPO) is the primary regulator of platelet production, supporting the survival, proliferation and differentiation of the platelet precursors, bone marrow (BM) megakaryocytes (MKs).[1–3] Hepatocytes are a major source of production and secretion of TPO into the blood. However, mechanisms regulating circulating TPO levels have been debated for decades. In one model, circulating TPO levels are maintained solely by its uptake and metabolism by high-affinity Mpl receptors on platelets and megakaryocytes.[4–8] In another model, circulating platelet levels are sensed, regulating TPO mRNA expression in liver and bone marrow (BM). [9–13] Although no physiological ligand-receptor pair capable of regulating steady-state TPO production has yet been identified, the pro-inflammatory cytokine interleukin-6 (IL-6) stimulates hepatic TPO liver synthesis, providing a regulated pathway to increase platelet production during acute inflammatory responses.[14–17]

Recent studies have highlighted the role of glycan modifications on platelet surface proteins in mediating platelet clearance.[18,19] Platelets with reduced α2,3-linked sialic acid during sepsis, after cold storage, or in mice lacking the sialyltransferase ST3GalIV are cleared by the hepatic endocytic Ashwell-Morell receptor (AMR).[20–23] The AMR is a transmembrane heteroligomeric glycoprotein complex composed of ASGPR1 (CLEC4H1, HL-1) and ASGPR2 (CLEC4H2, HL-2) subunits, which are highly conserved among mammalian species.[24] However, since its discovery four decades ago, the regulatory role of the hepatic AMR has remained unclear.

We report here that the circulatory lifespan of platelets is determined by sialic acid loss that triggers platelet removal by the hepatic AMR. This removal system drives hepatic TPO mRNA expression *in vivo* and *in vitro* via JAK2 and STAT3 to communicate with BMMKs. Disruption of AMR-desialylated platelet signaling by the JAK1/2 inhibitors AZD1480, TG101348 and BMS911543 adversely affect hepatic TPO mRNA expression and secretion, We conclude that this feedback mechanism posits the AMR-desialylated platelet pair as the critical control point for TPO homeostasis and contributes to our understanding why JAK1/2 inhibition commonly induces thrombocytopenia in patients with Myeloproliferative Neoplasms (MPN).[25,26]

Results

The AMR regulates platelet survival and thrombopoiesis *in vivo*

We hypothesized that circulating platelets become desialylated as they age and are removed by the hepatic AMR. We investigated platelet circulatory lifespan in mice lacking AMR function (*Asgr2*$^{-/-}$ mice) and contrasted that to *St3gal4*$^{-/-}$ mice, which are poorly sialylated due to genetic sialyltransferase loss.[21,23] Platelet counts were elevated by 50% in *Asgr2*$^{-/-}$ mice and the lifetime (T1/2) was increased by ~35% compared to that of platelets in WT mice (Table 1). Platelet volume and immature platelet fraction (IPF) (newly produced platelets), were decreased in *Asgr2*$^{-/-}$ mice, consistent with the notion that platelets in *Asgr2*$^{-/-}$ mice circulate longer and are older. Our results contrast with those of Grewal *et al* who reported normal platelet counts in *Asgr2*$^{-/-}$ mice.[23] Deficiency of the *St3gal4* gene induces a marked thrombocytopenia, due to rapid platelet clearance by the hepatic AMR.[21,23] Consistent with the rapid platelet clearance, platelet volume and IPF were increased in *St3gal4*$^{-/-}$ mice, reflecting high platelet turnover and young platelets. Platelets

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isolated from *St3gal4*$^{-/-}$ mice had a significant increase in terminal galactose, as determined by RCA-I and ECL lectin binding (Table 1). Platelets isolated from *Asgr2*$^{-/-}$ mice had a significant increase in terminal galactose consistent with prolonged lifetime in the absence of AMR removal system (Table 1). BMMK counts were decreased in *Asgr2*$^{-/-}$ and increased in *St3gal4*$^{-/-}$ mice (Table 1). Platelets count, size, half-life, IPF were normalized in *St3gal4*$^{-/-}$/*Asgr2*$^{-/-}$ mice (Table 1).[23] Our data shows that loss of AMR function allows desialylated platelets to circulate. Thus, platelets become normally desialylated as they circulate are removed by the AMR, i.e. the number of desialylated platelets depends primarily on the AMR as a removal mechanism.

Desialylated platelet uptake by the AMR regulates hepatic TPO mRNA expression *in situ*

We tested if uptake of desialylated platelets by the AMR influences steady state hepatic TPO mRNA expression and secretion by measuring the relative ratios of TPO/cycloA mRNA in livers isolated from *Asgr2*$^{-/-}$ and *St3gal4*$^{-/-}$ mice, compared to livers from WT mice (Fig. 1a). Hepatic TPO mRNA expression was reduced by ~45% in *Asgr2*$^{-/-}$ mice, thereby defining its constitutive threshold. In *St3gal4*$^{-/-}$ mice, liver TPO mRNA increased by as much as 40% when compared to WT livers. The difference in the TPO mRNA levels between the *St3gal4*$^{-/-}$ and *Asgr2*$^{-/-}$ mouse genotypes revealed that maximal uptake of *St3gal4*$^{-/-}$ platelets by the AMR resulted in a ~2.5-fold increase in liver TPO mRNA expression, compared to its constitutive threshold. As expected, *St3gal4*$^{-/-}$/*Asgr2*$^{-/-}$ livers had normalized TPO mRNA levels compared to WT livers (Fig. 1a).

Circulating plasma TPO levels, Mpl receptor expression, total and surface, and total platelet TPO were increased in *St3gal4*$^{-/-}$ mice, slightly reduced in *Asgr2*$^{-/-}$ mice, and normalized in *St3gal4*$^{-/-}$/*Asgr2*$^{-/-}$ mice (Table 1 and Supplementary Fig. 1). Because plasma TPO is regulated by internalization of Mpl upon TPO binding,[6] we investigated Mpl internalization following TPO stimulation. In control platelets, Mpl surface expression was maximally decreased to 78 ± 3% of resting values after incubation with 50 ng ml^{-1} TPO for 10 min, as evidenced by flow cytometry using an antibody directed against the extracellular domain of Mpl (Table 1). Mpl expression was 96 ± 6 % and 57 ± 6 % of resting values in platelets isolated from *Asgr2*$^{-/-}$ and *St3gal4*$^{-/-}$ deficient platelets at the same time point (Table 1). In *St3gal4*$^{-/-}$/*Asgr2*$^{-/-}$ mice, Mpl internalization was normalized to 83 ± 3%. We hypothesize that chronic thrombocytosis, as presented in *Asgr2*$^{-/-}$ mice, where platelets are older, is accompanied by impaired Mpl internalization, as reported for patients with Essential Thrombocythemia (ET) or in mouse-models of thrombocytosis.[27–29] In contrast, platelets with high turnover rates (young platelets) such as *St3gal4*$^{-/-}$ platelets, have high platelet Mpl expression and internalization. Consistent with plasma TPO levels, megakaryocyte numbers were significantly increased in *St3gal4*$^{-/-}$ mice, decreased in *Asgr2*$^{-/-}$ mice, and normalized in *St3gal4*$^{-/-}$/*Asgr2*$^{-/-}$ mice (Table 1).

Transfusion of endogenously desialylated platelets stimulates hepatic TPO mRNA expression *in situ*

To confirm that the uptake of desialylated platelets by the AMR can stimulate hepatic TPO mRNA expression, we infused WT mice or *Asgr2*$^{-/-}$ mice with endogenously desialylated platelets isolated from *Asgr2*$^{-/-}$ (*Asgr2*$^{-/-}$ platelets) or *St3gal4*$^{-/-}$ (*St3gal4*$^{-/-}$ platelets)

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mice. Desialylated platelets had decreased recoveries and survival rates when transfused into WT mice. As expected, the survival of WT, *Asgr2*$^{-/-}$ and *St3gal4*$^{-/-}$ platelets were prolonged in animals lacking a functional AMR (Fig. 1b). Mouse recipients were injected with 4×10^8 platelets, the minimum dose required to significantly increase liver TPO mRNA expression after 12 h (Fig. 1c). Injection of similar desialylated platelet numbers into rabbits induces *de novo* platelet production.[30] Critically, transfusion of *St3gal4*$^{-/-}$ or *Asgr2*$^{-/-}$ platelets into WT, but not into *Asgr2*$^{-/-}$ mice, significantly increased hepatic TPO mRNA expression by 12 h post-transfusion (Fig. 1d). Circulating TPO levels were initially (<24 h) decreased after injection of WT, *Asgr2*$^{-/-}$ and *St3gal4*$^{-/-}$ platelets into WT mice (Fig. 1e), consistent with previous reports.[6] Thereafter, plasma TPO concentrations of mice injected with desialylated, but not WT platelets significantly increased in parallel with TPO mRNA levels, peaking by day 2 post-infusion. TPO mRNA expression or TPO plasma concentrations did not increase above the pre-injection levels >24 h after transfusion of WT or desialylated platelets into *Asgr2*$^{-/-}$ mice (Fig. 1d,e). Consistent with the increase in liver TPO mRNA expression and secretion, transfusion of *St3gal4*$^{-/-}$ and *Asgr2*$^{-/-}$ platelets into WT mice, but not into *Asgr2*$^{-/-}$ mice, increased BMMK numbers at day 3 and platelet counts, which peaked at day 10 post-injection (Fig. 1f–h). A peak in platelet numbers at day 10 post-desialylated platelet treatment was consistent with observations in rabbits.[30] Transfusion of desialylated platelets into *Mpl*$^{-/-}$ mice had no effect on platelets counts (Supplementary Fig. 2), showing that the increase in platelet count is dependent on TPO and its receptor Mpl. MK and platelet numbers remained unaffected in transfused *Asgr2*$^{-/-}$ mice (Fig. 1f–h). Of note, splenectomy had no long-term effect on platelet count, terminal platelet surface β-galactose exposure and TPO plasma levels in WT and *Asgr2*$^{-/-}$ mice (Supplementary Fig. 3).

Uptake of desialylated platelets stimulates hepatic JAK2-STAT3 signaling *in vivo*

If platelet uptake by the AMR is able to induce TPO mRNA transcription, the appropriate signaling pathways must become active in hepatocytes. Hence, signals activated by the uptake of desialylated platelets by AMR were investigated. Major polypeptides of 60–70 and 125 kDa were highly tyrosine phosphorylated in WT liver lysates (Fig. 2a). Using a specific antibody directed against JAK2, we identified the 125-kDa polypeptide as the tyrosine kinase JAK2. The presence of additional phosphoproteins of 125 kDa cannot be excluded. Analysis of liver samples revealed a marked reduction in the phosphorylation of JAK2 in *Asgr2*$^{-/-}$ mice and significant increase in *St3gal4*$^{-/-}$ mice (Fig. 2a,b). We next investigated possible JAK2 downstream effectors, *i.e.* STAT proteins. STAT3 phosphorylation was significantly higher in livers obtained from *St3gal4*$^{-/-}$ mice, when compared to all other liver samples (Fig. 2a,b) and livers from *Asgr2*$^{-/-}$ mice revealed a marked reduction in the phosphorylation of STAT3 when compared to control livers, whereas Tyrosine phosphorylation of other STAT proteins, *e.g.* STAT1, STAT5A, STAT5B and STAT6, was not detected (data not shown). To directly demonstrate that platelet binding of the AMR induced an increase in TPO mRNA expression through JAK2 activation, we administrated the clinically used JAK1/2 inhibitor, AZD1480 or the JAK2 inhibitor TG101348 to WT mice for 2 days. Liver samples of mice treated with JAK inhibitors showed a significant decrease in total tyrosine phosphorylation (pTyr) (Fig. 2a), particularly in JAK2 and STAT3 (Fig. 2a,b and Supplementary Fig. 4). Treatment with AZD1480 and

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TG101348 blocked TPO mRNA expression in WT mice 12 h post-infusion of desialylated platelets from *St3gal4*$^{-/-}$ or *Asgr2*$^{-/-}$ mice (Fig. 2c). A slight but reproducible decrease in TPO mRNA expression was observed in the livers of WT mice treated with AZD1480 and TG101348 (Fig. 2c). Consistent with TPO mRNA liver data, expression of TPO protein was increased in *St3gal4*$^{-/-}$ and decreased in *Asgr2*$^{-/-}$ mice when compared to WT levels (Fig. 2d). AZD1480 and TG101348 treatment decreased the expression of TPO protein in WT mice (Fig. 2d and Supplementary Fig. 4).

Uptake of desialylated human platelets by HepG2 cells stimulates TPO mRNA expression via JAK2-STAT3 signaling *in vitro*

Human HepG2 cells ingest desialylated platelets [20,21] and produce TPO in response to IL-6.[14] We tested whether ingestion of desialylated human platelets by the AMR affects TPO mRNA expression and secretion by HepG2 cells. Human platelets were desialyated using α2-3,6,8 neuraminidase (sialidase) as confirmed by RCA-I binding (Fig. 3a, inset). Desialylation markedly accelerated the rate of platelet uptake by HepG2 cells (Fig. 3a), resulted in increased TPO mRNA (3-fold) and protein (1.8-fold) expression (Fig. 3b,c), and TPO protein secretion into the media by 6 h (Fig. 3d). TPO mRNA expression was slightly increased after 0.5 to 2 h of incubation with sialidase-untreated platelets (1.6-fold), and returned to below baseline levels within 6 h.

Tyrosine phosphorylation of JAK2 and its downstream effector, STAT3, was further investigated in HepG2 cells. Incubation of HepG2 cells with desialylated platelets induced a strong, transient JAK2 tyrosine phosphorylation at 0.5 h while the addition of control platelets induced a prolonged, but weaker JAK2 tyrosine phosphorylation (Fig. 3f). STAT3 tyrosine phosphorylation and its nuclear translocation were observed 1 h after addition of both, control and desialylated human platelets (Fig. 3e,g). TPO protein expression was increased as judged by immunofluorescence at 6 h (Fig. 3h). We observed that TPO was packaged into granular structures, indicative of secretory granules (Fig. 3h and inset). To determine the role of the AMR in TPO expression, we transfected HepG2 cells with small interference RNA (siRNA) targeting *ASGR2*. ASGR2 protein expression was down regulated to ~80% of normal values following transfection of *ASGR2* siRNA (Supplementary Fig. 4), a maneuver which inhibited STAT3 translocation to the nucleus (Fig. 3g), blocked TPO mRNA (Fig. 3j) and TPO protein increase (Fig 3h and Supplementary Fig. 4).

To investigate the effects of JAK2 inhibition on TPO protein expression, HepG2 cells were pre-treated for 24 h with AZD1480, TG101348 or BMS911543 before addition of platelets. Treatment with JAK1/2 inhibitors prior to addition of desialylated platelets inhibited the translocation of STAT3 to the nucleus (Fig. 3i and Supplementary Fig. 6), JAK2 phosphorylation (Supplementary Fig. 6), and increase in TPO mRNA (Fig. 3j), and TPO protein (Fig. 3i and Supplementary Fig. 6). Together, the data show that the hepatic AMR signals via JAK2-STAT3 to stimulate TPO mRNA expression *in vivo* and *in vitro*.

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Exogenously desialylated platelets stimulate hepatic TPO mRNA expression

As an alternative approach, WT platelet were desialylated *in vitro* using sialidase and transfused into WT and *Asgr2*$^{-/-}$ mice. Depletion of sialic acid was documented by RCA-I lectin binding (Fig. 4a; insert). As expected, desialylated platelets were rapidly removed from the circulation of WT mice and only ~15% remained after 1 h (Fig. 4a). By contrast, ~90% of the untreated platelets remain in the circulation at the same time point. Removal was highly dependent on a functional liver AMR, because desialylated platelets had higher recoveries and circulated longer in *Asgr2*$^{-/-}$ mice. Hepatic TPO mRNA expression increased by ~50% in WT mice 12 h after injection with desialylated platelets, whereas no changes in TPO mRNA expression were detected in WT mice injected with control platelets or in *Asgr2*$^{-/-}$ mice (Fig. 4b). Plasma TPO levels increased at day 2, platelet numbers at day 3 and BMMK at day 4, respectively (Fig. 4c–f). None of these changes were reported in *Asgr2*$^{-/-}$ mice injected with desialylated platelets. Similarly, direct injection of sialidase induced a severe (>95%) thrombocytopenia in WT mice after 24 h, which was incomplete (~60%) in *Asgr2*$^{-/-}$ mice (Fig. 4g). Sialidase injection induced an AMR-dependent increase in liver TPO mRNA expression, plasma TPO levels and BMMK numbers in WT, but not in *Asgr2*$^{-/-}$ mice (Fig. 4h–j).

Antibody-mediated thrombocytopenia does not stimulate liver TPO mRNA expression

The role of Mpl in regulation of TPO plasma levels is evident in animal models with induced acute immune thrombocytopenia, in which liver TPO mRNA expression remains unchanged but circulating TPO levels are elevated.[4] We tested whether hepatic TPO mRNA expression in *Asgr2*$^{-/-}$ and *St3gal4*$^{-/-}$ mice was regulated in a model of acute immune thrombocytopenia, independent of platelet sialic acid loss. Injection of rabbit anti-mouse platelet serum (RAMPS) induced a severe (>99%) thrombocytopenia in WT, *Asgr2*$^{-/-}$ and *St3gal4*$^{-/-}$ mice within 24 h (Fig. 5a). Platelets did not return to the circulation of these animals until 72 h post-RAMPS treatment. RAMPS-induced thrombocytopenia did not stimulate liver TPO mRNA expression in WT, *Asgr2*$^{-/-}$ and *St3gal4*$^{-/-}$ mice (Fig. 5b). Plasma TPO levels increased markedly within 24 h (Fig. 5c) and BMMK numbers at 72 h after RAMPS treatment, independently of the genotype (Fig. 5d).

Discussion

Our study shows that the AMR recognizes circulating desialylated platelets under steady state conditions and defines a direct feedback pathway between desialylated senile platelet removal and hepatic TPO mRNA expression and secretion via JAK2-STAT3 signaling (Supplementary Fig. 6). This regulatory system defines an unrecognized feedback mechanism for TPO production and adds novel insights why thrombocytopenia is a common clinically significant adverse event associated with JAK1/2 inhibition.

Circulating platelets lose sialic acid defining their lifespan, as evidenced by microthrombocytosis in *Asgr2*$^{-/-}$ mice despite increased platelet surface terminal β-galactose, consistent with exposure to sialidases during aging in circulation. Platelet survival also depends on the interplay between prosurvival and proapoptotic members of the Bcl-2 family, critical regulators of the intrinsic apoptotic pathway.[31–33] Whether members of the

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Bcl-2 family alter platelet surface sialic acid content is unclear. Interestingly, the primary platelet clearance site following administration of the pro-apoptotic ABT-737 is the liver,[34] whereas the spleen does not regulate senescent platelet life span.[32] This notion is confirmed by our study showing no significant differences in platelet count in WT and *Asgr2*$^{-/-}$ mice following splenectomy.

Hepatic TPO mRNA expression and secretion were stimulated by the AMR-mediated uptake of endogenously and exogenously desialylated platelets *in vivo* and *in vitro*. Human and mouse platelets do not contain TPO mRNA,[35] showing that TPO mRNA expression is intrinsic to both hepatocytes *in vivo* or HepG2 cells *in vitro*, following engagement of desialylated platelets with the AMR. *St3gal4*$^{-/-}$ mice had high liver TPO mRNA expression, due to increased platelet clearance by the AMR, whereas *Asgr2*$^{-/-}$ mice had significantly low liver TPO mRNA expression, as they were genetically unable to clear desialylated platelets through this receptor, thereby defining the constitutive threshold for steady state TPO production.

The mechanisms regulating steady state TPO production have been subject of discussion for decades. In one model, hepatic TPO production is constitutive and TPO serum levels are maintained solely by its uptake and metabolism by platelets and megakaryocytes.[4–8] This prevailing model posits that circulating TPO concentration is inversely proportional to the "Mpl mass" contributed by the total number of MKs and platelets. The reciprocal relationship between platelet number and circulating TPO level is clearly evident in bone marrow transplant patients,[3] and in *Mpl*$^{-/-}$ mice.[36,37] Further, serum TPO levels increase, while hepatic TPO mRNA levels remain unchanged in models of acute immune or chemotherapy induced thrombocytopenia.[4–8] In another model of TPO regulation, platelet counts are sensed by an unidentified mechanism, resulting in appropriately regulated levels of TPO expression in the liver and the bone marrow.[9–11 13] This hypothesis is based on the observations that liver TPO mRNA expression is not constitutive, as it can be stimulated by IL-6 during reactive inflammatory thrombocytosis or epithelial ovarian cancer,[15–17] or after selective liver irradiation.[38] However, the physiological signal and receptor necessary to stimulate hepatic TPO production had until now remained elusive. Our study identifies both desialylated platelets and the AMR as the physiological pair regulating steady state hepatic TPO mRNA production *in vivo*. In this platelet aging/injury sensing system, liver TPO mRNA expression is uncoupled from total platelet counts, and instead senses desialylated platelets as they age in blood or under conditions of increased desialylation such as sepsis.[22,23] Platelets cleared independently of the AMR, such as the antibody-mediated removal of platelets, failed to increase hepatic TPO mRNA expression, consistent with previous studies.[5–8]

Several human and mouse phenotypes lend credence to the assertion that platelet TPO metabolism is not the sole determinant of plasma TPO levels. Serum TPO levels are lower than expected in patients with Immune Thrombocytopenia (IT),[39–41] and high in patients with ET.[41–43] Serum TPO levels are normal in *Nfe2*$^{-/-}$ mice despite thrombocytopenia,[44] and mice deficient for Bak and Bax have normal to slightly increased serum TPO levels despite significantly increased platelet counts.[32] Circulating TPO concentrations are therefore likely to be regulated in a complex manner, a notion further supported by data

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showing that Mpl expression on megakaryocytes and platelets is not required for thrombopoiesis but is essential to prevent myeloproliferation.[37] The AMR-asialo-platelet induction of hepatic TPO mRNA expression could explain why TPO synthesis is increased in ET but decreased in IT. ET would lead to an increase in numbers of desialylated platelets, a condition that increases feedback for TPO expression. In contrast, platelet clearance by immune mechanisms involving macrophage Fcγ receptors and the circulation of young, sialylated platelets, would shunt platelet removal away from the AMR and reduce TPO expression. Thus, our data provides a partial explanation for the "plasma TPO-level discrepancies" observed in human pathologies with defects in platelet counts and liver disease. Regulation of liver TPO mRNA expression by the AMR does not exclude that total platelet counts and Mpl expression levels participate in regulating plasma TPO levels as a fine-tuning regulatory mechanism. Changes in Mpl expression, altered plasma TPO measured in *Asgr2*$^{-/-}$ and *St3gal4*$^{-/-}$ mice and BMMK numbers, reflect differences in platelet turnover kinetics and document that changes in TPO mRNA expression in the liver does not fully explain all data acquired.

Our data demonstrates that the AMR regulates gene expression, in addition to its known role as an endocytic receptor.[24] The AMR signaling cascade involves JAK2 phosphorylation and STAT3 phosphorylation and translocation to the nucleus. The characterized AMR signaling cascade shares similarities with that of IL-6.[45] Binding of IL-6 to its hepatic receptor (IL-6R) 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 is unknown. A tyrosine kinase of 127 kDa (JAK2?) constitutively associates with the AMR ASGPR1 subunit in HepG2 cells.[46] Importantly, IL-6 stimulates TPO mRNA expression in hepatocytes *in vivo* and in HepG2 and Hep3B cells *in vitro*.[12,14,16,17] Thus, our study suggests that both desialylated platelets and IL-6 lead to STAT3-mediated hepatic TPO mRNA expression downstream of the AMR-JAK2 and IL-6R-JAK1 signaling cascades, respectively.

The clinically used JAK1/2 inhibitor AZD1480 and JAK2 inhibitors TG101348 and BMS911543 blocked STAT3 activation and TPO mRNA production downstream of AMR binding, both in mice and in cultured human HepG2 cells, confirming that JAK1/2-STAT3 signals to TPO production *in vitro* and *in vivo*. Thrombocytopenia is a common adverse event of JAK1/2 inhibitor treatment, which is clinically used in MPN, consecutive or not of somatic JAK2 mutation V617F.[25,26] 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.[47,48] Our study indicates that inhibition of TPO production downstream of the hepatic AMR-JAK2 signaling cascade (and likely IL-6R-JAK1) could additionally contribute to the thrombocytopenia associated with JAK1/2 treatment.

In conclusion, our study identifies desialylated senile platelets and the AMR as the long elusive physiological ligand-receptor pair regulating hepatic TPO mRNA production, resolving the longstanding mystery of steady-state TPO regulation. The AMR-mediated removal of desialylated senile platelets regulates TPO synthesis in the liver by recruiting JAK2 and STAT3.

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Methods

Healthy volunteers

Approval to obtain whole blood samples from healthy volunteers was obtained from the institutional review boards of Brigham and Women's Hospital, and informed consent was approved according to the Declaration of Helsinki.

Mice

We have used WT, as well as transgenic *Asgr2*$^{-/-}$ and *St3gal4*$^{-/-}$ mice were obtained from the Jackson Laboratory and backcrossed onto C57BL/6J background for at least 10 generations. All studies were performed according to protocol approved by the Harvard Medical School, as set forth in the Animal Care and Use of Laboratory Animals Guide. 8–10 weeks old males and females were used in all experiments. We used 6 mice per each time point shown in all experimental mouse cohorts, unless otherwise indicated. No non-inclusion or exclusion parameters were used in our studies. No randomization procedure was implemented, however the production of the knockout mice and the administration of the relevant treatments were administrated randomly to the animals used in this study.

Platelet counts and preparation

Blood was obtained from anesthetized mice by retroorbital bleed. Mouse platelets and platelet poor plasma were prepared as described.[21,23] Venous blood was collected from healthy volunteers and platelets were prepared in the presence of 1 μg ml^{-1} PGE_1 (Sigma-Aldrich).[49] Platelet counts were determined by flow cytometry using 5.5 μm diameter SPHERO rainbow beads (Spherotech) as a reference.[21,23] Platelet counts and the immature platelet fraction (IPF%) were determined using Sysmex XT-2000iV system (Kobe, Japan). Platelets were treated with α2–3,6,8 neuraminidase from *C. perfringens* (Sigma-Aldrich) to remove terminal sialic acid.[49] Plasma TPO levels were quantified using a Mouse and Human Thrombopoietin Quantikine ELISA kit (R&D System). Mouse plasma was also depleted from Albumin and IgG using ProteoPrep® Immunoaffinity Depletion kit (Sigma-Aldrich) according to manufactor's instructions. Samples were then subjected to SDS-PAGE followed by immunoblot using anti-mouse TPO antibody (R&D System). All major study endpoints such as platelet counts, BMMK counts, TPO mRNA expression and TPO protein concentration measurements were performed in a blinded fashion.

Flow cytometry

Platelet surface β1–4 galactose exposure using FITC-conjugated *R. communis* agglutinin I (RCA-I, Vector Labs) and FITC-conjugated *E. cristagalli* lectin (ECL, Vector Labs), and Mpl expression were determined by flow cytometry.[20] To determine Mpl internalization platelets were incubated with 50 ng ml^{-1} TPO for 10 min at 37 °C or not (rest), then all samples were incubated with a rabbit antibody directed against the extracellular domain of Mpl (provided by Dr. Wei Tong, UPenn) or control rabbit IgG, followed by Alexa Fluor 488-labeled goat anti-rabbit IgG antibody, and analyzed by flow cytometry.

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Sialidase and RAMPS injections

Mice were injected intraperitoneally with 50 μl of rabbit anti-mouse platelet serum (RAMPS, Inter-cell Technologies) or intravenously by retroorbital injections with 50 mU of α2–3,6,8 neuraminidase (sialidase) from *C. perfringens* (Sigma-Aldrich).

Platelet transfusions

Endogenous and exogenous platelet survivals were determined by intravenous injections in mice of biotin-NHS and CMFDA-labeled mouse platelets, respectively.[20] Blood was collected by retroorbital bleed and the percentages of biotin- or CMFDA-positive platelets were determined by flow cytometry. The amount of fluorescent platelets at 5 min was normalized to 100%.[50]

AZD1480, TG101348 and BMS911543 (JAK1/2 inhibitors) experiments

Treatment of wild type (C57BL/6J) mice with vehicle (5% dimethyl acetamide, 0.5% methocellulose) or AZD1480 or TG101348 began 2 days before deislaylated platelet injections. Mice were treated with 50 or μg kg^{-1} of AZD1480 once a day or treated with 120 μg kg^{-1} of TG101348 twice a day, by oral gavage. Following vehicle, AZD1480 or TG101348 injections, mice were transfused with 4×10^8 platelets isolated from WT, *St3gal4*$^{-/-}$ or *Asgr2*$^{-/-}$ mice. Mice were sacrificed after 12 h of platelet injections and liver TPO mRNA measured as described below. HepG2 cells were incubated for 24 h with 2 μM of AZD1480 or 10 μM of TG101348 or 2 μM of BMS911543 or vehicle in the cell culture media before addition of sialidase treated or untreated human platelets.

Liver TPO mRNA expression

Mouse livers were obtained after blood collection, perfused with PBS, and kept at −80 °C prior to extract total RNA. Liver tissues (~20 mg) were extracted with Trizol® Reagent (Invitrogen), followed by cDNA preparation from 2 μg of total RNA with the SuperScript VILO cDNA Synthesis kit (Invitrogen). cDNA samples were quantified by real time PCR using the SYBR Green Mix (BioRad) on a MyiQ iCycler (BioRad).[51] Crossing point (Cp) values of TPO and cycloA sequences were measured using the iQ5 optical system software (BioRad). Relative expression was calculated according to the E-ΔCp formula. Cyclo A mRNA was detected in all samples at a median cycle number of 14.0 ± 5.6. The relative TPO/cyclo A mRNA ratio was determined at the indicated time points and compared to that of untreated WT livers. Primers (5′ to 3′) used were: TPO forward, CACAGCTGTCCCAAGCAGTA; TPO reverse, CATTCACAGGTCCGTGTGTC; cyclo A forward, GCCGATGACGAGCCCTTG; cyclo A reverse, TGCCGCCAGTGCCATTATG.

Immunofluorescence

HepG2 cells (purchased from ATCC-HB-8065) were grown at 4×10^5 cells/cm^2 in Permanox® (Nalge Nunc International) slides for 24 h in the presence of 2 μM AZD1480 or 10 μM of TG101348 (both Selleckchem) or 2 μM of BMS911543 (Chemie Tek) or vehicle before addition of human platelets treated or non-treated with sialidase. Immunofluorescence for anti-pSTAT3 (Cell Signaling Technology) was performed 1 h after platelet addition to HepG2 cells following antibody manufactor's protocol. After 6 h

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incubation with humans platelets treated or non-treated with sialidase, HepG2 cells were fixed in 4% PFA, followed by permeabilization in 2% Triton X-100. Cells were incubated for 30 min at 37 °C with 5% goat-serum to block unspecific binding and incubated overnight at 4 °C with a polyclonal anti-human TPO antibody (R&D Systems, USA) followed by secondary AlexaFluo®568 conjugated polyclonal goat-anti rabbit antibody (Invitrogen). Nuclei were visualized by inclusion of 4′, 6-diamino-2-phenyllidole (DAPI) (Invitrogen) in the mounting medium.

In vitro platelet phagocytosis by HepG2 cells

HepG2 cells were cultured at 37 °C in a 5% CO_2 incubator in DMEM supplemented with 10% FBS and 1% Pen-Strep. For the assays, 2.8×10^6 HepG2 cells were seeded into 60 mm dishes and incubated with 28×10^6 CM-Orange-labeled human platelets. At indicated time points, media was collected and depleted from platelets by centrifugation. TPO levels were quantified in 7X concentrated media using a Human Thrombopoietin Quantikine ELISA kit (R&D System). HepG2 cells were further dissociated from wells using 0.25% Trypsin-EDTA, washed with PBS, and platelet ingestion was evaluated on CM-Orange-positive events by flow cytometry.[52] HepG2 cells were also lysed directly with Trizol (Invitrogen) to quantify TPO mRNA expression, as above. Primers (5′ to 3′) used were: TPO forward, TGCAAATAAGTGCTGCCTTG; TPO reverse, GGATGCTTGTAGGAGGGTCCA; cyclo A forward, AGTCCATCTATGGGGAGAAATTTG; cyclo A reverse, GCCTCCACAATATTCATGCCTTC.

siRNA design, transfection and verification

Three small interference RNAs (siRNAs) for human ASGR2 gene and one scramble siRNA as negative control were designed and synthesized (OriGene technologies) (Supplementary Table 1). HepG2 cells were transfected using DharmaFECT-4 siRNA Transfection Reagent (Thermo Fisher Scientific, USA). The negative control received DharmaFECT-4 transfection reagent plus the scramble siRNA. HepG2 cells were incubated with 0, 10 or 30 μM of ASGR2 or scramble siRNA for monitoring of the protein silencing effect. Cells were harvested after 36 h and subjected to immunoblot blot analysis using anti-human ASGR2 antibody (Santa Cruz Biotechnology). HepG2Cells were transfected with ASGR2 siRNA at 30 μM final concentration, 36 h after transfection platelets treated or not with sialidase were added to cells and harvest occurred at the indicated time points.

Immunoblotting

Livers were isolated from WT, *Asgr2*$^{-/-}$, *St3gal4*$^{-/-}$ mice, and from WT mice treated with AZD1480 or TG101348 and lysed. HepG2 cells were harvested after 0, 0.5, 1 and 2 h incubation with sialidase treated or control platelets and lysed in 3x NP40 buffer. Liver and HepG2 lysates were subjected to SDS-PAGE and immunoblotting using anti-phosphotyrosine 4G10 (Millipore, USA), anti-pJAK2, anti-JAK2, anti-pSTAT3, anti-STAT3 (Cell Signaling Technology) antibodies and anti-TPO (both mouse and human from R&D systems). Band intensities from 4 individual western blots were quantified by densitometric analysis using software Image J.

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Bone marrow histology

Mouse femurs were fixed in 4% paraformaldehyde/PBS for 48 h, and decalcified in 0.5 M EDTA (Boston BioProducts), pH 7.8, for 5 days, exchanging EDTA twice. Tissues were paraffin-embedded, and sections were stained with hematoxylin and eosin.[53] Megakaryocytes were counted by 3 independent individuals in 20 randomly selected fields for each condition. All cohorts contain at least 6 mice.

Splenectomy

Platelet count was determined by flow cytometry prior to surgical splenectomy. Mice were anesthesized using 2–3% isoflurane. The left upper abdominal cavity was entered and exposed via median incision. After the spleen was identified, the organ was gently manipulated through the incision and covered with a moist saline gauze. Splenic arteries and venous supply were dissected and knots placed with 10-0 nylon suture, followed by spleen removal. The incision was closed with 5-0 prolene suture. Mice were monitored for 48 h and received buprenorphine analgesic for 72 h. After a recovery period of 14 d, platelet counts and surface β-galactose were measured by flow cytometry as described above. Plasma TPO levels were determined as described above.

Data analysis

Numeric data were analyzed using one-way (for single variant) or two-way (for multiple variants) ANOVA analysis of variance followed by Bonferroni adjustment for multiple comparisons. Two groups were compared by the two-tailed Student's unpaired *t*-test. The significance of data was assessed using the GraphPad Prism 5 software. Differences were considered as significant when $P<0.05$. Different levels of significance are indicated as $*P<0.05$, $**P<0.01$, $***P<0.001$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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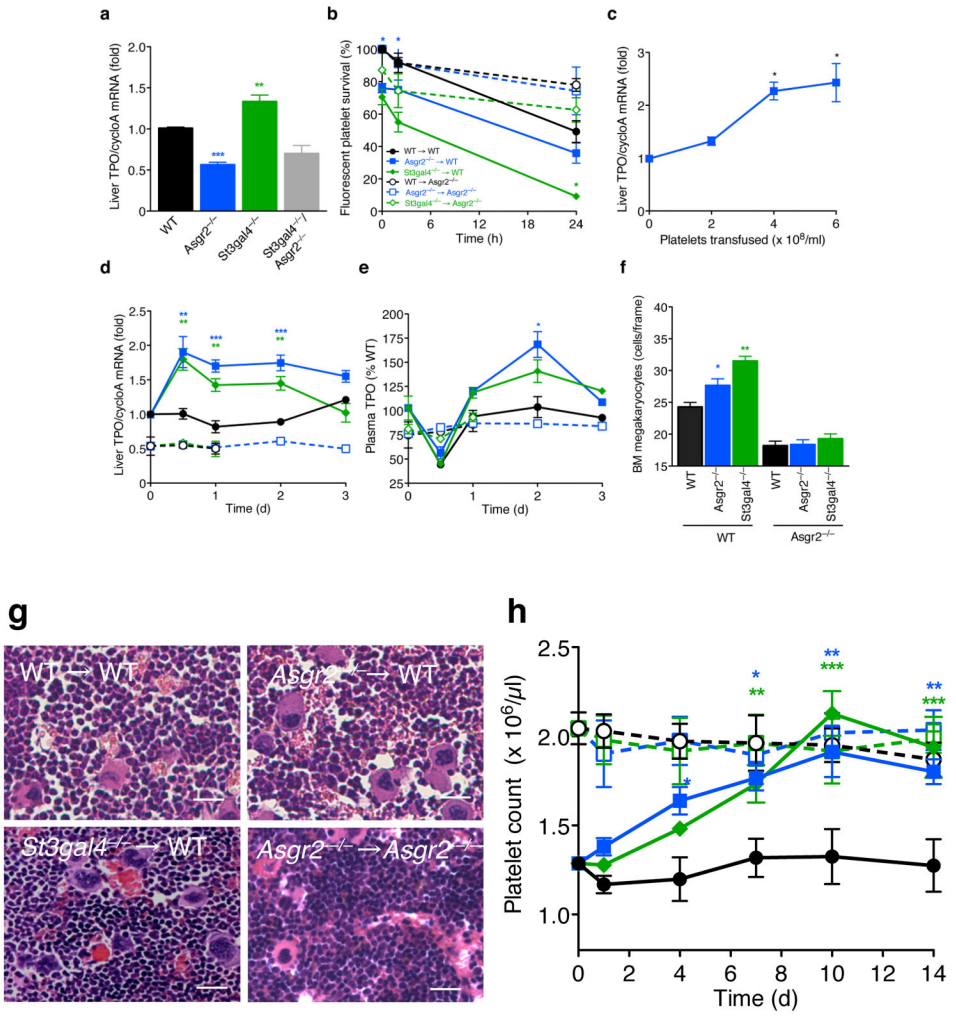

Figure 1. The Ashwell-Morell receptor regulates TPO homeostasis

(**a**) TPO mRNA expression in livers of WT, *Asgr2*$^{-/-}$, *St3gal4*$^{-/-}$ and *St3gal4*$^{-/-}$/*Asgr2*$^{-/-}$ mice. Data are mean ± SD of 15 WT, 15 *Asgr2*$^{-/-}$ 7 *St3gal4*$^{-/-}$ and 8 *St3gal4*$^{-/-}$/*Asgr2*$^{-/-}$ mice. (**b**) Survival of fluorescently labeled WT (black circles), *Asgr2*$^{-/-}$ (blue squares) and *St3gal4*$^{-/-}$ (green rhombus) platelets transfused into WT (closed symbols, solid lines) or *Asgr2*$^{-/-}$ (open symbols, dashed lines) mice. (**c**) Dose-response of liver TPO mRNA expression in WT mice 12 h after platelet transfusion of desialylated *Asgr2*$^{-/-}$ platelets (n=5). (**d**) Liver TPO mRNA expression, (**e**) plasma TPO levels, (**f**) BMMK numbers and (**h**) blood platelet counts of WT (closed symbols, solid lines) and *Asgr2*$^{-/-}$ (open symbols, dashed lines, n=4) mice transfused with WT (black circles), *Asgr2*$^{-/-}$ (blue squares) and *St3gal4*$^{-/-}$(green rhombus) platelets. (**g**) Representative bone marrow sections stained with hematoxylin and eosin. BMMK numbers were measured 3 days after injection. Each time point in each transfused animal cohort represents mean ± SD of 6 mice unless otherwise specified. **P*<0.05, ** P<0.01, ****P*<0.001.

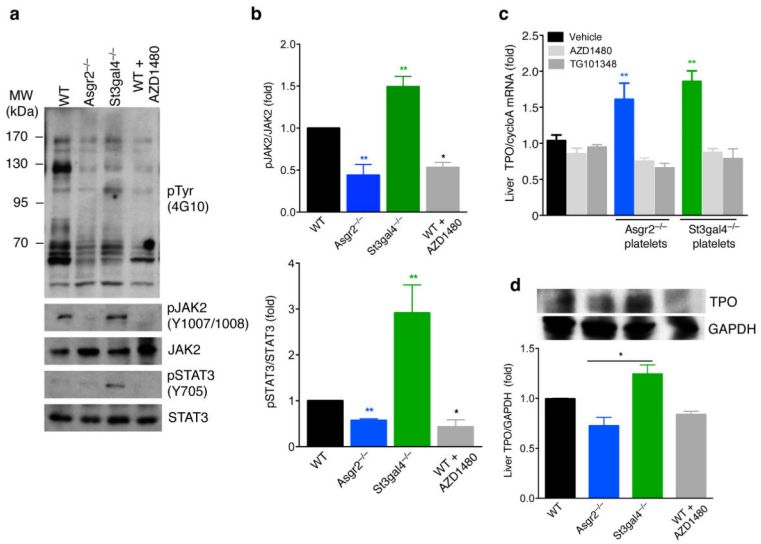

Figure 2. JAK2-STAT3 signaling regulates hepatic TPO mRNA and protein expression *in vivo*

(**a**) Western blots analysis of total liver lysates from WT, *Asgr2*$^{-/-}$, *St3gal4*$^{-/-}$ mice and WT mice treated with AZD1480 (WT + AZD1480) using monoclonal anti-pTyr (4G10), anti-pJAK2 (Y1007/1008), anti-JAK2, anti-pSTAT3 (Y705) and anti-STAT3 antibodies, (**b**) Densitometry of immunoblots for pJAK2/JAK2 (upper panel, 4 independent experiments) and pSTAT3/STAT3 (lower panel, 3 independent experiments). (**c**) TPO mRNA expression in livers isolated from WT vehicle (black bars), WT mice treated with AZD1480 (light grey bars) or TG101348 (dark grey bars) for 48 h and injected with *Asgr2*$^{-/-}$ or *St3gal4*$^{-/-}$ platelets. Liver TPO mRNA was measured 12 h after platelet injection. Each time point in each non-treated and transfused animals cohort represents mean ± SD of 6 mice, TG101348 experiments represents mean ± SD of 3 mice. (**d**) Representative immunoblots and respective densitometry of TPO protein expression in livers from WT, *Asgr2*$^{-/-}$, *St3gal4*$^{-/-}$ mice and WT mice treated with AZD1480 (WT + AZD1480) using GAPDH as loading control (n = 3 independent experiments). **P*<0.05, ***P*<0.01.

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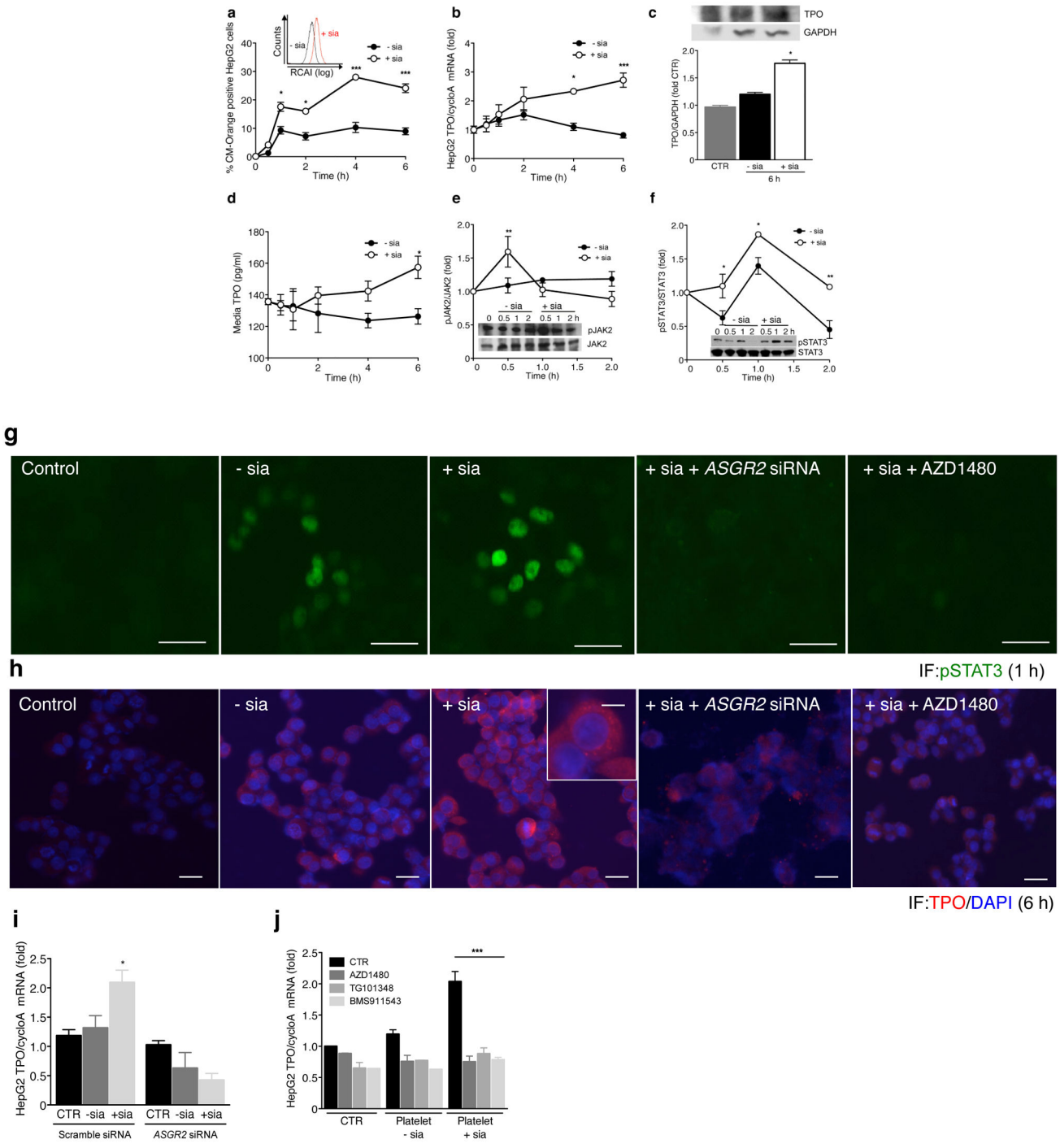

Figure 3. JAK2-STAT3 signaling regulates TPO mRNA expression *in vitro*

(**a**) Human platelets treated with sialidase (open circles) or control platelets (filled circles) were labeled with CM-Orange and incubated with HepG2 cells. Ingestion was evaluated as percentage of CM-orange positive HepG2 cells. Insert: Terminal surface β-galactose in untreated (black) and sialidase-treated (red) platelets as judged by RCA-I lectin binding. (**b**) TPO mRNA expression after incubation with untreated or desialylated platelets. (**c**)

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Quantification of TPO protein using a polyclonal anti-human TPO antibody in control HepG2 cells (CTR) and 6 h after addition of untreated (− sia) or desialylated (+ sia) platelets. GAPDH is shown as loading control. (**d**) TPO-protein quantification in the incubation media. (**e**) Representative immunoblots and densitometry analysis of total cell lysates using anti-pJAK2 and anti-JAK2 antibodies and (**f**) anti-pSTAT3 and anti-STAT3 monoclonal antibodies. n=3 (**g**) Immunofluorescences for pSTAT3 (1 h) and (**h**) human TPO-protein (6 h) of HepG2 cells incubated with untreated (− sia) and desialylated (+ sia) platelets or left untreated (Control); of HepG2 cells transfected with *ASGR2* siRNA prior to desialylated platelet addition (+ sia + *ASGR2* siRNA) and of HepG2 cells pretreated for 24h with AZD1480 before addition of desialylated platelets (+ sia + AZD1480). DAPI: nuclear counterstain. Scale: 20 μm. Insert: Increased magnification. Scale: 2 μm. (**i**) Quantification of TPO mRNA expression (6 h) in control (CRT) or cells transfected with scramble or *ASGR2* siRNA, or (**j**) in cells pretreated for 24 h with vehicle and JAK inhibitors AZD1480, TG101348 or BMS911543, after addition of untreated (− sia) and desialylated (+ sia) platelets. n=3 $*P<0.05$, $**P<0.01$, $***P<0.001$.

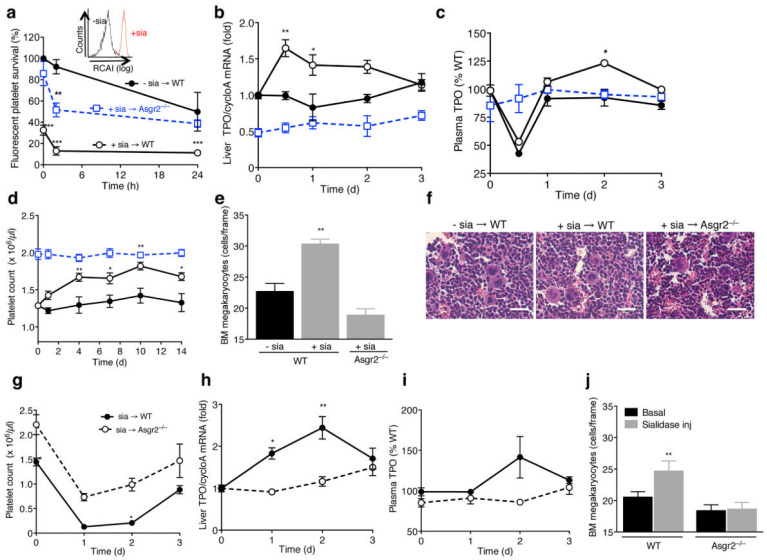

Figure 4. Injection of exogenously desialylated platelets stimulates hepatic TPO mRNA expression, TPO release and platelet production in mice

(**a**) Survival of fluorescently labeled WT platelets treated (open symbol) or not (closed symbol) with sialidase injected into WT (full black line) or *Asgr2*$^{-/-}$ (dashed blue line) mice. Insert: Terminal β-galactose exposure was evaluated in untreated (black, −sia) and sialidase-treated (red, +sia) WT platelets by flow cytometry using RCA-I lectin. (**b**) Liver TPO mRNA expression, (**c**) plasma TPO levels, (**d**) blood platelet counts of WT (full line) and *Asgr2*$^{-/-}$ (dashed line) mice injected with WT platelets treated (open symbols) or not (closed symbols) with sialidase. (**e**) BMMK numbers were measured 3 days after injection. (**f**) Representative bone marrow sections stained with hematoxylin and eosin. BMMK numbers. Data are mean ± SD of 6 WT and 4 *Asgr2*$^{-/-}$ recipients. *P<0.05. **Sialidase injections.** (**g**) Blood platelet counts, (**h**) liver TPO mRNA expression, (**i**) plasma TPO levels, and (**j**) BMMK numbers in WT and *Asgr2*$^{-/-}$ mice injected or not with sialidase. BMMK numbers were measured 3 days after injection. Data are mean ± SD of 5 mice. *P<0.05, **P<0.01.

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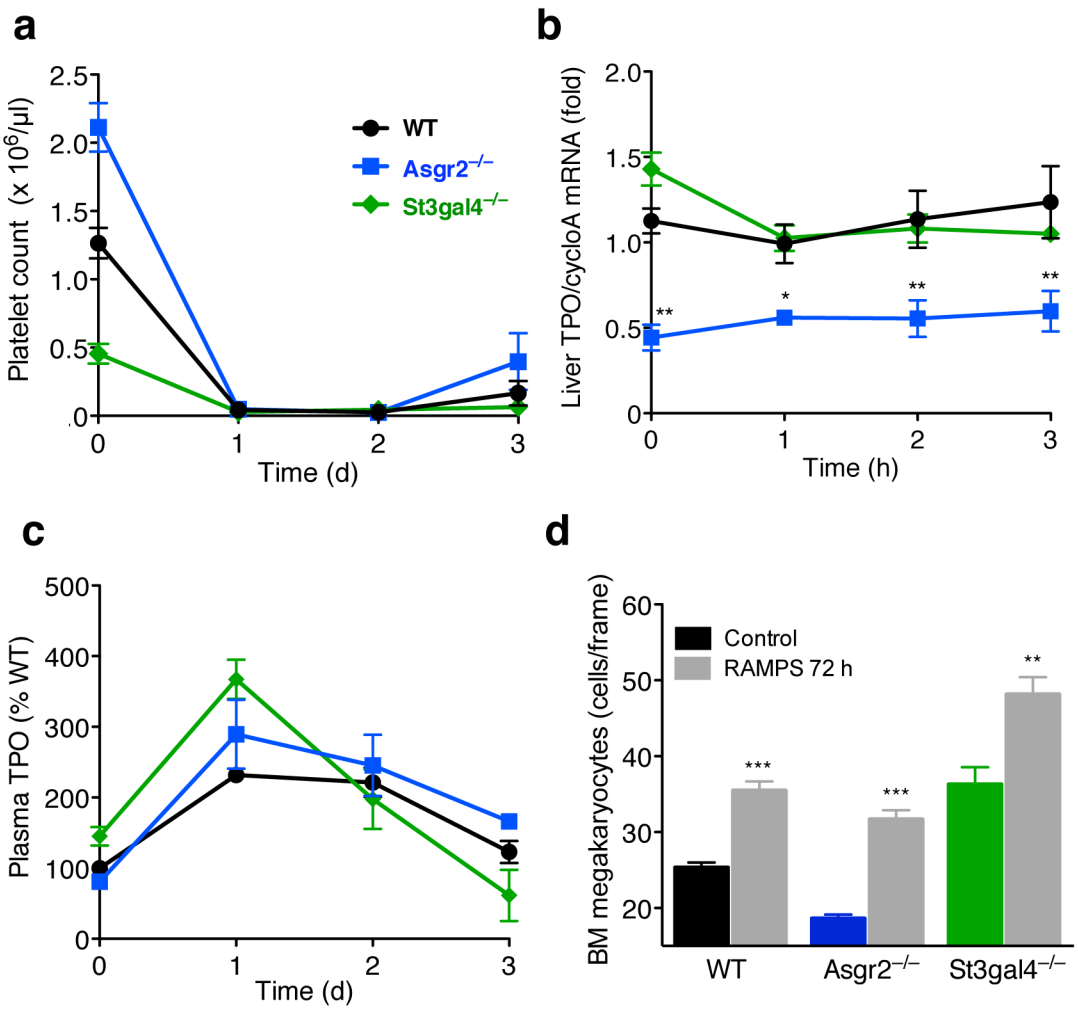

Figure 5. Antibody-mediated thrombocytopenia does not stimulate liver TPO mRNA expression

(**a**) Blood platelet counts, (**b**) liver TPO mRNA expression, (**c**) plasma TPO levels and (**d**) BMMK numbers in WT (black circle), *Asgr2*$^{-/-}$ (blue square) and *St3gal4*$^{-/-}$ (green rhombus) mice injected with a rabbit anti-mouse platelet serum (RAMPS). Bone marrow megakaryocyte numbers were measured 3 days after RAMPS injection. Data are mean ± SD of 5 mice. *P<0.05, **P<0.01, ***P<0.001.

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Table 1

Platelet homeostasis in WT, *Asgr2*$^{-/-}$, *St3gal4*$^{-/-}$ *and St3gal4*$^{-/-}$/*Asgr2*$^{-/-}$ mice.

Parameter	WT	*Asgr2*$^{-/-}$	*St3gal4*$^{-/-}$	*St3gal4*$^{-/-}$/*Asgr2*$^{-/-}$
Platelet count (10^6/μL)	1.42 ± 0.3	2.15 ± 0.6	0.4 ± 0.3	1.32 ± 0.3
FSC (size, fold WT)	1.0	0.78 ± 0.5*	1.67 ± 0.2**	0.83 ± 0.2
Platelet half-life (h)	47.5 ± 4.3	63.4 ± 3.6 ***	17.6 ± 2.9 ***	57.6 ± 2.9 ***
IPF (%)	5.9 ± 0.7	3.5 ± 0.7 *	20.1 ± 3.2 ***	4.1 ± 0.5
ECL (fold WT)	1.0	1.5 ± 0.2 *	1.8 ± 0.3 *	1.9 ± 0.2 *
RCA-I (Fold WT)	1.0	1.9 ± 0.2 *	4.5 ± 0.8 **	4.2 ± 0.6 **
Surface Mpl (rest, fold WT)	1.0	0.7 ± 0.1 **	1.8 ± 0.3 ***	0.8 ± 0.1
Surface Mpl + TPO (% resting MFI)	78 ± 3*	96 ± 6	57 ± 6**	83 ± 3
Total Mpl (fold WT)	1.0	0.8 ± 0.1	1.2 ± 0.1	1.06 ± 0.1
Plasma TPO (pg ml^{-1})	769 ± 37	621 ± 60	1175 ± 185 *	748 ± 72
Platelet TPO (pg/5×10^7 plts)	194 ± 37	121 ± 49 *	253 ± 51 **	201 ± 3
BMMKs (cells per frame)	23.3 ± 2.6	18.6 ± 4.8 *	36.3 ± 5.5 *	21.7 ± 2.5

Platelet count, size (FSC), immature platelet fraction (IPF), RCA-I and ECL binding to platelets and Mpl platelet surface expression were determined by flow cytometry. Mpl expression was measured on freshly isolated resting platelets and following TPO stimulation (Mpl + TPO). Data represent anti-Mpl minus control IgG mean fluorescence intensities, and are expressed as percentage of resting values at 10 min after TPO stimulation. Platelet half-life was determined in biotin-injected mice. Total Mpl expression in platelet lysates was determined by immunoblots. Plasma and platelet TPO levels were measured by ELISA. Data are mean ± SD of 15 WT, 15 *Asgr2*$^{-/-}$, 7 *St3gal4*$^{-/-}$ and 10 *Asgr2*$^{-/-}$/*St3gal4*$^{-/-}$ mice. Megakaryocyte numbers were quantified in H&E-stained bone marrow sections of 8–10 week old mice. Data represent mean megakaryocyte number per field of view from 10 fields per mouse. Data are mean ± SD of 12 mice per genotype.

* $P<0.05$,

** $P<0.01$,

*** $P<0.001$.