ABSTRACT

Primary immune thrombocytopenia (ITP) is an autoimmune disorder characterized by the decrease in peripheral blood platelet count below 100 x 10^9/L, and an increased bleeding risk when thrombocytopenia drops below 30 x 10^9/L. The mechanisms leading to ITP in adults, although not completely elucidated, involves an imbalance between effector and regulatory cells that results in a breakdown of the immune tolerance. Autoantibodies are considered the main responsible for thrombocytopenia, although direct T-cell cytotoxic effect and lysis by Complement attachment and activation could also contribute to platelet elimination from circulation. In addition to increased peripheral clearance, abnormalities in platelet production also favors platelet count reduction. This review is intended to describe some specific knowledge about peripheral and bone marrow mechanisms leading to thrombocytopenia in adult ITP.

Introduction

Primary immune thrombocytopenia (ITP) is an acquired autoimmune disorder characterized by chronic isolated low platelet count (<100 x 10^9/L), triggered by a loss of the immunological system equilibrium that tips the balance toward autoimmunity. Although in the last years the pathophysiology of ITP has been intensively studied, this field still remains incompletely understood. Another significant feature of ITP is the heterogeneity observed among patients, concerning not only their clinical profile but also their response to treatment, with a small proportion of patients classified as refractory. This heterogeneity is likely due to the participation of multiple pathophysiological contributing mechanisms, affecting individual patients in different degrees.

The presence of autoantibodies targeting platelet surface glycoprotein(s) (GP) has been demonstrated in patients with ITP with percentages of patients with positive autoantibodies varying according to different case series. Most of these antibodies are IgG, but IgM and IgA can also be detected, usually in association with IgG. Main autoantibody targets are GPIIbIIIa (integrin αIIbβ3), GPIbIX and GPIaIIa complex, but, autoantibodies against other GPs (i.e. the vitronectin receptor, integrin αvβ3), against thrombopoietin and/or its receptor c-Mpl were also detected in some patients. Therefore, humoral immunity has been classically considered the main cause of thrombocytopenia, although other mechanisms such as direct T-cell cytotoxic effect could contribute to platelet elimination as well. In addition, Complement activation/fixation capacity is enhanced...
in plasma samples from ITP. Indeed, Complement-mediated platelet destruction has been pointed as another mechanism of platelet elimination, mainly triggered by anti-platelet autoantibodies, but also observed in some ITP patients with no-detectable autoantibodies.

This review summarizes mechanisms leading to thrombocytopenia in ITP with special focus on peripheral platelet destruction and impaired platelet production.

Increased circulating platelet clearance

The first and most commonly accepted mechanism of platelet clearance in ITP is the binding of anti-platelet autoantibodies to their antigenic glycoprotein targets that leads to platelet elimination by monocytes/macrophages of the reticuloendothelial system through FcγRIIA and FcγRII-mediated recognition, mainly in the spleen and liver. However, additional pathological mechanisms including platelet apoptosis and loss of sialic acid from platelet membrane glycoproteins were described in recent years, that also contribute to platelet clearance from circulation.

Increased platelet apoptosis in ITP

Several studies have demonstrated the contribution of platelet apoptosis to ITP pathogenesis. Winkler and colleagues showed evidence of platelet apoptosis, including caspase 3, 8 and 9 activation, in children with acute ITP, which was ameliorated by intravenous immunoglobulin infusion. Concerning adult chronic ITP, increased platelet phosphatidylserine (PS) exposure, as a marker of platelet apoptosis, was shown by Catani and Alvarez Román.

In 2016, our group described increased mitochondrial membrane depolarization, active caspase 3 and membrane exposure of PS in platelets from ITP, as markers of apoptosis, while basal platelet activation assessed by PAC-1 binding and P-selectin externalization was ruled out as another possible cause of increased PS expression. Later, Deng and colleagues confirmed these results and showed higher expression levels of the proapoptotic molecules Bak and Bax, while the antiapoptotic factor Bcl-xL was decreased. Moreover, the same group demonstrated dysregulation of certain microRNAs related to apoptosis in platelets from ITP. Cells expressing PS on their membrane are known to be cleared through scavenger receptors in macrophages, thus, apoptotic platelets would also be eliminated from circulation by this mechanism. In our cohort of ITP, the inverse relationship found between apoptotic variables and peripheral platelet counts, further reinforces the relevance of platelet apoptosis in the development of thrombocytopenia in this disorder. Interestingly, platelet apoptosis was observed in all ITP patients carrying autoantibodies against the glycoproteins GPIIb/IIIa and GPIbIX, but was absent in those patients expressing anti-GPIIb/IIIa autoantibodies, suggesting a causal role for these specific antibodies in triggering this phenomenon.

In this study, PS externalization was not noticed in normal platelets incubated with ITP plasmas but it could be observed by adding normal autologous CD3+ lymphocytes to this system, suggesting an antibody-dependent cytotoxic effect. In the same system, anti-platelet antibodies-depleted ITP plasma had no effect on normal platelet apoptosis, while the IgG fraction of ITP plasma retained the apoptotic effect observed when whole plasma was used, confirming the involvement of auto-antibodies in the induction of apoptosis.

Interestingly, recent work described that autophagy is decreased in platelets from ITP and suggested this reduction could be linked to increased platelet apoptosis. Further studies are warranted to elucidate the interplay between autoantibodies, autophagy and apoptosis in this disorder.

Increased loss of sialic acid from platelet glycoproteins in ITP

In 2015, Li and colleagues described for the first time that plasma from ITP patients containing anti-GPIbIX autoantibodies induced loss of sialic acid capping carbohydrates from platelet glycoproteins. This mechanism is triggered by the ability of these autoantibodies to induce platelet activation, which promote neutaminidase externalization, the ultimate responsible for sialic acid cleavage from membrane glycoproteins. Platelets bearing asialoglycoproteins on their membrane are clarified in the liver by cooperation between macrophage galactose lectin (MGL) and the C-type lectin receptor CLEC4F, both expressed on Kupffer cells, and Ashwell-Morell receptor (AMR) in hepatocytes, as demonstrated by studies performed in mice. In addition, activation of the JAK2/STAT3 pathway downstream AMR after asialoglycoprotein binding, has been pointed as the mechanism triggering synthesis of thrombopoietin (Tpo), the main growth factor of the megakaryocytic lineage.

In a recent study, our group investigated several possible causes of thrombocytopenia in a cohort of ITP patients, including platelet desialylation. Results showed that loss of sialic acid from normal platelet glycoproteins occurs not only in the presence of anti-GPIbIX but also anti-GPIIb/IIIa ITP autoantibodies, as assessed by the increase in Ricinus communis agglutinin I (RCA-I), recognizing galactosyl (β-1,4) N-acetylgalcosamine (Galβ4GlcNAc) and Peanut agglutinin (PNA) which recognizes galactosyl (β-1,3) N-acetylgalactosamine (Galβ3GalNAc), both exposed after sialic acid loss. In addition, desialylation could be concomitantly observed with platelet apoptosis, although this was not a mandatory association. Recently, Manzano and colleagues reported increased apoptosis and loss of...
sialic acid in platelets from non-responsive ITP patients.

Interestingly, although a direct correlation was found between caspase activation and desialylation in the whole population included in the study (ITP patients who did not need treatment for at least six months, ITP patients responding to agonists of thrombopoietin receptors, and non-responders), levels of caspase activity between non-responders and thrombopoietin receptor agonists-responders were not different, supporting the notion that apoptosis and desialylation are not necessarily related. In addition, decreased platelet surface glycans in non-responders ITP patients was accompanied by a decrease in T regulatory lymphocyte population suggesting a link between this platelet abnormality and immune imbalance.

The observation that anti-GPIIbIIIa autoantibodies can also induce loss of sialic acid from platelet glycoproteins was recently confirmed by Marini and col. By blocking the FcγRIIA signaling, authors demonstrated that sialic acid loss is at least partially due to crosslinking of anti-GPIIbIIIa autoantibodies to FcγRIIA, which is responsible for the release of sialidase from the intracellular compartments of platelets.

Concerning circulating Tpo levels in ITP, it was suggested that they were lower than expected for the degree of thrombocytopenia. In the late ’90s, when Tpo synthesis by the liver was supposed to be constant, the cumulative mass and ploidy were normal. These results are in line with the known concept that the number of megakaryocytes in bone marrow from ITP patients is normal or increased. Besides, although it was described that circulating levels of thrombopoietin are low for the degree of thrombocytopenia in ITP, as mentioned before, they seem sufficient to maintain a proper megakaryocyte population in bone marrow.

**Impaired platelet production**

Platelet production is the final step of a complex series of events involving two well defined processes: megakaryopoiesis and thrombopoiesis. Megakaryopoiesis initiates when undifferentiated CD34+ hematopoietic progenitor commits to the megakaryocytic lineage and ends when megakaryocytes reach their full maturation state. Next, the process identified as thrombopoiesis involves cytoplasmic megakaryocytic reorganization, proplatelet extension and branching and, after these proplatelets go through the endothelial bone marrow barrier, platelets are released from their tips into the bloodstream.

**Megakaryopoiesis in ITP**

Early independent works from Chang and McMillan studied the effect of the addition of 10% of ITP plasma to the culture medium of normal hematopoietic progenitors induced to differentiate into megakaryocytes. Their results showed that plasma from ITP patients impaired megakaryocyte development, pointing to autoantibodies directed to GPIbIX and GPIIbIIIa as responsible for this effect. Unlike these previous studies that used mononuclear cells or 20-30% pure CD34+ progenitors as source of normal hematopoietic precursors, we reproduced these experiments using at least 93% pure CD34+ hematopoietic progenitors isolated from normal human cord blood. In these conditions, megakaryopoiesis was not inhibited by ITP samples. Moreover, the expression of CD42b, a marker of megakaryocyte maturation, was increased in the presence of 45% of the ITP plasma, while megakaryocyte size and ploidy were normal. These results are in line with the known concept that the number of megakaryocytes in bone marrow from ITP patients is normal or increased.

**Participation of apoptosis during megakaryocyte and platelet production**

Reports describing apoptosis in the megakaryocytic lineage performed on bone marrow samples from ITP patients are controversial. Uçar and col found no differences in the number of megakaryocytes and their apoptotic status between ITP and control children; Houwerzijl et al. found ultrastructural abnormalities compatible with apoptosis in adult ITP, and a recent study described a decrease of megakaryocyte apoptosis in bone marrow biopsies of ITP subjects compared to healthy controls. Although these discrepant results are intriguing and would encourage researchers to continue the investigations, bone marrow samples form ITP are not easily available because bone marrow examination is not generally needed to diagnose ITP.

Beyond megakaryopoiesis, data about the participation of apoptotic events during normal physiologic proplatelet formation and platelet production has also been controversial. While several studies support that localized apoptotic events are needed for mature megakaryocytes to rearrange their cytoplasmic architecture and proceed with proplatelet extension and platelet release, more recent information hold the notion that apoptosis must be
restrained during proplatelet formation, and only when platelets have been released, the remaining naked nuclei undergo apoptosis (reviewed by McArthur45).

Similarly, investigations evaluating the effect of ITP plasma on mature megakaryocyte apoptosis have led to discordant results. Yang et al46 described lower expression of tumor necrosis factor-related apoptosis-inducing ligand, higher expression of Bcl-xl and lower platelet release in normal megakaryocytes incubated with ITP plasma, pointing to decreased apoptosis as a contributing factor to reduced platelet production. In our hands, normal mature megakaryocytes incubated for 48 hours with 10% ITP plasma did not suffer caspase 3-7 activation, and the number of megakaryocytes displaying nuclear picnosis was slightly increased. However, proplatelet formation was significantly decreased as will be discussed later47. Then, although the number of megakaryocytes in bone marrow from ITP is normal or elevated, abnormalities in the apoptotic pathways of mature megakaryocytes could not be ruled out as possible factors leading to a lower number of megakaryocytes capable of producing proplatelets. Indeed, further work is necessary to clarify this topic.

Altered megakaryocyte functionality and impaired thrombopoiesis

Other important players participating in the proper functionality of megakaryocytes are extracellular matrix proteins present in the bone marrow milieu. Megakaryocyte interaction with different matrix proteins play specific roles in megakaryocytic physiology. Two of these essential molecules present in the vascular niche are fibrinogen and von Willebrand factor (vWF). Main receptors for these ligands are GPIIbIIIa, which binds both proteins, and GPIbIX, that recognizes vWF. In addition to their key role in platelet function, GPIIbIIIa and GPIbIXV complexes are involved in megakaryocyte development and platelet production. Proof of their participation in these events are the deep abnormalities observed in macrothrombocytopenia due to mutations leading to partial activation of GPIIbIIIa48, and mutations in GPIbIXV, responsible for Bernard-Soulier syndrome 49.

Grodzielski and col50 demonstrated that plasma and specifically the IgG fraction from ITP patients interfere with adhesion and spreading of megakaryocytes. This interference is specific for each receptor-ligand pair depending on the type of autoantibody present. Anti-GPIIbIIIa autoantibodies inhibit megakaryocytic interaction with fibrinogen, reducing adhesion, spreading and signal transduction downstream this receptor, as demonstrated by decreased β3 subunit phosphorylation. Abnormal glycoprotein function was also observed by impaired PAC-1 binding to megakaryocytes incubated with ITP plasmas bearing these autoantibodies47. Similarly, anti-GPIbIX autoantibodies impair megakaryocyte adhesion and spreading to vWF, and preclude Src phosphorylation downstream this receptor.

The main raison d'être of megakaryocytes is platelet production, and, as a consequence of autoantibody binding toGPIIbIIIa and/or GPIbIX complexes, proplatelet formation is inhibited 47,51. The participation of autoantibodies in abnormal thrombopoiesis was proved by reproducing the inhibitory effect of ITP plasma using purified IgG, and by its reversion when ITP plasma was immunodepleted from autoantibodies by incubation with normal platelets.

In addition to the decrease in the number of megakaryocytes producing proplatelets, morphological changes were observed in the architecture of proplatelets generated in the presence of ITP plasma. Most of the patient’s samples induced wider and shorter proplatelets, bearing lower numbers of bifurcation points and tips, and overall, less complex than those observed when megakaryocytes were cultured with the addition of normal plasma47. Considering that megakaryocytes must elongate their proplatelets through the bone marrow endothelial barrier in order to release platelets into the bloodstream, these morphological proplatelet features could hinder thrombopoiesis. Altogether, these results could imply that, in addition to the lower number of megakaryocytes capable of producing proplatelets in ITP, each proplatelet-bearing megakaryocyte would be unable to produce a proper number of platelets.

The osteoblastic niche of bone marrow is a type I collagen-enriched environment. Megakaryocyte binding to collagen through GPIaIIa complex inhibits proplatelet production52, precluding premature platelet release within this compartment. Our in vitro experiments demonstrated that plasma and purified IgG fractions from ITP patients containing anti-GPIaIIa autoantibodies, induced a decrease in megakaryocyte adhesion and spreading on type I collagen along with lower MLC2 phosphorylation downstream GPIaIIa50. In agreement with the fact that MLC2 phosphorylation is a key downstream effector mediating GPIaIIa-induced negative regulation of thrombopoiesis53, these autoantibodies interfered with normal inhibition of proplatelet formation on type I collagen, allowing megakaryocytes to extend proplatelets even on a type I collagen-coated surface47. These findings suggest that the presence of anti-GPIaIIa autoantibodies could lead to premature proplatelet production in the osteoblastic niche, hindering platelet release within the bloodstream.

Along with these abnormalities, Zeng and col.54 added autoantibodies targeting the vitronectin receptor, to the list of factors responsible for the interference between megakaryocyte adhesion to the vascular niche, and potentially contributing to impair platelet production in ITP.
In addition to platelets, megakaryocyte glycoproteins are also target for autoantibody desialylation. Unlike what was observed in platelets, loss of sialic acid from normal megakaryocytes was only found in the presence of ITP plasma containing anti-GPIbIX autoantibodies\textsuperscript{31}. Marini and col.\textsuperscript{33} demonstrated that desialylation induced by ITP autoantibodies (either anti GPIIbIIa or GPIbIX) inhibits megakaryocyte adhesion to the bone marrow extracellular matrix proteins fibrinogen and vWF, leading to limited cell differentiation and reduced ability to extend proplatelets. Thus, besides increasing platelet clearance, desialylation has also been established as a mechanism of impaired platelet production.

Overall, these studies demonstrate that ITP autoantibodies mainly affect specific and different intracellular pathways, depending on their target proteins, interfering with normal megakaryocytic functions within the bone marrow and leading to decreased platelet production.

**Conclusions**

The growing amount of knowledge concerning pathophysiological abnormalities in ITP and results that evaluated several of these mechanisms in individual patients, show that mechanisms of thrombocytopenia are multifactorial, and frequently involve concomitant defects in platelet production and clearance. One of the main conclusions of our work concerning platelet production is that ITP autoantibodies are more harmful on inhibiting thrombopoiesis than affecting megakaryopoiesis. Regarding platelet clearance, apart from the classical Fc-dependent mechanism, platelet apoptosis and desialylation are additional and independent processes contributing to lower platelet count (Figure 1). These findings provide a rational for the use of combination therapy, including drugs with different mechanisms of action, in patients with highly refractory disease.

![Figure 1: Mechanisms leading to low platelet count in Immune Thrombocytopenia.](image-url)

This graphical abstract includes mechanisms published in references 19, 24, 37 and 40. Impaired platelet production is likely due to abnormal megakaryocytic function, including altered interaction with extracellular matrix proteins from bone marrow and thrombopoiesis (reduced number of proplatelet-producing megakaryocytes and abnormal proplatelet structure), induced by autoantibody binding to megakaryocytic glycoproteins. Although autoantibody-induced abnormalities were also observed during megakaryopoiesis, they seem to contribute in lesser extent to the reduction in platelet production. Enhanced peripheral platelet clearance could be related to monocyte/macrophage phagocytosis induced by binding of platelet-autoantibody complex to Fcγ receptors IIa and IIIa and phosphatidylserine expressed on apoptotic platelets that binds to scavenger receptors. In addition, desialylated platelets could be clarified by cooperation between Kupffer cells and hepatocytes in the liver.
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