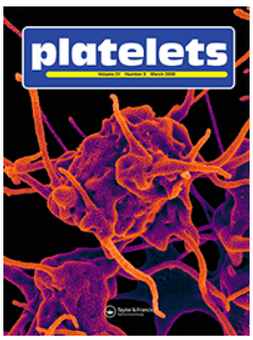

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Novel scientific approaches and future research directions in understanding ITP

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Abstract

Diagnosis of immune thrombocytopenia (ITP) and prediction of response to therapy remain significant and constant challenges in hematology. In patients who present with ITP, the platelet count is frequently used as a surrogate marker for disease severity, and so often determines the need for therapy. Although there is a clear link between thrombocytopenia and hemostasis, a direct correlation between the extent of thrombocytopenia and bleeding symptoms, especially at lower platelet counts is lacking. Thus, bleeding in ITP is heterogeneous, unpredictable, and nearly always based on a multitude of risk factors, beyond the platelet count. The development of an evidence-based, validated risk stratification model for ITP treatment is a major goal in the ITP community and this review discusses new laboratory approaches to evaluate the various pathobiologies of ITP that may inform such a model.

Keywords

Autoantibody, ITP, platelet, receptor, thrombocytopenia

History

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Introduction

Immune thrombocytopenia (ITP) is an acquired autoimmune disorder characterized by a moderate to a severe reduction in platelet count in peripheral blood and where there is no other cause or disorder associated with thrombocytopenia. In healthy individuals, the platelet count ranges from 150 to 400 × 10^9/L, however in ITP, the platelet count falls below 100 × 10^9/L [1]. In adults, the incidence of ITP is approximately two to four per 100,000 [2]; however, these numbers may be inaccurate. In fact, ITP is likely to be under or even misdiagnosed [3] as clinical diagnosis is by exclusion, both patient presentation and disease etiology are diverse and non-uniform, and in the absence of meaningful standardized tests, the simple platelet count remains the primary tool for diagnosis and evaluation of response to therapy. Distinguishing ITP from other forms of acquired thrombocytopenia such as drug-induced thrombocytopenia is crucial for diagnosis and for implementation of the appropriate medical treatment (Figure 1) [4]. New diagnostic approaches that can assess platelet quality and functional changes are desperately needed as novel clinical therapies become available. Further, application of these new research-based approaches to evaluate platelet and immune cell levels and function in samples from people with ITP in a measured and strategic fashion is likely to simultaneously improve our understanding of the etiology and pathology of ITP.

Contribution to the Special Focus Issue on Immune Thrombocytopenia

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Mechanisms of Disease in ITP

ITP is an acquired heterogeneous autoimmune disorder characterized by phagocytosis of autoantibody-coated platelets by splenic macrophages and complement- or T cell-mediated platelet destruction (Figure 2) [5]. The targets of autoantibodies, primarily of the immunoglobulin (Ig) G class, are specific glycoprotein (GP) receptors found on platelets and the parental bone marrow megakaryocyte. In ITP the major antigenic receptor targets are GPIb-IX-V (primarily the GPIbα subunit but also GPV) and αIIbβ3 [6–8], although antibodies against GPVI and α2β1 have also been reported [9, 10]. Binding of these autoantibodies to platelets triggers destructive processes often mediated by Fc receptor (FcR) on macrophages as well as inhibition of platelet function and platelet production by megakaryocytes. Levels of thrombopoietin (TPO), mostly a liver-derived hormone that drives megakaryocyte maturity and platelet production, are generally not different in ITP patients from levels measured in healthy donors [11, 12]. Although the measurement of TPO levels is unable to confirm a diagnosis of ITP, as this entity remains a diagnosis of exclusion, elevated TPO levels may help suggest an alternative cause for thrombocytopenia [12]. The observation that TPO levels are inappropriately modest in ITP suggests that this under-expression contributes to the pathogenesis of the disease. The onset of ITP has no clear underlying cause but seems to develop through loss or perturbation of B- and T-cell immune tolerance via diverse mechanisms. An abnormal T cell response, driven by splenic T follicular helper cells, stimulates the proliferation and differentiation of autoreactive B cells. Altered T helper cell ratios and changes to attendant cytokines such as interleukin (IL)-17 have also been reported to contribute to ITP pathology [13]. Cytotoxic T cells have been demonstrated targeting platelets in the periphery, as well as affecting megakaryocyte proplatelet formation in the bone marrow niche [14, 15].

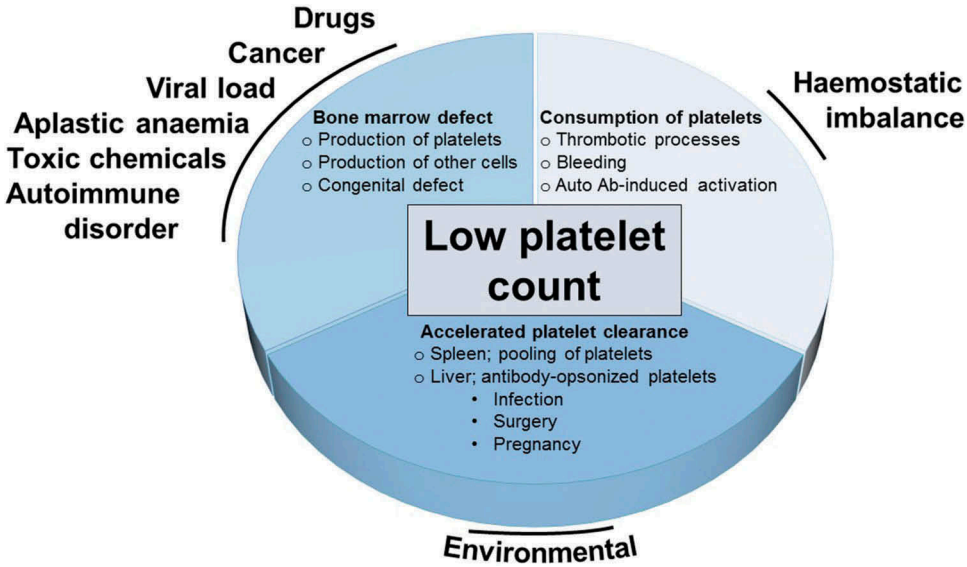

Figure 1. Factors that lead to a low platelet count.A low platelet count can result from one or more main elements; reduced production of platelets by megakaryocytes in the bone marrow, increased consumption of platelets in hemostatic processes and accelerated clearance of platelets. Exposure to toxic chemicals and chemotherapy, autoimmune disorders and other pathologies can disturb the bone marrow niche and platelet production by megakaryocytes. Certain drugs have been associated with the development of drug-dependent anti-platelet antibodies. Reduction in platelet count can also arise from extensive consumption of platelets in hemostatic processes. Furthermore, the production of anti-platelet autoantibodies can result in inappropriate platelet activation and opsonization leading to clearance via the spleen where antibody-opsonized platelets are removed by splenic macrophages or via the liver where Kupffer cells remove activated/desialylated platelets. External triggering factors such as infection, pregnancy and surgery can also trigger thrombocytopenia however the mechanisms have not been elucidated.

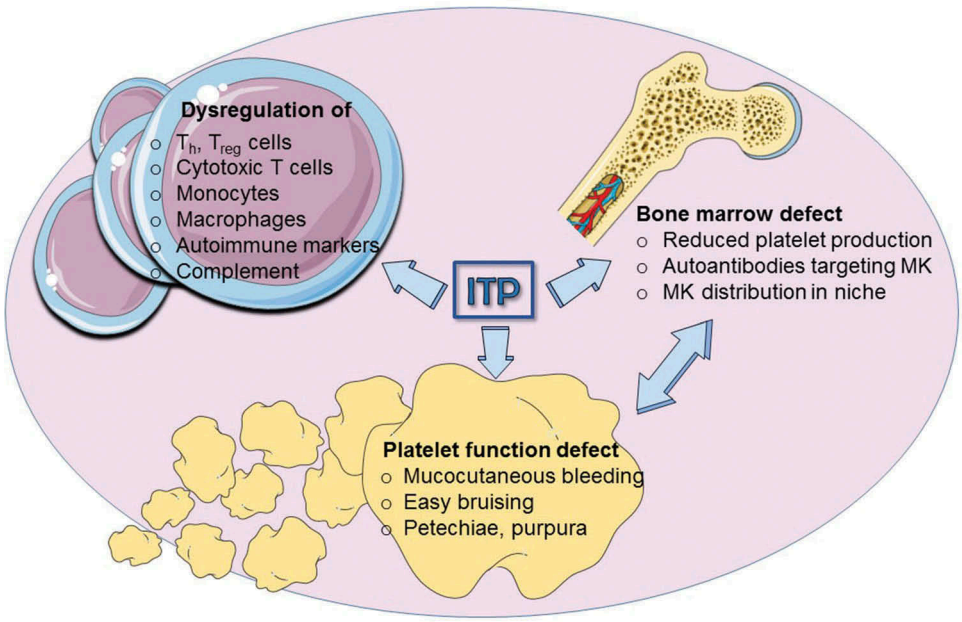

Figure 2. Pathophysiology of immune thrombocytopenia.The pathophysiology of ITP stems from dysregulation of the immune system and disturbed bone marrow and platelet function. Control of an appropriate T cell response is lost, with increased T helper 1 cells and decreased T regulatory cells leading to increased macrophage-mediated phagocytosis and presentation of platelet antigens to T and B cells. Autoantibodies produced by B cells opsonize platelets leading to complement-mediated and phagocytic clearance. Loss of platelet numbers and function through autoantibody binding to both platelets and bone marrow megakaryocytes can result in mild to severe bleeding symptoms. Immune cell dysregulation can also directly affect the bone marrow niche.

Current Means to Diagnose ITP

There is no diagnostic test or reliable panel of biomarkers to direct treatment, and few comparative studies to help management decisions around ITP, and this syndrome remains diagnosed by the exclusion of other known causes [2, 16]. Response to ITP-specific therapy, for example, intravenous immunoglobulin (IVIg) and intravenous anti-D, is supportive of the diagnosis, but a platelet response does not confirm a diagnosis either. As people with ITP often present with fatigue and unexplained mild bleeding, the platelet count in association with blood and bone marrow smears remain the major clinical tools to evaluate an isolated thrombocytopenia. Secondary causes for ITP need to be excluded with screening for autoimmune diseases, serology for hepatitis C virus, human immunodeficiency virus and in some regions *Helicobacter pylori* testing [16, 17].

A whole blood smear in combination with immunocytochemical staining using monoclonal antibodies (mAbs) and qualitative analysis by light microscopy can reveal changes in platelet morphology as well as reduction of platelet receptor levels and help rule-out disorders of macrothrombocytopenia [18]. Measurement of platelet diameter may be helpful to distinguish ITP from other inherited forms of thrombocytopenia [19].

Notwithstanding procedural nuances that can impact on the counting of platelets, including the choice of anticoagulant in which to collect the blood, and the mode of automated counter (optical or impedance) used to enumerate platelets, the platelet count remains just a broad guide of disease status. It does not predict treatment responses or duration of response or provide a means to stratify patients for bleeding risk. Some measurements are only available in reference centers including quantification of reticulated (new) platelets, serum thrombopoietin (TPO) and platelet sequestration studies which remain of disputed benefit from conflicting data [20–23].

Avenues to Investigate ITP Pathogenesis

As our understanding of the pathogenesis of ITP is strengthening, research-based approaches that focus on measuring the underlying causes of this syndrome may have utility to evaluate ITP pathology in patients and assist in the diagnosis of ITP (Figure 3). This will be particularly valuable if new approaches can enable stratification of patients into therapeutic groups, as well as identification of patients at risk of relapse and of bleeding.

Characterizing Antiplatelet Autoantibodies

Autoantibodies against platelet antigens are considered a diagnostic hallmark of ITP; however, reliable detection of pathological antibodies is challenging due to variable levels of free versus platelet-bound antibodies, and an absence of detectable anti-platelet autoantibodies in up to 40% of patients [24]. In some patients, antibodies recognize antigens derived from a single glycoprotein; whereas in others, antibodies recognize multiple glycoproteins [25]. Although the prognostic value of platelet antibodies to date is limited, it might be helpful to characterize the target of an antiplatelet autoantibody as specific types of antibody may predict responses to steroids or IVIg [26, 27] and may predict chronic disease and bleeding [28]. More recently, antibody-mediated desialylation of platelets in ITP has been reported to occur in patients with anti-platelet antibodies, raising the possibility that these patients could be treated with a neuraminidase inhibitor such as oseltamivir [29–31].

Direct and indirect forms of the Monoclonal Antibody-specific Immobilization of Platelet Antigen (MAIPA) assay using patient or donor platelets are designed to detect and characterize the autoantibody target [32]. Indirect MAIPA positivity at disease onset was observed to be associated with more severe hemorrhage and predicted a chronic course in adult ITP patients [28]. In this assay, platelets from donors or patients are isolated and washed, then mixed separately with mouse mAbs directed against various human platelet receptors, primarily GPIb-IX-V, αIIbβ3 or α2β1 which are the major platelet antigens in ITP. Platelets are then washed and solubilized by detergent-containing buffer and plated onto 96-well plates coated with anti-mouse IgG. The wells are washed and incubated with a peroxidase- or phosphatase-conjugated anti-human IgG antibody followed by substrate. Detection of a signal signifies the presence of autoantibodies in complex with the mAb-bound platelet receptor captured by the anti-mouse IgG. This assay requires specialist laboratory training and does not report on autoantibody binding affinity or avidity. Also, a lack of signal indicates only that antiplatelet autoantibodies

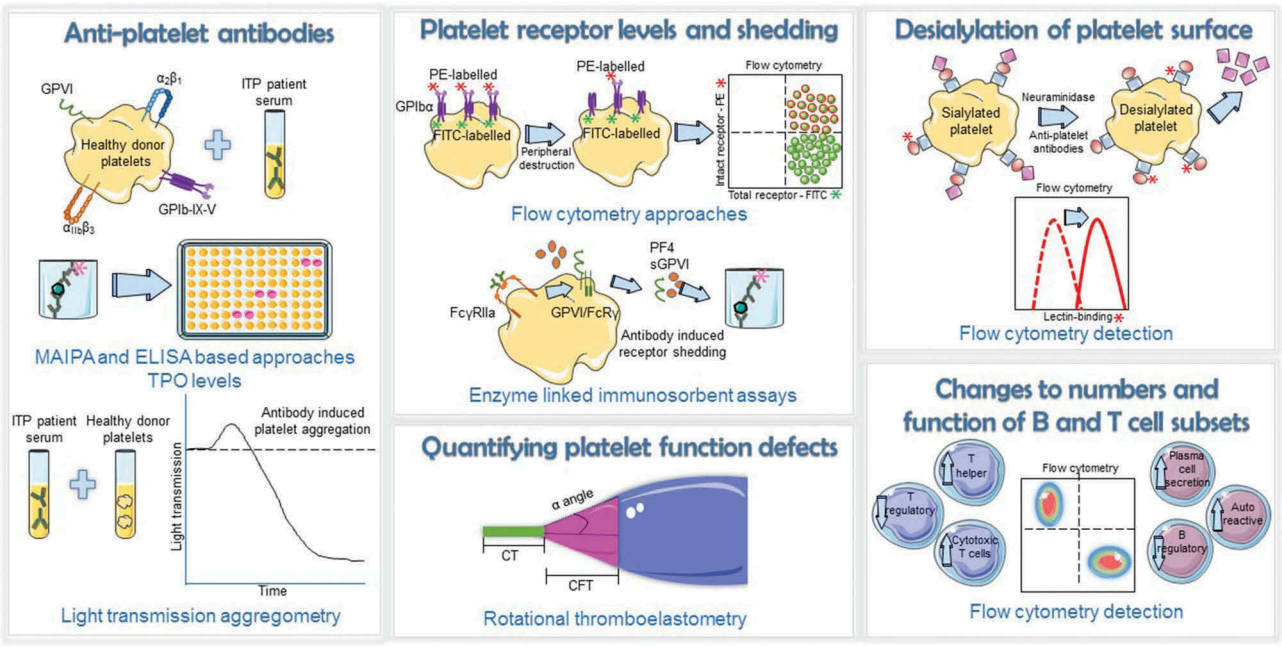

Figure 3. Some existing and new laboratory approaches to aid diagnosis of ITP.Some existing and new research-based methods that can be applied to evaluate ITP both at initial presentation and in a patient receiving treatment focus on evaluating antibody-induced changes to platelet function, detection and characterization of anti-platelet autoantibodies and enumerating immune cell subsets and specific functions (e.g. cytokine production). A new assay evaluates the levels of intact and total GPIbα present on circulating platelets, to gauge peripheral destruction of platelets. PF4 and sGPVI are released from antibody-bound platelets and can be batch analyzed by ELISA. Desialylation of the platelet surface (predominantly GPIbα) can be evaluated by flow cytometry using fluorescence-labeled lectins. Assessment of T and B cell subsets by flow cytometry and functional assays can also report on the extent of immune dysregulation. Ongoing and future work will evaluate the utility of each of these assays to inform clinical decisions, predict bleeding and assess responses to therapies.

that target the receptors specified are not detected, neglecting alternate epitopic specificity. Further, as mAbs against GPVI are generally not included in a standard MAIPA, autoantibodies that bind GPVI will not be detected. A version of the MAIPA using beads coated with mAbs against GPIbα or αIIb integrin that was mixed with patient washed platelet lysates followed by a radiolabeled anti-human IgG antibody has been used with reasonable specificity but only modest sensitivity [24].

Platelet-associated Immunoglobulins

Approaches that use flow cytometry to evaluate the platelet surface for the presence of antibodies (platelet-associated IgG; PaIgG) also have been described. Samples of patient whole blood are mixed with fluorescently tagged anti-human IgG antibodies and evaluated for fluorescence activity. This approach has the advantage of being able to directly interrogate samples of anticoagulated whole blood from patients, rather than use surrogate donor platelets, as flow cytometry is sufficiently sensitive to be able to monitor the platelet surface in thrombocytopenic samples even when the platelet count is extremely low ($<5 \times 10^9$/L). The disadvantage of this approach is the lack of specificity [33] as PaIgG may be elevated in both immune and nonimmune thrombocytopenia [34]. Interestingly, the population of PaIgG-positive platelets detected in peripheral blood is likely to be the 'surviving' platelets that have not been cleared by the reticuloendothelial system and may themselves be worthy of investigation.

Evaluating Age of Circulating Platelets as a Surrogate for Platelet Production

In healthy individuals, platelets circulate for 7–10 days unless consumed as part of a hemostatic response. Newly synthesized (reticulated) platelets are released from bone marrow megakaryocytes and these platelets are larger in size and contain ribonucleic acid (RNA) [35] which can be used by platelets for protein synthesis [36, 37]. RNA levels can be estimated by mixing whole blood samples with thiazole orange (TO) and measuring fluorescence activity in a flow cytometer, although a standard TO labeling protocol needs to be rigorously followed and TO^{bright} and TO^{dull} gates clearly defined. Values for healthy ranges of TO staining can be challenging to set as platelet autofluorescence contributes significant background signal in this assay. Modern blood analyzers also use this approach to calculate the immature platelet fraction (IPF) and the automated IPF has been shown to measure thrombopoiesis in real time and track treatment effectiveness in ITP [38, 39], predict bleeding [40] and helps distinguish between hypo- and hyper-proliferative thrombocytopenias [41]. Importantly, concordance between flow cytometric and automated analyzer approaches was achieved in ITP samples [42]; however, more work is required in larger patient cohorts to fully understand the implications of this measurement in predicting the course of the disease and in assisting decisions on therapy. Reticulated platelets have also recently been shown to carry high levels of human leukocyte antigen (HLA) I with good reproducibility [43]. This observation requires further detailed analysis to characterize the HLA-positive population as HLA I levels on platelets may be regulated by multiple means [44, 45].

Evaluating Receptor Levels on Circulating Platelets

Engagement of the platelet surface by antibodies targeting platelet proteins can lead to platelet activation. This is most readily seen in heparin-induced thrombocytopenia (HIT) where autoantibodies against platelet factor 4/heparin form an immune complex that can engage the antigen on the platelet surface via Fab regions and simultaneously interact with FcγRIIa on the platelet via the Fc portion resulting in platelet activation [46]. The binding and activation of FcγRIIa require that the pathological antibody binds to its antigenic target in an appropriate orientation that permits Fc interaction with platelet FcγRIIa. To what extent ITP-related antiplatelet autoantibody pathology triggers metalloproteolysis and other platelet activation and degranulation events remain to be determined; however, the Fc portion of PaIgG is likely to mediate platelet clearance by engaging with Fc receptor on monocytes and macrophages [5].

Using flow cytometry, levels of platelet receptors on circulating platelets can be measured in thrombocytopenic samples. In particular, altered levels of GPIbα, αIIb and β3 integrin subunits, GPVI and *P*-selectin may signal the presence of an activating anti-platelet antibody. Whilst the mechanisms by which these receptors are regulated *in vivo* remain unclear, a number of platelet receptors can be metalloproteolytically shed from platelets, particularly upon engagement of the platelet antigen by an antiplatelet autoantibody. Being able to assess one or more shed ectodomain fragments in plasma using enzyme-linked immunosorbent assays (ELISA) offers flexibility in sample acquisition and storage for batch analysis. Notably, a small number of ITP patients who presented with bleeding were shown to have an anti-GPVI autoantibody [9, 10], with evidence of platelet activation and metalloproteolysis of GPVI, resulting in the release of the GPVI ectodomain (sGPVI) and loss of collagen-related platelet function. Whether other platelet autoantibodies, particularly antibodies against GPIbα also trigger shedding of platelet receptors, and whether monitoring the loss of receptor ectodomains by flow cytometry of patient platelets in whole blood, or measurement of one or more shed ectodomain fragments in patient plasma has any value in assessing the presence of a platelet-activating antibody are open research questions. For example, a significant increase in soluble GPVI as quantified by ELISA signaled the presence of an acquired anti-GPVI autoantibody [47, 48]. These measurements explained the loss of collagen responsiveness in platelet aggregation assays as well as the observed bleeding that could not be explained by the platelet count [9, 10, 49].

In contrast to GPVI, GPIbα is constitutively shed from platelets, and whilst the shed portion of GPIbα (glycocalicin) can be monitored by ELISA, the value of this measurement in reporting on anti-platelet autoantibody-induced platelet activation is not clear. Plasma levels of glycocalicin vary considerably in healthy donors and loss of GPIbα can be triggered by one or more non-antibody mediated mechanisms [38,50–52]. A more useful approach might be to evaluate the extent of intact receptors loss (for example GPIbα) relative to the age of the platelet population as determined by TO uptake or other methods [38]. In this way, changes to the normal lifespan of circulating platelets, and increases in platelet production (proportion of TO+/GPIbα+ platelets) can be monitored for assessment of acute phases of ITP and responses to therapy.

Autoantibodies that target GPIbα seem to increase platelet clearance by triggering receptor clustering, platelet degranulation to expose *P*-selectin and phosphatidylserine, and to release neuraminidases which remove (desialylate) sialic acid residues that cap *N*- and *O*-linked carbohydrate moieties on the platelet surface [27,53–55]. Levels of platelet desialylation can be estimated by assessing the ability of glycan-binding lectins such as *Ricinus Communis* agglutinin-1 or wheat germ agglutinin to bind to exposed galactose or *N*-acetylglucosamine residues, respectively, on washed platelets. Along with an improved understanding of ITP pathology, these findings may also have clinical implications as in a murine model of ITP [56], mice with anti-GPIbα autoantibodies were resistant to intravenous immunoglobulin (IVIg) a common therapy used to treat ITP in patients refractory to

steroids. Further, in selected ITP patients with anti-GPIbα autoantibodies, recovery of the platelet count was observed after treatment with oseltamivir, a neuraminidase inhibitor suggesting that neuraminidase activity was a significant mediator of pathology in these individuals [29, 30]. Accurate identification of GPIbα autoantibodies, estimation of platelet desialylation or elevated platelet-associated or plasma neuraminidase activity in patients with ITP represent new approaches that may aid clinical decisions with regards to therapy. Such approaches require significant technical expertise, access to freshly drawn blood and sometimes washed platelets and a clear understanding of physiological and pathophysiological ranges for each parameter.

Evaluating Platelet and Megakaryocyte Dysfunction

Bleeding is observed in primary ITP, and often the extent of bleeding is out of step with the platelet count, implying that a platelet function defect has been acquired as part of the ITP etiology in many patients with bleeding issues [57]. However, all of the currently available approaches to evaluate platelet function in a clinical setting (light transmission and multiplate aggregometry, platelet function analyzer-100, thromboelastometry) are standardized for platelet counts greater than 100 x 10^9/L and require >30 x 10^9 platelets/L to achieve a blood clot as the measured endpoint. Thus, it can be difficult to ascertain the presence of a platelet functional defect and associated bleeding risk in ITP. However, in the research setting and in some clinical trials, viscoelastic testing (TEG, ROTEM, ClotPro), which provides a rapid assessment of clot formation and lysis in whole blood under low shear conditions, may provide meaningful data on thrombocytopenic samples [40,57–60]. Measurements including clot firmness and alpha angle have demonstrated utility in the context of ITP [57]. Furthermore, by subtracting clot amplitude parameters, such as A10, obtained from the fibrin-specific FibTEM parameters (wherein platelets are neutralized through the addition of cytochalasin-D), from the extrinsically activated EXTEM A10 value, an indication of platelet function can be obtained even in patients with a platelet count <20 x 10^9/L [61]. Of course, to assess the utility of thromboelastometry to identify a platelet defect in ITP, ranges for these values in thrombocytopenic samples with no platelet function defect must first be established. ROTEM has, however, been used to predict the onset of thrombocytopenia and hypofibrinogenemia after cardiac bypass surgery [62, 63] suggesting such an approach is feasible.

Since the target antigens of antiplatelet autoantibodies are present on both platelets and the precursor megakaryocytes, autoantibodies may also disrupt megakaryopoiesis and thrombopoiesis in ITP. The ability of anti-platelet autoantibodies to impact megakaryocyte function is established as a platelet-independent mechanism of thrombocytopenia [64, 65]. Megakaryocyte numbers are generally normal or increased in ITP however these bone marrow cells show signs of apoptosis and enhanced immune cell proximity [66, 67]. The plasma of ITP patients has been shown to inhibit mature megakaryocyte function, resulting in decreased platelet production despite normal megakaryocyte numbers [68]. With the advent of high fidelity megakaryocyte cell lines [69], it may be possible to design a simple screening assay to evaluate the effect of ITP patient plasma on megakaryocyte rates of maturation and proplatelet production [70]. This would allow discrimination between the contributions to thrombocytopenia of inhibited platelet production and peripheral platelet destruction and may help predict responsiveness to thrombopoietin mimetics.

Measuring and Identifying Changes in Immune Cell Subsets

In many ITP patients, autoantibodies drive premature destruction of antibody-coated platelets. However, a variety of T cell irregularities have also been described in patients with ITP. These abnormalities, facilitated by splenic T follicular helper cells, result in the proliferation and differentiation of autoreactive B cells which produce antiplatelet autoantibodies [13]. Reductions in both number and function of T-regulatory cells (Tregs), which suppress self-reactive lymphocytes and preserve immunological self-tolerance, have been recorded in ITP [71]. Along with the decreased function of anti-inflammatory IL-10-secreting B regulatory cells [72], a shift in the balance of T-helper cell (Th) types occurs, with decreased Th2 polarization resulting in an increased ratio of Th1 to Th2, which may enhance macrophage activation [73]. Numbers of CD8+ cytotoxic T cells are also elevated in peripheral blood and in the bone marrow of ITP patients [74]. Modern systems for polychromatic flow cytometry yield up to 20 distinct channels of data for a given cell and can rapidly process millions of cells per sample. Nonetheless, developing standardized methodology to accurately quantify and immunophenotype specific low-abundance subsets of immune cells involving highly multiplexed (minimally 16-color) flow cytometry panels remains extremely challenging [75]. The advent of mass cytometry [76, 77] and spectral flow cytometry [78] means that the resolution of antigen-specific T and B cell subsets is now possible; the application of these systems to the analysis of ITP patient blood remains a future possibility. More feasible is the standardization of assays to evaluate specific functional changes of ITP patient and control macrophage, monocyte, B and T cell subsets, such as cytokine production (e.g. IL-17) [13], phagocytic potential and detection of platelet-reactive T and B cells [79, 80].

Concluding Remarks and Outlook

ITP is a syndrome resulting from disturbances of one or more immune pathways that vary from patient to patient. Whilst the main triggering factor is often never determined, monitoring discrete changes in platelet biology and biochemistry as well as the innate and adaptive immune system will provide clues as to the pathogenesis and the course of the disease, and is also likely to identify subsets of patients who may be predisposed to respond to specific treatments. Evaluating the ascendancy of peripheral *versus* central mechanisms driving thrombocytopenia in patients will enable improved and tailored therapeutic strategies to treat autoantibody *versus* CD8 + T cell-mediated platelet destruction.

Authorship

S.M.H and E.E.G drafted the manuscript. All authors contributed to the review of the manuscript. Images were created with the help of https://smart.servier.com/

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Conflict of interest disclosure

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