



Emerging Concepts in Immune Thrombocytopenia

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Immune thrombocytopenia (ITP) is an autoimmune disease defined by low platelet counts which presents with an increased bleeding risk. Several genetic risk factors (e.g., polymorphisms in immunity-related genes) predispose to ITP. Autoantibodies and cytotoxic CD8⁺ T cells (Tc) mediate the anti-platelet response leading to thrombocytopenia. Both effector arms enhance platelet clearance through phagocytosis by splenic macrophages or dendritic cells and by induction of apoptosis. Meanwhile, platelet production is inhibited by CD8⁺ Tc targeting megakaryocytes in the bone marrow. CD4⁺ T helper cells are important for B cell differentiation into autoantibody secreting plasma cells. Regulatory Tc are essential to secure immune tolerance, and reduced levels have been implicated in the development of ITP. Both Fcγ-receptor-dependent and -independent pathways are involved in the etiology of ITP. In this review, we present a simplified model for the pathogenesis of ITP, in which exposure of platelet surface antigens and a loss of tolerance are required for development of chronic anti-platelet responses. We also suggest that infections may comprise an important trigger for the development of auto-immunity against platelets in ITP. Post-translational modification of autoantigens has been firmly implicated in the development of autoimmune disorders like rheumatoid arthritis and type 1 diabetes. Based on these findings, we propose that post-translational modifications of platelet antigens may also contribute to the pathogenesis of ITP.

Keywords: immune thrombocytopenia, immune thrombocytopenic purpura, autoantibodies, CD8⁺ T cells, autoimmunity, ITP

INTRODUCTION

Immune thrombocytopenia (ITP) is an autoimmune disease characterized by low platelet counts and increased bleeding risk (1–4). The initial event(s) leading to anti-platelet autoimmunity remains unclear, but strong evidence exists that autoantibodies and autoreactive CD8⁺ cytotoxic T cells (Tc) trigger enhanced platelet destruction and impair platelet production by megakaryocytes (MKs) in the bone marrow. We will briefly discuss the clinical aspects of this heterogeneous disease, followed by an overview of the mechanisms and pathways by which autoreactive B and Tc engage in anti-platelet immunity, with a particular focus on their specificity for platelet autoantigens. We will postulate a general model for ITP pathophysiology and finally highlight opportunities in ITP research, which can be derived from studies on other autoimmune diseases.

Epidemiology and Clinical Features

Immune thrombocytopenia is a diagnosis of exclusion: patients who develop thrombocytopenia (defined as platelet counts below 100,000 platelets per microliter) with no clear underlying cause

are currently diagnosed with (isolated) primary ITP (1, 4). Secondary ITP is defined as an ITP induced by other diseases or treatments. These include autoimmune disorders, lymphoproliferative disorders, infectious agents, transfusion, or induction by drugs, accounting in total for 20% of ITP cases (5, 6). In total, the incidence of ITP is approximately 1.9–6.4 per 100,000 children/year and 3.3–3.9 per 100,000 adults/year (6–8), and this number is increasing (6, 9). ITP can be classified in a transient form termed newly diagnosed ITP (up until 3 months), or persistent ITP (up until 12 months) that is more prevalent in children, or a chronic form (longer than 12 months) that does not resolve on itself and is more prevalent in adult patients (1, 6, 7). The acronym “ITP” should not be confused with the outdated definition of “idiopathic thrombocytopenic purpura” that has been used previously (1, 4). ITP is no longer considered an idiopathic disease and a proportion of patients do not present with purpura (see below). In this review, we discuss both adult and pediatric ITP studies and highlight discrepancies between both groups where necessary.

Bleeding symptoms in ITP patients typically present as either a mild form, such as bleeding in skin and mucosal regions, or a more severe, life-threatening form, such as bleeding in gastrointestinal or intracranial areas (6, 10). Patients with ITP have varying platelet counts as a result of the disease. Those with platelet counts above 50,000 per microliter rarely bleed, but below this “threshold” value, there are large differences in clinical phenotypes between patients that are as of yet unexplained (2, 3, 10). Platelet function testing appears successful in predicting bleeding risk in patients (11–13). However, no clear-cut diagnostic tools exist as associations between biomarkers and ITP remain limited, and no markers exist that may predict treatment responses (2, 3). The most common therapeutic options are based on immunosuppression [by corticosteroids, intravenous immunoglobulin (IVIg), or rituximab], or stimulation of platelet production [by thrombopoietin receptor agonists (TPO-RAs), see below].

Platelet Life Cycle

On average, the human body produces around 100 billion platelets per day resulting in a concentration of ~150,000–400,000 platelets per microliter blood (14). Platelets circulate for approximately 7–10 days, slowly undergoing age-related changes in morphology, activation, and surface receptor density (15–18). Platelets are produced by MKs in the bone marrow (19). MKs are polynuclear cells that protrude extensions in the blood, termed proplatelets, and eventually bud off platelets from these extensions (20, 21). Recent findings show that MKs may also reside in the lung, facilitating platelet production in lung tissues (22), although the relevance of platelet production at this site is currently unclear.

Thrombopoietin (TPO) is the key hormone responsible for platelet production. It is primarily synthesized in the liver and promotes MKs to produce platelets in the bone marrow *via* the TPO receptor, Mpl (23–25). As newly made TPO is released in the bloodstream by hepatocytes, it is also incorporated into circulating platelets *via* Mpl. This constitutes an inhibitory feedback loop in which platelet counts inversely correlate with the amount of TPO reaching the bone marrow to stimulate new platelet production (23, 26). Recent evidence suggests that the Ashwell-Morrell receptor (AMR) on hepatocytes plays an important role

in this physiological process. Normally, as platelets age terminal sialic acid is gradually lost from the surface, which exposes the underlying galactose residues. This allows for their clearance by the AMR (27). AMR-mediated platelet clearance triggers hepatic TPO transcription and translation, and new TPO is released (27). Several other physiological clearance mechanisms exist that control platelet numbers, such as platelet apoptosis (28) and possibly phagocytosis by α M β 2 integrins on hepatic and splenic macrophages [for a review, see Ref. (29)].

In ITP, this normal platelet life cycle is disturbed by autoantibodies and platelet-reactive CD8⁺ Tc as summarized in **Figure 1**. Autoantibodies and CD8⁺ Tc may interfere with multiple aspects of the platelet life cycle, including their production and clearance that result in thrombocytopenia. In such thrombocytopenic conditions, the small amount of circulating Mpl-containing platelets often leads to high TPO levels (30, 31). Interestingly, only slightly elevated TPO levels are observed in ITP; likely because platelets with incorporated TPO are rapidly cleared (31). Therefore, one of the therapeutic options for ITP patients involves stimulation of the TPO receptor on MKs by TPO-RAs, which proves to be successful in many patients (32). Not all patients are equally responsive to TPO-RAs and poor responders likely suffer from a prolonged autoimmune response against platelets that cannot be resolved by increasing the platelet production.

Genetic Risk Factors

As mentioned, autoreactive B and Tc have been firmly implicated in the pathophysiology of ITP. Consequently, many studies have reported associations between ITP and single nucleotide polymorphisms (SNP) in immunity-related genes. Polymorphisms in genes encoding specific cyto- or chemokines, such as interleukin (IL)-1, IL-2, IL-4, IL-6, IL-10, IL-17, TNF- α , TGF- β , and IFN- γ , have been associated with ITP (33–37). Several studies have also investigated whether specific HLA class I or II alleles are elevated in patients with ITP (38–45); current findings suggest that polymorphic sites within the HLA locus are not linked to ITP as studies have reported both significant and nonsignificant findings (37–44). The variation in studies could potentially be explained by small sample size, ethnic variability, or differences in diagnosis, yet does not allow to reach a consensus. New biomarkers such as miRNAs regulating levels of cytokines or other immune components are also increasingly recognized as potential risk factors for ITP (46). Classically, polymorphisms in Fc γ receptors (Fc γ R) have been associated with the onset and pathogenesis of ITP (47–54) and are therefore further discussed below. Most of the reported association studies performed in ITP patients were conducted in small cohorts and in specific ethnic subgroups, and thus should be interpreted with caution. Additionally, many of the identified associations are not found in all patients and are commonly observed in other autoimmune diseases as well and are therefore general predisposing factors and not specific for ITP. Advances in (epi)genomics are likely to identify additional genetic risk factors for the development of ITP (55, 56).

Environmental Risk Factors

For a long time, the occurrence of specific infections has been associated with ITP, particularly in children (5–7). Some of

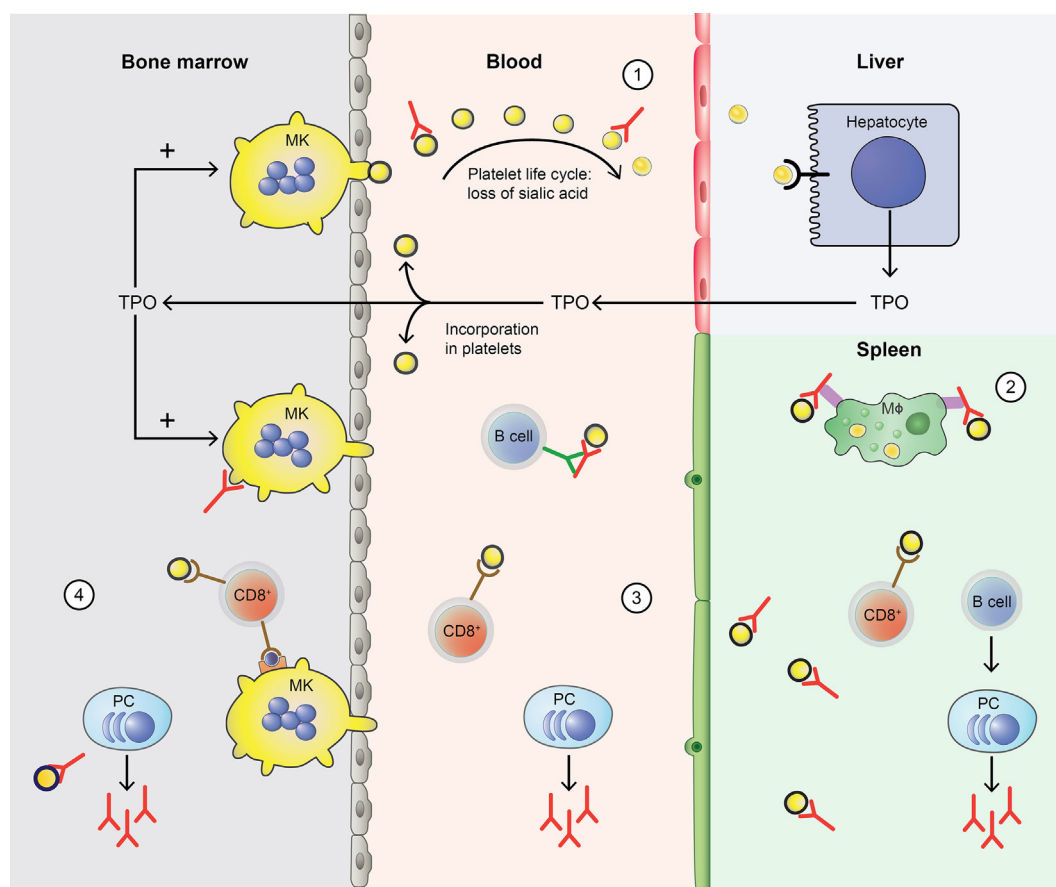


FIGURE 1 | Disturbance of the platelet life cycle in immune thrombocytopenia (ITP). (1) Platelets (yellow) are normally produced by megakaryocytes (MKs, yellow) in the bone marrow. Aging platelets undergo apoptosis but also gradually lose terminal sialic acid from the surface (indicated by black circles). This allows for their clearance in the liver. Liver-mediated platelet clearance triggers hepatic TPO transcription and translation, and new TPO is released. This process is disrupted by autoantibodies in ITP, which are hypothesized to enhance platelet desialylation leading to enhanced clearance. (2) Macrophages (M ϕ , green) can phagocytose platelets; meanwhile, platelet antigens are presented in the spleen to immune cells, such as CD4⁺ T helper (Th) cells. With CD4⁺ T cell help, B cells (B cell, dark blue) are able to differentiate into platelet-reactive plasma cells (PC, light blue) that can secrete autoantibodies (red). Cytotoxic T cells (Tc) (CD8⁺, red) can directly lyse platelets. (3) In peripheral blood, plasma cells and cytotoxic Tc further induce autoimmune responses against platelets. Cytotoxic Tc may also induce desialylation leading to enhanced clearance. In addition, platelet-reactive memory B cells may be present in the blood. (4) Plasma cells and cytotoxic Tc are also present in the bone marrow, where they can inhibit platelet production by targeting MKs.

the most occurring and most studied infectious agents are *Helicobacter pylori* (57, 58), Hepatitis C virus (59, 60) and human immunodeficiency virus (61–67). Evidence also exists for Cytomegalovirus (68, 69), Epstein Barr Virus (69), and some other viruses (70, 71). Although individual cases of ITP have been reported after vaccination, this is exceedingly rare (72, 73). One of the suggested mechanisms by which infections lead to autoimmunity is the occurrence of molecular mimicry. In this case, viral proteins resemble platelet receptors to evade the immune system (74). In case of an immune response against these viral proteins, cross reactivity may occur against platelet receptors, which subsequently lead to autoantibodies specific for both the viral protein and platelet receptors. This could explain the initiation of ITP in some cases (60–63, 66), which can be resolved by clearance of the infectious agent after which autoantibodies diminish (57, 58). Besides a transient decrease in platelet counts, infections sometimes elicit strong immune responses that can perpetuate and develop into chronic ITP, resulting in sustained platelet clearance.

Toll-like receptors are present on various innate immune cells, including platelets, and are suggested to mediate some of the microbial-platelet interactions that can trigger and/or aggravate autoimmunity (75). Immune-mediated thrombocytopenia may also occur as a result of other autoimmune diseases, drugs, transfusion, and in lymphoproliferative disorders (76, 77). Often, these cases are also diagnosed as secondary ITP, but may greatly differ in etiology. As our review focuses on primary ITP, we refer readers to Ref. (77) for more information on the underlying pathophysiology of these forms of secondary ITP.

ETIOLOGY

Autoantibodies

In approximately 60% of all ITP patients, autoantibodies are found, predominantly against platelet glycoprotein (GP) IIb/IIIa (~70%) and/or the GP Ib–IX–V complex (~25%) (78–81). Antibodies against GPIa–IIa or GPVI are also detected in

sporadic cases (~5%) (80, 82, 83). While it is not entirely clear how autoantibodies against platelet antigens are generated, their effect on platelet clearance and production have now been fully elucidated (**Figure 1**). When microbial-antigens mimicking platelet autoantigens, or the platelet antigens themselves, are presented to B cells, these can develop into autoantibody-secreting plasma cells. The spleen has been implied as an organ where immune cells are primarily presented with platelet autoantigens, and where platelet clearance takes place most (84, 85). Particularly splenic macrophages and dendritic cells (DCs) can present platelet antigens to T helper (Th) cells that provide help to B cells that differentiate into antibody-secreting plasma cells (86, 87). Plasma cells secreting platelet-reactive autoantibodies are present in peripheral blood and bone marrow, where they can further generate autoantibodies that can sequester platelets and MKs (88–90). In addition, memory B cells activated in the spleen are also released in the circulation (**Figure 2**) (85). Autoantibodies accelerate platelet clearance by removal *via* splenic macrophages and DCs (87), complement deposition (91–93) and platelet apoptosis (94), or by inhibiting megakaryocytic platelet production (88–90).

Autoantibody and B Cell Classification

Initial studies investigating autoantibodies in ITP identified high levels of platelet-associated IgGs (PAIgGs) in nearly all patients, and they were soon thought to be the causative factor

of the autoimmune response. However, it was found that PAIgGs bound nonspecifically to platelets and were detected in other non-ITPs as well (95), likely because platelets themselves can bind circulating IgG *via* FcγRIIa (96). PAIgGs thus proved to be a poor predictor of the disease [for a review, see Ref. (95)]. Although it is interesting that PAIgGs levels are higher in ITP and other thrombocytopenic patients (consisting of different IgG subclasses compared to healthy individuals), their usefulness in investigating ITP remains limited and can be largely subscribed to the state of thrombocytopenia rather than the autoimmune conditions. Following the introduction of the MAIPA and immunobead assays in 1987 (79, 97), investigators were able to detect and further study platelet-specific autoantibodies in ITP (98, 99).

Most autoantibodies found in chronic ITP patients are of the IgG class, but IgM and sporadically IgA antibodies are also detected (100–102). IgM antibodies were shown to fix complement on platelets which could facilitate clearance, but this has not been further investigated; IgG autoantibodies seem to be the main mediator of antibody-driven autoimmunity (100). Most prevalent are IgGs of the IgG1 subclass, and while IgG2, IgG3, and IgG4 subclass autoantibodies can be also found in patients, they are often accompanied by IgG1 antibodies (103, 104). Autoantibody allotypes and Fc-glycosylation are important determinants in antibody-mediated immunity and immunological disorders related to ITP (105–107), yet have been scarcely investigated.

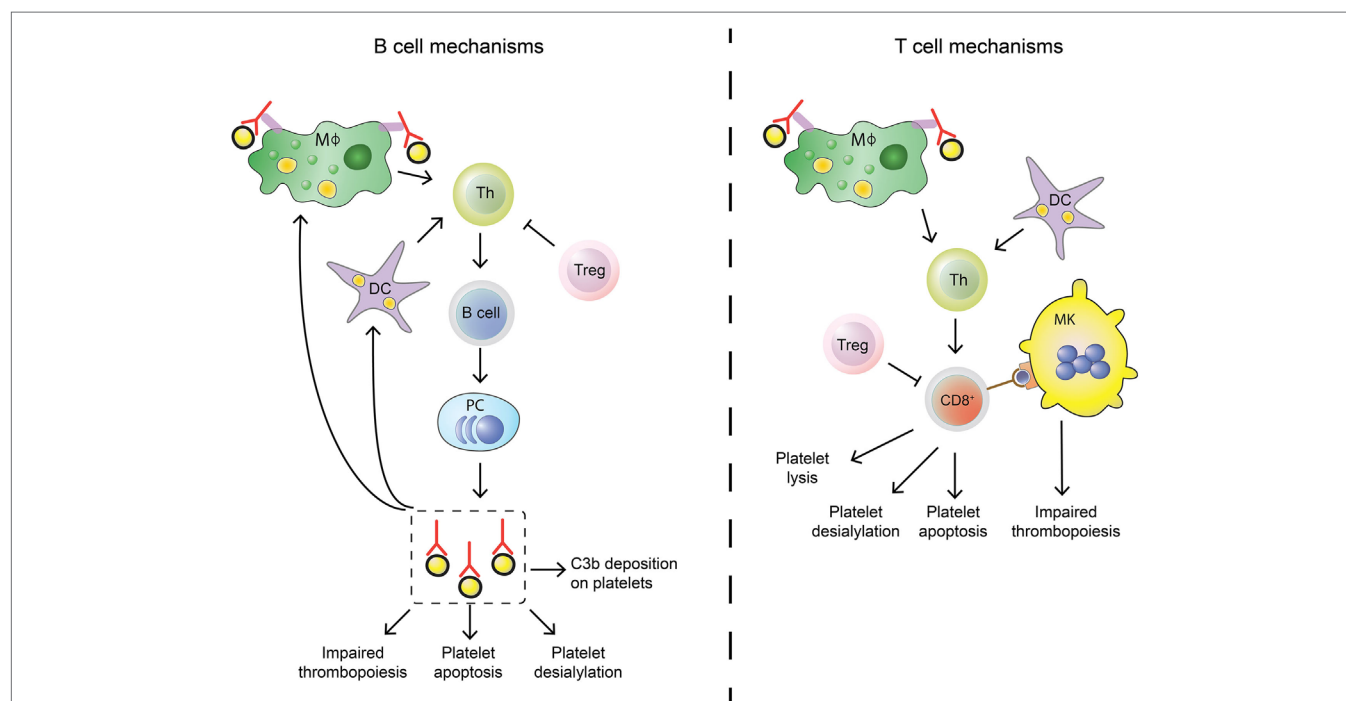


FIGURE 2 | Differences in B cell and T cell mechanisms in immune thrombocytopenia (ITP). B cells (left) differ from cytotoxic T cells (Tc) (right) in their autoimmune response against platelets in ITP. Stimulation of the adaptive immune response is similar: splenic macrophages (green) and dendritic cells (DCs, purple) can phagocytose platelet fragments to present to T helper (Th, light green) cells. Th cells are able to induce B cell development into autoantibody secreting plasma cells and can also stimulate cytotoxic Tc effector mechanisms. This process is regulated by regulatory Tc (Treg, pink), but regulatory Tc cell levels are imbalanced in ITP patients which leads to insufficient control of the autoimmune response. Shared effector functions of B cell-produced autoantibodies and cytotoxic Tc include impairing thrombopoiesis by targeting megakaryocytes (MKs), inducing platelet apoptosis and enhancing platelet desialylation. Autoantibodies can further stimulate C3b deposition on platelets to initiate complement activation, while cytotoxic Tc can directly lyse platelets.

In the majority of ITP patients, B cells producing platelet-binding antibodies have been identified in clinical samples from different sources, such as peripheral blood, spleen, and bone marrow (108–115). However, not all patients have platelet-reactive B cells (108, 109, 112–114), suggesting that B cell independent autoimmune mechanisms (such as CD8⁺ T cell mediated autoimmunity) exist. A landmark study by Roark and co-workers employed repertoire cloning to clone platelet autoantibodies from the spleen of two patients with chronic ITP (110). Sequence analysis of Ig heavy chain arrangements revealed that these anti-platelet antibodies evolved from a restricted number of B cell clones and provided evidence for extensive modification of heavy chain segments by somatic hypermutation (110). Overall, these findings provide evidence for a CD4⁺ T cell-driven antigen-specific response in patients with ITP. Evidence for the selective incorporation of the VH3-30 variable heavy chain gene segment was noted in this study providing additional evidence for a restricted, oligoclonal B cell response targeting a limited number of epitopes on platelet antigens in ITP patients (110).

Autoepitope Specificity of Antibodies

As the predominant source of epitopes for autoantibodies in ITP, the GP IIb/IIIa receptor, or integrin $\alpha_{IIb}\beta_3$, has been studied most frequently. Reports have shown that autoantibodies can bind epitopes in both the extracellular- and cytoplasmic domain of GPIIb/IIIa (80, 116). However, autoantibodies targeting the cytoplasmic domain are likely to be generated during platelet destruction rather than being pathogenic, but their significance remains unclear (80). Subsequent studies have shown that autoantibodies particularly bind to the IIB subunit (117, 118), or contradictory, the IIIa subunit of the dimer (119). Eventually, several investigators have demonstrated that specific portions of the protein are preferred autoepitopes in ITP, often near ligand binding sites (81, 120). The vitronectin ($\alpha_v\beta_3$) receptor shares the β_3 integrin with GPIIb/IIIa and was shown to be an important autoantigen in ITP as well (121). However, this has not been further investigated.

Less is known about relevant autoepitopes on GP complex Ib-IX-V, although most antibodies are directed against the GPIb part of the receptor complex (78, 98, 122). Interestingly, patients with autoantibodies against GPIb are often less responsive to immunosuppressive therapy with corticosteroids or IVIg when compared to patients with GPIIb/IIIa autoantibodies (122–124). This could be explained by specific epitopes on GPIb, relative receptor abundance on the platelet surface or differences between both protein complexes.

B Cell Help by CD4⁺ T Cells

B cells require help by CD4⁺ Tc to efficiently develop into antibody-secreting plasma cells (Figure 2). As the development of autoantibodies is a hallmark of ITP, several studies have explored the involvement of CD4⁺ Tc in the pathogenesis of ITP. Initial observational studies showed that cytokines necessary for Th functions (such as IL-2, IL-10, IFN- γ) are increased in ITP patients (125, 126). Further evidence came from studies that identified a T cell imbalance in ITP: patients have a disturbed Th1/Th2 subset ratio, which trends toward a Th1 phenotype (127–129). Both rituximab and splenectomy seemed to resolve this polarization

in responding patients, indicating the importance of balancing different populations of CD4⁺ Tc in ITP (128, 130). Pioneering work by Kuwana and co-workers have provided firm evidence for the presence of auto-reactive CD4⁺ Tc that target epitopes on GPIIb/IIIa (131, 132).

Recently, pro-inflammatory Th17 cells have emerged as a critical player in development of autoimmunity (133). Higher levels of Th17 cells were observed in several ITP cohorts (134, 135), but not in all studies (136). Several studies have found higher levels of both Th1 and Th17, compared to Th2 (137–141). The potential involvement of another subset of Th cells, Th22, was also investigated in ITP. Th22 cells typically promote protective and regenerative responses with predominant effects on epithelial cells (142–144). Increased levels IL-22 and elevated levels of the Th22 T cell subset have been observed in patients with ITP suggesting a role for this population of Tc in ITP pathogenesis (145, 146). In line with its established role in B cell help, splenic follicular Th (T_{FH}) cells have also been implicated in the pathogenesis of ITP (147). These findings show that multiple Th populations including Th1/Th17/Th22/T_{FH} contribute to the pathogenesis of ITP (127, 129, 135, 137, 145–147). We anticipate that the observed skewing toward Th1/Th17/Th22/T_{FH} populations is not specific for ITP as similar Th polarization profiles are observed in other autoimmune diseases.

CD8⁺ T Cells

Besides autoreactive B cells, CD8⁺ Tc have also been implicated in ITP pathogenesis (126, 148, 149). Evidence from association studies shows that patients with ITP more often present with polymorphisms in CD8⁺ related cytokines (126, 150, 151), have increased granzyme levels (152), and have imbalanced ratios of CD8⁺ Tc cell subsets (137, 140). As CD8⁺ Tc are also dependent on help of CD4⁺ Th cells to efficiently perform effector functions, the polarization of CD4⁺ Th cells probably also affects the CD8⁺ Tc cell response (137, 140).

T cells are part of cell-mediated immunity and have different effector functions compared to antibody-secreting B cells. In ITP, B cells and Tc thus elicit different forms of anti-platelet immunity (Figure 2). CD8⁺ Tc have been shown to directly lyse platelets (148, 153–155), induce platelet apoptosis (153), and inhibit thrombopoiesis by MKs (156). CD8⁺ Tc can further inhibit platelet production by inhibiting MK apoptosis (157).

Increased levels of CD8⁺ Tc were found in patients without autoantibodies (154), suggesting that CD8⁺ Tc cell-mediated autoimmunity can be elicited separately from autoantibody-mediated autoimmunity. Evidence of a T cell response separate from antibody-mediated autoimmunity was further shown in ITP patients who did not respond to the anti-CD20⁺ B cell-depleting antibody rituximab, in whom increased levels of splenic CD8⁺ Tc were detected (158). In contrast, CD8⁺ Tc were found to be protective and required for effective steroid therapy in a murine model of ITP, although these findings are counterintuitive and not supported by observations in other autoimmune diseases (159).

It is unclear how the B cell depletion and repopulation effects of rituximab alter T cell subsets in responding patients. Possibly, the altered cytokine environment as a result of B cell depletion affects T cell subsets, as the B-T cell interplay is essential in a

systemic autoimmune response (160). A recent study showed that rituximab could suppress murine CD8⁺ T-cell mediated immune responses (161), suggesting that B cells may regulate the CD8⁺ T-cell response in ITP. In fact, ITP patients present with lower levels of regulatory B cells (162). However, the effect of rituximab treatment in ITP remains difficult to interpret as B cell depletion may also affect CD20⁺ regulatory B cells, which can secrete IL-10 and other suppressive cytokines to induce immune tolerance (163), as suggested previously.

As of yet, the target peptides expressed on MHC class I recognized by platelet specific CD8⁺ Tc have not been identified. Interestingly, no clear HLA association is found in ITP patients (38–45), as opposed to other autoimmune diseases. In *H. pylori*-mediated ITP, HLA associations were also unclear (114, 164). Platelets are capable of presenting non-renewable MK-derived peptides on MHC class I, and it is likely that these peptides are being recognized by CD8⁺ Tc that develop in patients with ITP (165). More recently, it was proposed that platelets have the propensity to activate naïve CD8⁺ Tc and that platelets can present pathogen-derived peptides in the context of MHC class I (166). In this context, it is interesting to note that following dengue infection the MHC class I density on platelets increases, suggesting an active role of platelets in combatting infections (166, 167). Under resting conditions, platelets do not express MHC class II molecules on their surface, but several reports suggested platelets to express MHCII complexes during infection (164, 168). Whether antigen presentation on MHC class I by platelets has a role in the pathogenesis of ITP has not been demonstrated. In view of the established role of CD8⁺ Tc in this autoimmune disorder, this will be an interesting area for further research.

Regulatory T Cells

Tregs are a crucial checkpoint to limit immunity and secure immune tolerance. As such, they are important regulators that keep both B- and T cell-mediated autoimmunity in check (**Figure 2**). The importance of Tregs for the pathogenesis of ITP is evidenced by their reduced numbers and function in patients (169–173). The pivotal role of immune regulation in ITP, particularly by Tregs, was further shown by phenotypic and Treg profiling studies of treated versus untreated ITP patients. Treatment with corticosteroids and/or rituximab in responding patients both improved Treg levels as well as their activity (130, 174–178), indicating that loss of tolerance is essential for the pathogenesis of ITP. In an experimental murine model of ITP, Tregs were retained in the thymus. This was resolved by IVIg treatment, which normalized Tregs in the periphery (179). Additionally, transferring retained thymocytes delayed the onset of ITP, suggesting Tregs actively prevent ITP development at least in mice (179). Interestingly, TPO-RAs improved Treg activity indicating that platelets could directly or indirectly play a regulatory role in ITP by affecting Treg levels (175). As such, it is clear that ITP patients present with lower Treg levels which are restored upon successful treatment (see above). However, it is still unclear whether restoring Treg functionality directly alleviates the disease or is simply a marker of restored immune tolerance. Potentially involved pathways are further discussed below.

Tregs can interact with DCs to induce a tolerogenic phenotype. Two studies found that the interplay between Tregs and DCs is impaired in ITP (178, 180). As Treg levels are lower in ITP, this leads to a reduced expression of immunomodulatory enzyme indoleamine 2,3-dioxygenase 1 (IDO1) by DCs, and increased levels of mature DCs that can present (auto)antigens to other immune cells (178, 180). The important role of tolerance induction by DCs in ITP was further suggested by another study, in which IVIg was shown to mediate its effect *via* DCs in a murine model (181). The interplay between Tregs and DCs and immunomodulation *via* IL-10 is not only important in ITP but was also found essential in antibody-mediated acute lung injury (182, 183). As such, the Treg-DC-axis may be particularly important in autoantibody-mediated ITP, but this remains to be investigated.

Other Immune Cells

Several other immune cells may modulate autoimmune responses in ITP but have been investigated sparsely. Neutrophils have been found to line MKs in ITP bone marrow (184), but their role in ITP has not been further investigated. A subset of CD16⁺ monocytes derived from patients with ITP has shown to promote the proliferation of IFN- γ ⁺ CD4⁺ Tc (185). Shifts in the balance of inhibitory and activating Fc γ Rs were observed on monocytes following treatment with high-dose corticosteroid dexamethason as well as following *H. pylori* eradication in ITP (186, 187) (further discussed below). Additionally, they were found to be involved in T cell development (185). Both increased and decreased levels of NK cells have been found in ITP patients (188–190). The significance of these observations is unclear since NK cells are not able to lyse platelets (148).

Finally, platelets themselves may be able to affect the autoimmune response in ITP, as they are increasingly recognized as mediators of immunity and inflammation [for a recent review, see Ref. (191)]. Evidence for such an autoregulatory loop was found in ITP patients responding to TPO-RAs, who not only had increased platelet counts but also correlating higher TGF- β plasma levels (175). Presumably, increased plasma TGF- β levels derive from an increased platelet mass (175). Furthermore, TPO-RAs reduced both autoantibody and T cell responses in a mouse model, which also lead to elevated TGF- β plasma levels (192). Interestingly, TPO-RAs may induce remission in a subset of patients whom then no longer needed therapy to maintain platelet levels (193–195). This would imply that immune tolerance can be restored in certain patient subsets by enhancing platelet numbers. Another mechanism by which platelets regulate immune responses occurs *via* CD40L. Activated Tc can stimulate B cell proliferation and differentiation *via* CD40L interactions with CD40 on B cells (196). Platelets normally express CD40L only upon activation, but higher baseline levels are observed in ITP patients (13). Furthermore, activated platelets from ITP patients were shown to stimulate autoreactive B cells by CD40L (197). Interestingly, CD40L inhibition was successful in suppressing T cell-assisted B cell-mediated autoantibody production in ITP, even in treatment of refractory ITP (198, 199). However, whether this is similarly successful affecting a potential B cell-platelet interaction remains unknown.

PATHWAYS INVOLVED IN PLATELET CLEARANCE

Fc γ R-Mediated Eradication of Platelets

Fc γ Rs have long been implicated in ITP etiology. These receptors are differentially expressed on immune cells and are the primary receptor for IgG. Fc γ Rs mediate different functions, including phagocytosis, antibody dependent cellular cytotoxicity, and release of cytokines [reviewed in detail in Ref. (96)]. Most Fc γ Rs are involved in activating the immune system, whereas Fc γ RIIb is the only inhibitory Fc γ R. Platelets only express Fc γ RIIa on their surface, while myeloid cells, such as granulocytes, monocytes, macrophages, and DCs express several Fc γ Rs (96). In liver and particularly spleen, monocytes and macrophages have been suggested to bind and phagocytose Ig-opsonized platelets by Fc γ Rs, explicitly contributing to platelet clearance and autoantigen presentation (85, 87). As such, polymorphisms in several Fc γ Rs have been associated with ITP (47–54). The low affinity Fc γ RIIa, Fc γ RIIb on granulocytes, and Fc γ RIIIa on NK cells, monocytes, and macrophages all contain SNP that affect binding affinity to IgG (200).

For Fc γ RIIa, one polymorphism at position 131 (R/H, with H having higher affinity) most strongly or exclusively affects IgG2 binding (200), and the higher-affinity allele was found to be associated with ITP (48, 51–54). However, these studies had inconsistent outcomes. A recent meta-analysis indicated that the R131H polymorphism might be associated with a subgroup of childhood-onset ITP, but this should be interpreted with caution (54). In accordance with the notion that Fc γ RIIIa⁺ splenic monocytes are particularly important for the clearance of platelets, only the higher affinity-allele of the Fc γ RIIIa polymorphism at position 158 [F/V, with 158 V having higher affinity for IgG1 and IgG3 (200)] has been found to be associated with ITP (48, 50–53). Intriguingly, one study found that a polymorphism in the transmembrane region of the inhibitory Fc γ RIIb (232I/T) is associated with the onset of newly diagnosed ITP in children (49). This polymorphism (232T) has been found to negatively affect the capacity of this receptor to downregulate immune responses (201) and could point at an immunomodulatory role of Fc γ RIIb. Intriguingly, eradication of *H. pylori* (a potential molecular mimicry causative of the onset of ITP) was found to shift monocyte Fc γ R expression toward an inhibitory Fc γ RIIb phenotype (187). Finally, the Fc γ RIIc has also been associated with ITP (50). Fc γ RIIc is a pseudogene in most individuals, but having Fc γ RIIc most likely predisposes individuals to stronger immune responses (202, 203). While the extracellular IgG-binding domain of Fc γ RIIc is identical to the inhibitory Fc γ RIIb, the intracellular tail is identical to Fc γ RIIa and contains an activating motif (202). Due to the proposed expression of Fc γ RIIc on B cells, it may downregulate the negative feedback provided by Fc γ RIIb (202). Interestingly, Fc γ Rs are known to crosstalk with Toll-like receptors, particularly during bacterial infections. This leads to T cell polarization (204), but it is unclear if this crosstalk is in any way relevant for platelets and/or in the context of ITP. Considering the strong correlation with infections in the onset of ITP, investigating the Fc γ R-TLR crosstalk could be interesting.

Additional evidence that Fc γ R-mediated pathways are important in ITP pathogenesis was shown by the therapeutic use of IVIg, which may bind Fc γ Rs by its Fc-portion (205), and is one of the successful cornerstone treatments for ITP to rapidly increase platelet counts. It was recently shown that IVIg does not modulate Fc γ R expression directly but inhibits the phagocytic capabilities of splenic macrophages (206). In addition, a previous pilot study has also shown that Syk-inhibitors, which affect downstream Fc γ R signaling, can improve ITP (207). While IVIg does not work in all patients, the efficacy may be predicted by specific Fc γ R polymorphisms (208). As such, various Fc γ R polymorphisms provide the most compelling evidence that genetics may affect ITP, both by predicting higher risk of disease development and treatment outcomes. In addition, the role of Fc γ Rs on platelets and other immune cells has now been firmly implicated in ITP pathogenesis. Nevertheless, Fc γ R-independent mechanisms may exist as well.

Fc γ R-Independent Eradication of Platelets

A recent study has implicated a Fc γ R-independent pathway in an experimental mouse model (209), which was hypothesized to occur simultaneously aside Fc γ R-mediated clearance by splenic macrophages. Autoreactive antibodies against GPIIb were hypothesized to induce platelet activation and degranulation, which leads to sialidase release (210). This induces desialylation of platelet membrane glycans, which can subsequently lead to recognition of platelets by the AMR in the liver thereby accelerating platelet clearance (27, 209). Interestingly, there are a few cases of ITP patients with abnormal platelet surface sialic acid levels (211, 212). Oseltamivir, which is a sialidase inhibitor used to treat influenza, has been found to increase platelet sialic acid content (213) and in two cases was successful in ameliorating thrombocytopenia whereas conventional therapy was not (212, 214). Platelet desialylation was also found to correlate with non-responsiveness to first-line therapies in ITP (215). Finally, CD8⁺ Tc have also been suggested to induce platelet desialylation and to facilitate platelet clearance similar to the earlier mentioned mechanisms (155). While the importance of sialic acid in the platelet life cycle has long been established (14), it is unclear whether the experimental findings in mice can be translated to a human and/or clinical setting. Ongoing studies are needed to establish the importance of platelet desialylation in ITP.

C-Reactive Protein and Reactive Oxygen Species

Recently, a role for inflammatory acute-phase protein C-reactive protein (CRP) has also been implied in ITP pathogenesis (216). CRP levels were elevated in ITP patients and enhanced platelet phagocytosis in presence of anti-platelet antibodies *in vitro* and *in vivo*. This effect was ameliorated by IVIg treatment, suggesting that this mechanism may at least in part be mediated *via* Fc γ Rs (216). Phosphorylcholine, a CRP ligand present on cell surfaces, was exposed after antibody-induced oxidative stress. Oxidative stress induced by ITP autoantibodies has also been shown in two separate studies on ITP (217, 218) and appears to be a suitable biomarker for ITP (219). Additionally, the pathophysiological role of reactive oxygen species has long been implied in a model of

HIV-initiated ITP (64, 65, 67). In this model, reactive oxygen species induced by platelet antibodies were able to directly lyse platelets, leading to platelet fragmentation. This appears to involve the platelet NADPH pathway and is complement independent (65). Interestingly, treating platelets with dexamethasone was shown to inhibit NADPH oxidase components that partially prevented induction of reactive oxygen species (67). Further studies will be required to elucidate the exact role of CRP, oxidative stress, and autoantibodies or autoreactive CD8⁺ Tc in ITP.

MODEL FOR ITP PATHOGENESIS

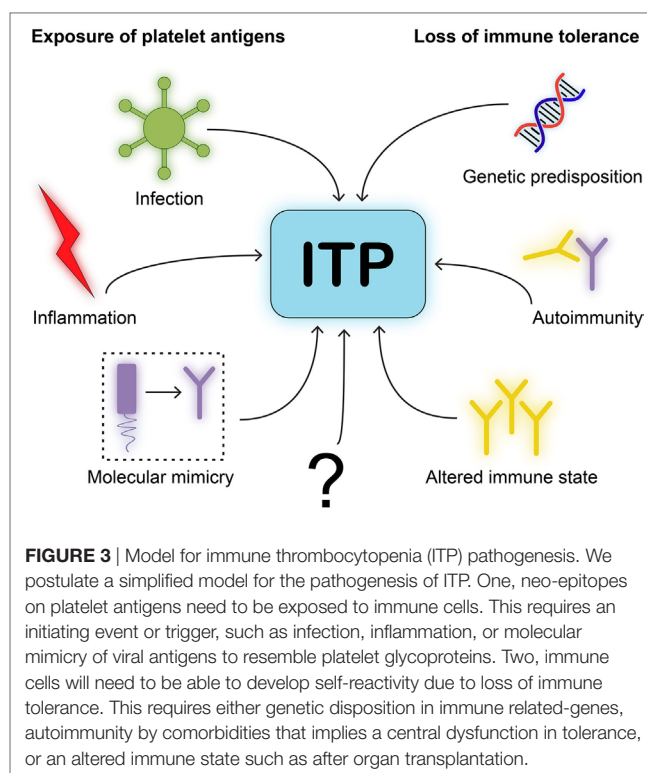
As knowledge on the pathogenesis of ITP develops, definitions become outdated, and lines between primary and secondary ITP are beginning to blur. In other autoimmune diseases, infections are increasingly recognized as one of the primary initiating events that can lead to an autoimmune response. This is not the case for ITP, where it is regarded as a secondary form. However, even in what is called primary ITP, there must be some sort of initiating event that triggers the autoimmune response and exposes platelet antigens. This initiating event will obviously still have consequences for clinical treatment of ITP, whether it is an infection, blood transfusion, drug, or an unknown other trigger. Nonetheless, infections should no longer simply be regarded as a secondary form considering their potential as an initiating event or trigger to expose platelet antigens.

The number of people developing ITP directly after an infection is small, which suggests that additional factors have to be present during an infection to develop persistent autoimmunity. Individuals with a known autoimmune disease are more prone to develop ITP, indicating that dysregulation of immune homeostasis may contribute to the onset of ITP. Interestingly, most pediatric patients only develop transient thrombocytopenia, which is eventually resolved when the viral antigen is cleared. Meanwhile, similar to other autoimmune disorders, chronic ITP is more prevalent in adult patients, and the incidence increases with age. Based on the currently available data, we propose a simplified model of ITP in which both exposure of platelet antigens and loss of tolerance are required to induce ITP (Figure 3). The specific type of trigger likely determines whether a CD4⁺ T cell-assisted B cell response develops or whether CD8⁺ Tc targeting platelets are induced. Transient forms of ITP may develop if insufficient CD4⁺ T cell help is available for the generation of class-switched, fully affinity matured, strongly binding anti-platelet antibodies. Such antibodies are likely produced by bone marrow-residing plasma cells in a fully developed CD4⁺ T cell-assisted B cell response. We furthermore propose that platelet directed CD8⁺ T cell responses develop following presentation of pathogen-derived peptides on MHC class I that may evoke the formation of CD8⁺ Tc that (cross) react with peptides presented on MHC class I on platelets.

FUTURE RESEARCH

Emerging Concepts and Opportunities to Unravel the Pathogenesis of ITP

Limited information is available on the autoantigens in ITP and their importance for recognition by immune cells once bound by



autoantibodies. Epitopes targeted by platelet autoantibodies seem to differ between patients, coinciding with different responses to therapy and different bleeding phenotypes. The molecular basis for the variable bleeding diathesis in patients with ITP has not yet been fully elucidated. Investigators have primarily made use of ITP sera or plasmas to study the role of autoantibodies. However, these can contain multiple autoantibodies, some potentially undetected by the current methods. Similar to the elegant studied by Roark and co-workers (110), specific autoantibodies should be isolated to further study their effects on platelets, possibly combining characteristics like subclass characterization, epitope specificity, and glycosylation patterns.

ITP Versus Other Autoimmune Diseases: Lessons to Be Learnt

In other autoimmune disorders such as rheumatoid arthritis, systemic lupus erythematosus, or type 1 diabetes, it has been shown that post-translational modifications of autoantigens can elicit the formation of CD4⁺ T cell responses as well as create neo-epitopes that are recognized by B cells. In view of the common mechanisms involved in loss of tolerance against self, these findings may open novel avenues for dissecting pathways contributing to the onset of ITP.

Evidence has been obtained for post-translational modifications of platelet proteins. Phosphorylation and particularly glycosylation of platelets have been well studied (29, 210, 220, 221), and the importance of platelet glycans is increasingly appreciated. Furthermore, platelets and peripheral blood contain different glycosyltransferases to modify platelet glycans (221), but their relevance in normal platelet physiology is still unclear and their

potential relevance for the onset of ITP has not been established. A role for desialylation triggered by platelet autoantibodies or CD8⁺ Tc has been postulated, but it is unknown if this can also lead to the generation of neo-epitopes on the platelet surface (155, 209). Recently, it was also shown that formation of oxidative stress induced neo-epitopes on platelets promotes binding of the acute phase protein CRP resulting in enhanced phagocytosis of IgG-coated platelets (216). It is unclear whether the autoantibodies found in ITP patients are able to recognize such neo-epitopes in similar fashion.

Besides post-translational modifications, platelet membranes are highly dynamic with respect to the expression of cell-surface receptors. GP expression on the platelet surface is tightly regulated by different metalloproteases, such as ADAM10 and ADAM 17 that facilitate receptor shedding (222, 223). Additionally, platelet granules release their content to rapidly increase receptor density on the membrane, such as the well-established activation marker P-selectin (223). These processes are important in both health and disease (224); however, it is unknown if the dynamic shuffling of receptors on the platelet surface is in any way relevant to formation of neo-epitopes in ITP.

The difference between post-translational modifications in other autoimmune diseases and ITP is that most of the modifications mentioned above are induced by autoantibodies in ITP, while modifications in for example the autoantigens that are implicated in rheumatoid arthritis precede the formation of autoantibodies (225–227) and are postulated to be one of the key events that triggers their generation. In fact, infection-induced post-translational modifications of target proteins, such as citrullination of fibrin, are thought to initiate a continuous inflammatory environment, which eventually leads to autoimmunity (225–227). Interestingly, the autoantigens in rheumatoid arthritis are usually located on “static” long-lived cartilage and/or joint proteins, such as fibrin. This is different in ITP, where the autoantigens are located on GPs on platelets that have a limited lifespan. Currently, no information is available with respect to the potential of post-translational modifications of platelet antigens to trigger autoimmune responses. In view of the prominent role of post-translational modifications

in the onset of autoimmunity, we speculate that this will provide a novel and interesting avenue for future research to dissect the mechanisms that contribute to the onset of ITP.

CONCLUSION

We suggest a simplified model of ITP in which both exposure of platelet antigens and loss of tolerance are required for the onset of ITP, thereby promoting CD4⁺ T cell-assisted B cell responses against platelets. Additionally, we propose that infections resulting in the presentation of pathogen-derived peptides on MHC class I may induce the formation of CD8⁺ Tc that (cross) react with peptides presented on MHC class I on platelets. Specific triggers likely determine the type of autoimmune response against platelets. We speculate that post-translational modifications of platelet antigens harbor potential to generate neo-epitopes that trigger autoimmune responses in ITP, as they do in other autoimmune disorders. Future studies interrogating these hypotheses may yield novel insights into the mechanisms that underlie the development of ITP.

AUTHOR CONTRIBUTIONS

MS wrote the manuscript. MR, JV, GV, FL and AJ provided input, made suggestions for improvement, and approved the final version for submission.

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