REVIEW ARTICLE

Immune thrombocytopenia (ITP): Pathophysiology update and diagnostic dilemmas

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Abstract

Immune thrombocytopenia (ITP) is a common autoimmune bleeding disorder. The understanding of ITP pathogenesis is rapidly evolving. We now recognize ITP as a complex and heterogeneous syndrome that results from a combination of humoral and cell-mediated attacks on platelets peripherally and megakaryocytes in the bone marrow. Autoantibody-mediated ITP also varies in the pathway used to clear platelets, which depends on the platelet glycoprotein being targeted. Moreover, ITP patients present with variable bleeding severities and treatment responses that do not closely correlate with platelet count. A gold standard diagnostic test for ITP is lacking, and biomarkers to assess disease severity are in their infancy. This review provides an update on the immunopathogenesis of ITP and summarizes currently available tests for ITP diagnosis, prediction of disease severity, and treatment responses. Given the heterogeneous pathogenesis and clinical presentation of ITP, we highlight the need for the development of diagnostic and prognostic tests that would allow for the individualized management of a complex disease.

KEYWORDS
autoantibodies/autoimmune disease, bleeding score, dog, hemorrhage, immune thrombocytopenia, immunology, platelet

1 | INTRODUCTION

Immune thrombocytopenia (ITP) is the most commonly acquired disorder of primary hemostasis in dogs. ITP remains a diagnosis of exclusion in both human and veterinary medicine; however, recent advances in the understanding of disease pathogenesis and new treatment options are changing the management of ITP in people. The challenge for veterinarians is to adapt these findings to improve the outcomes of canine ITP patients.

Current concepts related to human ITP pathogeneses extend beyond antibody-mediated platelet destruction to include the contribution of cellular immunity and ineffective thrombopoietic responses. This expanded understanding has translated into the use of thrombopoietin (TPO) mimetic drugs early in the course of treatment. Yet uncertainties remain in choosing the optimal treatment

plan for each patient, and efforts continue to develop evidence-based management strategies and clinically relevant ITP biomarkers for individualized treatment. Included among these areas of research are greater disease classification standardization and the evaluation of clinical and laboratory predictors of ITP severity.

2 | ITP NOMENCLATURE AND PATHOGENESIS UPDATE

Throughout this review, we will refer to immune thrombocytopenia as ITP. In the medical literature, ITP historically stood for **i**diopathic **t**hrombocytopenic **p**urpura. However, this terminology was deemed inappropriate by the International Working Group (IWG) of human ITP experts since bleeding signs are usually absent or minimal in most people with

ITP.[1] The disease was retermed "immune thrombocytopenia" to emphasize its immune-mediated mechanism, but the same acronym was maintained (**i**mmune **t**hrombocyto**p**enia).[1] In the veterinary literature, there was a brief trend to use the term "**i**mmune-**m**ediated **t**hrombocytopenia," which was abbreviated, IMT. However, the IWG recommendations are applicable for the animal disease counterpart, and we recommend the consistent use of the acronym ITP in veterinary medicine.

ITP is an autoimmune disease characterized by both platelet destruction and impaired megakaryocyte and platelet production.[2] The pathogenesis of the immune dysregulation resulting in ITP is incompletely understood, and likely quite complex. Very little is known about the pathogenesis of ITP in dogs; and therefore, the following review will reflect on what is known about the human disease counterpart. Since Harrington's seminal 1951 experiment in which the transfusion of ITP plasma into healthy people induced thrombocytopenia, ITP has been known as a humoral disease.[3,4] In ITP, autoantibodies targeting platelet surface glycoproteins (GPs) lead to platelet clearance by the mononuclear phagocytic system, primarily that of the spleen.[2] However, it is now recognized that antibody-mediated destruction is not the sole mechanism of platelet clearance. T cells play a central role in platelet destruction in ITP.[2,5-7] A proinflammatory Th1, Th17, and Th22 cytokine milieu predominates in many ITP patients that together promote macrophage function, autoreactive B-cell development, and T-cell cytotoxicity.[2,4] Furthermore, immune regulators that normally serve to maintain self-tolerance, like T and B regulatory cells, are dysfunctional in ITP, allowing the autoimmune response to persist.[2,8,9] Several studies have found a reduction in regulatory T cell (Treg) number and function in people with ITP, and one pilot study found a decrease in Treg numbers in ITP dogs compared with those of healthy controls.[10-12] Interestingly, Treg numbers recover when human and canine ITP patients are in remission even if patients are only treated with TPO mimetics, suggesting that platelets, perhaps through their role as transforming growth factor-β reservoirs, are themselves immunomodulatory (Figure 1).[4,11-14]

Although antibody-mediated platelet destruction is often central in ITP pathogenesis, some human ITP patients lack detectable platelet autoantibodies and instead have cytotoxic T lymphocytes that induce platelet destruction via apoptosis and perforin/granzyme-mediated cytotoxicity.[2,7,15,16] In some patients, antibody and T-cell attacks occur at the level of the megakaryocytes, resulting in decreased platelet production that further compounds platelet destruction (Figure 2).[15,17] Complement could also contribute to the immune-mediated destruction of both platelets and megakaryocytes.[18]

In addition to immune targeting of megakaryocytes and platelets, TPO levels are often inappropriately normal in human patients with ITP. TPO is the major regulator of platelet production that is necessary for survival, proliferation, and differentiation of megakaryocytes to platelets.[19-21] Until recently, TPO was thought to be produced constitutively by the liver and cleared by binding to its receptor, the myeloproliferative leukemia protein (Mpl) receptor, on platelets and megakaryocytes.[21] Thus, TPO concentrations should be inversely proportional to the platelet or "Mpl" mass.[21] Although the Mpl mass plays a role in TPO regulation, a more elegant system has
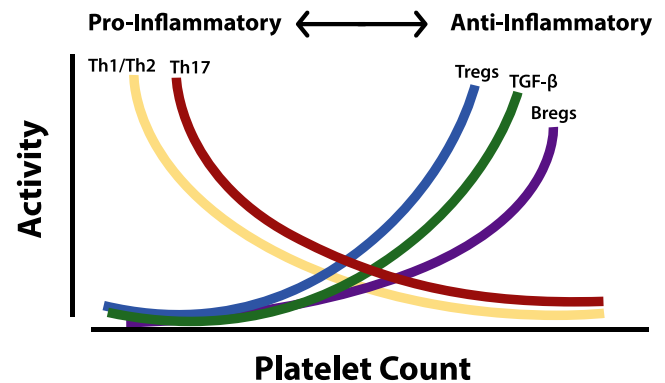

FIGURE 1 Loss of self-tolerance is central in immune thrombocytopenia (ITP) development. In this model, a reduced number and/or function of regulatory B (Breg) and T (Treg) cells allows for the development of a Th1/2 imbalance and activation of Th17 cells. Ultimately, the dysfunction of Tregs and Bregs enables autoreactive B cell and cytotoxic T-cell survival. Circulating platelets are a large source of transforming growth factor β (TGFβ), and the return of TGFβ with platelet count recovery could play a role in disease remission. Adapted with permission from John Semple (unpublished)
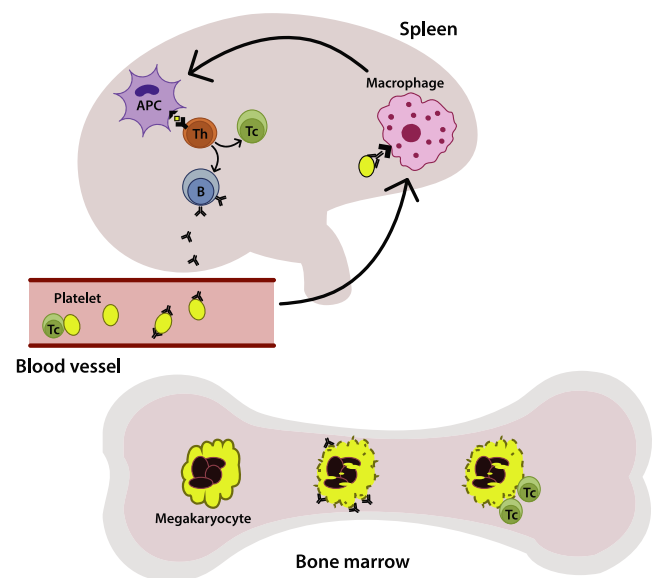

FIGURE 2 Immune thrombocytopenia (ITP) is a heterogeneous disease with a complex pathogenesis sustained by a positive feedback loop. After the loss of self-tolerance, T helper cells (Th) promote the generation of autoantibody-producing B cells and/or autoreactive cytotoxic T cells (Tc). Autoantibodies can opsonize platelets, leading to their splenic and hepatic clearance by macrophages of the reticuloendothelial system. Macrophages can then serve as one type of antigen presenting cell (APC), propagating the cycle. Dendritic cells can also serve as APCs. Tc can destroy platelets in the blood or spleen. Autoantibodies and autoreactive Tc might target megakaryocytes in the bone marrow, inhibiting platelet production. Thus, ITP is a disorder of both platelet destruction and decreased platelet production

been recognized that might explain the normal levels of circulating TPO documented in human ITP patients. When platelets age, they become desialylated and are subsequently recognized and cleared by

the hepatic Ashwell-Morell receptor.[21] This removal, in turn, drives hepatic TPO expression providing a feedback mechanism; as more platelets are cleared, more TPO is produced (Figure 3). However, in a murine model of ITP, platelets opsonized with an antiplatelet antibody are cleared by the reticuloendothelial cells of the spleen and liver, thus shunting platelets away from the Ashwell-Morell receptor, failing to trigger hepatic TPO production.[21] Inappropriately normal circulating TPO levels in people with ITP fail to effectively stimulate platelet production.[19] TPO levels in dogs with ITP have not been established as there are no available assays for circulating canine TPO.

Megakaryocytes might also play a direct role in the pathogenesis of ITP. Platelets have long been recognized as immune cells with the ability to promote leukocyte and endothelial activation, stimulate neutrophil extracellular trap formation, detect and clear pathogens, and promote inflammation.[22] However, the concept of megakaryocytes as immune cells is just starting to emerge.[22] Megakaryocytes express immune-sensing receptors, like TLRs and IgG Fc receptors, and they can modulate other bone marrow hematopoietic cells via release of mediators.[22] Megakaryocytes can stimulate Th17 expansion and also promote inflammation by the release of IL-1 rich microparticles.[22] Perhaps, most relevant for ITP, it has been recently shown that megakaryocytes can present antigens on their surface in association with MHC Class I molecules, activating specific CD8+ T cells.[23] Even more relevant for ITP, these MHC class I molecules presenting foreign antigen can be transferred to pro-platelets.[23] Using a murine ITP model (the CD61 active model described below), megakaryocytes were also shown to present endogenous CD61 (platelet GPIIIa) antigen, leading to a CD8+ T-cell–mediated immune response in the bone marrow that, in turn, caused thrombocytopenia.[23] Thus, megakaryocytes could play a role in antigen presentation and activation of CD8+ T cells in ITP.

Genetic susceptibility to autoimmunity is recognized in human medicine; however, familial ITP is very rare, complicating discovery of susceptibility loci in human ITP.[24] A breed predisposition to developing ITP is consistently reported in the veterinary literature, with an overrepresentation of Cocker Spaniels and Old English Sheepdogs.[25-32] Given this breed predisposition, it is very likely that genetic factors contribute to ITP pathogenesis.[25-27] ITP development is presumptively the result of genetic predisposition combined with some environmental or infectious trigger, which often remains unidentified.[2] Indeed, the trigger for the initial loss of self-tolerance in ITP is not understood.

3 | CONTRIBUTION OF MURINE MODELS TO UNDERSTANDING ITP PATHOGENESIS

Murine models have been instrumental in our understanding of ITP pathobiology and the mechanisms of ITP therapeutics. As such, we
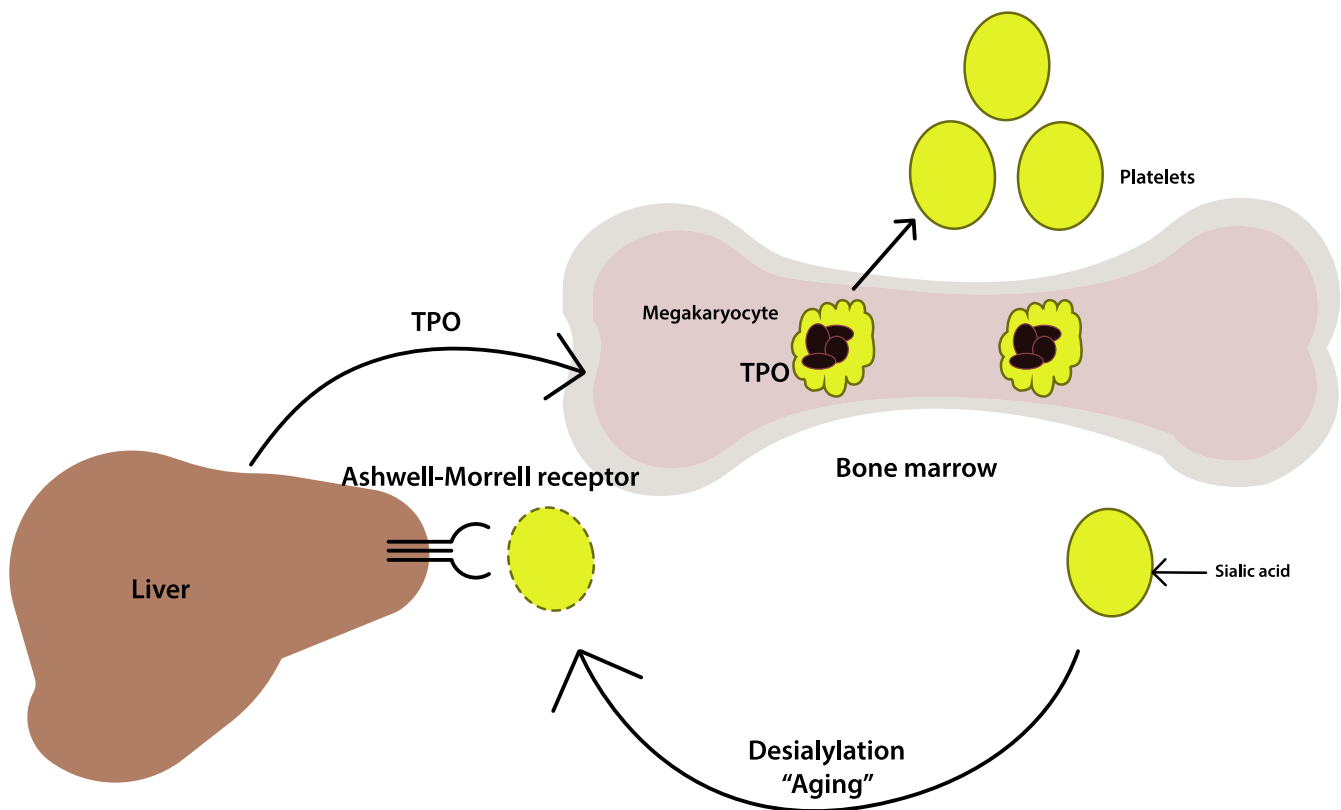

FIGURE 3 The normal clearance of aged, desialylated platelets. As platelets age, membrane sialic acid residues become desialylated by circulating sialidases. Desialylated platelets are then cleared by the hepatic Ashwell-Morell receptor, which in turn, triggers hepatic thrombopoietin (TPO) production. TPO binds to the myeloproliferative leukemia protein (Mpl) receptor on megakaryocytes, stimulating the release of young sialic acid-rich platelets

have briefly summarized some characteristics of murine models that have contributed to the current understanding of ITP. Mice apparently do not develop spontaneous ITP, and inherent platelet and immunologic differences exist between mice and people and dogs. Nevertheless, murine models excel at enabling researchers to dissect the consequences of dysfunction in individual immune system compartments.[33,34] Murine models include the passive model of ITP, secondary ITP (reviewed elsewhere) and platelet-induced models.[33,35] The passive transfer model involves the injection or continuous infusion of mice with platelet-specific antibodies to induce/maintain thrombocytopenia. Although passive models do not mimic the loss of self-tolerance that initiates ITP, they have provided valuable information, especially regarding treatment mechanisms and efficacy.[36] Intravenous immunoglobulin (IVIG), a frontline ITP therapy, can protect against passive ITP by blocking Fc receptors of the reticuloendothelial system.[37] The passive murine model was one of the first to demonstrate that IVIG was effective against anti-GPIIbIIIa antibodies, but could not ameliorate thrombocytopenia caused by GPIbα antibodies suggesting that anti-GPIbα antibodies initiate thrombocytopenia via an Fc-independent pathway.[38] Recognizing that there is a difference in the platelet clearance pathway depending on the autoantibody target was an important breakthrough in the understanding and treatment of ITP (see Question 5 below). Similarly, the passive model has shown that the mechanism of IVIG depends on the presence of dendritic cells.[35] The passive murine model is also being used to evaluate the efficacy of novel ITP therapeutics.[39,40]

Several "active" murine models have been developed that help in the understanding of the immune imbalance that initiates ITP. For example, the role of T cells in ITP pathogenesis was further elucidated by a murine model of severe ITP. In this model, CD61 knockout mouse mice are immunized against CD61+ (GPIIIa) platelets, and splenocytes from these mice are then transferred to SCID CD61+ mouse recipients, successfully inducing both thrombocytopenia and bleeding in recipient mice.[36] Using this model, lymphocyte depletion before splenocyte transfer showed that CD4+ T helper cells were essential for the induction of thrombocytopenia, production of antiplatelet antibodies, and bleeding. Furthermore, the role of both CD19+ B cell antibody and CD8+ T-cell–mediated effector mechanisms was documented.[36] This model also demonstrated that IVIG therapy is effective against antibody-mediated platelet destruction but not cell-mediated disease.[36] This model also demonstrates that the peripheral Treg deficiency that enables ITP development may be due to thymic retention of these cells.[41] Another adoptive transfer model has confirmed the importance of Tregs in ITP development.[33,42] In this model, adoptive transfer of Treg-depleted T cells into nude mice leads to prolonged thrombocytopenia and elevated levels of antiplatelet antibody; the ITP can be rescued by adoptive transfer of Tregs.[42]

A recent platelet-induced ITP model utilizing CD41 (GPIIb) knockout mice in place of CD61 provides better disease reproduction because CD41 is expressed exclusively on platelets and megakaryocytes in contrast to the multiple cell types, including endothelial cells that express CD61. This platelet/megakaryocyte specificity of the active CD41 model avoids the complications of placental defects and postnatal hemorrhage demonstrated by the CD61 ITP model, presumably due to antibody-mediated vessel damage.[43] Using lymphocyte depletion prior to splenocyte transfer, the CD41 active model has shown that CD8+ T cells are likely responsible for impairing megakaryocyte maturation in ITP.[43]

4 | GUIDELINES FOR DIAGNOSIS AND DISEASE CLASSIFICATION

As a result of its complex disease pathogenesis and the variable contribution of many different immunologic players in individual patients, ITP is a clinically heterogeneous disease in both people and dogs.[44,45] Not all ITP patients bleed and platelet count alone does not reliably predict bleeding outcomes.[27,46] It is uncertain whether all ITP patients require treatment, and there is a growing recognition that ITP treatment should be individualized to match disease severity. As a result of disease variability, evaluation of suspect ITP cases should include testing to identify any inciting disease and improve predictions of disease progression, and response to treatment.

Unfortunately, an ITP diagnosis remains a diagnosis of exclusion in both human medicine and veterinary medicine. A recent state-of-the-art review on ITP management in people stated that "There is no diagnostic test, no biomarkers to direct treatment and few comparative studies to help management decisions."[44] Before the reader is too discouraged, there are many tests with potential utility for assisting in diagnoses, determining disease severities, and predicting treatment responses that are described below. However, the diagnostic approach to ITP, remains, as described in an international consensus report in the management of adult and childhood ITP, a combination of history, physical examination, and routine clinicopathologic tests to rule out other causes of thrombocytopenia.[47] In both human and veterinary medicine, we lack a diagnostic "gold standard" test for ITP.[47]

The IWG emphasized the need to standardize terminology and apply consistent criteria for evaluating severity and treatment response.[1] The development and use of comparable standards are applicable for veterinary medicine to facilitate multi-institution clinical trials and comparisons of treatment modalities.

5 | ITP AS A DIAGNOSIS OF EXCLUSION: WHAT SHOULD BE EXCLUDED?

In human medicine, primary ITP is defined as a platelet count of under 100,000/µl in the absence of other causes or disorders that may be associated with thrombocytopenia.[48] Other causes of thrombocytopenia include platelet consumption and decreased platelet production. Primary ITP must also be distinguished from secondary ITP due to infections, medications, or neoplastic causes.

The first step in a diagnostic ITP workup is to confirm that the thrombocytopenia is real by performing a manual platelet count

based on examination of a stained blood smear and ruling out platelet clumping as a cause of the thrombocytopenia. Congenital macrothrombocytopenia should be suspected in dogs with chronic thrombocytopenia in the absence of bleeding signs. Congenital macrothrombocytopenia due to a β1 tubulin gene mutation has been identified in the Cavalier King Charles Spaniel, Norfolk and Cairn Terriers, and other breeds.[49] Auburn University and several commercial laboratories offer DNA testing that can be employed to identify the causative mutation. A consumptive cause of thrombocytopenia should be ruled out with hemostasis tests to identify a disseminated intravascular coagulation process.

In a recent study of 61 thrombocytopenic dogs, 57% of dogs were determined to have primary ITP, 28% secondary ITP due to lymphoid/myeloid neoplasia (9.8%), infectious disease (9.8%), liver disease (5%), or drug exposure (3%), and 15% had non-immune thrombocytopenia attributed to bone marrow aplasia or consumptive coagulopathy.[45,50] Investigations for secondary causes of ITP include medication history review, blood chemistry panel and urinalysis for systemic disease, vector-borne disease screening, and imaging to rule out neoplasia. Thrombocytopenia in conjunction with antiplatelet antibodies has been described in dogs infected with *Anaplasma phagocytophilum*,[51] *Ehrlichia canis*,[52,53] *Babesia gibsoni*,[54] and *Leishmania infantum*;[53,55] thus, these diseases should be screened for in endemic areas. We recommend both PCR and serology for vector-borne disease testing as one study documented that combining these methodologies increased testing sensitivity by up to 58%.[56]

In people, testing for *Helicobacter pylori* is suggested in endemic regions and in patients with symptoms beyond thrombocytopenia.[48] Treatment of positive patients is recommended since people can develop ITP secondary to *H pylori* infection, with about half of the patients experiencing ITP remission following *H pylori* eradication.[48,57] It is speculated that there is molecular mimicry between *H pylori* cytotoxic-specific gene A, a virulence factor, and platelet surface glycoproteins leading to the generation of cross-reactive antibodies.[57,58] No studies have been performed to date to determine whether non-*H pylori* helicobacters that infect dogs cause secondary ITP, but testing for helicobacter could be considered in dogs with any gastrointestinal signs concurrent with thrombocytopenia. The role of helicobacter, if any, in canine ITP, warrants further study.

6 | BUILDING THE EVIDENCE FOR A DIAGNOSIS OF ITP

While there is no specific gold standard diagnostic test for ITP, other tests can provide supportive evidence. Here, we review the role of other investigations in the diagnosis of ITP.

1. Does the platelet count help?

In several studies, platelet counts were significantly lower in dogs with primary thrombocytopenia than dogs with other causes of thrombocytopenia.[45,53,59] Even when comparing primary vs secondary ITP, thrombocytopenia was more severe in dogs with primary disease.[45,53] Although platelet counts overlap, severe (<20 000 platelets/μL) thrombocytopenia is suggestive of primary ITP.

2. Does the platelet size provide useful information?

Circulating platelets demonstrate size heterogeneity and automated hematology analyzers provide measures of this variability in the mean platelet volume (MPV) and platelet distribution width (PDW). A high MPV is generally a feature of young and more active platelets; however, appropriate validation studies have not yet demonstrated a diagnostic utility of platelet size indices to differentiate the causes of thrombocytopenia in people.[60] Dogs with ITP were found to have significantly higher MPVs compared with healthy controls in one clinical study;[61] however, no differences were found in a second study using a different hematology analyzer.[62] Yet another study found MPV to be lower in dogs with primary ITP compared with dogs with non-immune thrombocytopenia,[53] which could reflect the presence of microparticles in some dogs with ITP and the fact that some dogs with ITP might have megakaryocyte-targeted destruction as described in people. One retrospective study in dogs found that an MPV >12 fL had a 96% positive predictive value of a normal or hyperplastic bone marrow megakaryocyte population.[63] However, an MPV of ≤12 fL was not strongly predictive of an inadequate bone marrow response (negative predictive value of 57%).[63] Overall, the authors believe that an increased MPV supports an ITP diagnosis, but a normal to low MPV does not exclude the diagnosis.

On the other end of the spectrum, MPVs could help differentiate ITP from hereditary thrombocytopenias. One human study found that an MPV of >12.4 fL differentiated hereditary macrothrombocytopenia from ITP with reasonable sensitivity (83%) and specificity (89%).[64] While the discriminatory value of MPV has not been explored in dogs, the determination of plateletcrit as a measure of total platelet mass has been described as a useful test to differentiate clinically relevant thrombocytopenia from hereditary macrothrombocytopenia in Cavalier King Charles spaniels.[65]

3. What is the utility of measuring reticulated platelets and immature platelet fraction?

Platelets newly released from megakaryocytes contain small amounts of RNA and are referred to as reticulated platelets. Analogous to reticulocytes denoting erythropoiesis, the presence of circulating reticulated platelets is a marker of megakaryopoiesis. Reticulated platelets were first characterized using flow cytometric techniques to detect thiazole orange staining of intraplatelet RNA. Although methodologically challenging to standardize, reticulated platelet assays have shown diagnostic utility for differentiating hypoproliferative thrombocytopenia from platelet consumptive and destructive disorders.[66] More recently, advanced, fully automated hematology analyzers (Sysmex XE and XN series, Sysmex Corporation; Abbott CELL-DYN Sapphire,

Abbott Diagnostics) can measure reticulated platelets in a parameter referred to as the immature platelet fraction (IPF) or percent reticulated platelets, respectively. These automated assays can be standardized and are better suited for routine and high throughput testing. Some clinical studies of thrombocytopenic patients have shown increased IPF fractions in ITP patients; however, these studies have also revealed differences between analyzers that can limit the usefulness of the assay.[67] A recent, well-designed prospective study assessing immature platelets indices on both the Sysmex XE and Abbott CD Sapphire found that while the Sysmex (not the Sapphire) demonstrated a significant increase in immature platelets in ITP patients compared with those with bone marrow failure, both machines demonstrated a broad overlap between the patient groups.[67] The authors speculated that this overlap in immature platelets between ITP etiologies is because of one, a shift toward immature platelets that occurs in any thrombocytopenic state regardless of etiology, analogous to the anemia "reticulocyte shift" that requires a reticulocyte count correction and two, a decrease in platelet production that occurs in many ITP patients.[67,68]

A canine study compared a flow cytometric, reticulated platelet assay with the Sysmex automated IPF assay in healthy and thrombocytopenic dogs.[62] The two methodologies demonstrated a fair correlation and expected increases in immature platelet fractions in dogs with hyperproliferative thrombocytopenia; however, short (<18 hours) sample stabilities were noted for both methods along with high CVs. Another prospective study of dogs with thrombocytopenia determined that having a reticulated platelet percentage >8% (as determined by flow cytometry) combined with positive direct or indirect platelet-associated antibody testing distinguished primary ITP from other causes of thrombocytopenia with high sensitivity but low specificity.[69] Overall, the utility of immature platelet fractions/reticulated platelets as an ITP diagnostic tool appears low in both people and dogs.

However, absolute IPF could have diagnostic utility in determining whether the main mechanism of thrombocytopenia in an individual ITP patient is reduced production vs increased destruction, which could guide individual treatment selection.[70] This was explored in a pilot study of human ITP patients where absolute IPF served as a reliable indicator of the patient's thrombopoietic state.[70] As the mechanism of the treatment effects is incompletely understood for many ITP therapeutics, absolute IPF could also be used to discern whether treatments ameliorate platelet destruction or increase platelet production. Treatments that stimulate platelet production would result in an increased platelet count along with a substantial increase in the absolute IPF while those that interfere with platelet destruction would increase the platelet count but without an accompanying increase in the absolute IPF.[70]

4. Is a bone marrow examination necessary for an ITP diagnosis?

Bone marrow examination is not recommended in the initial evaluation of uncomplicated adult or childhood ITP; however, bone marrow examination is considered potentially useful for patients with refractory disease, atypical presentation, or ages greater than 60 years.[47]

Combined morphologic and flow cytometric assessments are performed in these patients and could reveal causes of secondary thrombocytopenia, lympho/myeloproliferative disease, and other bone marrow disorders.[44] In clinical studies, bone marrow examinations are not typically included in the diagnostic workup of canine ITP.[27,31,71] A retrospective study of dogs with thrombocytopenia concluded that bone marrow cytology did not provide diagnostic or prognostic information in dogs with severe thrombocytopenia.[72] Similarly, a large retrospective study of dogs with ITP found that megakaryocyte hypoplasia did not impact the likelihood of survival or time to platelet count recovery.[27] One recent retrospective study of thrombocytopenic dogs contradicted these studies, showing that the finding of megakaryocytic hypoplasia on bone marrow examinations was associated with bleeding and nonsurvival.[73] However, this study was small and statistical comparisons were not performed between the groups. The authors do not routinely perform bone marrow evaluations in presumptive ITP patients unless there is a suspicion of underlying marrow disease such as the presence of multiple cytopenias, poor response to therapy, aged dogs where the suspicion of underlying disease is high, or in a dog with concerning abnormalities on abdominal imaging that cannot be safely sampled, but the marrow provides a safe site to search for possible lymphoproliferative disease. When marrow evaluation is warranted, a sternal marrow aspirate can be considered as a less invasive alternative to the humerus or ilium.[74]

5. Is there value in measuring platelet autoantibodies to support an ITP diagnosis?

Autoantibodies directed against platelet antigens play a key role in platelet destruction; however, the presence of antiplatelet antibodies is not included in current consensus criteria for the routine diagnosis of human ITP given that assays lack sensitivity and specificity.[47] Different assay configurations detect circulating antiplatelet antibodies (indirect methods) and membrane-bound, platelet-associated antibodies (direct, PAIg). Direct assay methods are generally more sensitive than indirect assays; however, platelets constitutively express immunoglobulin receptors that cause nonspecific antibody binding and complicate interpretation of a "positive" test.[75,76] Assay sensitivity is impeded by the fact that ITP is not always an antibody-mediated process. Up to 40% of human ITP patients lack detectable antibodies, presumably due to cytotoxic T-cell–mediated platelet destruction.[44] In human medicine, assay specificity is greatly enhanced by measuring platelet glycoprotein-specific autoantibodies. Platelet antigens primarily targeted by autoantibodies include GPIIb/IIIa (CD41/CD61, fibrinogen receptor), GPIb/IX (CD42c/CD42a, von Willebrand factor receptor), and GPV (CD42d).[75] The monoclonal antibody-specific immobilized platelet antigen assay (MAIPA) and bead-immobilized monoclonal antibody assays are configured to define each ITP patient's autoantibody specificity. The assays are based on detection of trimolecular complexes formed by a monoclonal antibody to a known platelet glycoprotein, the patient's autoantibodies, and the platelet membrane that carries the respective epitopes of each of these antibodies.[75,76] MAIPA specificity for an ITP diagnosis is reported to be as high as 98% but still
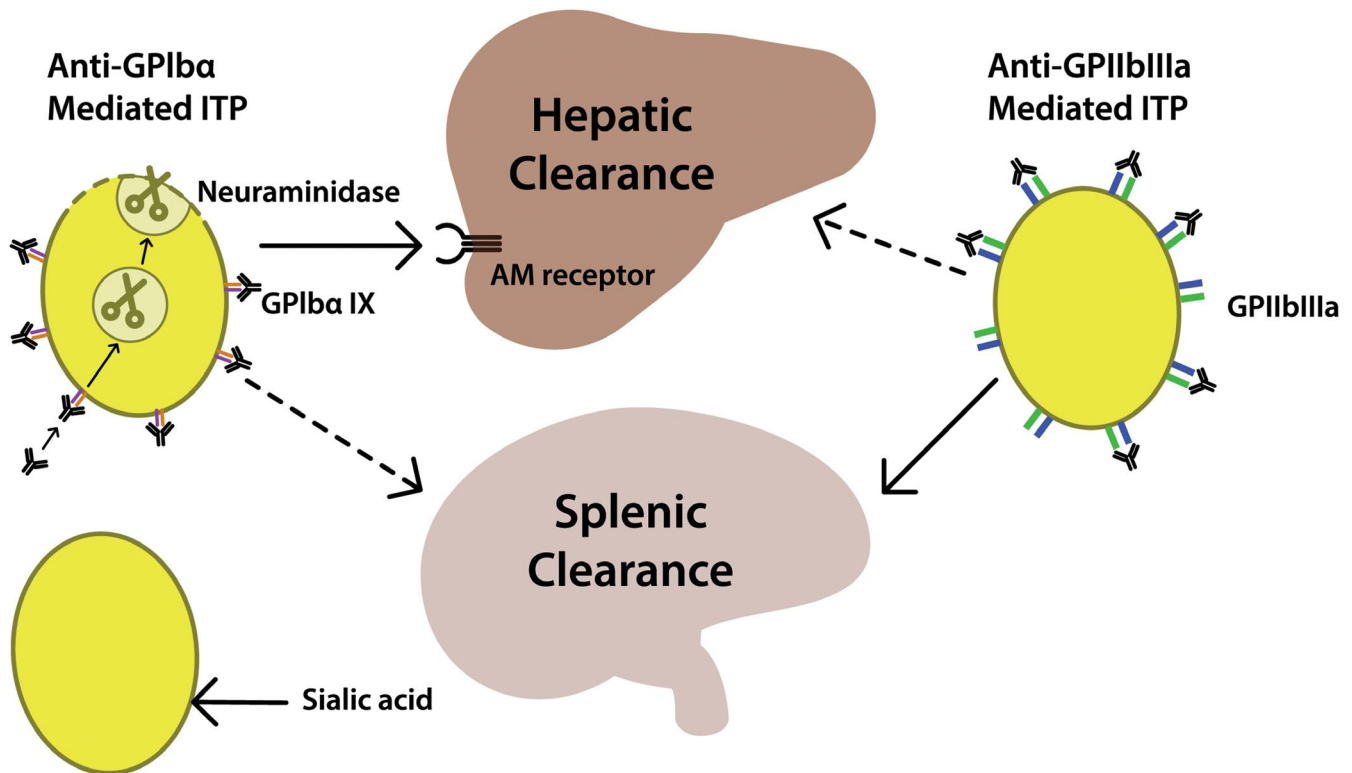
FIGURE 4 Autoantibody specificity determines the pathway of platelet clearance in immune thrombocytopenia (ITP). Anti-GPIIbIIIa antibodies lead to Fc-receptor-mediated reticuloendothelial cell clearance in the spleen and, less so, in the liver. Anti-GPIbα antibodies result in platelet activation and neuraminidase-1 surface translocation. Neuraminidase-1, in turn, cleaves sialic acid residues from GPIbα and leads to Fc-independent hepatic clearance of the desialylated platelets via the Ashwell-Morell receptor (AM)

lacks sensitivity (81% sensitive), potentially due to T cell as opposed to antibody-mediated autoimmunity in some patients.[75] Surveys of ITP patients reveal that autoantibodies targeting GPIIb/IIIa are the most common; found in approximately 70-80% of patients, with anti-GpIb complex antibodies found in 20-40% of patients.[4] The only study describing the target antigen in dogs with ITP identified anti-GPIIb and IIIa antibodies in the serum of 4/17 (24%) of affected dogs.[77]

While PAIg assays are not used for diagnosis, recent studies demonstrated their potential value in optimizing treatment and monitoring response. Whereas antibodies to GPIIb/IIIa lead to macrophage clearance of platelets by the Fc receptor, anti-GPIb antibodies trigger platelet desialylation via platelet neuraminidase-1, which results in clearance via the hepatic Ashwell-Morell receptor, the normal pathway for clearance of aged, desialylated platelets (Figure 4).[4,21] Patients with anti-GPIb antibodies appear to be refractory to IVIG therapy due to non-Fc receptor-mediated platelet clearance.[78] As demonstrated in recent case reports, these patients could instead respond to sialidase inhibitors like oseltamivir.[4,79] In addition, persistent high PAIg titers have been shown to be predictive of poor responses to the anti-B cell drug, rituximab.[80] Although glycoprotein-specific PAIg and platelet desialylation testing are performed in research laboratories, these assays are not routinely performed in human medicine and have not been described in canine studies.

Flow cytometric assays to detect PAIg without defining target platelet antigen have been described in case series of canine thrombocytopenia.[53,54,69] As in human medicine, the diagnostic utility of canine PAIg assays for ITP has not been clearly demonstrated, mostly due to the lack of sensitivity and specificity. A recent prospective study of dogs with thrombocytopenia determined that the sensitivity and specificity of direct and indirect platelet-associated IgG and IgM assays combined were 76.5% and 65.5%, respectively, for distinguishing primary ITP from other causes of thrombocytopenia.[69] Limitations of PAIg assays include a lack of standardization and defined controls and non-specific antibody binding during sample storage. Furthermore, PAIg are well documented in many cases of canine secondary ITP including anaplasmosis, babesiosis, ehrlichiosis, leishmaniasis, leptospirosis, sepsis, hepatitis, lymphoma, and histiocytic sarcoma, thus limiting the ability of PAIg testing to differentiate primary from secondary ITP.[53,54] Assays to define the targeted platelet glycoprotein could prove useful in dogs as in people to increase diagnostic specificity and potentially guide treatment choices. To date, the development of these assays is hampered by the limited availability of commercial antibodies that recognize the main canine platelet glycoproteins.[81]

Although PAIg testing is not specific for primary ITP, the finding of a bound antibody is supportive of antibody-mediated platelet destruction, and persistent high PAIg could help guide treatment in cases where the patient has not responded to first-line drug therapy as expected.[44] The decision to begin tapering immunosuppressive therapy could also be guided by measuring PAIg.[75]

In addition to detecting PAIg, canine studies have included a qualitative assessment of platelet membrane antigens, platelet size, and microparticle production. Reduced CD61 (GPIIIa) expression in dogs with ITP has been described in several studies[54,69]; however, the clinical relevance of this abnormality has not been explored. One study found that CD61 expression was significantly lower in dogs with primary (3.3%) compared with secondary (50.4%) ITP.[69] However, in a preliminary study, the authors found a similar reduction in CD61 expression for dogs with primary and secondary ITP compared with dogs with non-immune thrombocytopenia and healthy control dogs (unpublished data). In human ITP, reduced expression of platelet membrane antigens has been attributed to antiplatelet antibody coating,[82] and antibody-mediated platelet activation with changes in membrane glycosylation.[78] The diagnostic potential of measuring CD61 expression warrants further exploration.

6. Does measurement of thrombopoietin levels aid in ITP diagnosis?

The recognition that many human ITP patients have inappropriately normal TPO levels and will respond to TPO mimetic drugs has revolutionized human ITP treatment. The diagnostic utility of TPO testing, however, has not been established,[47] and TPO assays are primarily performed in research laboratories. Surveys of TPO levels in thrombocytopenic patients have revealed an overlap between the values of consumptive thrombocytopenias and controls but increases up to several 1000-fold in patients with hypoproliferative thrombocytopenias.[19] While the utility of TPO levels for an ITP diagnosis is uncertain, a potential prognostic relevance has been identified. Patients with relatively high TPO, defined as >95 pg/mL, were found to have only transient or partial responses to TPO mimetic therapy.[83] Assays to measure canine TPO are not currently available. However, preliminary studies in the authors' laboratories show cross-reactivity of some anti-human TPO antibodies with canine native and recombinant TPO, providing the basis for developing immunoassays to detect canine TPO in clinical serum samples.

7 | TREATMENT NECESSITY AND GOALS

While extensive discussion of treatment is beyond the scope of this review, we will briefly discuss treatment goals in the context of ITP diagnostics. Briefly, immunosuppression with glucocorticoids, often in combination with adjunctive immunosuppressive agents, is the mainstay of ITP treatment in dogs. In people with ITP, frontline treatments include short courses of corticosteroids and IVIG, while second-line therapies include rituximab, an anti-CD20 antibody, splenectomy, and TPO receptor agonists.[48]

As described above, ITP is a heterogeneous disease both in terms of pathogenesis and clinical presentation. Variable bleeding severity in the face of similar platelet counts might represent the combined and variable influence of platelet activation by autoantibodies,[84] interference of antibodies with platelet function,[85,86] alterations in endothelial integrity secondary to thrombocytopenia,[87,88] and the presence of circulating procoagulant microparticles.[89,90]

The risk of spontaneous bleeding theoretically occurs with platelet counts less than 30 000/μL.[44] Although platelet counts give some indication of bleeding risk, not all patients with ITP bleed with low platelet counts, while others bleed excessively. Immunosuppressive therapy is associated with side effects, including potentially fatal secondary infections and bone marrow suppression. Much of the overall disease burden of ITP is due to the lack of prognostic criteria and resultant uniform administration of high intensity and long-term immunosuppressive therapy. Indeed, in severe human ITP, mortality results equally from refractory hemorrhage and secondary infections in immunosuppressed patients.[91] The question then becomes, which patients require aggressive therapy or even therapy at all?

While veterinarians routinely aim to normalize platelet counts, the American Society of Hematology (ASH) guidelines do not recommend treating adult ITP patients unless platelet counts are less than 30 000 platelets/μL or when there is active bleeding.[48] Additionally, ASH guidelines recommend, in the absence of bleeding, a treatment goal of over 30 000 platelets/μL should be achieved.[48] Reconsidering ITP treatment goals in veterinary medicine to better align with human recommendations is likely to be warranted.

8 | PREDICTORS OF SEVERITY AND RESPONSE

8.1 | The necessity of bleeding assessment tools

In human medicine, the platelet count is now recognized as an imperfect predictor of bleeding severity, and maintenance of normal platelet counts is an unreasonable treatment goal. A standard clinical metric to gauge bleeding severity in ITP patients has been developed through a consensus and has been described as an "ITP-specific Bleeding Assessment Tool" or ITP-BAT.[92] In addition to standardization, the metric aims to simplify data collection by categorizing bleeding manifestations into three anatomic sites (skin, mucosa, organ) and apply numeric scores based on the severity of hemorrhage. Similarly, "DOGiBAT," a bleeding assessment tool based on the human ITP Bleeding Scale,[46] has recently been developed for classification of bleeding severity in canine ITP with the goal of facilitating patient stratification and monitoring response to therapy across institutions and treatment trials.[45] The DOGiBAT scoring system assigns bleeding severity grades at nine anatomic sites. In a pilot study of ITP, increasing DOGiBAT scores, which denoted more severe clinical signs, were found in dogs with more severe thrombocytopenia. However, unlike platelet counts, the DOGiBAT score at admission correlated with transfusion requirements and the duration of hospital stay.[45] Although further validation studies are required, these preliminary results suggest that DOGiBAT scoring could prove clinically useful as a prognostic indicator. Using DOGiBAT in future studies could also help identify other predictors of disease severity and response to treatments, and could ultimately help to improve our understanding of why ITP patients present with variable bleeding tendencies.

8.2 | The identification of bleeding severity and relapse predictors

The ability to identify patients at risk of severe bleeding or relapse would allow the individualization of therapy to match treatment intensity with disease severity. In human medicine, a few hemostatic assays have shown promise in predicting bleeding in ITP patients but are not routinely used.[44] In children with ITP, platelet function as assessed by flow cytometry was predictive of future bleeding severity.[93] Specifically, elevated resting platelet P-selectin expression, reduced agonist-stimulated P-selectin expression, and activated GPIIbIIIa were associated with bleeding severity. The study authors speculated that a strong response of platelets to agonist stimulation might protect against severe bleeding, and partially activated circulating platelets (high resting P-selectin expression) could have a reduced ability to become activated.[93] Flow cytometric assessment of platelet function is technically challenging and is not widely available for people or dogs. However, platelet function could help predict bleeding risk and guide ITP patient management. Similarly, thromboelastometry measures of clot firmness were significantly and inversely correlated with the bleeding score in one study of adult and pediatric ITP patients, suggesting a potential role for global hemostatic assays in guiding ITP management.[94] Absolute IPF could also have utility in predicting bleeding outcomes as absolute IPF measured with the Sysmex XE-2100 was inversely correlated with bleeding scores in the same study.[94]

Bleeding predictors have not been identified in dogs, though a few disease severity markers have been recognized. In a retrospective study of 73 dogs, dogs with elevated BUN concentrations on admission were less likely to survive to discharge (57%) compared with dogs that had BUN concentrations within the reference range (85%).[27] Similarly, dogs with melena on admission were much less likely to survive to discharge (60%) compared with dogs without melena (90%) and were more likely to require transfusions.[27]

9 | SUMMARY

ITP is a heterogeneous disease both in terms of pathogenesis and variable bleeding presentation. Understanding the complex and varied pathogenesis of ITP has recently advanced so that we can now recognize in human ITP that:

1. Platelet destruction involves not only antibodies but also cytotoxic T cells
2. Antiplatelet antibody specificity influences the pathway of platelet clearance and treatment response
3. ITP is not only a disease of platelet destruction but also one of decreased production that involves T-cell and antibody-mediated megakaryocyte attack and inappropriately normal TPO levels.

Despite all of these advances in our understanding, much about ITP pathogenesis remains unknown, especially what leads to the lack of self-tolerance that initiates the disease process. This has resulted in the lack of a diagnostic test for ITP and minimal biomarkers that can predict disease severity and guide ITP treatment. Future research is needed to continue unraveling the pathogenesis, which will ultimately enable better testing and safer, more targeted and individualized treatments based on disease pathogenesis and severity. Comparative genomics is one area where veterinary medicine has a distinct advantage over human medicine. The clear breed predispositions for ITP will hopefully allow identification of genetic polymorphisms that are associated with ITP. Ultimately, this knowledge could help us have a greater understanding of ITP disease pathogeneses and allow us to develop more effective diagnostic tests for this disease.

DISCLOSURE

The authors have indicated that they have no affiliations or financial involvement with any organization or entity with a financial interest in, or in financial competition with, the subject matter or materials discussed in this article.

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