ABSTRACT

LE VINE, DANA NICOLE. A Novel Model of Canine Immune Thrombocytopenia and Early Investigations of Mechanisms of Bleeding in Immune Thrombocytopenia. (Under the direction of Samuel L. Jones.)

Immune thrombocytopenia (ITP) is an autoimmune bleeding disorder in which platelet autoantibodies lead to platelet destruction, thrombocytopenia, and, sometimes, severe bleeding. Animal models have been instrumental in our current understanding of ITP. However, many unanswered questions remain, including why some ITP patients bleed while others with similarly low platelet counts do not. There are no large animal models of ITP, and available rodent models have many shortcomings. More importantly, though, rodents do not develop naturally-occurring ITP. The dog represents an ideal translational model since canines develop spontaneous ITP with a similar disease course and response to treatment as humans. Furthermore, human platelet counts, volume, and signaling are more similar to those of dogs than those of mice.

An induced canine model allows the study of ITP without the confounding comorbidities of co-existing diseases and treatment effects that are present in naturally-occurring disease. In Chapter 2, we describe a novel canine model of ITP induced by infusing dogs with a murine antibody to a component of the fibrinogen receptor, a frequent autoantibody target in ITP. We determined that this model is very analogous to spontaneous canine and human ITP, most notably in the heterogeneity of bleeding demonstrated by the dogs despite similar platelet counts.

In both canine and human patients with immune thrombocytopenia (ITP), bleeding risk is difficult to predict, which leads to potential over-treatment of patients with low risk of hemorrhage. Therapy for ITP is not benign. ITP mortality is equally attributed to fatal hemorrhage and to secondary infections from immunosuppressive therapy or splenectomy, current mainstays of ITP treatment. Understanding mechanisms of bleeding in ITP is necessary to tailor ITP patient management to actual bleeding risk and ultimately to develop novel therapeutics to stop thrombocytopenic bleeding. In Chapters 3 and 4, using our novel canine model of ITP, we investigate the relative roles of platelet hemostatic function and platelet vascular stabilizing function in thrombocytopenic bleeding. In Chapter 3, we show that alterations do occur in thrombocytopenic endothelium, specifically reduction in pinocytotic vesicles, suggesting that endothelial membrane damage occurs when platelets are absent. The most dramatic endothelial alterations were present in the ITP model dogs that experienced the most prominent cutaneous bleeding. Chapter 4 discusses our inability to find a relationship between decreased platelet hemostatic function and bleeding in the model dogs. Our data argue against the importance of platelet hemostatic function in predicting thrombocytopenic hemorrhage. Instead, we determined that platelet reactivity increases during the recovery period of ITP and might be related to observed thromboembolic tendencies that have been recently reported in some ITP patients.

Overall, our early investigations of mechanisms of bleeding in ITP suggest that platelet vascular stabilizing function may be more important than platelet hemostatic function in determining bleeding outcome in ITP. Our data represent the early stages of development of bleeding predictors in canine and human ITP patients. Our novel induced canine ITP

model has great potential for further investigations into mechanisms of bleeding in ITP and for developing and testing novel ITP therapeutics for canines and humans alike.

A Novel Model of Canine Immune Thrombocytopenia and Early Investigations of Mechanisms of Bleeding in Immune Thrombocytopenia

by
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BIOGRAPHY

Dana LeVine grew up in Port Jefferson, New York. In childhood, she developed interests in both biological sciences and companion animals that led her to study Molecular Biochemistry and Biophysics at Yale University and then veterinary medicine at Cornell University. After obtaining a Doctor of Veterinary Medicine degree in 2004, she began advanced training in companion animal internal medicine through a rotating clinical internship at the University of Georgia.

In 2005, in order to pursue a career as a clinician-scientist, she entered the Clinician Investigator program at North Carolina State University (NCSU), a combined Small Animal Internal Medicine residency and doctoral program. In 2008 she completed her residency at the NCSU College of Veterinary Medicine and in 2009 she became a board-certified small animal internist as a Diplomate of the American College of Veterinary Internal Medicine.

Dr. LeVine started her PhD studies in Comparative Biomedical Sciences at NCSU in 2008. Interaction with coagulation experts during veterinary school had instilled an interest in veterinary hematology. This led to graduate research in hemostasis, examining an immune-mediated disorder of platelets in dogs. Her PhD research was conducted in coagulation laboratories at the University of North Carolina at Chapel Hill, first at the Francis Owen Blood Research Laboratory with Dr. Thomas Fischer and later in the laboratory of Dr. Nigel Key.

Dr. LeVine plans to continue to combine clinical small animal medicine and research in veterinary hematology as a clinician-scientist at Iowa State University. On completing her PhD, she will begin a position there as an Assistant Professor of Small Animal Internal Medicine.

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Introduction to Immune Thrombocytopenia: Impact and Definitions

Primary immune thrombocytopenia (ITP) is an acquired immune-mediated bleeding disorder characterized by isolated thrombocytopenia in the absence of an obvious initiating or underlying cause [1]. Although it is relatively uncommon, with an average annual prevalence of 12.1 per 100,000 adults, the chronicity of the disease, the lack of highly effective treatments, and the side-effects associated with many current ITP therapies make ITP a disease of significant patient morbidity and one that places heavy economic and resource burdens on the health care system [2, 3]. In one United States study, the average cost of ITP-related hospitalization was higher, the length of stay longer, and in-hospital mortality greater on average than those all other hospitalized patients [4]. The mortality rate of ITP is up to 2.3 times that of the general population [5].

ITP has been defined by an International Working Group consensus panel as a platelet count less than 100 $x10^9$/L in the absence of other causes or disorders that may be associated with thrombocytopenia [6]. ITP can be categorized depending on disease duration as newly diagnosed (diagnosis to 3 months), persistent (3 to 12 months from diagnosis), or chronic (lasting for more than 12 months) [6]. ITP is a diagnosis of exclusion of other causes of thrombocytopenia as there are no specific tests for ITP and there is a lack of disease-defining features other than thrombocytopenia with or without associated bleeding manifestations [7].

Both children and adults are affected by ITP, but the disease has different manifestations in these two populations. Childhood ITP is often acute, preceded by viral illness, and in the majority of cases, resolves spontaneously within 6 months [2, 8]. Here, we focus on adult ITP, which, in contrast, tends to be chronic with an insidious onset and rarely

resolves spontaneously [2, 8]. Adult ITP can be of long duration, with some patients being followed as long as 30 years [8].

ITP is an old disease that was first reported in 1557 in a boy with "dark macules, resembling flea bites, had no fever and for several days had bloody discharges, eventually recovering" [7]. Our modern understanding of ITP began in 1951 when William Harrington injected blood from ITP patients into healthy volunteers, who, within hours, developed thrombocytopenia [9]. This seminal experiment established that the blood of ITP patients contains a "thrombocytopenic factor," which has since been determined to be autoantibodies directed against platelet glycoproteins [9]. For the 60 years following Harrington's study, the mechanism of platelet destruction in ITP was attributed to platelet autoantibodies opsonizing platelets and subsequent platelet clearance by macrophages of the reticuloendothelial system [1]. Recently, this paradigm has been revised and platelet autoantibodies are understood to be the tip of the immunologic ITP iceberg. Autoantibodies are the result of a complex loss of self-tolerance, including formation of autoreactive B and T cells, autoreactive cytotoxic T cells, and decreased regulatory T cells [8, 10]. Furthermore, in addition to accelerated platelet destruction, ineffective platelet production is now understood to be central in disease pathogenesis: thrombopoietin levels are inappropriately low (normal to minimally elevated) and altered megakaryocyte physiology results in impaired platelet production [8, 11, 12]. We are beginning to recognize that ITP is probably a heterogeneous syndrome with a final common pathway of platelet autoantibodies and platelet destruction [7].

Although we have come a long way from the famous 1808 ITP treatment recommendation of "moderate exercise in the open air, a generous diet, and the free use of wine" [7], we are really only just beginning to understand this complex disease.

Overview of Current Animal Models of Immune Thrombocytopenia

Animal models have provided great insights into the pathophysiology of ITP and the development of novel therapeutics. Here we review laboratory animal models of ITP, how they have governed our understanding of ITP, and how our present understanding of the complexity of ITP immunopathogenesis demands development of better animal models.

Rodents, namely mice, have been the most heavily used species in ITP research. We review the benefits and limitations of mice as ITP models and introduce the dog, a large animal which develops spontaneous ITP, as an excellent, highly translational ITP model.

Passive ITP Models

Animal models can be divided into spontaneous and induced. Spontaneous models are those diseases that occur naturally within an animal as opposed to induced models in which some treatment of the animal initiates the disease [13]. Most animal models of immune thrombocytopenia are induced passive immune thrombocytopenia models. In passive models of ITP, anti-platelet antiserum or monoclonal anti-platelet antibodies are injected into recipients to induce thrombocytopenia [13]. When monoclonal antibodies are used, their targets are usually the fibrinogen receptor, glycoprotein IIbIIIa (GPIIbIIIa), or the von Willebrand receptor, GPIb/IX/V, as autoantibodies in human patients with ITP are most commonly directed against epitopes on these receptors [14]. A wide range of species has been used in passive thrombocytopenia models including mice, rats, rabbits, and guinea pigs, with mice being the most frequently employed [15-18].

Passive immune thrombocytopenia models have been integral in laying the foundation for much of what we know about ITP today, including the importance of anti-platelet antibodies in targeting platelets for clearance [15], the efficacy of glucocorticoids in raising platelet counts in ITP [19], and the central role of thrombopoietin in stimulating platelet production [20-22]. Recently, it has been recognized that ITP is not just a disorder of increased platelet destruction, but also one of impaired platelet production. The determination, through a passive murine ITP model, that that thrombopoietin (TPO) is actually inappropriately normal in ITP stimulated the development of a major treatment modality in human ITP today: the TPO mimetics [11, 23]. In murine ITP models, TPO was cleared at an increased rate by uptake and degradation by the high platelet turnover [11].

Passive models have also established the efficacy of intravenous immunoglobulin (IVIG) infusion in treatment of ITP and determined potential mechanisms of IVIG's action [13]. IVIG is a highly purified immunoglobulin preparation containing IgG and trace

amounts of IgM and IgA that is prepared from human donors [24]. Infusion of IVIG is currently a first-line treatment option for adult ITP patients with an initial response rate of up to 80% [1]. One of the first demonstrations of the effect of IVIG in ITP was that of Tsubakio and colleagues who showed that human IVIG could raise platelet counts in a passive rat model of ITP [25]. Subsequent work in murine models has revealed many mechanisms by which IVIG may exert its effects [26]. In mice, IVIG has been shown to induce surface expression of the inhibitory Fc receptor (FcγRIIB) on effector cells that would otherwise clear antibody-opsonized platelets by phagocytosis [27, 28]. IVIG is now understood to shift the balance of activating and inhibitory Fc receptors, thereby down-regulating platelet phagocytosis [13].

Passive ITP models are elegant demonstrations of what transpires in ITP once anti-platelet antibodies have formed. However, a major limitation of passive transfer models is that they do not reflect the complex process of autoantibody development and the non-antibody mediated destruction of platelets that occurs in some ITP patients [26]. The production of autoantibodies is the end result of poorly understood immune dysregulation [29]. Patients with ITP have recently been shown to have central tolerance defects including deficiency in regulatory T and B cells [30, 31]. Not all ITP patients have anti-platelet antibodies and other mechanisms of platelet destruction may be transpiring such as destruction by cytotoxic T cells [32]. Indeed, in Harrington's original ITP model, thrombocytopenia resulted from infusion of only 70% of the ITP patient plasma into healthy volunteers [9]. This suggests that in some ITP patients, a mechanism of platelet destruction other than autoantibodies exists [9]. More complex murine models have been recently developed to better capture the complex pathogenesis of autoimmunity in ITP.

Knockout ITP Models

Probably the greatest advantage of the murine species as an ITP model is the ability to manipulate the mouse genome to recapitulate the immune pathogenesis of ITP. The best demonstration of the use of knockout technology is Semple and colleagues' murine model that utilizes GPIIIa knockout mice (KO). In this model, splenocytes from GPIIIa KO mice

that have been immunized against GPIIIa+ platelets are transferred to severe combined immunodeficient GPIIIa+ mouse recipients [33]. Recipient mice demonstrate both antibody and cell-mediated platelet destruction. Interestingly, using this model, Semple et al. have been able to show that only the antibody-mediated destruction is responsive to IVIG therapy; cell-mediated disease is unaffected [33]. This model even replicates the peripheral regulatory T cell deficiency that is observed in human ITP [34, 35]. However, this transgenic model has limitations. It still requires immunization of knockout mice with wild-type platelets to initiate the immune response. Furthermore, the splenocyte recipients must be irradiated prior to transfer causing a transient irradiation-induced thrombocytopenia [33].

Another murine model that exploits knockout technology is a model of fetal neonatal alloimmune thrombocytopenic purpura in which GPIIIa knockout mice dams are immunized with transfusions of wild-type platelets and then mated with wild-type male mice [36]. Surviving GPIIIa positive fetuses subsequently develop thrombocytopenia and bleeding.

In contrast to passive ITP models which infuse exogenous antibody, antibody generated in these transgenic models is endogenous. Thus these transgenic models have potential to address the role of cytotoxic T cells and regulatory T and B cells in the initiation and propagation of the autoimmune response in ITP [32].

Secondary ITP Models

Some animal models recapitulate secondary ITP in which ITP is due to an underlying infection, disease, or drug exposure [13]. One such murine model is of note because it occurs spontaneously: the New Zealand black mouse (NZWxBXSB)F_1 strain develops lupus nephritis spontaneously and with age shows thrombocytopenia that is due to anti-platelet antibodies [37]. This model, though unique in being the only spontaneous rodent model, only models secondary ITP associated with an underlying immune disorder and does not allow for understanding of how primary ITP spontaneously develops [13].

Induced secondary ITP models have been important in demonstrating how infectious agents contribute to ITP. Examples of induced secondary ITP models include infusion of passive ITP murine models with lipopolysaccharide to exacerbate platelet phagocytosis and

thrombocytopenia [38] and enhancement of autoantibody pathogenicity in passive ITP models by simultaneous infection with common murine viruses [39]. These models have been important in demonstrating that infection can induce thrombocytopenia in patients with clinically silent autoimmune disease [38]. Indeed, similar exacerbation of ITP by co-infection has since been demonstrated in human ITP patients with *Helicobacter pylori* (*H. pylori*) infection. *H. pylori* induced down-regulation of the monocyte/macrophage inhibitory FcγRIIB receptor leads to enhanced platelet phagocytosis [40]. Eradication of *H. pylori* in infected patients suppressed the activated monocyte phenotype and improved platelet counts [40]. Testing for *H. pylori* is now a standard component of the work-up of adults with ITP [1].

The Mouse as a Model

The advantages of murine models are clear. Because of their relatively low cost and their short gestation period, it is easy to use large numbers of animals when working with mice [41]. Furthermore, there is ready availability of reagents like antibodies that recognize a vast array of murine proteins. As previously mentioned, our ability to manipulate the murine genome via knocking out or in expression of specific gene products provides a powerful tool.

However, murine models have their limitations. Here we address some of the limitations of the mouse as an ITP animal model. Because of our interest in utilizing the dog as a spontaneous and induced model of ITP, we highlight how the limitations of the murine model contrast with the dog model (Table 1).

One major disadvantage is that mice never develop spontaneous primary ITP, making translation of any murine model to the naturally-occurring disease challenging. In contrast, dogs do develop spontaneous ITP. Indeed, thrombocytopenia is the most common acquired hemostatic disorder and the most common cause of spontaneous bleeding in dogs [42]. As companion animals, dogs also more readily reflect the environmental conditions of the human target populations that may influence the development of an immune-mediated disease. Genetically defined and uniform rodent strains fail to model the genetic, dietary, and environmental diversity of people [43].

Quite simply, a mouse is not a small person. The normal platelet count of mice (1 million platelets/μl) is on average three times greater than that of people (150,000-400,000/μl) or dogs (200,000-300,000 platelets/μl) [13, 44, 45]. Additionally, murine platelets (mean platelet volume 4.7 ± 0.3 fl) are smaller than human platelets (7.5-10 fl) or canine platelets (7.9-13.8 fl) [44, 46]. Murine platelet lifespan is about 5 days, about one-half that of human platelets, and unlike in people, bone marrow platelet production is probably assisted by splenic megakaryocytes in mice [44, 47-49]. The very high murine platelet count compared to humans dampens the mouse's role as an ITP model given that a mouse could be considered thrombocytopenic at a platelet count that would be normal for a person or a dog. It should also be noted that platelet-leukocyte ratios are very different between humans and mice with mice having only 500 to 1000 neutrophils/μl compared to the 2500 to 7500/μl in humans or 3000 to 9,000/μl in dogs [50]. The greater platelet: leukocyte ratio in mice compared to humans necessitates caution in extrapolating findings from a murine model of inflammatory thrombocytopenic hemorrhage that we later describe [50].

The small size of mice provides a distinct challenge. Mice become readily anemic without much sampling, introducing a confounder into studies [41]. Only 0.2 ml of blood can be safely taken from a 25 g mouse whereas over 400 times that volume (85 ml) can be safely sampled from a 10 kg dog [41, 51]. Greater sampling volume obviously allows more studies to be performed with fewer animals. In measuring mediators that are influenced by hematocrit, multiple mice may be needed to generate a time course of samples, whereas the same study could be performed in one dog.

Allometric scaling of small animals provides some challenges in that mice have much higher metabolic rates than do people [52]. In animals smaller than rabbits, capillary density increases with decreasing body weight in order to provide oxygen delivery adequate to sustain the high metabolic rate of these animals [52]. High capillary density may influence rheodynamics and thus hemostasis and thrombosis [43]. The short blood vessel lengths of rodents also alter their hemorheology compared to that of humans. Studies of canine and human hemorheologic factors such as erythrocyte aggregation and deformability showed greatly similarity between humans and dogs than between rats and humans [53]. Murine

aortic wall shear stresses are an order of magnitude higher than human wall shear stresses, which may alter expression of endothelial adhesion factors and subsequent hemostasis [54].

Overall murine and human platelets are very similar, making mice a very useful ITP model, however, there are some differences that must be acknowledged. Unlike human platelets, murine and rat platelets show a greater maximal response to ADP than to thrombin in a flow cytometric assay [55]. Canine and human platelets show reduced responsiveness to ADP compared to thrombin [55]. In contrast, ADP fails to elicit maximal murine platelet dense body release when used at concentrations comparable to those used with human platelets, suggesting that ADP may be less effective at inducing signal transduction leading to secretion in murine platelets than human platelets [44, 47, 56]. Murine platelets also do not respond as well as human platelets to epinephrine or serotonin, again likely due to differences in signal transduction [47]. Gender differences in the responsiveness of murine platelets to different agonists have been described which must be considered when designing a murine model of a bleeding disorder [44, 57]. Finally, thrombin receptor expression differs between murine and human platelets: human platelets express protease-activated receptors (PAR) 1 and PAR4 which both function as thrombin receptors while murine platelets lack PAR1 and instead express PAR3 and PAR4. [44, 58, 59] PAR3 behaves as a cofactor for PAR4 and cannot, by itself, promote thrombin signaling [44, 58, 59]. Canine platelets express PAR receptors 1,3 and 4 but likely signal, like humans, through PAR 1 and 4 (James Catalfamo, personal communication, November 8, 2012), [60]. Species differences in platelet receptors and signaling pathways have implications for clot formation and might result in differences in the bleeding phenotype of ITP mouse models compared to human ITP patients. Furthermore, in human platelets, signaling through PAR receptors determines whether pro (PAR 1) or antiangiogenic (PAR 4) factor containing alpha granules are released. PAR-mediated differential granule release may impact how platelets effect vascular integrity and module thrombocytopenic hemorrhage as discussed below [61]. This PAR-granule release pattern likely differs in mice since they do not have the same PAR receptors.

There are also significant immunological differences between mouse and man. Humans possess Fcγ receptors that are absent in the mouse genome [62]. Mice lack the

FcγRIIa and any platelet IgG Fc receptor, while human macrophages and platelets express the FcγRIIa activating receptor [62-64]. The FcγRIIa on macrophages is thought to play a major role in platelet clearance in human ITP [62]. Although mice clear antibody-opsonized platelets via their FcγRI and FcγRIII receptors, to more accurately represent human disease, transgenic mice have been created in which murine FCR γ receptors are knocked out (resulting in absent functional FcγRI and FcγRIII on splenic macrophages) and human FcγRII is instead expressed on murine platelets and leukocytes at human physiologic levels [62]. Minimally, canine neutrophils have been documented to express the FcγRIIa [65]. Unpublished work suggests that canine platelets may, like murine, lack the platelet FcγRII (Mike Scott, personal communication, October 31, 2012).

Finally, passive murine models in which antibodies against the fibrinogen receptor (GPIIbIIIa) are infused lead to incompletely understood acute systemic reactions, incoordination, and hypothermia [15, 66]. Canine and human ITP patients with antibodies to the fibrinogen receptor, one of the most common ITP antibody targets, do not display such systemic reactions [14, 67]. Similarly, as demonstrated in Chapter 2, an induced canine model using an antibody to GPIIb does not result in any systemic reactions. Thus, murine models utilizing antibodies to GPIIbIIIa fail to recapitulate the naturally-occurring disease in which predominant clinical manifestations, if any, are mucocutaneous bleeding and fatigue [68]. Murine ITP models utilizing anti-GPIb antibodies do not demonstrate such systemic effects [15].

Mice provide very useful tools for studying ITP, however their hemostatic and immunologic differences from humans must be acknowledged when interpreting results garnered from murine models.

Canine Immune Thrombocytopenia

There are no examples of spontaneous primary ITP in vivarium research animals [13]. However, various domestic animals including dogs, cats, and horses do develop primary ITP [69, 70]. Of these species, primary ITP occurs most commonly in dogs. ITP is well-

characterized in dogs and appears analogous to adult ITP with a similar disease course and response to treatment [67].

Epidemiology

Thrombocytopenia is reported to the most common acquired hemostatic disorder and the most common cause of spontaneous bleeding in dogs [42]. Unfortunately, precise epidemiological data regarding the incidence of canine ITP are not available and, therefore, a direct comparison with the reported incidence of 3.3 per 100,000 per year for adult ITP cannot be made [71]. In a survey of cases presented to a United States veterinary referral hospital between 1983 and 1989, 5.2% of canine patients were thrombocytopenic and 5% of those were classified as having ITP [72]. A recent study of dogs presented to a European university clinic reported similar findings: 6.7% incidence of thrombocytopenia in the hospitalized population with 5.6% due to ITP [73]. These prevalence studies are likely vast underestimates of the number of dogs with ITP since routine complete blood counts are not commonly performed in veterinary medicine and thus ITP dogs without bleeding signs would go unrecognized and not be presented to a veterinary referral hospital. Furthermore, the classification of dogs with ITP by Botsch et al. was too stringent, requiring a positive Coombs test for inclusion and thus missing many dogs with ITP [73]. In both studies, dogs with ITP had more severe thrombocytopenia than dogs with thrombocytopenia of other causes [72, 73].

ITP affects mostly middle-age dogs, with a median age of presentation of 6 years, although it has been reported in dogs below one year of age and in elderly dogs [67, 74-76]. A high prevalence of ITP is reported in Cocker Spaniels, Miniature, Toy, and Standard Poodles, and Old English Sheepdogs [67, 74, 76-78]. These clear breed predispositions suggest that genetic factors are likely involved in the development of canine ITP. Genetic polymorphisms have been identified in genes associated with T-cell activation in human ITP, though genetic studies of this disease remain in their infancy [26, 79]. Some studies show an overrepresentation of female dogs with ITP; between 30 to 70 years, women are more often affected with ITP than men [8, 72, 76].

Diagnosis and Clinical Signs

As in people, ITP diagnosis in dogs is typically one of exclusion [80]. Other causes of thrombocytopenia like disseminated intravascular coagulation along with causes of secondary ITP such as neoplasia, drug administration, and infection must be ruled out [74]. Appropriate response to immunosuppressive therapy can be included in the diagnostic criterion [75]. Some veterinarians consider a positive antiplatelet antibody test as necessary for ITP diagnosis [76]. However, antiplatelet antibody testing is not widely available and is relatively insensitive and nonspecific [81]. Anti-platelet antibodies have also been documented in dogs with infectious diseases (Ehrlichia, Babesia, Leishmania, sepsis), neoplasia (lymphoma and histiocytic sarcoma), and chronic renal insufficiency, highlighting the lack of specificity of the test [82, 83]. Furthermore, in human ITP, platelet auto-antibodies are not detectable in up to 50% of patients [81, 84]. This may be because of assay design: some human anti-platelet antibody tests only detect antibodies against GPIIb/IIa or GPIb/IX or it may be because of there are alternate mechanisms causing the thrombocytopenia [81]. Thus, if a dog has anti-platelet antibodies, a diagnosis of primary ITP is not confirmed until all secondary causes are excluded. And, if a dog lacks anti-platelet antibodies, a diagnosis of ITP cannot be excluded. Anti-platelet antibody testing is not routinely recommended for people with ITP [80].

The major clinical manifestation of ITP is mucocutaneous bleeding. Fatigue is described in people with ITP in the absence of bleeding but is not reported dogs [68]. Dogs, like people, have heterogeneous bleeding manifestations despite similarly low platelet counts. Many dogs with severe thrombocytopenia do not bleed and the degree of hemorrhage for a given platelet count is unpredictable [67, 72]. However, since screening tests like complete blood counts are performed less frequently in veterinary than human medicine, nonclinical thrombocytopenia is often overlooked and about 70% of dogs with ITP do present with signs of mucocutaneous bleeding[67, 75, 76]. Bleeding is most commonly represented by petechiae and ecchymoses, but can also include melena, epistaxis, and hematochezia [67, 75, 76]. In human studies, 23 to 52% of adults with newly diagnosed ITP present with clinical signs of bleeding [85, 86]. Interestingly, dogs do not develop wet

purpura, a common manifestation of thrombocytopenia in people [87]. The reason for this difference is not known.

Paradoxically, despite the increased risk of bleeding in ITP, both human and canine patients with chronic ITP have also been reported to have increased risk for thromboembolism [74, 88, 89]. In one study, once their platelet counts exceeded 40,000 platelets/µl, dogs with ITP had increased maximal amplitude (MA) on thromboelastogram (TEG) tracings, which is consistent with hypercoagulability [90]. However, these dogs were all administered corticosteroids as part of their ITP therapy which can cause similar TEG alterations, so the hypercoagulability in the canine patients may be due to therapy, not the disease [90, 91]. Venous thromboembolism is reported in human patients who are not receiving steroid therapy [89].

Treatment and Prognosis

Similarities between canine and human ITP extend to response to treatment. Front-line therapy for ITP in dogs, just like in people, consists of immunosuppression with glucocorticoids [81]. Most dogs with ITP have platelet counts greater than 40,000/µl within two weeks of immunosuppressive glucocorticoid treatment [67, 92]. As in humans, IV administration of human immunoglobulin is effectively used in canine ITP when a rapid increase in platelet count is required or when patients are not responsive to corticosteroids [24, 81]. Vincristine is often administered in canine patients to stimulate platelet release from bone marrow megakaryocytes since one veterinary study demonstrated that administration of vincristine and prednisone shortens the interval to platelet recovery compared with prednisone used alone [92]. Vincristine is not routinely used as a human ITP therapy. For those human patients who do not achieve safe platelet counts with initial corticosteroid or immunoglobulin therapy, second-line therapies exist. In people, splenectomy is the most effective therapeutic option for patients with persistent thrombocytopenia with initial complete response rates of 65 to 70% [81, 93, 94]. Splenectomy has had variable success in management of canine ITP, but is not currently favored in veterinary medicine [1, 67, 77, 95]. Other second-line human ITP treatments challenge splenectomy's role as the second-line

therapy of choice for adults: rituxamib and TPO receptor-agonists [81]. Rituxamib is an anti-CD20 monoclonal antibody that targets B cells, including those producing anti-platelet antibodies, leading to transient B cell depletion [81, 96]. Rituxamib is not employed in canine ITP due to lack of cross-reactivity with canine CD20 [97]. TPO-receptor agonists increase stimulation of megakaryopoiesis which is impaired in human ITP [81]. This treatment is not used in veterinary medicine because whether TPO levels are inappropriately low is canine ITP is unknown. Also, TPO agonists might not cross-react with the canine TPO (mpl) receptor. Furthermore, there is concern that antibodies could form with chronic use of a foreign protein. However, a peptide agonist of the TPO receptor (GW395058) has been successfully used to reduce thrombocytopenic effects of carboplatin in dogs without adverse effects and might have potential future use in managing dogs with ITP [98].

Treatment strategies for ITP differ in human and veterinary medicine. Veterinarians aim to obtain normal platelet counts and only taper therapy gradually when that goal is achieved. In contrast, rather than aiming to restore normal platelet counts, treatment for chronic or refractory adult ITP is aimed at preventing life-threatening hemorrhage by achieving hemostatically safe platelet counts (above 20,000 to 30,000/µl) while minimizing adverse side effects of medication [67, 74, 99]. Treatment is considered unnecessary in newly diagnosed adults with platelet counts greater than 30,000 platelets/µl in the absence of bleeding [80]. Differences in treatment strategies do not reflect underlying differences in the disease, only differences in medical and veterinary culture. Due to these different treatment goals, dogs are usually treated for longer periods of time with glucocorticoids than are people. For this reason, unlike in human medicine, a second immunosuppressant such as cyclosporine or azathioprine is usually included in the initial treatment of canine ITP patients for steroid-sparing effects [74]. As in people, transfusion support is provided to dogs as needed to control life-threatening hemorrhage [67].

Prognosis for canine ITP varies from good to guarded with an overall mortality rate during initial hospitalization or after relapse of 10 to 30% [67, 74-76, 92]. Long-term mortality is sometimes due to treatment cost and side effects of long-term immunosuppression. Mortality is lower in human ITP. In one Danish population study, ITP

mortality risk was 2.2 that of the general population with a rate of 53.0 per 1000 person-years compared with 29.1 for members of the general population [5]. Another recent study reported a 6.6 % mortality rate for chronic ITP [80].

One negative survival predictor that has been identified in canine ITP is elevated BUN [74]. This is likely a reflection of gastrointestinal bleeding in these dogs, and thus serves as an indirect marker of serious bleeding. Interestingly, uremia is a predictor of bleeding in human ITP, but probably for different reasons. Uremia is associated with vasculitis and also with platelet dysfunction, both of which challenge the patient's thrombocytopenic state [100].

Immunopathogenesis

In people autoantibodies are often directed against some component of the fibrinogen receptor (GPIIbIIIa) or, less frequently, the von Willebrand factor receptor (GPIb/IX) [14, 81, 101]. One study in dogs detected anti-platelet antibodies against GPIIb and/or IIIa, however the study did not look for antibodies against other platelet glycoproteins [78]. The prevalence of antiplatelet antibodies is similar in dogs (77%) and people (60%) with ITP and in both species, the majority of these antibodies are IgG [70, 78, 83, 102, 103]. Whether the target antigen of the autoantibodies determines disease severity or response to treatment remains controversial. Human patients with antibodies to both GPIb and GPIIb/IIIa may have more severe disease than those with antibodies to just GPIIb/IIIa [101, 104]. This remains to be explored in canine patients. In people, the spleen is the primary site for antibody production and also for clearance of immunoglobulin-coated platelets, except in a minority of patients where hepatic clearance predominates [105-107]. The site of antibody production is not known in dogs, but clearance appears to occur via both liver and spleen [108]. An *ex vivo* study in dogs showed that canine platelets coated with antiplatelet antibodies were phagocytosed by neutrophils; but the role of neutrophils *in vivo* platelet destruction in dogs with ITP is unknown [109]. In people, macrophages are thought to be the most important antigen presenting and phagocytic cell in ITP [81]. Splenic macrophages take up opsonized platelets via their FcγRI and can induce proliferation of GPIIb/IIIa reactive T cells [81, 110].

Our understanding of the pathogenesis of ITP in canine patients that leads to the end-result of autoantibody formation is, unfortunately, limited. Briefly we summarize what is known about the immunopathogenesis of human ITP literature, all of which remains to be studied in canine ITP. Dysfunctional cellular immunity is important in ITP pathophysiology and T-cell abnormalities in chronic ITP appear central to the pathogenesis of the disorder [29, 111]. Several human ITP studies have shown the presence of autoreactive T cells that recognize autologous platelet antigens and are responsible for driving the production of platelet reactive autoantibodies by B cells [111-113]. Interestingly, one study demonstrated that autoreactive T cells directed against GPIIb/IIIa are present in the blood of all healthy people, emphasizing the importance of functional peripheral tolerance mechanisms to prevent these autoreactive T cells from being activated [81, 114].

One way that autoreactive T cells may go unchecked is through altered regulatory T cells. CD4+ CD25+ regulatory T cells (Tregs) are the lymphocytes that are essential for maintaining peripheral tolerance [29]. Human ITP patients have decreased number and suppressive activity of their Tregs compared to healthy controls [30]. Interestingly, different therapies that raise platelet counts in ITP such as dexamethasone, rituximab, intravenous immunoglobulin, and even TPO receptor agonists may do so through normalization of the Tregs [29, 35, 115, 116]. Treg numbers or activity in canine ITP have not yet been characterized.

Adult ITP is the manifestation of a type-1 polarized immune response with elevated Th1 cytokines (IL-2, IFN-γ) and decreased Th2 cytokines (IL-4 and IL-5)[111, 117]. IL10 serum levels have been reported to be reduced in ITP patients with active disease compared with those that are stable clinically or with healthy controls [111, 118]. Another study did not recognize such a difference in IL10 levels between patients and controls [119]. ITP patients also tend to have TGF-β1 levels that vary inversely with disease activity [118]. Interestingly, lower platelet counts have been found to correlate with higher Th1/Th2 ratios [111]. Additionally, Th17 cells along with the pro-inflammatory and Th17 promoting cytokine IL17 are significantly increased in human ITP patients and likely play a pathogenic role in the

disease [26, 120, 121]. To date the cytokine profile of canine ITP has not been assessed. This must be performed in order to confirm that canine ITP is indeed analogous to adult ITP.

Cytotoxic T cells against autologous platelets have been reported in human studies of chronic ITP and are speculated to play a part in some patients with ITP, perhaps those who do not have detectable autoantibodies [10, 81]. Again, presence of cytotoxic T cells against autologous platelets has not yet been assessed in canine ITP.

Whether decreased platelet production is a component of canine ITP pathogenesis as it is in human ITP is unclear [81]. In one study, bone marrow aspirates of dogs with ITP show decreased numbers of megakaryocytes in about 1/3 of dogs, suggesting that ineffective thrombopoiesis may play a role in canine ITP [67, 77]. Anti-platelet antibodies have been shown to bind to and damage canine megakaryocytes in the bone marrow of ITP patients [122]. However, another study found an increase in megakaryopoiesis in all dogs with suspected primary ITP [82]. Studies have also reported increased reticulated and large platelets in circulation of canine ITP patients, suggestive of a robust production of new platelets [67, 72, 83]. Megathrombocytosis in a thrombocytopenic dog has been determined to be a good predictor of a normal or hyperplastic bone marrow megakaryocyte population (adequate bone marrow response) [123]. There are conflicting studies that report microthrombocytosis in dogs with ITP [82, 124]. Thus, it is unknown whether there ineffective platelet production in ITP dogs. In human ITP there is clear evidence that platelet production is inadequate. *In vitro* experiments have shown that autoantibodies from human ITP patients can suppress megakaryocyte production and maturation [81, 125]. Circulating TPO is normal to minimally elevated in people with ITP, which in inappropriate in the face of thrombocytopenia [11]. More studies are required to better evaluate platelet production in canine ITP, namely measuring circulating TPO in dogs.

Applications

Our current knowledge of canine ITP suggests that it is highly analogous to adult ITP. However, more exploration of the immunopathogenesis of the disease is required to understand the extent of the analogy. Regardless, there is clearly a great opportunity to learn

from this spontaneous companion animal model. One potential powerful application of the spontaneous dog model is in investigating the genetics of ITP. Familial ITP is reported in people and a heritable component of the disease is suspected [126]. Dogs with spontaneous disease provide an excellent resource for family-based genetic linkage studies. These are challenging to perform in human ITP as familial cases of ITP are rare [79]. Linkage studies would be much easier to perform in dogs where there is a documented breed-predisposition for ITP combined with the availability of the complete dog genome sequence and ready accessibility of purebred pedigrees that would identify affected and unaffected littermates [79].

There is precedent for using spontaneous canine models in hemostasis research. Canine model of hemophilia A, hemophilia B, Glanzmann's thrombasthenia, and von Willebrand disease have high phenotypic similarity with the corresponding human disease [43, 127, 128]. Response to therapy in these canine models is similar to that of the human patient and these models have been central in the development of treatment modalities for the corresponding human disease [43, 127].

Induced Models of Canine ITP

Given that spontaneous canine ITP is analogous to adult human ITP, development of an induced canine model would provide a model with ready translatability to the spontaneous disease in both species. The model would not have the confounding co-morbidities often present in clinical patients and could be studied in the absence of treatment effects. Studies such as assessing TEG values during platelet recovery in the absence of glucocorticoids could be performed in model dogs with induced ITP, whereas dogs with spontaneous disease could not be deprived of such therapy.

Historically there are a few induced primary canine ITP models described in the literature, but none of them have been well-characterized [108, 129, 130]. In these models, thrombocytopenia was induced to investigate one target question; characterization of the model was not the study goal [129]. Furthermore two of these three studies employed rabbit antiserum to induce thrombocytopenia which may induce nonspecific effects related to other

components of the serum [129, 130]. Use of platelet specific monoclonal antibodies avoids potential confounders associated with anti-platelet antisera such as batch variability and nonspecific effects from other serum-derived proteins [13].

Hosono et al. induced thrombocytopenia in dogs using NNKY2-11, a murine monoclonal antibody to GPIIb/IIIa in order to determine the kinetics of platelets in thrombocytopenia [108]. The authors infused dogs with radiolabeled platelets prior to inducing thrombocytopenia and demonstrated radiolabeled platelet accumulation in the spleen and liver following antibody administration, indicating that that opsonized platelets are cleared by these organs [108]. In similar studies in human ITP patients, platelets are predominantly sequestered in the spleen except in a minority of patients or those patients with failed splenectomies where hepatic clearance predominates [81, 105]. Hosono's passive canine model had great potential, however, the antibody is no longer available (Makoto Hosono, personal communication, May 12, 2009). Furthermore, NNKY2-11 blocks ADP and collagen-dependent aggregation of canine platelets making it both a thrombocytopenia and thrombopathia-inducing antibody [108]. Thus, if the model had been further developed, it would be challenging to know whether to attribute NNKY2-11's *in vivo* effects to thrombopathia or thrombocytopenia or both.

Animal models have revealed much of what we know today about basic immunology of ITP, however many important questions remain with regard to pathogenesis such as why patients present with variable disease phenotypes such as heterogeneous bleeding and how genetics predispose patients to ITP development. Both induced and spontaneous canine models hold great promise in answering these questions.

Clinical Diversity of Bleeding Phenotype in Immune Thrombocytopenia

As a sequel of thrombocytopenia, patients with ITP may develop spontaneous hemorrhage that can be life-threatening. However, other patients with equally low platelet counts do not bleed [131]. Although bleeding is more likely at platelet counts below 30,000 platelets/µl, some patients with higher platelet counts bleed significantly, while many with platelet counts below 5,000 platelets/µl do not bleed at all [1, 74, 87, 92, 132, 133]. Platelet count does not

correlate with bleeding risk and platelet number alone is an insufficient predictor of bleeding tendency [134, 135]. The majority of patients with severe thrombocytopenia do not develop spontaneous bleeding [132]. This heterogeneity of bleeding in thrombocytopenic patients suggests that although severe thrombocytopenia is permissive for spontaneous bleeding it is not sufficient and factors besides thrombocytopenia contribute to bleeding tendency [132].

The inability to predict which ITP patients will bleed presents a clinical conundrum. The treatment of ITP is not benign and it would be ideal to only treat those patients at risk of bleeding, while monitoring the others. Indeed, in recent years, the threshold platelet count at which treatment for ITP is initiated has decreased from 50,000 to 30,000 platelets/μl [80, 136]. If bleeding risk could be predicted, this threshold could be lowered even further. Additionally, if factors that contribute to thrombocytopenic bleeding were identified, they could be exploited pharmacologically for treatment of bleeding in thrombocytopenia.

As death due to hemorrhage is rare in ITP patients, in many patients the morbidity and mortality of treatment-related side effects exceed the problems of ITP itself [8]. Immunosuppression with glucocorticoids is the mainstay of treatment for ITP, with splenectomy being recommended for those patients who have failed front-line therapy for ITP. In one study, patients severe ITP refractory to treatment had a mortality risk four times that of the general population, and mortality was equally due to fatal bleed (cerebrovascular hemorrhage) as it was to secondary infection from ITP treatment [137]. Splenectomized patients have an increased risk for overwhelming infections and septicemia [80, 81, 138]. ITP patients with platelet counts over 30,000 post-splenectomy experienced no mortality from bleeding. Deaths (5.3%) were instead due to complications from ITP treatment [80, 139]. Hematologic malignancies are also increased among ITP patients, which may be the result of long-term exposure to immunosuppressive medications [5].

Side-effects of corticosteroids include weight gain, anxiety, hypertension, immunosuppression, diabetes, and osteoporosis [140]. A study of health-related quality of life (HRQOL) showed that the HRQOL of adult ITP patients is significantly worse than that of the general population and that of patients with arthritis and cancer [141]. In general, patients are more concerned with their side-effects of corticosteroid treatment than they are

with their risk of bleeding [140]. The treatment is often considered worse than the disease [140].

Given the complications of treating ITP, it would be ideal to aggressively treat only those patients at risk of bleeding. However, the reasons for a heterogeneous bleeding phenotype in ITP are unknown and bleeding predictors have yet to be identified. The role of platelets in preventing thrombocytopenic-bleeding is two-fold. The first, traditionally accepted platelet function is that of primary hemostasis, the formation of the platelet plug. The second platelet function, maintenance of vascular endothelial barrier integrity, is less understood.

Two theories may explain the differential bleeding observed in thrombocytopenia:

1. Differences in platelet hemostatic function may occur in the thrombocytopenic patients.
2. Differences in vascular integrity may occur in the thrombocytopenic patients.

In the latter, alterations in vascular integrity may be due to differences in platelet vascular stabilizing function of the remaining platelets or due to the presence of conditions such as inflammation that disrupt vascular integrity.

There are other factors that may contribute to bleeding in thrombocytopenia, but these are usually easy to identify and variable bleeding occurs in the absence of these factors. These factors include uremia, hypoalbuminemia, coagulopathy, leukocytosis, and iatrogenic factors like drugs [142].

Platelet Primary Hemostatic Function

The classical role of platelets is to mediate primary hemostasis, or the formation of the platelet plug. Faced with vascular damage, platelets begin the process of primary hemostasis: subendothelial adhesion, granule release, aggregation, and finally platelet plug formation [143]. Formation of the platelet plug is all that is necessary to arrest bleeding from damaged small vessels [144].

Without sufficient platelets to form a platelet plug at sites of vascular injury, bleeding can occur. Variability in platelet primary hemostatic function could lead to variability in

bleeding tendency in the face of thrombocytopenia. If a patient has platelets with lower functionality in conjunction with thrombocytopenia, that patient might have higher bleeding risk than a patient with a similar platelet count but more robust platelet function. The primary hemostatic capacity of circulating platelets can vary. Platelets circulate in a spectrum of different activation states from quiescent to highly activated and differ in their *ex vivo* reactivity to agonists. Inter-individual differences exist in platelet hemostatic function and reactivity have been documented even in healthy people [145].

Platelet function in thrombocytopenia has not been extensively evaluated and the contribution of platelet function to bleeding risk in thrombocytopenic patients is not well understood [132]. Methodology has been a major limiting factor in understanding platelet function in ITP as many tests of platelet function are impacted by platelet count. Light aggregometry, for example, the gold standard test for platelet function evaluation, cannot be used if platelet count is less than 100,000/μL [146]. Flow cytometry is one of the few tools that can evaluate platelet function separately from platelet number [132].

Many studies have shown that autoantibodies present in ITP serum can enhance or inhibit normal platelet function, depending on the antibody [147, 148]. However, these studies have not investigated the function of ITP patients' own platelets [149]. Of the few flow cytometric studies that have been performed directly on ITP patients' platelets, results have been confusing and contradictory. There are conflicting descriptions of the degree of circulating (unstimulated) platelet activation state in ITP. Some investigators have described increased platelet activation in ITP, while others have not detected platelet activation [117, 132, 149-152]. Even amongst the studies that report increased platelet activation in ITP, the relevance of platelet activation state to bleeding risk is disputed [132, 149].

Traditionally, the larger, younger platelets of ITP patients have been viewed as more functional than normal platelets. Platelet aggregation has been shown to be positively correlated with platelet volume in healthy people with normal platelet counts [153]. Indeed, several studies have shown that thrombocytopenic patients without bleeding had higher MPVs [134, 135]. The young, larger platelets in ITP patients have been speculated to account for why ITP patients seem to bleed less than patients with thrombocytopenia due to bone

marrow failure [132, 153-155]. However, these assumptions have never been validated and do not explain why some ITP patients do bleed severely.

Interestingly, Psaila and colleagues found that ITP patients with bleeding had lower platelet reactivity in response to agonists than those with no bleeding [132]. This implies that platelet reactivity protects against bleeding. However, the same study also found the opposite to be true in thrombocytopenic patients with acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS). In these patients, platelet reactivity was higher in those that bled than in those that did not [132]. The investigators could not explain these conflicting findings. Similarly, platelets of patients with chemotherapy induced thrombocytopenia (CIT) are less reactive than platelets of ITP patients. This may explain why severely CIT patients have more gastrointestinal, urinary, and pulmonary hemorrhage than ITP patients [156]. However, it does not explain why ITP patients have more cutaneous and oral bleeding [156].

Some of the conflicting reports regarding platelet function in ITP probably reflect diversity in ITP autoantibodies. Some autoantibodies in ITP can bind to receptors essential for adhesion or aggregation, impairing platelet function while others bind to sites that induce platelet activation [157-161]. The diversity in circulating autoantibodies in ITP may prevent any overarching conclusions regarding platelet activation state in ITP. Despite the heterogeneity of the ITP syndrome, a relationship between platelet hemostatic function and bleeding symptoms could still be determined.

Finally, high concentrations of circulating platelet derived-microparticles (PMPs), which are associated with activated platelets and may also facilitate coagulation, have been shown to protect against bleeding in ITP [162].

Differences in hemostatic platelet function may explain differences in bleeding phenotypes in ITP, but the contradictions in current literature underscore the need for further investigations of the relationship between platelet hemostatic function and thrombocytopenic bleeding.

Platelet Maintenance of Vascular Integrity

In addition to the primary hemostatic role of platelets, it is now recognized that platelets are essential mediators of vascular integrity, supporting the semi-permeable function of the endothelium [50]. Platelets stabilize the endothelial barrier, preventing vascular permeability and erythrocyte extravasation. Indeed, petechial hemorrhage, the classic presentation of thrombocytopenia, has been described microscopically as extravasation of red blood cells at interendothelial cell junctions in the absence of trauma [143]. As with the primary hemostatic function of platelets, variability in platelet vascular-stabilizing function could account for the variability in bleeding phenotype in thrombocytopenia.

The details of the vascular stabilizing function of platelets are largely undefined. However, the "nurturing" effect of platelets on the endothelium was recognized as early as 1969 when Gimbrone et al. demonstrated that platelets maintained microvascular barrier function in isolated perfused canine thyroid glands [163]. When perfused with platelet poor plasma in contrast to platelet rich plasma, endothelial degeneration was observed in the thyroid glands and vascular permeability increased with extravasation of fluid, plasma proteins, and erythrocytes [163].

Interestingly, platelet stabilization of the endothelium has been shown to occur with less than 7,100 platelets/µl blood volume/day, which is too low for statistically meaningful platelet-platelet hemostatic interactions [164-166]. This suggests that platelets sustain vascular integrity by a mechanism discrete from the their hemostatic function [165]. Several mechanisms have been proposed by which platelets may support the vascular endothelium. These include physically blocking gaps in the endothelial lining, maintaining endothelial ultrastructure such as the integrity of endothelial cell-cell junctions, and counteracting vascular damaging effects of leukocytes [50].

Vascular permeability is determined by a complex interaction of inter-endothelial junctions, caveolae, vesiculo-vacuolar organelles (VVOs), and the endothelial glycocalyx [167]. Platelets have been proposed to reduce vascular permeability through maintenance of inter-endothelial cell adherens junctions (AJs) [143]. Thrombocytopenic bleeding typically occurs from capillaries and postcapillary venules of the skin and mucosa, where AJs are the

most abundant intercellular connection [143]. AJs are dynamic endothelial cell-cell adhesion complexes composed of homotypic interactions of membrane-spanning vascular endothelial (VE)-cadherin molecules (Figure 1). These cadherins are coupled intracellularly to β-catenins, which in turn associate indirectly with actin, stabilizing cell-cell junctions [168]. Endothelial permeability is regulated in part by the opening and closing of AJs. Phosphorylation of components of the AJ alters the integrity of the junctional machinery [143]. The inflammatory cytokine, TNFα, for example, induces tyrosine phosphorylation of VE-cadherin, leading to disassembly of the AJ complex [169-171]. AJ complex disassembly results in increased vascular permeability, and, potentially, if cell-cell gaps are large enough, red cell extravasation to tissues [143].

Ultrastructural studies have provided conflicting evidence as to the importance of AJs in the mechanism of platelet vascular barrier maintenance. Thrombocytopenic guinea pigs have been shown to develop widened endothelial intercellular junctions and disrupted adjacent endothelial basement membrane that in turn allow red cells to extravasate [172]. Similarly, when venous pressure was increased in cremaster muscles (via constriction) of thrombocytopenic but not normal rats, colloidal carbon and erythrocytes could be seen within inter-endothelial cell junctions [173]. These findings suggest that platelets might support the endothelial barrier specifically by maintaining intercellular junctions [172]. Yet others have found that red cells passed through the cytoplasm of vascular endothelial cells during thrombocytopenia [174].

In a widely referenced paper, Kitchens and Weiss demonstrated thinning and fenestration of the capillaries and post-capillary venules of thrombocytopenic rabbits [175]. These changes correlated with an increased in microvascular permeability to the contrast agent Thorotrast within 6 hours of platelet depletion [175, 176]. No junctional changes were observed in this study and Thorotrast was believed to be transported across endothelial cells via vesicles [175]. Kitchens and Pendergast later described similar ultrastructural abnormalities in the capillary endothelium of human ITP patients, again with absent junctional changes [177]. Still other studies have not been able to replicate the findings of Kitchens et al. and have found no ultrastructural changes in capillaries associated with

thrombocytopenia in hamsters, guinea pigs, rabbits, or rats [178, 179]. Some of these animals may not have been severely thrombocytopenic enough to enable thrombocytopenia-induced endothelial changes.

Thus, ultrastructural evidence for platelets effecting vascular maintenance via endothelial junctions is conflicting. It is also possible that when structural changes in endothelial junctions are absent, functional changes are still present.

Studies investigating the mechanisms by which platelets maintain vascular integrity have suggested that the supportive role of platelets is mediated by factors released by platelets. Experiments with cultured endothelial cells have shown that platelet lysates can replicate the ability of platelets to decrease permeability [50, 180]. This could be explained by platelets releasing soluble factors or platelet-derived microparticles that maintain vascular integrity [50]. Given the low level of platelets required to provide endothelial stabilization and the overall surface area of capillary beds, each platelet stabilizes several hundred microns of capillary bed length [164]. Platelets releasing vasoactive mediators that exert a paracrine effect on the endothelium is consistent with this geometric requirement.

Platelets release and/or generate over three hundred proteins and small molecules [181]. Several of these molecules have potential functions in maintaining the endothelial barrier, including sphingosine 1-phosphate (S1P) [182], angiopoietin-1 (Ang1) [183], and endostatin [184] (Figure 1). The relative importance of these factors or others to the barrier-enhancing effect of platelets remains unknown [50].

S1P is a biologically active lipid that is released upon platelet activation [185, 186]. S1P induces reorganization of the endothelial cytoskeleton into a strong cortical actin ring, and leads to the assembly and stabilization of the AJ complex [182]. AJ stabilization tightens the endothelial barrier against paracellular fluid and solute translocation [187, 188]. *In vivo*, S1P reduces vascular leakage in murine and canine acute lung injury models, preventing associated pulmonary edema [189].

Ang1 is another endothelial-stabilizing factor localized in platelets and released on platelet stimulation[190]. Ang1 decreases the baseline phosphorylation of VE-cadherin, increasing VE-cadherin's association with β-catenin, and strengthening AJs [191]. *In vivo*,

transgenic overexpression of Ang1 protects blood vessels against plasma leakage in mice challenged with mustard oil or vascular endothelial growth factor (VEGF) [183, 192].

Endostatin, stored within α granules of platelets, is released upon thrombin ligation of PAR4 receptors [61, 184, 193, 194]. Endostatin's vascular stabilizing effects appear to be limited to opposing vascular permeability induced by other mediators, without having obvious vasomodulatory effects alone [184]. *In vivo*, endostatin decreases Evans blue dye leakage from retinas in mouse eyes treated with VEGF by reducing vasopermeability [184].

Although it is clear that these platelet-derived vasoactive mediators can stabilize the vasculature, their role in preventing thrombocytopenic hemorrhage is unknown.

A correlation between inflammation, thrombocytopenia, and hemorrhage has recently been demonstrated by Goerge et al. (Figure 1B) [166]. Goerge et al. observed that only mice confronted with concomitant thrombocytopenia and inflammation bled [166]. Since inflammation disrupts the vascular barrier, the relationship between thrombocytopenia, inflammation, and hemorrhage gives further evidence for the importance of platelets in barrier maintenance. Highlighting the difference in the platelet vascular "nurturing" function and the platelet hemostatic function, murine platelets that are defective in traditional platelet hemostatic functions (aggregation and adhesion) can still effect barrier stabilization and correct inflammatory thrombocytopenic hemorrhage [166]. The work of Goerge et al. also contradicted previous hypotheses that the anti-permeability effects of platelets might be purely mechanical, with platelets filling gaps in the endothelial lining [195]. Using a dorsal skinfold chamber, these investigators observed that platelets could protect against bleeding in inflamed skin vessels by rolling through the vessels without forming platelet plugs. Again, this suggests that platelets effect their vascular stabilizing function through a process distinct from primary hemostasis [166]. Of note, less than 5% of the normal murine platelet count is needed to exert the vascular protective action of platelets during inflammation [50, 166].

Recent work suggests that inflammatory thrombocytopenic hemorrhage may require both inflammation-induced vascular permeability and damage to the vessel by the leukocytes themselves [196]. Ho-Tin-Noé et al. recently found that inflammatory thrombocytopenic hemorrhage in mice only occurs in the presence of neutrophils; it does not occur when

neutrophils have been depleted or their chemotactic ability inhibited [196]. This group also showed that while VEGF, a pro-permeability factor that disrupts AJs, caused increased vascular leakage of Evans blue dye in thrombocytopenic mice, it did not cause any bleeding [196]. Thus, although disruption of AJs can lead to fluid and protein leakage into tissues, this does not appear to be sufficient to cause bleeding. Additional damage to the endothelial basement membrane may also be required to allow red blood cells to extravasate. Leukocytes likely cause this damage to the basement membrane, explaining why inflammatory cells must be present for bleeding to occur [50]. Platelets may prevent inflammatory hemorrhage by inhibiting or healing leukocyte-mediated vascular injury [50].

The platelet-derived vasoactive mediator S1P may also have anti-inflammatory function. S1P reduces neutrophil expression of the CXC chemokine receptor-1 and subsequent chemotaxis in response to IL-8 [197]. S1P also reduces cytokine-induced leukocyte endothelial adhesion [198]. Thus, S1P has the potential to mediate the platelet endothelial "nurturing" effect directly via barrier stabilizing effects and indirectly via anti-inflammatory effects. Alternatively, platelet granules contain factors like tissue inhibitors of metalloproteinases and reactive oxygen species scavengers that may be responsible for counteracting injurious effects of leukocytes [50, 199, 200]. Platelets are also important in the transcellular synthesis of lipoxins which can reduce neutrophil-mediated tissue injury [201].

A correlation between inflammation and hemorrhage in ITP has not been investigated in clinical patients. However, a recent ITP case report described petechial hemorrhage in the face of mild thrombocytopenia only in a region of local inflammation secondary to sunburn [202]. In this patient, it appears that inflammation may have induced thrombocytopenic bleeding [50, 202]. The observation that bleeding is first seen in the skin and mucosa in clinical thrombocytopenic patients also suggests a relationship between inflammation and bleeding. These organs likely have subclinical local inflammation because of their exposure to pro-inflammatory microbial and environmental irritants [166].

Briefly we note one scenario in which platelets actually drive vascular permeability: in the vasculature of the inflamed rheumatoid arthritis joints [203, 204]. Increased joint

vasculature permeability occurs via platelet-derived dense granule serotonin [203]. Serotonin enhances the formation of interendothelial cell gaps in joints in arthritis models and permeability in inflamed joints is abrogated if platelets are absent [204]. However, the effects of serotonin appear to be vascular bed and species-specific [205]. In rats and mice, serotonin increases vascular permeability and the formation of endothelial gaps [206-208]. In contrast Shepro et al. have shown that serotonin infusion can rescue hamsters from thrombocytopenia-induced hemorrhage [209]. Yet other *in vitro* studies have shown that endothelial monolayer permeability was not influenced by the application of serotonin [180, 205]. These different findings may be explained by the fact that there are 13 different serotonin receptor subtypes that can mediate distinct functions and the pattern of receptor expression in a given vascular bed can influence serotonin's permeability effects [204].

The serotonin example indicates that contributions of platelet-derived factors to the maintenance of the vascular barrier may vary with the experimental animal model or the location of the vascular bed or the source of endothelial cells being studied [50, 205]. Additionally, platelets can respond to their agonist milieus, selectively releasing granules containing pro (VEGF) and antiangiogenic (endostatin) peptides in response to different agonists [61, 204].

Although mechanistic details remain to be established, it is clear that platelets support the vascular barrier function of the endothelium and that this function is most important in the face of vascular-disrupting factors like inflammation. Furthermore, these vascular-protective effects are distinct from platelets' primary hemostatic ability. Variability in platelet vascular stabilizing function or the degree of local or systemic inflammation may account for variability of bleeding in ITP patients.

Thesis Overview

In this study we aimed to develop a novel induced canine model of immune thrombocytopenia that would be highly translational in that the dog develops spontaneous ITP analogous to adult ITP. Using this model we set out to investigate the relative roles of

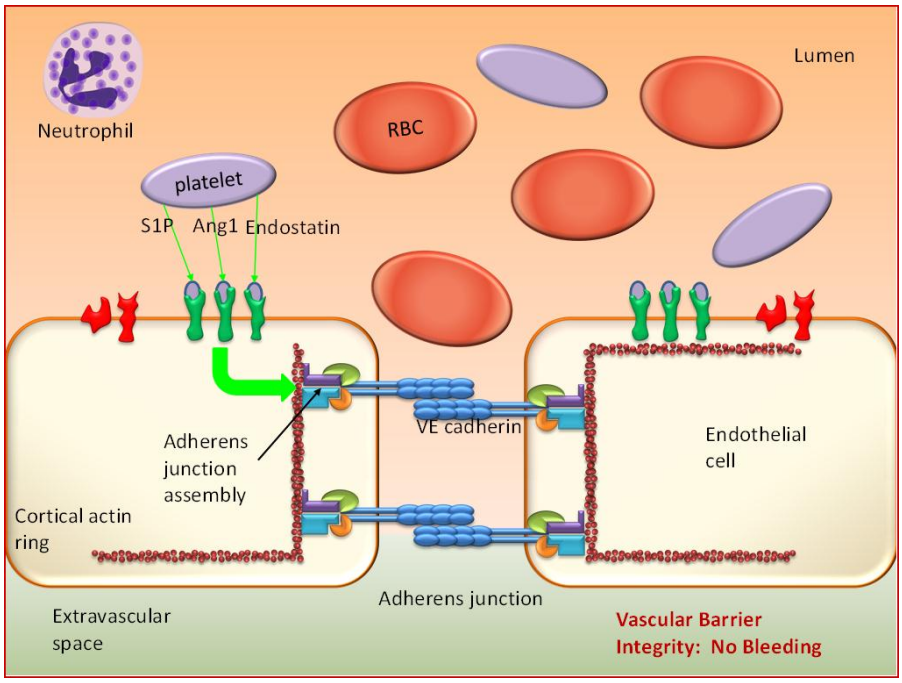
platelet hemostatic function and platelet vascular stabilizing function in bleeding in immune thrombocytopenia.

Table 1. Comparisons between human, canine, and murine platelets and advantages of dogs compared to mice as an animal model of ITP.

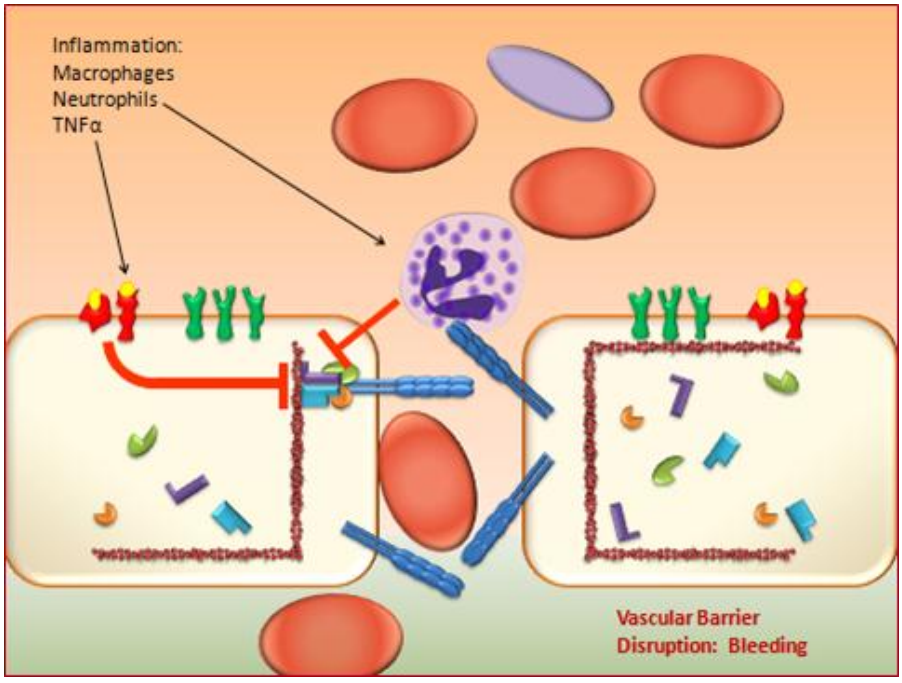
	Humans	Mice	Dogs
Spontaneous ITP?	Yes	No [13]	Yes [67]
Platelet counts	150-400 x 10^3/µl	1 million/µl [44]	190-468 x 10^3/µl
Platelet volumes	7.5-10 fl	4.7 ± 0.3 fl [44]	7.9-13.8 fl
Platelet protease activated-receptors (PAR)	PAR 1 and 4 [44]	PAR 3 and 4 [44]	PAR 1 and 4 [60]
Blood volume that can be safely sampled	N/A	0.2 ml [51] (25 g mouse)	85 ml [51] (10 kg dog)
Survival studies possible?	N/A	No	Yes
Result of ITP models utilizing antibodies to the fibrinogen receptor (GPIIbIIIa)	Variable bleeding, no systemic signs other than fatigue (with spontaneous disease)	Acute systemic reactions, hypothermia, incoordination [15]	Variable bleeding, no systemic signs

Figure 1. Adherens Junctions determine vascular permeability. A. Adherens Junctions (AJs) between adjacent endothelial cells maintain the vascular integrity of the endothelium. Homotypic interactions of (VE) cadherins from adjacent endothelial cells comprise AJs. Cytoplasmic tails of cadherins interact with an intracellular complex including β-catenin, actin binding proteins, and ultimately the actin cytoskeleton, which stabilizes the AJs. Platelet-derived factors like sphingosine 1-phosphate (S1P), endostatin, and angiopoietin-1 (Ang1) signal through their receptors to maintain AJ stability. B. In the face of thrombocytopenia, platelet-vascular stabilizing factors are reduced and AJ disassembly can occur. Inflammatory cells and cytokines can directly disrupt the AJs. Disruption of AJs results in increased vascular permeability and possibly red cell extravasation. Figure is adapted from Nachman and Rafil [143].

A.


Lumen
Neutrophil
RBC
platelet
S1P
Ang1
Endostatin
Adherens junction assembly
VE cadherin
Endothelial cell
Cortical actin ring
Adherens junction
Extravascular space
Vascular Barrier Integrity: No Bleeding

B.


Inflammation:
Macrophages
Neutrophils
TNFα
Vascular Barrier Disruption: Bleeding

References

1. Provan, D., et al., *International consensus report on the investigation and management of primary immune thrombocytopenia.* Blood, 2010. **115**(2): p. 168-86.

2. Terrell, D.R., et al., *Prevalence of primary immune thrombocytopenia in Oklahoma.* Am J Hematol, 2012. **87**(9): p. 848-52.

3. Saleh, M.N., M. Fisher, and K.M. Grotzinger, *Analysis of the impact and burden of illness of adult chronic ITP in the US.* Curr Med Res Opin, 2009. **25**(12): p. 2961-9.

4. Danese, M.D., et al., *Cost and mortality associated with hospitalizations in patients with immune thrombocytopenic purpura.* Am J Hematol, 2009. **84**(10): p. 631-5.

5. Norgaard, M., et al., *Long-term clinical outcomes of patients with primary chronic immune thrombocytopenia: a Danish population-based cohort study.* Blood, 2011. **117**(13): p. 3514-20.

6. Rodeghiero, F., et al., *Standardization of terminology, definitions and outcome criteria in immune thrombocytopenic purpura of adults and children: report from an international working group.* Blood, 2009. **113**(11): p. 2386-93.

7. Arnold, D.M., *Immune thrombocytopenia: getting back to basics.* Am J Hematol, 2012. **87**(9): p. 841-2.

8. Gernsheimer, T., *Epidemiology and pathophysiology of immune thrombocytopenic purpura.* Eur J Haematol Suppl, 2008(69): p. 3-8.

9. Harrington, W.J., et al., *Demonstration of a thrombocytopenic factor in the blood of patients with thrombocytopenic purpura.* J Lab Clin Med, 1951. **38**(1): p. 1-10.

10. Olsson, B., et al., *T-cell-mediated cytotoxicity toward platelets in chronic idiopathic thrombocytopenic purpura.* Nat Med, 2003. **9**(9): p. 1123-4.

11. Chang, M., et al., *Tissue uptake of circulating thrombopoietin is increased in immune-mediated compared with irradiated thrombocytopenic mice.* Blood, 1999. **93**(8): p. 2515-24.

12. McMillan, R., et al., *Suppression of in vitro megakaryocyte production by antiplatelet autoantibodies from adult patients with chronic ITP.* Blood, 2004. **103**(4): p. 1364-9.

13. Semple, J.W., *Animal models of immune thrombocytopenia.* Annals of Hematology, 2010. **89**(Suppl 1): p. S34-S44.

14. Beardsley, D.S. and M. Ertem, *Platelet autoantibodies in immune thrombocytopenic purpura.* Transfus Sci, 1998. **19**(3): p. 237-44.

15. Nieswandt, B., et al., *Identification of critical antigen-specific mechanisms in the development of immune thrombocytopenic purpura in mice.* Blood, 2000. **96**(7): p. 2520-7.

16. Hansen, R.J. and J.P. Balthasar, *Pharmacokinetics, pharmacodynamics, and platelet binding of an anti-glycoprotein IIb/IIIa monoclonal antibody (7E3) in the rat: a quantitative rat model of immune thrombocytopenic purpura.* J Pharmacol Exp Ther, 2001. **298**(1): p. 165-71.

17. Arnott, J., P. Horsewood, and J.G. Kelton, *Measurement of platelet-associated IgG in animal models of immune and nonimmune thrombocytopenia.* Blood, 1987. **69**(5): p. 1294-9.

18. Chieffi, O., et al., *[Action of cortisone on neonatal thrombopenia provoked in the guinea pig with heterologous immune serum]*. Atti Accad Fisiocrit Siena Med Fis, 1964. **13**(1): p. 463-70.

19. Rossolini, A., I. Pieri, and F. Malandrini, *[On the action of cortisone in thrombopenia induced in the guinea pig with heterologous immune serum. Note I]*. Riv Clin Pediatr, 1960. **65**: p. 238-44.

20. Nakeff, A. and K.J. Roozendaal, *Thrombopoietin activity in mice following immune-induced thrombocytopenia.* Acta Haematol, 1975. **54**(6): p. 340-44.

21. Dassin, E., et al., *Partial purification of a thrombocytopoiesis stimulating factor present in the serum of thrombocytopenic rats.* Acta Haematol, 1983. **69**(4): p. 249-53.

22. McDonald, T.P. and G.D. Kalmaz, *Effects of thrombopoietin on the number and diameter of marrow megakaryocytes of mice.* Exp Hematol, 1983. **11**(2): p. 91-7.

23. McMillan, R., *The pathogenesis of chronic immune thrombocytopenic purpura.* Semin Hematol, 2007. **44**(4 Suppl 5): p. S3-S11.

24. Bianco, D., P.J. Armstrong, and R.J. Washabau, *A prospective, randomized, double-blinded, placebo-controlled study of human intravenous immunoglobulin for the acute management of presumptive primary immune-mediated thrombocytopenia in dogs.* J Vet Intern Med, 2009. **23**(5): p. 1071-8.

25. Tsubakio, T., et al., *[Mechanism of increase in platelet count by intravenous gammaglobulin in thrombocytopenic rats]*. Nihon Ketsueki Gakkai Zasshi, 1987. **50**(5): p. 1031-7.

26. Semple, J.W. and D. Provan, *The immunopathogenesis of immune thrombocytopenia: T cells still take center-stage.* Curr Opin Hematol, 2012. **19**(5): p. 357-62.

27. Crow, A.R., et al., *Mechanisms of action of intravenous immunoglobulin in the treatment of immune thrombocytopenia.* Pediatr Blood Cancer, 2006. **47**(5 Suppl): p. 710-3.

28. Samuelsson, A., T.L. Towers, and J.V. Ravetch, *Anti-inflammatory activity of IVIG mediated through the inhibitory Fc receptor.* Science, 2001. **291**(5503): p. 484-6.

29. Semple, J.W., et al., *Recent progress in understanding the pathogenesis of immune thrombocytopenia.* Curr Opin Hematol, 2010. **17**(6): p. 590-5.

30. Liu, B., et al., *Abnormality of CD4(+)CD25(+) regulatory T cells in idiopathic thrombocytopenic purpura.* Eur J Haematol, 2007. **78**(2): p. 139-43.

31. Li, X., et al., *Defective regulatory B-cell compartment in patients with immune thrombocytopenia.* Blood, 2012. **120**(16): p. 3318-25.

32. Zehnder, J.L., J.W. Semple, and P. Imbach, *Future research in ITP: an ICIS consensus.* Ann Hematol, 2010. **89**(Suppl 1): p. S19-23.

33. Chow, L., et al., *A murine model of severe immune thrombocytopenia is induced by antibody- and CD8+ T cell-mediated responses that are differentially sensitive to therapy.* Blood, 2010. **115**(6): p. 1247-53.

34. Aslam, R., et al., *Thymic retention of CD4+CD25+FoxP3+ T regulatory cells is associated with their peripheral deficiency and thrombocytopenia in a murine model of immune thrombocytopenia.* Blood, 2012. **120**(10): p. 2127-32.

35. Bao, W., et al., *Improved regulatory T-cell activity in patients with chronic immune thrombocytopenia treated with thrombopoietic agents.* Blood, 2010. **116**(22): p. 4639-45.

36. Ni, H., et al., *A novel murine model of fetal and neonatal alloimmune thrombocytopenia: response to intravenous IgG therapy.* Blood, 2006. **107**(7): p. 2976-83.

37. Oyaizu, N., et al., *(NZW x BXSB)F1 mouse. A new animal model of idiopathic thrombocytopenic purpura.* J Exp Med, 1988. **167**(6): p. 2017-22.

38. Tremblay, T., et al., *Picogram doses of lipopolysaccharide exacerbate antibody-mediated thrombocytopenia and reduce the therapeutic efficacy of intravenous immunoglobulin in mice.* Br J Haematol, 2007. **139**(2): p. 297-302.

39. Musaji, A., et al., *Enhancement of autoantibody pathogenicity by viral infections in mouse models of anemia and thrombocytopenia.* Autoimmun Rev, 2005. **4**(4): p. 247-52.

40. Asahi, A., et al., *Helicobacter pylori eradication shifts monocyte Fcgamma receptor balance toward inhibitory FcgammaRIIB in immune thrombocytopenic purpura patients.* J Clin Invest, 2008. **118**(8): p. 2939-49.

41. de Jong, M. and T. Maina, *Of mice and humans: are they the same?—Implications in cancer translational research.* J Nucl Med, 2010. **51**(4): p. 501-4.

42. Couto, C.G., *Primary hemostatic defects*, in *Small animal internal medicine*, R.W. Nelson and C.G. Couto, Editors. 2003, Mosby: St. Louis, Mo. p. 1190-1194.

43. Brooks, M.B., T. Stokol, and J.L. Catalfamo, *Comparative hemostasis: animal models and new hemostasis tests.* Clin Lab Med, 2011. **31**(1): p. 139-59.

44. Ware, J., *Dysfunctional platelet membrane receptors: from humans to mice.* Thromb Haemost, 2004. **92**(3): p. 478-85.

45. Levin, J. and S. Ebbe, *Why are recently published platelet counts in normal mice so low?* Blood, 1994. **83**(12): p. 3829-31.

46. Corash, L., *The relationship between megakaryocyte ploidy and platelet volume.* Blood Cells, 1989. **15**(1): p. 81-107.

47. Tsakiris, D.A., et al., *Hemostasis in the mouse (Mus musculus): a review*. Thromb Haemost, 1999. **81**(2): p. 177-88.

48. Ault, K.A. and C. Knowles, *In vivo biotinylation demonstrates that reticulated platelets are the youngest platelets in circulation.* Exp Hematol, 1995. **23**(9): p. 996-1001.

49. Schmitt, A., et al., *Of mice and men: comparison of the ultrastructure of megakaryocytes and platelets.* Exp Hematol, 2001. **29**(11): p. 1295-302.

50. Ho-Tin-Noe, B., M. Demers, and D.D. Wagner, *How platelets safeguard vascular integrity.* J Thromb Haemost, 2011. **9 Suppl 1**: p. 56-65.

51. Diehl, K.H., et al., *A good practice guide to the administration of substances and removal of blood, including routes and volumes.* J Appl Toxicol, 2001. **21**(1): p. 15-23.

52. Schmidt-Nielsen, K. and P. Pennycuik, *Capillary density in mammals in relation to body size and oxygen consumption.* Am J Physiol, 1961. **200**: p. 746-50.

53. Son, K.H., et al., *Inter-species hemorheologic differences in arterial and venous blood.* Clin Hemorheol Microcirc, 2010. **44**(1): p. 27-33.

54. Suo, J., et al., *Hemodynamic shear stresses in mouse aortas: implications for atherogenesis.* Arterioscler Thromb Vasc Biol, 2007. **27**(2): p. 346-51.

55. Nylander, S., C. Mattsson, and T.L. Lindahl, *Characterisation of species differences in the platelet ADP and thrombin response.* Thromb Res, 2006. **117**(5): p. 543-9.

56. Rosenblum, W.I., et al., *Some properties of mouse platelets.* Thromb Res, 1983. **30**(4): p. 347-55.

57. Leng, X.H., et al., *Platelets of female mice are intrinsically more sensitive to agonists than are platelets of males.* Arterioscler Thromb Vasc Biol, 2004. **24**(2): p. 376-81.

58. Kahn, M.L., et al., *Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin.* J Clin Invest, 1999. **103**(6): p. 879-87.

59. Nakanishi-Matsui, M., et al., *PAR3 is a cofactor for PAR4 activation by thrombin.* Nature, 2000. **404**(6778): p. 609-13.

60. Boudreaux, M.K., J.L. Catalfamo, and M. Klok, *Calcium-diacylglycerol guanine nucleotide exchange factor I gene mutations associated with loss of function in canine platelets.* Transl Res, 2007. **150**(2): p. 81-92.

61. Italiano, J.E., Jr., et al., *Angiogenesis is regulated by a novel mechanism: pro- and antiangiogenic proteins are organized into separate platelet alpha granules and differentially released.* Blood, 2008. **111**(3): p. 1227-33.

62. McKenzie, S.E. and M.P. Reilly, *Heparin-induced thrombocytopenia and other immune thrombocytopenias: lessons from mouse models.* Semin Thromb Hemost, 2004. **30**(5): p. 559-68.

63. McKenzie, S.E., et al., *The role of the human Fc receptor Fc gamma RIIA in the immune clearance of platelets: a transgenic mouse model.* J Immunol, 1999. **162**(7): p. 4311-8.

64. Bruhns, P., *Properties of mouse and human IgG receptors and their contribution to disease models.* Blood, 2012. **119**(24): p. 5640-9.

65. Lilliehook, I., A. Johannisson, and L. Hakansson, *Expression of adhesion and Fcgamma-receptors on canine blood eosinophils and neutrophils studied by anti-human monoclonal antibodies.* Vet Immunol Immunopathol, 1998. **61**(2-4): p. 181-93.

66. Nieswandt, B., et al., *Targeting of platelet integrin alphaIIbbeta3 determines systemic reaction and bleeding in murine thrombocytopenia regulated by activating and inhibitory FcgammaR.* Int Immunol, 2003. **15**(3): p. 341-9.

67. Lewis, D.C. and K.M. Meyers, *Canine idiopathic thrombocytopenic purpura.* J Vet Intern Med, 1996. **10**(4): p. 207-18.

68. Newton, J.L., et al., *Fatigue in adult patients with primary immune thrombocytopenia.* Eur J Haematol, 2011. **86**(5): p. 420-9.

69. Garon, C.L., et al., *Idiopathic thrombocytopenic purpura in a cat.* J Am Anim Hosp Assoc, 1999. **35**(6): p. 464-70.

70. Dodds, W.J., *Animal model: canine and equine immune-mediated thrombocytopenia, and idiopathic thrombocytopenic purpura.* Am J Pathol, 1977. **86**(2): p. 489-91.

71. Terrell, D.R., et al., *The incidence of immune thrombocytopenic purpura in children and adults: A critical review of published reports.* Am J Hematol, 2010. **85**(3): p. 174-80.

72. Grindem, C.B., et al., *Epidemiologic survey of thrombocytopenia in dogs: a report on 987 cases.* Vet Clin Pathol, 1991. **20**(2): p. 38-43.

73. Botsch, V., et al., *Retrospective study of 871 dogs with thrombocytopenia.* Vet Rec, 2009. **164**(21): p. 647-51.

74. O'Marra, S.K., A.M. Delaforcade, and S.P. Shaw, *Treatment and predictors of outcome in dogs with immune-mediated thrombocytopenia.* J Am Vet Med Assoc, 2011. **238**(3): p. 346-52.

75. Huang, A.A., G.E. Moore, and J.C. Scott-Moncrieff, *Idiopathic immune-mediated thrombocytopenia and recent vaccination in dogs.* J Vet Intern Med, 2012. **26**(1): p. 142-8.

76. Putsche, J.C. and B. Kohn, *Primary immune-mediated thrombocytopenia in 30 dogs (1997-2003).* J Am Anim Hosp Assoc, 2008. **44**(5): p. 250-7.

77. Williams, D.A. and L. Maggio-Price, *Canine idiopathic thrombocytopenia: clinical observations and long-term follow-up in 54 cases.* J Am Vet Med Assoc, 1984. **185**(6): p. 660-3.

78. Lewis, D.C. and K.M. Meyers, *Studies of platelet-bound and serum platelet-bindable immunoglobulins in dogs with idiopathic thrombocytopenic purpura.* Exp Hematol, 1996. **24**(6): p. 696-701.

79. Bergmann, A.K., R.F. Grace, and E.J. Neufeld, *Genetic studies in pediatric ITP: outlook, feasibility, and requirements.* Ann Hematol, 2010. **89**(Suppl 1): p. S95-103.

80. Neunert, C., et al., *The American Society of Hematology 2011 evidence-based practice guideline for immune thrombocytopenia.* Blood, 2011. **117**(16): p. 4190-207.

81. Stasi, R., *Immune thrombocytopenia: pathophysiologic and clinical update.* Semin Thromb Hemost, 2012. **38**(5): p. 454-62.

82. Dircks, B.H., H.J. Schuberth, and R. Mischke, *Underlying diseases and clinicopathologic variables of thrombocytopenic dogs with and without platelet-bound antibodies detected by use of a flow cytometric assay: 83 cases (2004-2006).* J Am Vet Med Assoc, 2009. **235**(8): p. 960-6.

83. Wilkerson, M.J., et al., *Platelet size, platelet surface-associated IgG, and reticulated platelets in dogs with immune-mediated thrombocytopenia.* Vet Clin Pathol, 2001. **30**(3): p. 141-149.

84. McMillan, R., L. Wang, and P. Tani, *Prospective evaluation of the immunobead assay for the diagnosis of adult chronic immune thrombocytopenic purpura (ITP).* J Thromb Haemost, 2003. **1**(3): p. 485-91.

85. Kuhne, T., et al., *Newly diagnosed immune thrombocytopenia in children and adults: a comparative prospective observational registry of the Intercontinental Cooperative Immune Thrombocytopenia Study Group.* Haematologica, 2011. **96**(12): p. 1831-7.

86. Aledort, L.M., et al., *Prospective screening of 205 patients with ITP, including diagnosis, serological markers, and the relationship between platelet counts, endogenous thrombopoietin, and circulating antithrombopoietin antibodies.* Am J Hematol, 2004. **76**(3): p. 205-13.

87. Psaila, B., et al., *Intracranial hemorrhage (ICH) in children with immune thrombocytopenia (ITP): study of 40 cases.* Blood, 2009. **114**(23): p. 4777-83.

88. Norgaard, M., et al., *Risk of arterial thrombosis in patients with primary chronic immune thrombocytopenia: a Danish population-based cohort study.* Br J Haematol, 2012. **159**(1): p. 109-11.

89. Severinsen, M.T., et al., *Risk of venous thromboembolism in patients with primary chronic immune thrombocytopenia: a Danish population-based cohort study.* Br J Haematol, 2011. **152**(3): p. 360-2.

90. O'Marra, S.K., S.P. Shaw, and A.M. Delaforcade, *Investigating hypercoagulability during treatment for immune-mediated thrombocytopenia: a pilot study.* J Vet Emerg Crit Care (San Antonio), 2012. **22**(1): p. 126-30.

91. Rose, L.J., et al., *Effect of prednisone administration on coagulation variables in healthy Beagle dogs.* Vet Clin Pathol, 2011. **40**(4): p. 426-34.

92. Rozanski, E.A., et al., *Comparison of platelet count recovery with use of vincristine and prednisone or prednisone alone for treatment for severe immune-mediated thrombocytopenia in dogs.* J Am Vet Med Assoc, 2002. **220**(4): p. 477-81.

93. Kojouri, K., et al., *Splenectomy for adult patients with idiopathic thrombocytopenic purpura: a systematic review to assess long-term platelet count responses, prediction of response, and surgical complications.* Blood, 2004. **104**(9): p. 2623-34.

94. Vianelli, N., et al., *Efficacy and safety of splenectomy in immune thrombocytopenic purpura: long-term results of 402 cases.* Haematologica, 2005. **90**(1): p. 72-7.

95. Feldman, B.F., P. Handagama, and A.A. Lubberink, *Splenectomy as adjunctive therapy for immune-mediated thrombocytopenia and hemolytic anemia in the dog.* J Am Vet Med Assoc, 1985. **187**(6): p. 617-9.

96. Stasi, R., et al., *Rituximab chimeric anti-CD20 monoclonal antibody treatment for adults with chronic idiopathic thrombocytopenic purpura.* Blood, 2001. **98**(4): p. 952-7.

97. Impellizeri, J.A., et al., *The role of rituximab in the treatment of canine lymphoma: an ex vivo evaluation.* Vet J, 2006. **171**(3): p. 556-8.

98. Case, B.C., et al., *The pharmacokinetics and pharmacodynamics of GW395058, a peptide agonist of the thrombopoietin receptor, in the dog, a large-animal model of chemotherapy-induced thrombocytopenia.* Stem Cells, 2000. **18**(5): p. 360-5.

99. Stasi, R. and D. Provan, *Management of immune thrombocytopenic purpura in adults.* Mayo Clin Proc, 2004. **79**(4): p. 504-22.

100. Schattner, E. and J. Bussel, *Mortality in immune thrombocytopenic purpura: report of seven cases and consideration of prognostic indicators.* Am J Hematol, 1994. **46**(2): p. 120-6.

101. Mehta, Y.S., et al., *Influence of auto-antibody specificities on the clinical course in patients with chronic and acute ITP.* Platelets, 2000. **11**(2): p. 94-8.

102. Wilkins, R.J., A.I. Hurvitz, and W.J. Dodds-Laffin, *Immunologically mediated thrombocytopenia in the dog.* J Am Vet Med Assoc, 1973. **163**(3): p. 277-82.

103. Stasi, R., et al., *Idiopathic thrombocytopenic purpura: current concepts in pathophysiology and management.* Thromb Haemost, 2008. **99**(1): p. 4-13.

104. Kiefel, V., et al., *Platelet autoantibodies (IgG, IgM, IgA) against glycoproteins IIb/IIIa and Ib/IX in patients with thrombocytopenia.* Ann Hematol, 1996. **72**(4): p. 280-5.

105. Ballem, P.J., et al., *Mechanisms of thrombocytopenia in chronic autoimmune thrombocytopenic purpura. Evidence of both impaired platelet production and increased platelet clearance.* J Clin Invest, 1987. **80**(1): p. 33-40.

106. McMillan, R., et al., *Immunoglobulin synthesis in vitro by splenic tissue in idiopathic thrombocytopenic purpura.* N Engl J Med, 1972. **286**(13): p. 681-4.

107. Kuwana, M., et al., *Spleen is a primary site for activation of platelet-reactive T and B cells in patients with immune thrombocytopenic purpura.* J Immunol, 2002. **168**(7): p. 3675-82.

108. Hosono, M., et al., *Kinetics of platelets in dogs with thrombocytopenia induced by antiglycoprotein IIb/IIIa receptor monoclonal antibody.* Nucl Med Biol, 1995. **22**(1): p. 71-6.

109. Shebani, O.I. and N.C. Jain, *Mechanisms of platelet destruction in immune-mediated thrombocytopenia: in vitro studies with canine platelets exposed to heterologous and isologous antiplatelet antibodies.* Res Vet Sci, 1989. **47**(3): p. 288-93.

110. Kuwana, M., Y. Okazaki, and Y. Ikeda, *Splenic macrophages maintain the anti-platelet autoimmune response via uptake of opsonized platelets in patients with immune thrombocytopenic purpura.* J Thromb Haemost, 2009. **7**(2): p. 322-9.

111. Panitsas, F.P., et al., *Adult chronic idiopathic thrombocytopenic purpura (ITP) is the manifestation of a type-1 polarized immune response.* Blood, 2004. **103**(7): p. 2645-7.

112. Kuwana, M., J. Kaburaki, and Y. Ikeda, *Autoreactive T cells to platelet GPIIb-IIIa in immune thrombocytopenic purpura. Role in production of anti-platelet autoantibody.* J Clin Invest, 1998. **102**(7): p. 1393-402.

113. Kuwana, M., et al., *Immunodominant epitopes on glycoprotein IIb-IIIa recognized by autoreactive T cells in patients with immune thrombocytopenic purpura.* Blood, 2001. **98**(1): p. 130-9.

114. Filion, M.C., et al., *Presence in peripheral blood of healthy individuals of autoreactive T cells to a membrane antigen present on bone marrow-derived cells.* Blood, 1996. **88**(6): p. 2144-50.

115. Stasi, R., et al., *Analysis of regulatory T-cell changes in patients with idiopathic thrombocytopenic purpura receiving B cell-depleting therapy with rituximab.* Blood, 2008. **112**(4): p. 1147-50.

116. Ling, Y., et al., *Circulating dendritic cells subsets and CD4+Foxp3+ regulatory T cells in adult patients with chronic ITP before and after treatment with high-dose dexamethasome.* Eur J Haematol, 2007. **79**(4): p. 310-6.

117. Semple, J.W., et al., *Differences in serum cytokine levels in acute and chronic autoimmune thrombocytopenic purpura: relationship to platelet phenotype and antiplatelet T-cell reactivity.* Blood, 1996. **87**(10): p. 4245-54.

118. Andersson, P.O., et al., *A transforming growth factor-beta1-mediated bystander immune suppression could be associated with remission of chronic idiopathic thrombocytopenic purpura.* Ann Hematol, 2000. **79**(9): p. 507-13.

119. Lazarus, A.H., et al., *Comparison of platelet immunity in patients with SLE and with ITP*. Transfus Sci, 2000. **22**(1-2): p. 19-27.

120. Rocha, A.M., et al., *The levels of IL-17A and of the cytokines involved in Th17 cell commitment are increased in patients with chronic immune thrombocytopenia.* Haematologica, 2011. **96**(10): p. 1560-4.

121. Cao, J., et al., *[Imbalance of Th17/Treg cells ratio in peripheral blood of patients with immune thrombocytopenia]*. Zhongguo Shi Yan Xue Ye Xue Za Zhi, 2011. **19**(3): p. 730-3.

122. Joshi, B.C. and N.C. Jain, *Detection of antiplatelet antibody in serum and on megakaryocytes of dogs with autoimmune thrombocytopenia.* Am J Vet Res, 1976. **37**(6): p. 681-5.

123. Sullivan, P.S., K.L. Manning, and T.P. McDonald, *Association of mean platelet volume and bone marrow megakaryocytopoiesis in thrombocytopenic dogs: 60 cases (1984-1993).* J Am Vet Med Assoc, 1995. **206**(3): p. 332-4.

124. Northern, J., Jr. and H.W. Tvedten, *Diagnosis of microthrombocytosis and immune-mediated thrombocytopenia in dogs with thrombocytopenia: 68 cases (1987-1989).* J Am Vet Med Assoc, 1992. **200**(3): p. 368-72.

125. Takahashi, R., N. Sekine, and T. Nakatake, *Influence of monoclonal antiplatelet glycoprotein antibodies on in vitro human megakaryocyte colony formation and proplatelet formation.* Blood, 1999. **93**(6): p. 1951-8.

126. Karpatkin, S., M. Fotino, and R. Winchester, *Hereditary autoimmune thrombocytopenic purpura: an immunologic and genetic study.* Ann Intern Med, 1981. **94**(6): p. 781-2.

127. Nichols, T.C., et al., *Protein replacement therapy and gene transfer in canine models of hemophilia A, hemophilia B, von willebrand disease, and factor VII deficiency.* ILAR J, 2009. **50**(2): p. 144-67.

128. Boudreaux, M.K. and D.L. Lipscomb, *Clinical, biochemical, and molecular aspects of Glanzmann's thrombasthenia in humans and dogs.* Vet Pathol, 2001. **38**(3): p. 249-60.

129. Tocantins, L.M. and H.L. Stewart, *Pathological anatomy of experimental thrombopenic purpura in the dog.* Am J Pathol, 1939. **15**(1): p. 1-24 9.

130. Joshi, B.C. and N.C. Jain, *Experimental immunologic thrombocytopenia in dogs: a study of thrombocytopenia and megakaryocytopoiesis.* Res Vet Sci, 1977. **22**(1): p. 11-17.

131. Nieswandt, B., *How do platelets prevent bleeding?* Blood, 2008. **111**(10): p. 4835.

132. Psaila, B., et al., *Differences in platelet function in patients with acute myeloid leukemia and myelodysplasia compared to equally thrombocytopenic patients with immune thrombocytopenia.* J Thromb Haemost, 2011. **9**(11): p. 2302-10.

133. Cines, D.B., et al., *The ITP syndrome: pathogenic and clinical diversity.* Blood, 2009. **113**(26): p. 6511-21.

134. Kenet, G., et al., *Cone and platelet analyser (CPA): a new test for the prediction of bleeding among thrombocytopenic patients.* Br J Haematol, 1998. **101**(2): p. 255-9.

135. Eldor, A., et al., *Prediction of haemorrhagic diathesis in thrombocytopenia by mean platelet volume.* Br Med J (Clin Res Ed), 1982. **285**(6339): p. 397-400.

136. George, J.N., et al., *Idiopathic thrombocytopenic purpura: a practice guideline developed by explicit methods for the American Society of Hematology.* Blood, 1996. **88**(1): p. 3-40.

137. Portielje, J.E., et al., *Morbidity and mortality in adults with idiopathic thrombocytopenic purpura.* Blood, 2001. **97**(9): p. 2549-54.

138. Schilling, R.F., *Estimating the risk for sepsis after splenectomy in hereditary spherocytosis.* Ann Intern Med, 1995. **122**(3): p. 187-8.

139. McMillan, R. and C. Durette, *Long-term outcomes in adults with chronic ITP after splenectomy failure.* Blood, 2004. **104**(4): p. 956-60.

140. Guidry, J.A., et al., *Corticosteroid side-effects and risk for bleeding in immune thrombocytopenic purpura: patient and hematologist perspectives.* Eur J Haematol, 2009. **83**(3): p. 175-82.

141. McMillan, R., et al., *Self-reported health-related quality of life in adults with chronic immune thrombocytopenic purpura.* Am J Hematol, 2008. **83**(2): p. 150-4.

142. Friedmann, A.M., et al., *Do basic laboratory tests or clinical observations predict bleeding in thrombocytopenic oncology patients? A reevaluation of prophylactic platelet transfusions.* Transfus Med Rev, 2002. **16**(1): p. 34-45.

143. Nachman, R.L. and S. Rafii, *Platelets, petechiae, and preservation of the vascular wall.* N Engl J Med, 2008. **359**(12): p. 1261-70.

144. Topper, M.J. and E.G. Welles, *Hemostasis*, in *Clinical Pathology*, K.S. Latimer, E.A. Mahaffey, and K.W. Prasse, Editors. 2003, Blackwell Publishing: Ames, IA. p. 99-135.

145. Panzer, S., L. Hocker, and D. Koren, *Agonists-induced platelet activation varies considerably in healthy male individuals: studies by flow cytometry.* Ann Hematol, 2006. **85**(2): p. 121-5.

146. Misgav, M., et al., *Differential roles of fibrinogen and von Willebrand factor on clot formation and platelet adhesion in reconstituted and immune thrombocytopenia.* Anesth Analg, 2011. **112**(5): p. 1034-40.

147. Yanabu, M., et al., *Influences of antiplatelet autoantibodies on platelet function in immune thrombocytopenic purpura.* Eur J Haematol, 1991. **46**(2): p. 101-6.

148. Olsson, A., et al., *Serum from patients with chronic idiopathic thrombocytopenic purpura frequently affect the platelet function.* Thromb Res, 2002. **107**(3-4): p. 135-9.

149. Panzer, S., et al., *Platelet function to estimate the bleeding risk in autoimmune thrombocytopenia.* Eur J Clin Invest, 2007. **37**(10): p. 814-9.

150. Haznedaroglu, I.C., et al., *Thrombopoietin, interleukin-6, and P-selectin at diagnosis and during post-steroid recovery period of patients with autoimmune thrombocytopenic purpura.* Ann Hematol, 1998. **77**(4): p. 165-70.

151. Cahill, M.R., et al., *Protein A immunoadsorption in chronic refractory ITP reverses increased platelet activation but fails to achieve sustained clinical benefit.* Br J Haematol, 1998. **100**(2): p. 358-64.

152. Chong, B.H., et al., *Plasma P-selectin is increased in thrombotic consumptive platelet disorders.* Blood, 1994. **83**(6): p. 1535-41.

153. Karpatkin, S., *Heterogeneity of human platelets. VI. Correlation of platelet function with platelet volume.* Blood, 1978. **51**(2): p. 307-16.

154. Karpatkin, S., *Heterogeneity of human platelets. II. Functional evidence suggestive of young and old platelets.* J Clin Invest, 1969. **48**(6): p. 1083-7.

155. Rinder, H.M., et al., *Differences in platelet alpha-granule release between normals and immune thrombocytopenic patients and between young and old platelets.* Thromb Haemost, 1998. **80**(3): p. 457-62.

156. Psaila, B., et al., *Comparison of Platelet Function and Bleeding in Thrombocytopenic Patients with Immune Thrombocytopenia Purpura (ITP) and Chemotherapy-Induced Thrombocytopenia (CIT)*, in *ASH Annual Meeting Abstracts*. 2007.

157. Ahn, Y.S., et al., *Vascular dementia in patients with immune thrombocytopenic purpura.* Thromb Res, 2002. **107**(6): p. 337-44.

158. Kristensen, A.T., D.J. Weiss, and J.S. Klausner, *Platelet dysfunction associated with immune-mediated thrombocytopenia in dogs.* J Vet Intern Med, 1994. **8**(5): p. 323-7.

159. Niessner, H., et al., *Acquired thrombasthenia due to GPIIb/IIIa-specific platelet autoantibodies.* Blood, 1986. **68**(2): p. 571-6.

160. Yanabu, M., et al., *Platelet activation induced by an antiplatelet autoantibody against CD9 antigen and its inhibition by another autoantibody in immune thrombocytopenic purpura.* Br J Haematol, 1993. **84**(4): p. 694-701.

161. Gardiner, E.E., et al., *Compromised ITAM-based platelet receptor function in a patient with immune thrombocytopenic purpura.* J Thromb Haemost, 2008. **6**(7): p. 1175-82.

162. Jy, W., et al., *Clinical significance of platelet microparticles in autoimmune thrombocytopenias.* J Lab Clin Med, 1992. **119**(4): p. 334-45.

163. Gimbrone, M.A., Jr., et al., *Preservation of vascular integrity in organs perfused in vitro with a platelet-rich medium.* Nature, 1969. **222**(5188): p. 33-6.

164. Hanson, S.R. and S.J. Slichter, *Platelet kinetics in patients with bone marrow hypoplasia: evidence for a fixed platelet requirement.* Blood, 1985. **66**(5): p. 1105-9.

165. Roy, A.J. and I. Djerassi, *Effects of platelet transfusions: plug formation and maintenance of vascular integrity.* Proc Soc Exp Biol Med, 1972. **139**(1): p. 137-42.

166. Goerge, T., et al., *Inflammation induces hemorrhage in thrombocytopenia.* Blood, 2008. **111**(10): p. 4958-64.

167. Dvorak, H.F., *Vascular permeability to plasma, plasma proteins, and cells: an update.* Curr Opin Hematol. **17**(3): p. 225-9.

168. Lampugnani, M.G. and E. Dejana, *Interendothelial junctions: structure, signalling and functional roles.* Curr Opin Cell Biol, 1997. **9**(5): p. 674-82.

169. Dejana, E., F. Orsenigo, and M.G. Lampugnani, *The role of adherens junctions and VE-cadherin in the control of vascular permeability.* J Cell Sci, 2008. **121**(Pt 13): p. 2115-22.

170. Angelini, D.J., et al., *TNF-alpha increases tyrosine phosphorylation of vascular endothelial cadherin and opens the paracellular pathway through fyn activation in human lung endothelia.* Am J Physiol Lung Cell Mol Physiol, 2006. **291**(6): p. L1232-45.

171. Esser, S., et al., *Vascular endothelial growth factor induces VE-cadherin tyrosine phosphorylation in endothelial cells.* J Cell Sci, 1998. **111 (Pt 13)**: p. 1853-65.

172. Gore, I., M. Takada, and J. Austin, *Ultrastructural basis of experimental thrombocytopenic purpura.* Arch Pathol, 1970. **90**(3): p. 197-205.

173. Dale, C. and J.V. Hurley, *An electron-microscope study of the mechanism of bleeding in experimental thrombocytopenia.* J Pathol, 1977. **121**(4): p. 193-204.

174. Van Horn, D.L. and S.A. Johnson, *The mechanism of thrombocytopenic bleeding.* Am J Clin Pathol, 1966. **46**(2): p. 204-13.

175. Kitchens, C.S. and L. Weiss, *Ultrastructural changes of endothelium associated with thrombocytopenia.* Blood, 1975. **46**(4): p. 567-78.

176. Kitchens, C.S., *Amelioration of endothelial abnormalities by prednisone in experimental thrombocytopenia in the rabbit.* J Clin Invest, 1977. **60**(5): p. 1129-34.

177. Kitchens, C.S. and J.F. Pendergast, *Human thrombocytopenia is associated with structural abnormalities of the endothelium that are ameliorated by glucocorticosteroid administration.* Blood, 1986. **67**(1): p. 203-6.

178. Shepro, D., H.E. Sweetman, and H.B. Hechtman, *Experimental thrombocytopenia and capillary ultrastructure.* Blood, 1980. **56**(5): p. 937-9.

179. Miles, R.G. and J.V. Hurley, *The effect of thrombocytopenia on the ultrastructure and reaction to injury of vascular endothelium.* Microvasc Res, 1983. **26**(3): p. 273-90.

180. Haselton, F.R. and J.S. Alexander, *Platelets and a platelet-released factor enhance endothelial barrier.* Am J Physiol, 1992. **263**(6 Pt 1): p. L670-8.

181. Smyth, S.S., et al., *Platelet functions beyond haemostasis.* J Thromb Haemost, 2009.

182. McVerry, B.J. and J.G. Garcia, *In vitro and in vivo modulation of vascular barrier integrity by sphingosine 1-phosphate: mechanistic insights.* Cell Signal, 2005. **17**(2): p. 131-9.

183. Thurston, G., et al., *Angiopoietin-1 protects the adult vasculature against plasma leakage.* Nat Med, 2000. **6**(4): p. 460-3.

184. Brankin, B., et al., *Endostatin modulates VEGF-mediated barrier dysfunction in the retinal microvascular endothelium.* Exp Eye Res, 2005. **81**(1): p. 22-31.

185. Schaphorst, K.L., et al., *Role of sphingosine-1 phosphate in the enhancement of endothelial barrier integrity by platelet-released products.* Am J Physiol Lung Cell Mol Physiol, 2003. **285**(1): p. L258-67.

186. Polzin, A., et al., *Sphingosine-1-phosphate is thromboxane-dependently released from platelets and modulates chemotaxis of human monocytes*, in *American Heart Association*. 2010: Chicago, IL.

187. McVerry, B.J. and J.G. Garcia, *Endothelial cell barrier regulation by sphingosine 1-phosphate.* J Cell Biochem, 2004. **92**(6): p. 1075-85.

188. Garcia, J.G., et al., *Sphingosine 1-phosphate promotes endothelial cell barrier integrity by Edg-dependent cytoskeletal rearrangement.* J Clin Invest, 2001. **108**(5): p. 689-701.

189. McVerry, B.J., et al., *Sphingosine 1-phosphate reduces vascular leak in murine and canine models of acute lung injury.* Am J Respir Crit Care Med, 2004. **170**(9): p. 987-93.

190. Li, J.J., et al., *Thrombin induces the release of angiopoietin-1 from platelets.* Thromb Haemost, 2001. **85**(2): p. 204-6.

191. Gamble, J.R., et al., *Angiopoietin-1 is an antipermeability and anti-inflammatory agent in vitro and targets cell junctions.* Circ Res, 2000. **87**(7): p. 603-7.

192. Thurston, G., et al., *Leakage-resistant blood vessels in mice transgenically overexpressing angiopoietin-1.* Science, 1999. **286**(5449): p. 2511-4.

193. Troy, G.C., et al., *Endostatin and vascular endothelial growth factor concentrations in healthy dogs, dogs with selected neoplasia, and dogs with nonneoplastic diseases.* J Vet Intern Med, 2006. **20**(1): p. 144-50.

194. Ma, L., et al., *Platelets modulate gastric ulcer healing: role of endostatin and vascular endothelial growth factor release.* Proc Natl Acad Sci U S A, 2001. **98**(11): p. 6470-5.

195. Danielli, J.F., *Capillary permeability and oedema in the perfused frog.* J Physiol, 1940. **98**(1): p. 109-29.

196. Ho-Tin-Noe, B., et al., *Innate immune cells induce hemorrhage in tumors during thrombocytopenia.* Am J Pathol, 2009. **175**(4): p. 1699-708.

197. Rahaman, M., et al., *Neutrophil sphingosine 1-phosphate and lysophosphatidic acid receptors in pneumonia.* Am J Respir Cell Mol Biol, 2006. **34**(2): p. 233-41.

198. Kimura, T., et al., *Sphingosine 1-phosphate receptors mediate stimulatory and inhibitory signalings for expression of adhesion molecules in endothelial cells.* Cell Signal, 2006. **18**(6): p. 841-50.

199. Radomski, A., et al., *Identification, regulation and role of tissue inhibitor of metalloproteinases-4 (TIMP-4) in human platelets.* Br J Pharmacol, 2002. **137**(8): p. 1330-8.

200. McGarrity, S.T., et al., *Inhibition of neutrophil superoxide anion generation by platelet products: role of adenine nucleotides.* J Leukoc Biol, 1988. **44**(5): p. 411-21.

201. Serhan, C.N. and J. Savill, *Resolution of inflammation: the beginning programs the end.* Nat Immunol, 2005. **6**(12): p. 1191-7.

202. Carbo, C., I. del Conde, and D. Duerschmied, *Petechial bleeding after sunburn in a patient with mild thrombocytopenia.* Am J Hematol, 2009. **84**(8): p. 523.

203. Boilard, E., P. Blanco, and P.A. Nigrovic, *Platelets: active players in the pathogenesis of arthritis and SLE.* Nat Rev Rheumatol, 2012. **8**(9): p. 534-42.

204. Cloutier, N., et al., *Platelets can enhance vascular permeability.* Blood, 2012. **120**(6): p. 1334-43.

205. Shepard, J.M., et al., *Platelets decrease albumin permeability of pulmonary artery endothelial cell monolayers.* Microvasc Res, 1989. **37**(3): p. 256-66.

206. Fujii, E., et al., *Possible role of nitric oxide in 5-hydroxytryptamine-induced increase in vascular permeability in mouse skin.* Naunyn Schmiedebergs Arch Pharmacol, 1994. **350**(4): p. 361-4.

207. De Clerck, F., et al., *Platelet-mediated vascular permeability in the rat: a predominant role for 5-hydroxytryptamine.* Thromb Res, 1985. **38**(4): p. 321-39.

208. Majno, G. and G.E. Palade, *Studies on inflammation. 1. The effect of histamine and serotonin on vascular permeability: an electron microscopic study.* J Biophys Biochem Cytol, 1961. **11**: p. 571-605.

209. Shepro, D., S.L. Welles, and H.B. Hechtman, *Vasoactive agonists prevent erythrocyte extravasation in thrombocytopenic hamsters.* Thromb Res, 1984. **35**(4): p. 421-30.

A NOVEL CANINE MODEL OF IMMUNE THROMBOCYTOPENIA

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Introduction

Immune thrombocytopenia (ITP) is an autoimmune bleeding disorder in which platelet autoantibodies lead to platelet destruction, thrombocytopenia, and, sometimes, severe bleeding [1]. Though ITP is a relatively uncommon disease, the persistence of chronic ITP and lack of curative treatment results in high ITP associated health care costs and significant patient morbidity [2, 3]. Most patients do not die of hemorrhage, but significant morbidity is associated with bleeding episodes, treatment side effects, and symptoms of fatigue [4]. Mortality is up to 2.3 times higher in chronic ITP patients compared to the general population [5], and mortality is equally attributed to fatal hemorrhage and to secondary infections from immunosuppressive therapy or splenectomy, current mainstays of ITP treatment [6].

Although our understanding of the immunopathogenesis of ITP has advanced greatly, often as a result of what we have learned through animal models, many questions remain. One major unanswered question in ITP is why patients with ITP show different bleeding

tendencies: some platelets with ITP will hemorrhage, while others, with equally low platelet counts will not. Given the side-effects of ITP therapy, treatment is aimed at maintaining a hemostatically safe platelet count while minimizing untoward effects of therapy [3, 7]. This delicate treatment balance is complicated by the fact that platelet count does not accurately inform bleeding risk so that clinicians cannot predict which patients will bleed and thus which patients need more aggressive treatment [8-10]. Investigations are necessary to better understand and predict bleeding phenotype.

There are currently no large animal models of ITP. We have learned a great deal from murine models of ITP, but striking differences exist between mice and humans including significantly higher platelet counts (1 million platelets/μl) in mice compared to humans (150,000-400,000/μl) [11-13], species differences in platelet volume (murine platelets are about half the size of human platelets) and platelet signaling pathways [12], and immunologic differences between mice and humans. Mice lack the Fc gamma RIIa receptor that is thought to play a central in clearing opsonized platelets in human ITP patients [14].

Why a dog model? First and foremost, dogs develop spontaneous ITP with a similar disease course, heterogeneous bleeding phenotype, and response to treatment as adult ITP [15]. The dog can thereby be used as both an induced and spontaneous model, and what we learn from both can be readily applied to veterinary and human patients. Dogs are much closer in size to humans than are mice resulting in more similar rheology [16, 17] and metabolic rates [18], and dogs and humans have similar platelet counts and platelet volumes. Four hundred times the blood volume can be safely sampled from dogs compared to mice [19] and non-terminal studies are possible in dogs, but not in mice. Platelet signaling pathways are more analogous between dogs and humans, with canine and humans platelets signal through thrombin protease-activated receptors (PAR)1 and PAR4 while murine platelets utilize PAR3 and 4 [12, 20-22].

An induced canine ITP model is desirable in addition to the spontaneous dog model because it represents ITP without the confounding co-morbidities or treatment effects that are present in clinical cases. Furthermore, manipulations like biopsies can be performed in the induced model that are not possible in client-owned animals.

As such, we sought to develop a novel canine ITP model that would be highly translational because of greater species similarities between dogs and people and the fact that dogs develop the spontaneous disease. We employed 2F9, a murine anti-platelet antibody targeting canine platelet glycoprotein IIb (GPIIb), to model the autoantibody-induced clearance of platelets in ITP. We selected an anti-GPIIb antibody since autoantibodies in human patients with ITP are most commonly directed against epitopes on GPIIbIIIa, the fibrinogen receptor, and GPIb-IX-V, the von Willebrand factor receptor [23]. Anti-GPIIbIIIa antibodies are the only antibodies that have been described in canines with spontaneous ITP, but a search for other epitopes has not been performed [24]. Passive murine models in which antibodies against GPIIbIIIa are infused lead to incompletely understood acute systemic reactions and hypothermia [1, 25]. Thus, these murine models fail to recapitulate the naturally-occurring disease in which predominant clinical manifestations, if any, are mucocutaneous bleeding and fatigue [26].

Induced canine ITP models have been previously described but have not been well-characterized and the platelet-depleting antibodies or heterologous serum employed in these models are no longer available (Makoto Hosono, personal communication, May 12, 2009) [27-29].

The main aim of this study was to develop and characterize a novel canine model of ITP. Ultimately this large animal model will be used to investigate reasons for differential bleeding tendency in people and dogs with ITP.

Materials and Methods

Animals

Eight healthy adult (median age 2 years old; range 1-4) intact male mixed breed dogs were used (28.4 ± 5.6 kg) for development of the experimental ITP model. Research dogs were loaned from Francis Owen Blood Research Laboratory at the University of North Carolina at Chapel Hill or Laboratory Animal Resources at the North Carolina State College of Veterinary Medicine (NCSU). If coming from outside of NCSU they were quarantined and

allowed to acclimate for 72 hours. Dogs from Chapel Hill were also treated with fenbendazole (50 mg/kg per day x 3 days) prior to the study because of presence of Giardia in some of the stock dogs at that facility. Dogs were housed in the institutional laboratory animal facility at NCSU and returned to Chapel Hill or NCSU Laboratory Animal Resources on study completion.

To compare our experimental model findings with spontaneous canine ITP, blood samples were also obtained from dogs with naturally-occurring ITP or thrombocytopenia of other causes. These dogs were patients at the College of Veterinary Medicine, NCSU from December 2011 through September 2012. Owner consent was obtained prior to obtaining samples. Enrollment criterion was a platelet count of <30,000 platelets/µl as we focused on the platelet count range at which variable bleeding often occurs. Exclusion criteria consisted of weight less than 10 lbs. or transfusion during that hospitalization prior to study enrollment of a platelet-containing blood product (fresh whole blood, platelet concentrate, or platelet rich plasma). Diagnosis of primary ITP was based on thrombocytopenia with failure to identify an underlying disease process on physical examination, CBC, biochemistry profile, thoracic radiography, abdominal ultrasound, and vector borne disease testing (NCSU vector borne disease panel or Idexx Snap 3DX test).

All protocols were approved by the Institutional Animal Care and Use Committee of North Carolina State University.

Production of Monoclonal Antibodies

2F9 is a murine monoclonal IgG2a recognizing canine GPIIb [30]. The 2F9 producing hybridoma was a gift of David Wilcox (Medical College of Wisconsin, Milwaukee, WI). The isotype control murine IgG2a anti-yellow fever (αYFA or CRL-1689.1) producing hybridoma was a gift of Gregg Dean (Colorado State University College of Veterinary Medicine, Fort Collins, CO)[31].

Both antibodies were produced from their respective hybridomas by the Tissue Culture Facility, University of North Carolina, Chapel Hill using disposable bioreactors (CELLine, Integra Biosciences, Hudson, NH). Hybridomas were maintained in Protein Free

Hybridoma Medium II (Gibco, Grand Island, NY). To remove phenol red from the medium, antibody was dialyzed a minimum of four times against 0.9% sodium chloride using the bioreactors as sterile dialysis chambers.

Concentration of antibodies was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL). The purity of the antibody preparations was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining of the gel. Antibodies were tested for Mycoplasma using a rapid detection DNA probe test (Gen-Probe, San Diego, CA) and endotoxin using the Gel Clot LAL assay (Lonza, Walkersville, MD) according to manufacturers' instructions and determined to be mycoplasma-free and endotoxin-free to the limits of the detection (100,000 organisms/ml and 0.03 EU/ml, respectively).

Modification of Antibodies

For use in flow cytometry studies, 2F9 and αYFA were fluoresceinated by standard methods with FITC (Sigma-Aldrich, St. Louis, MO) in anhydrous DMSO and separated from free FITC by dialysis against citrated saline in 12,000-14,000 molecular weight dialysis tubing (Spectrum Laboratories, Inc., Rancho Dominguez, CA).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot Analysis

Western blot analysis was performed to confirm the specific reactivity of 2F9 with GPIIb as this had been suggested but not adequately confirmed [30]. Platelets were prepared from control dogs and Otterhounds with Glanzmann's thrombasthenia (GT) and lysed as previously described [22]. Washed platelets were solubilized in Laemmli sample buffer containing pepstatin A (1 µg/mL, Thermo Scientific, Rockford, IL), 5 mM EDTA(Thermo Scientific) and 1x Halt^{TM} protease and phosphatase inhibitor cocktail (Thermo Scientific). Oxidized or reduced (5 % β–ME, Sigma-Aldrich) platelet cell lysates (40 µg, 20 µg, and 10 µg protein per lane) were loaded onto pre-cast 10% polyacrylamide gels (Mini-PROTEAN TXG, BIO-RAD, Hercules, CA) and separated by SDS-PAGE (200 V, 30 min, RT) using 1x

Tris-glycine SDS electrophoresis buffer (BIO-RAD) then electro-blotted to PVDF membranes for 1 hour at 2-8 ^{0}C using 1x Tris-glycine transfer buffer (BIO-RAD) containing 20% methanol (Sigma-Aldrich). PVDF membranes were blocked, with shaking (300 rpm) for 1 hour, at room temperature in 5% human serum albumin (Talecris, Clayton, NC) in PBS-T (0.2% Tween-20, Sigma-Aldrich). The membranes were first probed with 2F9 (1.0 µg/ml), then washed 3x with PBS-T(0.2%) and reacted with a goat anti-murine HRP antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:100,000 in PBS-T(0.2%) that contained StepTactin HRP conjugate (BIO-RAD) diluted to 1:100,000 required for detection of Precision Plus Protein™ WesternC™(BIO-RAD) molecular weight standards. Immuno-reactive platelet proteins on PVDF membranes were visualized by chemiluminescence using an ECL+Plus ^{TM} kit (GE Healthcare Pittsburgh, PA) as directed. Digital images were captured and analyzed using a ChemiDoc™ XRS+ imaging system with Image Lab™ analysis software (BIO-RAD).

Flow Cytometry

Assessment of the Effect of 2F9 on Platelet Activation and Reactivity

To determine the effects of 2F9 on platelet activation state or reactivity flow cytometry assays were performed to assess baseline and thrombin-induced platelet P-selectin externalization and phosphatidylserine (PS) exposure after blood was incubated with 2F9. Whole blood from a healthy research dog was collected via cephalic venipuncture into a syringe containing acid citrate dextrose (ACD; 10% v/v). The whole blood was partitioned and incubated with 2F9 (2 µg/ml) or αYFA (2 µg/ml) or HEPES saline (10 mM HEPES, 150 mM NaCl, pH 7.5) for 1 hour at room temperature. Platelet-rich plasma (PRP) was generated from the whole blood by combining it with an equal volume of buffered saline glucose citrate (BSGC; 129 mM NaCl, 14 mM Na_3citrate, 11 mM glucose, 10 mM NaH_2PO_4, pH 7.3) and centrifuging at 275g for 5 minutes. Resulting PRP was utilized in the following reaction mixtures, running each reaction in triplicate using PRP from each of the three different whole blood preparations:

- P-selectin studies: Activation reactions were carried out in 5mL polystyrene tubes (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA) in a 100 µL (total) assay volume containing 1 µl PRP in a reaction mixture containing HEPES saline and 0.4mM gly-pro-arg-pro-NH2. Platelets were stimulated with varying concentrations of thrombin (none, 1, 0.1, 0. 05, 0. 025 U/ml final concentration; bovine thrombin, King Pharmaceuticals, Bristol, TN), for 15 minutes at room temperature. Fluorescent conjugated antibodies were added directly to the reaction tubes to identify platelets based on constitutive membrane GPIb/IX (142.2 alexa 488, kind gift of David Wilcox, Milwaukee, WI, 0.13 µg/100 µL reaction) and to detect stimulated expression of P-selectin (CD62P-phycoerythrin [PE], 8 mL/100 µL reaction; clone AC1.2, BD Biosciences, San Jose, CA). Additional control reaction tubes were also labeled with the appropriate isotype controls (BD Biosciences). After labeling for 20 minutes in the dark, samples were fixed for 15 minutes by the addition of $1/10^{th}$ the volume of 4% paraformaldehyde. After fixation, samples were quench diluted with 0.5 ml of HEPES saline.
- PS studies: Reactions were carried out in annexin binding buffer (ABB; 10 mM HEPES, 0.15 M NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, pH7.4) instead of HEPES saline and platelets were stimulated with only 1 U/ml of thrombin. After stimulation, antibodies were added as above in addition to annexin-V APC (E-Biosciences, 5 µl/100 µL reaction) to detect externalized PS. One control reaction was set up in HEPES saline (no calcium) which would not allow AV binding. After labeling for 20 minutes, these samples were not fixed but were quench-diluted in 0.5 ml of ABB and analyzed immediately on the flow cytometer.

Samples were collected and analyzed with a FACSCalibur cytometer and CELLQUEST software (Becton Dickinson), using logarithmic gain settings for light scatter and fluorescence. After initial optimization and compensation, the same settings were used throughout the study. Samples were gated to collect 10,000 events in the characteristic forward (FSC) and side (SSC) location.

For analysis, platelets were first discriminated based on their 142.2-alexa 488 fluorescence, then based on their characteristic FSC-SSC properties, and finally all 142.2-positive events with platelet light scatter properties were assessed for FL2 positivity to identify the proportion of platelets binding CD62P-PE, denoting P-selectin expression. Activated platelets were distinguished from resting platelets by use of an isotype control antibody. AV positivity was determined based on the percent 142.2 positive events that fluoresced on FL3. Gating for PS negativity was determined with the calcium negative control.

The percentage of platelets (identified by 142.2-alexa 488 on FL1) that expressed P-selectin (identified by CD62P-PE on FL2) was determined for each thrombin dose. A dose-response curve was plotted and the concentration of thrombin inducing half-maximal activation (ED50 thrombin) was calculated with GraphPad Prism 5 (San Diego, CA).

To determine the impact of 2F9 on platelet activation state and reactivity, baseline P-selectin and P-selectin ED50 and the baseline PS and stimulated PS exposure were compared among PRP generated from blood incubated with saline, 2F9, or αYFA.

All flow data analysis was performed with FlowJo (Tree Star, Inc., Ashland, OR).

Assessment of 2F9 Specificity

The affinity of 2F9 for canine platelets was assessed with a dose titration study via flow cytometry. A dilution series of FITC-2F9 beginning at a final concentration of 4 µg/ml down to 4 pg/ml was incubated with PRP prepared as above in 100 µl reaction volumes for 30 minutes at room temperature prior to quenching. Platelets were identified based on FSC and SSC properties and 2F9 binding was assessed via FL1 positivity on a flow cytometer (CyAn, Beckman Coulter, Fullerton, CA).

Flow cytometry studies were also performed to determine if 2F9 or αYFA binds to any other component of whole blood. Canine whole blood from healthy control dogs was lysed in red cell lysis buffer (1 mM EDTA, 10 mM sodium bicarbonate, 155 mM ammonium chloride, pH 7.3). Cells were washed in citrated saline (146 mM NaCl, 5.4 mM Na_3citrate) and labeled with different concentrations of FITC αYFA or 2F9 (40, 4, 0.4, or 0.04 µg/ml in

100 µl reaction mixtures), fixed in paraformaldehyde as above, washed (1000 g x 5 minutes) and analyzed on the FACSCalibur as above using linear FSC and SSC gates and looking for FL1 positivity of any cell population.

As we observed 2F9 binding of neutrophils in whole blood, similar studies were performed with isolated canine neutrophils that were prepared as previously described via centrifugation over Histopaque 1077 and erythrocyte lysis [32]. To determine 2F9 binding of freshly isolated canine neutrophils, cells were incubated with FITC-2F9 at the above concentrations in 1× PBS containing 10% FBS on ice for 30 min. After incubation, cells were washed twice and 2F9 labeling (FL1 positivity) was analyzed on a LSR II flow cytometer (BD Biosciences, Mountain View, CA) using FACSDiva software (BD Biosciences, Mountain View, CA). Neutrophils were identified based on their FSC and SSC characteristics.

Assessment of Duration of 2F9 Circulation in Experimental Dogs

To determine the duration of 2F9 platelet binding following 2F9 infusion in experimental dogs, PRP prepared from ACD blood from 2F9 and αYFA-treated dogs was analyzed daily. Reactions were set up in HEPES saline as above, activation was not performed, and platelets were labeled with rat anti-mouse IgG2a FITC (clone R19-15, BD Biosciences, 10 µg/ml final concentration) and fixed as above. After quenching, reactions were washed by centrifugation at 1000g for 10 minutes and resuspended in 0.5 ml of HEPES saline. Samples analyzed on the FACSCalibur and were gated for platelets based on FSC and SSC and percent platelets with 2F9 bound determined by percent FL1 positive platelets. Due to severe thrombocytopenia in some of these samples, a minimum of 500 events were collected in the platelet gate by collecting the entire contents of each tube. In samples with more platelets, 10,000 platelet events were collected. A gate for non-2F9 bound platelets was set by using an appropriate isotype control (Rat FITC IgG_1 κ, BD Biosciences).

In Vivo Studies: ITP Model Development

2F9 Dose Titration

Previously, one proof of concept dog received 300 µg/kg of 2F9 which induced absolute thrombocytopenia for 3 days. To model the clinical disease in which patients demonstrate variable bleeding tendencies (5,000 to 30,000 platelets/µl) we performed a dose titration study in two dogs with the goal of reaching a target nadir between 5,000 to 30,000 platelets/µl.

In the first dose titration dog, 2F9 was administered at a starting dose of 15 ng/kg i.v. and this dose was increased by a factor of ten up to every 2 hours until the platelet count fell in the target nadir, with platelet count being assessed hourly after antibody administration. Time zero was when the platelet count first fell into the target platelet nadir range.

The second dose titration dog received a starting dose of 1.5 µg/kg and this dose was increased by a factor of ten until a decrease in platelet count was observed and then the additional dose required to reach the target platelet nadir was calculated based on the amount of antibody given to achieve this initial decrease in platelet count.

Subsequently, three dogs were employed in a dose repeatability study, receiving starting 2F9 doses of 50 µg/kg based on the dose titration study results, and additional doses as needed were based on their decrease in platelet count in response to the first dose.

Three dogs served as controls. They received αYFA, the isotype control antibody, via infusion at the highest cumulative effective dose of 2F9 administered (167 µg/kg).

The first administration of each antibody was given as a slow intravenous infusion via a preplaced cephalic catheter through a syringe filter (Fisher Scientific, Dublin, Ireland) over 20 minutes. Subsequent doses were administered as intravenous boluses. Demeanor, temperature, pulse, and respiration were monitored throughout antibody infusion and before and after bolus antibody administration.

Blood Sampling and Preparation

At baseline (17-24 hours prior to antibody administration), a 20-guage cephalic catheter (Terumo, Elkton, MD) was placed. Dogs were then sedated with dexmedetomidine (Pfizer,

NY, NY) which was administered intravenously to effect (median 11.0 µg/kg; range 8.0-21.9 µg/kg) and a 19-gauge indwelling central catheter (BD Intracath; Becton Dickinson, Franklin Lakes, NJ) was placed in the jugular or lateral saphenous vein of each dog for blood sampling throughout the study. If needed, dogs were reversed with atipamezole (Antisedan; Pfizer) on completion of catheter placement.

Blood was obtained via indwelling catheters. Catheters were first flushed with 6 ml of 0.9% sodium chloride. A 5 ml catheter purge sample was then collected into 1 ml 0f 0.9% sodium chloride and set aside. Blood was collected into a plain syringe and transferred immediately to EDTA tube (Tyco Healthcare Group, Mansfield, MA) (2 ml) for platelet and complete blood count and an additive-free tube (Tyco) (3 ml) for serum preparation, a syringe containing 0.2 ml ACD (2ml) for platelet rich plasma preparation, and a syringe containing 0.38 % sodium citrate (final concentration; 4 ml) for plasma preparation. The initial purge sample was then returned to the dog and the catheter flushed with 6 ml of 0.9% sodium chloride. Sodium citrate blood was spun at room temperature at 2440 g for 15 minutes and supernatant spun again at 3500 g for 15 minutes to generate platelet free plasma (PFP) which was then stored at -80°C for future coagulation parameter analysis. Serum was generated after allowing the additive-free tube to clot at room temperature for a minimum of 20 minutes by centrifugation at 1000 g for 15 minutes. Serum was harvested and stored at -80°C for future cytokine analysis. To generate PRP for flow cytometry reactions, the ACD blood was combined with an equal volume of BSGC and centrifuged at 275g for 5 minutes.

If the indwelling catheter was not patent at the sampling time, blood was obtained via cephalic vein venipuncture through a 21 gauge butterfly needle (Terumo). Patient samples were obtained through indwelling sampling catheters if present or careful direct venipuncture if not.

Blood was drawn at the following time points: baseline, time zero (when platelet count first fell into the target nadir range or 1 hour after control antibody administration), 2, 4, 6, 8, 12, 24 hours, and then every 24 hours until platelet count recovered and new bleeding stopped, whichever came later (168-240 hours; termed recovery). In hospital patients, blood was drawn one time on study enrollment and only for serum and PFP preparation.

Platelet Counts and Complete Blood Counts

For determination of platelet counts and complete blood counts (CBC), EDTA whole blood was submitted for CBCs to the Clinical Pathology Laboratory at NCSU. Automated counts were performed with an Advia 120 (Siemens Healthcare Diagnostics, Inc., Norwood, MA). Differential cellular counts were determined by examination of Wright-Giemsa stained slides by trained veterinary clinical pathology laboratory technologists. Automated platelet counts were always confirmed by comparison with a slide estimate. If automated platelet counts were below 10,000 platelets/µl (limit of machine linearity), platelet count was instead determined by hemocytometer counts after red blood cell lysis in ammonium chloride (1:100 dilution, Acros Organics, Geel, Belgium). The diluted blood sample was allowed to settle for 10 minutes in a Neubauer hemocytometer (Hausser Scientific, Horsham, PA) and platelets were counted under a phase contrast microscope at 600x magnification.

Buccal Mucosal Bleeding Time

At baseline and time zero, while dogs were sedated with dexmedetomidine, buccal mucosal bleeding times (BMBTs) were performed in all experimental dogs. We measured bleeding time after incision of the buccal mucosa with a template device (Surgicutt, Jorgensen Laboratories, Loveland, CO) according to previously described methods [33]. If bleeding continued for over 15 minutes, the test was stopped and pressure was applied to the site. A value of >15 minutes was recorded.

Bleeding Score

At the time of each blood draw, bleeding was assessed by history (for clinical patients), observation in the time period since the last bleeding score (in experimental dogs), and physical examination using a bleeding scale that quantifies bleeding at 8 anatomic sites adapted from the human ITP bleeding score [34] (Table 1). The fecal occult blood test was performed using o-toluidine tablets (Hematest, Siemens Diagnostics, Tarrytown, NY). Microscopic hematuria was assessed with urine dipsticks (Siemens).

Cytokine and Chemokine Analysis

The inflammatory nature of the ITP model and the cytokine profile of ITP in dogs with naturally-occurring disease were analyzed by using a multiplex cytokine/chemokine assay. Cytokine and chemokine concentrations in the sera were measured by Milliplex Map canine cytokine magnetic bead panel (Milllipore, Billerica, MA) per the manufacturer's instructions that was read using a Bioplex 200 (BioRad, Hercules, CA). Analytes evaluated included interleukin 8 (IL-8), canine orthologue of Chemokine (C-X-C motif) ligand 1 (CXCL1 orthologue or KC-like), Tumor Necrosis Factor-α (TNF-α), monocyte chemoattractant protein-1 (MCP-1), IL-6, IL-10, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-2, interferon-inducible protein 10 IP-10 (CXCL10), interferon gamma (IFN-γ), IL-7, IL-15, and IL-18.

The dynamic range (lower and upper limits of detection in pg/ml) for cytokines were: IL-8 (218.306, 52841.549), IL-6 (11.867, 51919.926), IL-7 (10.194, 51200.999), IL-2 (11.954, 52343.73), IL-10 (11.857, 49655.509), IL-15 (10.161, 51199.813), IL-18 (11.333, 50535.442), CXCL1 orthologue (12.274, 41428.856), TNF-α (12.419, 53967.053), GM-CSF (12.649, 12452.237), IP-10 (12.289, 10347.254), IFN-γ (159.731, 50320.889), and MCP-1 (52.885, 2812.664) respectively.

Normal values were determined from the baseline values of the 8 healthy experimental dogs.

IL-8 findings were confirmed by analyzing serum samples using an R&D Quantikine canine IL-8 ELISA (Minneapolis, MN) as directed. Absorbances were read using a VersaMax plate reader (Molecular Devices, Sunnyvale, CA) and curve analysis performed with Softmax Pro 5 (Molecular Devices).

Fibrinogen and D-dimer Analysis

To ensure that the model was not pro-thrombotic, PFP samples were analyzed in the Comparative Coagulation Laboratory (Animal Health Diagnostic Laboratory, Cornell University) for determination of Clauss fibrinogen concentration [35] and D-dimer concentrations. Clottable (Clauss) fibrinogen was performed using an automated clot

detection instrument (STA Compact, Diagnostica Stago, Parsippany, NJ) and reaction conditions as previously described [35]. Pooled canine plasma (prepared from 20 healthy, adult dogs) was used as the fibrinogen assay standard. D-dimer concentration in ng/mL was measured using a quantitative, immunoturbidometric method as previously described [36], using a commercial kit and the manufacturer's human D-dimer standards (HemosIL, D-dimer Calibrator, Instrumentation Laboratory, Bedford, MA).

Statistical Analysis

In cases where cytokine concentrations were below the assay Lower Limit of Detection (LLOD), values were assigned that were one half of the LLOD (defined above) for the purpose of statistical analysis [32].

To test for differences in outcomes across all time points by treatment group (2F9 or control antibody), repeated measures generalized linear modeling (analysis of variance or ANOVA) was performed, where time point and the variable of interest were entered into the model. For all variables, the ANOVA models were checked for Gaussian residuals and equality of variance. The P-value for each parameter was evaluated and tested for significance. To control for multiple testing, a Bonferroni correction (for each set of hypotheses) was used to determine the alpha level for statistical significance that ensured a family-wise error rate of 0.05, meaning that a comparison was considered to be statistically significant if $P<0.002$.

To identify candidate bleeding predictors, the association between bleeding scores and different mediators was determined by univariate association between variables using a t-test for correlations with the appropriate degrees of freedom. Bleeding scores were treated as ordinate variables as described by Mantel-Haenszel. Again, a Bonferroni correction was applied as above so $P<0.002$ was considered significant.

To screen for differences in cytokines, chemokines, D-dimers, and fibrinogen, platelet count, HCT, and WBC count, and bleeding score in the dogs with spontaneous ITP, healthy dogs, and experimental dogs, a repeated measures ANOVA was performed. The secondary ITP dog was not considered in these analyses as there was just one patient in that group.

When differences between groups were identified (P<0.002;IL-8, hematocrit, platelet count, bleeding score) the Wilcoxon rank sum test was used to compare pairs of different groups of dogs (dogs with spontaneous ITP, experimental ITP dogs at 0 and 24 hours, healthy dogs, and control dogs at 0 and 24 hours). The Wilcoxon rank sum test was also used to compare nadir platelet counts for control and 2F9 treated dogs. Results were considered significant if P<0.05.

All calculations were performed using Stata version 10 (Statacorp LP). Graphs were drawn with GraphPad PRISM 5.0.

Results

Ex Vivo Characterization of 2F9

2F9 Recognizes GPIIb

Before administering 2F9 *in vivo* we aimed to further characterize the antibody, as the only study describing its specificity found that it reacted with a 140 kDa protein on a western blot of canine platelet lysate (non-reducing conditions) and based on the size of human GPIIb it was speculated that this protein might represent canine GPIIb [30]. Since that time it has been taken as dogma that 2F9 is an anti-canine GPIIb antibody.

In our Western blot (Figure 1), we determined that 2F9 does not recognize any protein bands in canine Glanzmann's thrombasthenia (GT) platelets, indicating that 2F9 must bind to either GPIIb or GPIIIa as GT platelets lack detectable amounts of only these two glycoproteins [37]. GPIIbIIIa is a noncovalently associated calcium-dependent heterodimer [38]. In humans, the GPIIIa subunit consists of one peptide while the GPIIb subunit consist of 2 peptides, a large 125kDa α chain and small 24 kDa β chain, linked by a disulfide bond [38]. 2F9 reacts with a band in the non-reduced state that is most prominent at 124 kDa with weaker areas extending to 148 kDa and 110 kDa. This band is close to the predicted size of GPIIb. Nonreduced human GPIIb migrates between 136 and 128 kDa (depending on the report, though 136 kDa is most commonly described) on a one-dimensional gel [39, 40]. Differences in canine GPIIb and human GPIIb could be accounted for by differences in

glycosylation of canine GPIIb. The predicted molecular weight of canine GPIIb based on proteomic data is 116 kDa, which does not account for post-translational modifications like glycosylation and is close to our observed strong band at 124 kDa [41]. 2F9 may be preferentially binding to the large 125 kDa subunit of GPIIb [38].

Reduced samples have a faint band at 108kDa suggesting either a weak reaction with GPIIIa (which has a predicted molecular weight of 100 kDa) or some GPIIb associated with IIIa despite the presence of 5 mM EDTA in the lysis buffer [42]. Reduction appears to eliminate the reactivity of 2F9 with GPIIb. According to the human literature, dissociated GPIIb should migrate at 125 kDa and 25 kDa under reduced conditions [38]. We do not see any bands of these sizes in reduced conditions.

Our results are most consistent with 2F9 binding to GPIIb. If 2F9 recognized GPIIIa, we should have seen a 100 kDa band under both reducing and nonreducing conditions, as GPIIIa does not dissociate under reducing conditions [42]. We did not observe such bands. We were also unable to replicate the original 141 kDa band observed by Burstein et al., however the MW standards used in that report did not have the same resolution as ours, thus 141 kDa may not have been an exact molecular weight [30]. The 210 kDa band likely represents aggregated GPIIb as can occur when heating integral membrane proteins above 37°C.

2F9 Is Not an Activating or Inhibitory Antibody

Based on our *ex vivo* flow cytometry studies, 2F9 does not alter baseline platelet activation state or platelet responsiveness to thrombin. Figure 2 shows that the thrombin dose response curves for P-selectin expression are superimposable for the PRP prepared following incubation of whole blood with saline, isotype control antibody, or 2F9. Thrombin ED50 for P-selectin was unchanged by 2F9. Baseline PS exposure was 4% in all PRP samples and similar in all samples following stimulation with 10 U/ml of thrombin (19% in saline and isotype control PRP, 25% in 2F9 PRP). At least by flow cytometric evaluation, 2F9 is neither an activating nor an inhibitory antibody. The concentration of 2F9 selected for these

experiments was based on the dose of 2F9 that induced thrombocytopenia in the model dogs: 150 µg/kg, which using an 80 ml/kg canine blood volume converts to 2 µg/ml [19].

2F9 Reacts with High Affinity with Canine Platelets and Low Affinity with Canine Neutrophils

Titration studies showed that 2F9 binding (100%) to platelets can be detected by flow cytometry at a concentration of 2F9 as low as 0.4 µg/ml.

Studies to confirm 2F9's platelet specificity were a little less clear. Previous studies stated that 2F9 does not react with cells other than platelets in the peripheral blood, but it was unclear how this was determined [30]. Our flow cytometry studies showed that while αYFA at the concentrations tested does not bind to cells present in whole blood, 2F9 does very weakly (6.25% 2F9 positive) label isolated canine neutrophils when present at 4 µg/ml, 10 times the minimum concentration of 2F9 required to label canine platelets (Supplemental Figure 1). 2F9 binding of neutrophils seems to require the presence of platelets as 2F9 labels neutrophils in whole blood much more strongly (100% 2F9 positive) at 4 µg/ml 2F9 compared to the 6.25% positivity of isolated neutrophils (data not shown). The isolated neutrophil population also likely contained a few platelets. Similarly, after whole blood incubation with 2F9, platelet-specific antibodies label neutrophils that do not label neutrophils in the absence of 2F9 (data not shown). As GPIIb is located exclusively on platelets and megakaryocytes [43], we postulate that 2F9 labeling of neutrophils is through an indirect mechanism in which 2F9 induces neutrophil-platelet cross-linking of 2F9 labeled platelets through the neutrophil Fc receptor.

In Vivo ITP Model Development

2F9 Induces a Severe, Dose-Dependent Thrombocytopenia

To determine the effective dose of 2F9 necessary to reach a target platelet nadir of 5,000 to 30,000 platelets/µl, we performed a dose titration study in two dogs and a repeatability study in the subsequent three dogs. Within 2 hours of a median cumulative 2F9 administration of 63 µg/kg (range 50.0-166.6 µg/kg), all dogs developed profound thrombocytopenia (range

11-28 × 10^3/µl) (Figure 3). Compared to the isotype control group, platelet nadir was significantly lower (median (range): 6 (4-11) × 10^3/µl vs. 200 (179-209) × 10^3/µl; P=0.036) and change in platelet count from baseline to nadir was significantly greater in the 2F9-treated group (median (range): 238 (179-325) × 10^3/µl vs. 4 (0-10) × 10^3/µl; P=0.036). Platelet nadir was in our target range and platelet count remained less than 40 × 10^3/µl in all 2F9-treated dogs for 24 hours. Nadir platelet counts were on average 3% of baseline counts. Dosing was predictable: in the dose-repeatability dogs, after an initial dose of 50 µg/kg 2F9, the second dose needed to reach the target nadir could be accurately calculated from the initial platelet decrease. Due to the dose titration component of the study, dogs received up to 6 doses of 2F9 within a period of 3 days. Platelet counts recovered in between 5 to 10 days (median of 6 days).

Dogs remained bright, alert, and responsive throughout the study. Temperature, pulse, and respiration were unchanged during antibody administration and remained within normal limits at all study time points (not shown). This is in sharp contrast to observations in mice where the administration of antibodies to GPIIbIIIa leads to acute systemic reactions, hypothermia, and anemia [1].

ITP Model Dogs Demonstrate Variable Bleeding

Experimental dogs demonstrated a range of mild bleeding from few cutaneous petechiae and/or ecchymoses to transient microscopic hematuria or transient melena. Bleeding scores ranged from 0 to 4 out of a possible 16 (Supplemental Figure 2). Overall, bleeding was clinically mild and required no supportive care as dogs never demonstrated clinical signs of anemia or bleeding into critical areas. Interestingly, two dogs developed large delayed ecchymoses (up to 8.5 by 13 cm) when their platelet count was already recovering (active bruising at 48 hours through 120 hours in one dog and 120 hours through 192 hours in the second dog). Figure 4 demonstrates one such ecchymosis and the platelet counts at which these bruises developed and continued to enlarge. Histologic examination of these bruises following cutaneous biopsies did not demonstrate any explanations other than bleeding for the ecchymoses such as vasculitis or a thrombus (data not shown).

BMBTs were variably prolonged at time zero in 2F9-treated dogs from 4.80 ± 2.09 times baseline compared to 2.15 ± 0.32 times baseline in control dogs. Interestingly, the longest time zero BMBTs were in the two dogs that later developed the delayed ecchymoses (>15 minutes and 13.13 minutes).

White blood cell counts (P=0.9957) and hematocrits (P=0.198) were not different between control and treated dogs over time, though hematocrits did decrease slightly in both groups due to repeated phlebotomy (not shown). One 2F9 treated dog became transiently neutropenic for two hours at time zero and one developed a severe delayed neutropenia (nadir on day 6 of 99 neutrophils/µl, duration 12 hours through 240 hours) when his platelet count was already recovering (Supplemental Figure 3). The cause of the prolonged delayed neutropenia could not be determined: the dog was thoroughly evaluated for sepsis (though he showed no clinical signs of sepsis), had no 2F9 binding of his neutrophils, and had no canine IgG, IgM, or complement binding of his neutrophils (data not shown). The neutropenia resolved following administration of GM-CSF (Neupogen, Amgen, Thousand Oaks, CA). The transient neutropenia may have been the result of non-specific 2F9 neutrophil binding through neutrophil Fc receptors as described above. The delayed neutropenia may have been the result of generalized stimulation of the reticuloendothelial system secondary to 2F9 administration.

Phenotypes of Circulating Platelets

In the 2F9 treated dogs, circulating platelets were partially (16 to 63% at 6 hours) opsonized with 2F9 as demonstrated by flow cytometric detection of surface-bound murine IgG2a (Figure 5) for an average of 24 hours. This is in contrast to the murine model in which rat antibodies targeting murine GPIIbIIIa result in a paradoxical loss of surface expression of GPIIbIIIa and minimal surface-bound rat IgG is detected on platelets from antibody-treated mice [1]. The presence of anti-platelet antibody is consistent with what is observed in about 60% of humans and 77% of dogs with spontaneous ITP [44-47].

Induced ITP Model Is Not Pro-Thrombotic

Both fibrinogen and D-dimers increased slightly over time in both treated (n=5) and control (n=3) groups but no difference (P=0.268 fibrinogen; P=0.457 D-dimers) was noted between experimental groups, indicating that, as expected, this is not a model of disseminated intravascular coagulation (Supplemental Figure 4).

Experimental ITP Cytokine/Chemokine Profile Models the Spontaneous Canine Disease and Identifies Platelets as an Important Source of Serum IL-8

2F9 infusion generated negligible systemic inflammation, as assessed by white blood cell count and serum cytokine measurement. Changes over time in TNF-α, MCP-1, IL-6, IL-10, GM-CSF, IL-2, CXCL10, IFN-γ, IL-7, IL-15, CXCL-1 orthologue, and IL-18 were not significantly different between 2F9 and αYFA treated dogs (Supplemental Figure 5). The dog that received GM-CSF was excluded from cytokine analysis at subsequent time points. Unexpectedly, however, serum IL-8 tracked faithfully with platelet count, demonstrating that platelets are a major source of serum IL-8 in dogs (Figure 6A-B). IL-8 was significantly different in 2F9-treated compared to control dogs (P=0.000). Our initial finding of IL-8 tracking with platelet count on the multiplex assay was confirmed with the IL-8 specific ELISA. The absolute values of serum IL-8 were higher using the multiplex assay (4439.58 ± 2019.28 pg/ml in baseline samples) than the ELISA (1499.50 ± 525.84 pg/ml) but the dramatic IL-8 decrease with platelet depletion was consistent (not shown). Although α granules are known to contain IL-8 [48], platelets have not been previously described as a significant serum IL-8 source. Since IL-8 is an important neutrophil chemokine, our finding may illuminate a novel mechanism of platelet-neutrophil cross-talk.

In light of our unexpected IL-8 findings, we investigated whether the same pattern of decreased IL-8 was present in dogs with spontaneous ITP by surveying dogs with the naturally-occurring disease. Ten dogs with naturally-occurring thrombocytopenia were evaluated including 5 with primary ITP, one with secondary ITP due to Ehrlichiosis, and four with thrombocytopenia of other causes (pancytopenia of unknown cause, lymphoma and possible chemotherapy induced-thrombocytopenia, suspected myelodysplastic syndrome, and

DIC). Their signalment information is listed in Table 2 and their cytokine/chemokine data are shown in Table 3. Normal values for healthy dogs were determined from the baseline samples from our 8 experimental dogs (treated and control dogs combined). Our IL-8 findings were repeatable in dogs with naturally-occurring disease: significantly decreased levels of serum IL-8 were found in all dogs with naturally-occurring thrombocytopenia (primary ITP, P=0.0016; other causes of thrombocytopenia, P=0.0162) compared to healthy control dogs as shown in Figure 6C. When a repeated measures ANOVA was performed to screen for differences between the groups of dogs in Table 3 (excluding the one secondary ITP dog), no other cytokine/chemokine differences were detected. The cytokine and chemokine profile of dogs with spontaneous primary ITP was similar to that of our experimental dogs at platelet nadir and 24 hours as shown in Table 3, suggesting that the ITP model recapitulates the naturally-occurring disease.

Identification of Candidate Bleeding Predictors

As a preliminary survey to identify candidate bleeding predictors, we looked for correlation between the mediators measured and bleeding scores. No bleeding correlations were found in experimental dogs, which is not surprising given their relatively low range of bleeding scores. In dogs with spontaneous primary ITP, IL-10 levels were directly correlated with bleeding score (R^2=0.6053, P<0.022), but this would not hold up to multiple testing corrections. This warrants further investigation in more patients.

Discussion

We have developed an induced canine model of ITP which is highly analogous to the naturally-occurring disease in dogs and humans. In this study, using GT platelets, we better characterized 2F9 as an antibody that targets a component of the GPIIb/IIIa complex, with an immunoblotting pattern most consistent with GPIIb (Figure 1). This confirmed the previous suggestion by Burstein et al. that 2F9 might target GPIIb [30]. The GPIIb specificity of 2F9 made it a logical choice for development of an ITP model as the majority of platelet autoantibodies in ITP patients target epitopes on the fibrinogen receptor (GPIIbIIIa) and von

Willebrand factor receptor (GPIb-IX-V) [23]. We also demonstrated that 2F9, as assessed by flow cytometry, is not an activating or inhibitory antibody (Figure 2). This makes 2F9 an excellent choice for development of an ITP model that could later be used to study platelet reactivity in ITP since the antibody itself does not influence platelet function.

Our *in vivo* studies demonstrated that dosing of 2F9 was repeatable and could be tailored to reach a desired platelet nadir. After a starting dose of 50 μg/kg, initial platelet count decrease could be used to calculate the additional dose necessary to reach a clinically relevant target nadir of 5,000 to 30,000 platelets/μl, the platelet count at which ITP patients most commonly demonstrate heterogeneous bleeding. Interestingly, the median effective cumulative 2F9 dose of 63 μg/kg combined with a median starting platelet count of 243,000 platelets/μl translates to about 10,000 molecules of 2F9 per platelet. This parallels what has been described in the spontaneous disease: human ITP patients have on average 4,000 IgG molecules per platelet [49] and canine ITP patients have a median of 10,000 IgG molecules per platelet [24]. Monocytes have a threshold of 1,000 IgG molecules per platelet to recognize and bind IgG-coated platelets through their Fc receptors [49].

At the platelet nadir, a median of 63.3% of platelets were opsonized with 2F9 (Figure 5). Again, this recapitulates spontaneous human and canine ITP where circulating antibodies are present in 60% and 77% of cases, respectively [44-47]. This contrasts with available murine models using antibodies to target GPIIbIIIa in which surface expression of GPIIbIIIa on platelets is lost and only minimal platelet-surface bound antibodies can be detected [1].

Just as with the spontaneous disease, experimental dogs demonstrated a range of mucocutaneous bleeding phenotypes from few cutaneous petechiae and or ecchymoses to transient microscopic hematuria and melena [10, 50-54]. Dogs also had prolonged buccal mucosal bleeding times, as has been described in dogs with spontaneous thrombocytopenia [55]. Overall, bleeding in these dogs was quite mild, which may have reflected the short duration of thrombocytopenia. It is unclear why the two dogs developed delayed ecchymoses as platelet count was recovering. The ecchymoses may be the result alterations in endothelial integrity due to previous severe thrombocytopenia as platelets have vascular stabilizing

effects [56-58], (Chapter 3). As best as possible, we ruled out thrombosis or vasculitis causing these lesions with histologic examination of cutaneous biopsies (not shown).

Most significantly, our model is not pro-inflammatory and the cytokine profile in the experimental ITP dogs matched that of dogs with spontaneous primary ITP (Table 3). Similarly, adult ITP is not a pro-inflammatory disorder. Adult ITP is, however, marked by a type-1 immune response with elevated Th1 cytokines (IL-2, IFN-γ) and decreased Th2 cytokines (IL-4 and IL-5)[59, 60]. Reduced IL-10 serum levels have been described in ITP patients with active disease [59, 61, 62]. The cytokine profile in spontaneous canine ITP has not been previously evaluated to our knowledge. We did not observe a Th1 profile in our experimental or clinical dogs, but our sample size may have been too small to detect this pattern.

Our experimental dog model sharply contrasts with passive murine models utilizing antibodies against GPIIbIIIa. In these models, through incompletely understood mechanisms involving platelet-activating factor, mice develop acute systemic reactions, uncoordinated movements, and hypothermia [1, 25]. Like our experimental dogs, patients with naturally-occurring disease due to antibodies targeting a variety of platelet epitopes including GPIIbIIIa do not demonstrate acute systemic reactions, only variable mucocutaneous bleeding and fatigue [26]. In terms of clinical presentation, the canine model more accurately represents the spontaneous disease than do the passive anti-GPIIbIIIa murine models.

The relationship between IL-8 and platelets suggested by our study is intriguing (Figure 6). IL-8 is a CXC chemokine that serves as a potent chemoattractant and neutrophil activator [63, 64]. Many cells produce IL-8, including endothelial cells, neutrophils, fibroblasts, mast cells, monocytes, macrophages, and T lymphocytes [63, 65-72]. Although platelets and megakaryocytes have been documented to contain IL-8 in their α granules, they have never been considered to be a major source of IL-8 [48, 73, 74]. In our study, serum IL-8 was not correlated with other blood cell counts like neutrophils (not shown). Our study demonstrates that, at least in dogs, platelets provide the major source of serum IL-8. Our ITP model results led us to examine canine cases of naturally-occurring ITP and we determined

that the same marked decrease in serum IL-8 is also present in the spontaneous canine disease (Figure 6C).

We do not propose that IL-8 is a major player in ITP pathogenesis, but rather that our model uncovered a potentially novel mechanism of platelet-neutrophil interaction. Platelets are recognized as important players in inflammatory processes and diseases, recruiting leukocytes into inflamed tissues. Indeed, reduced numbers of leukocytes are observed in the inflamed organs of thrombocytopenic mice [57, 75]. The leukocyte-recruiting effect of platelets can be both beneficial and deleterious [57]. Leukocyte recruitment by platelets assists in pathogen clearance [57, 76]. However, platelet-mediated leukocyte recruitment also promotes unwanted tissue damage like lung injury in models of acute lung injury and atherosclerosis [73, 77, 78]. Understanding how platelets interact with leukocytes is essential for therapeutically manipulating these beneficial and deleterious effects. Multiple mechanisms for platelet-leukocyte recruitment have been described including direct platelet-leukocyte interactions via platelet P-selectin [73, 75], platelet secretion of cytokines like RANTES [73, 79], and platelet-mediated up-regulation of adhesion molecules on the endothelium [57, 73, 80]. However, platelet secretion of IL-8 has not, to our knowledge, been described as an important mechanism of leukocyte recruitment. As IL-8 is a major neutrophil chemokine [64], this putative mechanism of platelet-neutrophil cross-talk warrants further investigation. Importantly, mice lack IL-8, so platelet contribution of IL-8 would be missed in murine ITP models [81]. Rodents even lack a direct homologue as IL-8; instead three chemokines are considered functional rodent homologues of IL-8 homologues: CXCL1/KC, CXCL2/MIP-2, and CXCL5-6/LIX [81].

While it is possible that the IL-8 assays employed were recognizing the structurally similar platelet-derived β-thromboglobulin [64], this is unlikely given that we confirmed our multiplex assay results with a different manufacturer's ELISA assay. Interestingly, serum IL-8 levels in healthy dogs (~1,500 pg/ml) are orders of magnitude greater than serum IL-8 levels in healthy people (~3 pg/ml), so species differences in IL-8 distribution may exist [63]. However, we also found a significant correlation (R=0.52; P=0.041) between platelet count and serum IL-8 in human ITP patients (data not shown).

In the dogs that we examined with naturally-occurring primary ITP, bleeding as quantified by bleeding score, was directly correlated with IL-10 levels. In some studies but not others, serum IL-10 levels have been observed to be decreased in human ITP patients compared to healthy controls and to increase during disease recovery [59, 61, 62]. A genetic polymorphism that results in low IL-10 production has been associated with more severe thrombocytopenia in adult ITP patients [82] and this low IL-10 producing haplotype is a predictor of which children with acute ITP will go on to develop the chronic disease [83]. To our knowledge, there has been no previous study relating IL-10 to bleeding tendency. Our results warrant further investigation of the relationship between IL-10 and bleeding tendency in more canine and human ITP patients. Ultimately, IL-10 could be administered locally or systemically to model dogs to further explore the relationship between IL-10 and hemorrhage.

We recognize that our passive canine model has limitations similar to any passive model of ITP in that it does not recapitulate the immunopathogenesis that leads to endogenous autoantibody production in patients with ITP. In spontaneous ITP, endogenous antiplatelet antibodies are produced and circulate continuously [14]. Despite this, the model has many potential applications for investigation of the disease once platelet autoantibody production has commenced. This model can be used to determine the efficacy and safety of novel ITP therapeutics. Once efficacy of a treatment in model dogs is demonstrated, it can be easily translated to clinical canine and human patients. If the duration of thrombocytopenia is inadequate to assess treatment effects, repeated 2F9 administration or constant rate infusions of 2F9 could be administered as repeated exposure to 2F9 over a 3 day period was well-tolerated in our dogs. An alternate strategy would be to employ a higher initial 2F9 dose similar to that administered to our proof of concept dog (300 µg/kg) that induced absolute thrombocytopenia for 3 days (data not shown). This higher dose may have enabled 2F9-megakaryocyte binding and thereby reduced platelet production as has been described with autoantibodies in human ITP [84].

The dog model can also be used to test theories relating to differential bleeding. One suggested mechanism by which thrombocytopenic bleeding occurs is through inflammation that destabilizes the vasculature. In a murine model of thrombocytopenia, only mice that had

concurrent inflammation and thrombocytopenia developed hemorrhage [85]. We can capitalize on our knowledge that our model is non-inflammatory by assessing how bleeding phenotype differs when inflammation is induced locally or systemically in model dogs.

One limitation of our study that must be recognized is the small sample size. Differences between control and 2F9-treated dogs could have been missed and some candidate bleeding predictors in clinical patients might have been overlooked. Future studies involving more experimental and clinical dogs are indicated.

The weak neutrophil-2F9 binding that we observed was unexpected but does not detract from our model. We believe that the neutrophil-2F9 binding occurred indirectly through the neutrophil Fc receptor for three reasons: 1. GPIIb, the 2F9 target, is exclusively located on megakaryocytes and platelets [43]. 2. The presence of platelets greatly increased 2F9 labeling of neutrophils, suggesting the mechanism of 2F9 binding is indirect. We propose that 2F9 molecules that are specifically binding platelets through their Fab region bind to neutrophils via the neutrophil Fc receptor. 3. Presence of 2F9 enables a platelet-specific antibody to label neutrophils that does not label neutrophils in the absence of 2F9 (not shown). One dog became transiently neutropenic for one hour after 2F9 administration (Supplemental Figure 3A), likely through this indirect binding mechanism causing neutrophils to be cleared with platelets. This did not detract from the dog's suitability as an ITP model. A second dog developed severe profound neutropenia (Supplemental Figure 3B) when platelet count was recovering. No antibodies, 2F9 or autoantibodies, could be detected on the dog's neutrophils. We believe this dog's neutropenia was spurious and the result of generalized stimulation of the reticuloendothelial system. No other dogs demonstrated neutrophil count decreases. In general, we don't expect the weak and indirect 2F9-neutrophil interactions to compromise the accuracy of our ITP model.

In summary, we have developed the only large animal model of ITP. This model is highly translational and representative of the naturally-occurring disease in canines and humans. Indeed, using our model we have already identified IL-8 as an exciting putative mechanism of neutrophil-platelet cross-talk, at least in canines. Such a mechanism could not have been elucidated in mice due to their lack of IL-8.

Table 1. ITP Bleeding Score. Bleeding was graded at 8 different anatomic sites and the grade at each site summed for a total bleeding score out of a possible 16. This bleeding score was adapted from human ITP bleeding score that has been validated for use in people [34].

Site	**Bleeding grade**		
	0	***1***	***2***
Skin (PE)	None	≤1 bruise, scattered petechiae	>1 bruise and/or diffuse petechiae
Oral mucosa (PE)	None	Petechiae present, but ≤10	>10 petechiae and/or gingival bleeding
Intraocular (PE)	None	N/A	Hyphema
Epistaxis (observed)	None	Bleeding <5 min (per episode)	Bleeding ≥ 5 min (per episode)
Gastrointestinal (observed)	None	Occult blood (stool o-toluidine test)	Gross blood
Urinary (observed)	None	Microscopic (positive on dipstick)	Macroscopic
Pulmonary (suspected/observed)	None	N/A	Yes
Intracranial hemorrhage (suspected/observed)	None	N/A	Yes
Total Score (out of possible 16)			

Table 2. Signalment of naturally-occurring canine thrombocytopenia cases. *FS indicates spayed female; MC, neutered male.

Dog Number	Breed	Age (yrs)	Sex*	Diagnosis
1	Doberman Pinscher	6	FS	Primary ITP
2	Labradoodle	6	MC	Other (pancytopenia of unknown cause)
3	Greyhound	3	FS	Primary ITP
4	Maltese	10	MC	Primary ITP
5	Rottweiler	8	MC	Primary ITP
6	Labrador Retriever	9	FS	Other (lymphoma and suspected chemotherapy-induced thrombocytopenia)
7	Soft Coated Wheaten Terrier	4	FS	Other (suspected myelodysplastic syndrome)
8	Cocker Spaniel	5	FS	Primary ITP
9	German Shepherd	4	MC	Secondary ITP (Ehrlichiosis)
10	Irish Setter	12	FS	Other (Hemangiosarcoma and DIC)

Table 3. Experimental ITP dogs and dogs with spontaneous primary ITP have similar cytokine/chemokine profiles and other laboratory parameters. Comparison of laboratory parameters [median (range)] between dogs with naturally-occurring thrombocytopenia (primary ITP, secondary ITP, and thrombocytopenia due to other causes), experimental dogs and time-matched controls, and healthy dogs. Number (n) in each group is indicated at top of column or in individual cell if different than column header. The secondary ITP case was not employed in statistical comparisons. In the paired analyses (exact Wilcoxon rank sum test), healthy dogs were not compared with control or experimental dogs since healthy dogs were these same dogs at baseline; instead control and experimental were compared to each other at matching time points. NS = not statistically significant; S = significant as shown in relevant cells.

Laboratory Parameter	Healthy (n=8)	Primary ITP (n=5)	Secondary ITP (n=1)	Thrombocytopenia (other) (n=4)	Experimental ITP at time 0 (n=5)	Experimental ITP at 24 hr (n=5)	Control at time 0 (n=3)	Control at 24 h (n=3)	Differences
GM-CSF (pg/ml)	34.06 (3.57-1135.58)	59.53 (22.82-145.65)	5664.22	1.91 (1.91-68.41)	6.32 (3.57-1022.53)	6.32 (0.25-698.8)	74.31 (6.32-163.99)	98.6 (16.88-129.88)	NS
IFN-gamma (pg/ml)	132.67 (79.87-347.16)	35.46 (35.46-283.3)	1890.82	35.46 (35.46-153.02)	212.3 (42.84-562.2)	173.26 (79.87-878.1)	79.87 (79.87-132.67)	42.84 (42.83-287.13)	NS
IL-2 (pg/ml)	27.61 (5.98-581.42)	55.97 (26.78-67.87)	4513.74	14.89 (2.05-42.41)	6.03 (5.98-456.68)	6.03 (5.98-329.25)	48.00 (10.56-73.77)	55.99 (43.98-106.83)	NS
IL-6 (pg/ml)	43.86 (2.71-328.49)	39.97 (4.59-103.58)	4369.50	29.25 (14.36-76.59)	21.37 (2.71-407.39)	5.93 (5.93-238.87)	43.89 (24.27-65.38)	65.38 (54.73-96.54)	NS
IL-10 (pg/ml)	5.93 (5.93-445.10)	4.06 (2.14-37.62)	175.16	21.54 (2.14-66.64)	5.93 (5.93-1,142.66)	5.93 (5.93-133.25)	5.93 (5.93-5.93)	5.93 (1.05-5.93)	NS
IL-8 (pg/ml)	3724.23(1954.34-6881.63)	74** (20.55-373.53) ***P=0.0016 vs. healthy*	446.83	249.40* (37.75-2613.42) **P=0.0162 vs. healthy*	109.15* (109.15-692.03) **P=0.0357 vs. control 0*	242.72* (71.93-1594.39) **P=0.0357 vs. control 24*	4354.90 (2609.74-5527.64)	3102.75 (2984.24-5684.68)	S

Table 3 Continued

MCP-1 (pg/ml)	225.80 (26.43-704.06)	161.53 (74.33-185.19)	1016.47	305.31 (163.97-1,430.81)	227.50 (26.43-2,741.65)	86.13 (26.43-512.86)	256.63 (129.28-365.59)	319.93 (158.56-432.63)	NS
TNF-alpha (pg/ml)	6.21 (6.21-17.39)	9.65 (2.09-36.03)	1181.03	3.58 (2.09-6.58)	6.21 (0.86-15.32)	6.21 (0.00-6.21)	6.21 (6.21-6.21)	6.21 (6.21-6.21)	NS
IL-7 (pg/ml)	43.73 (5.10-297.33)	42.02 (9.71-80.99)	6970.06	7.48 (2.04-62.67)	5.10 (5.10-216.32)	21.74 (5.10-155.28)	102.18 (5.10-201.01)	191.46 (5.10-201.01)	NS
IL-15 (pg/ml)	161.48 (5.08-2,255.74)	85.76 (10.04-215.79)	9356.16	39.45 (23.50-177.69)	92.60 (5.08-2,450.65)	30.48 (5.08-1,443.25)	197.53 (137.00-412.32)	379.44 (197.53-488.03)	NS
IP-10 (pg/ml)	6.14 (2.93-24.53)	20.56 (4.41-35.04)	19.65	1.98 (1.98-45.26)	6.14 (0.82-18.17)	6.54 (1.39-21.38)	6.14 (6.14-22.09)	6.14 (6.14-27.94)	NS
CXCL-1 orthologue (pg/ml)	320.10 (84.78-617.93)	150.61 (104.80-1,330.12)	717.70	712.79 (100.24-1,408.88)	157.26 (37.77-785.26)	82.41 (30.09-151.32)	248.21 (89.52-354.10)	162.07 (97.75-420.09)	NS
IL-18 (pg/ml)	86.00 (0.58-1,600.21)	95.45 (83.60-237.63)	8761.73	128.36 (20.62-236.83)	20.19 (6.25-1,705.85)	42.25 (11.85-1,219.44)	77.85 (5.67-148.55)	88.76 (6.25-186.46)	NS
Fibrinogen (mg/dl)	341.50 (295.00-473.00)	621.00 (322.00-1,146.00); n=4		820.50 (790.00-851.00); n=2	389.00 (270.00-471.00)	389.00 (323.00-500.00)	373.00 (340.00-550.00)	414.00 (336.00-519.00)	NS
D-dimers (ng/ml)	163.00 (0.00-290.00)	212.50 (78.00-411.00); n=4		2,335.50 (649.00-4,022.00); n=2	231.00 (109.00-2,450.00)	133.00 (0.00-456.00)	224.00 (125.00-269.00)	269.00 (149.00-273.00)	NS
WBC (cells x 10^3/µl)	8.98 (6.92-11.73)	14.52 (6.72-40.23)	15.38	1.31 (0.35-35.71)	8.19 (3.66-14.16)	9.32 (4.94-12.93)	7.45 (7.26-11.07)	7.28 (7.24-10.28)	NS

Table 3 Continued

HCT (%)	48.80 (45.90-51.70)	40.50 (21.10-58.70)	46.6	20.70** (15.70-29.10) ***P=0.0040 vs. healthy*	45.70 (32.90-47.60)	37.80 (31.50-48.70)	43.40 (42.10-45.70)	44.80 (41.30-46.90)	S
Platelets (cells x 10^3/µl)	209.50 (183.00-329.00)	0.50** (0.00-16.00) ***P=0.0016 vs. healthy*	16	26.50** (0.00-29.00) ***P=0.0040 vs. healthy*	14.00* (11.00-28.00) **P=0.0347 vs control 0*	32.00* (23.00-36.00) **P=0.0119 vs. pITP *P=0.0357 vs. control 24*	208.00 (174.00-211.00)	189.00 (174.00-189.00)	S
Bleeding score	0.00 (0.00-2.00)	3.00** (1.00-8.00) *P=0.0055 vs. healthy*	1	3.50** (2.00-10.00) *P=0.0064 vs. healthy*	1.00* (0.00-1.00) *P=0.0254 vs. pITP*	2.00* (0.00-4.00) *P=0.0306 vs. control 24*	0.00 (0.00-0.00)	0.00 (0.00-0.00)	S

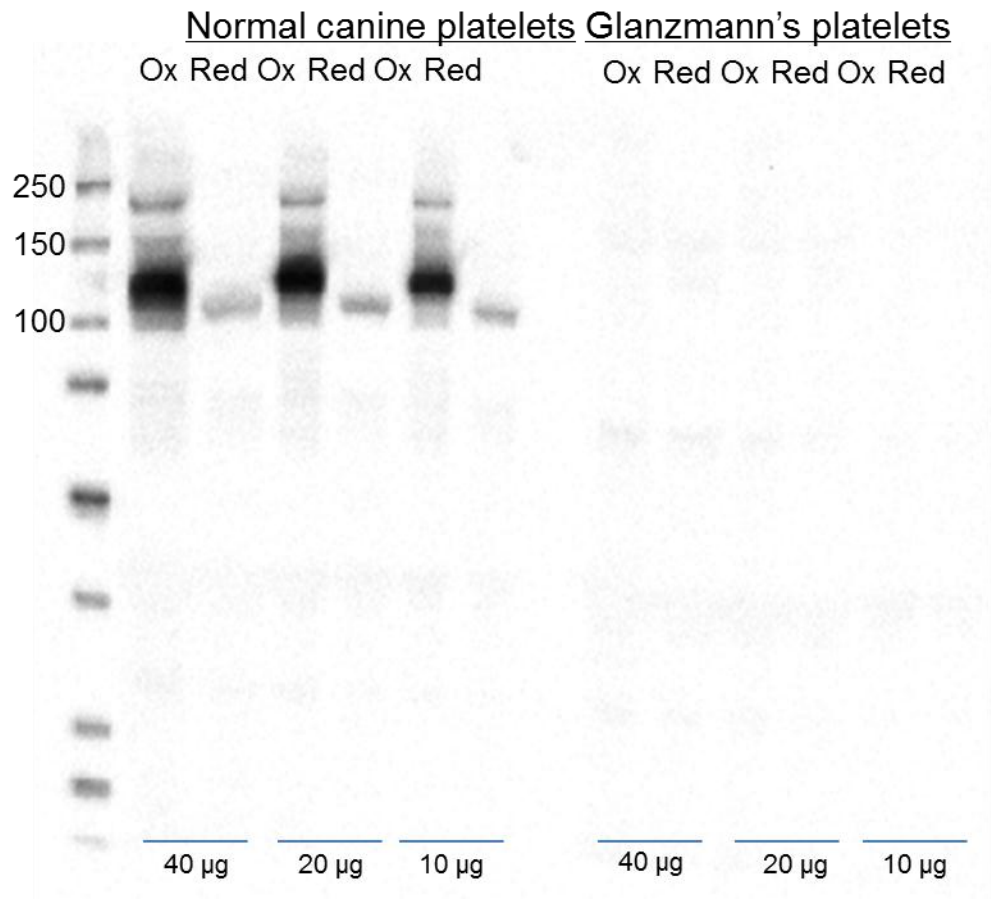


Figure 1. Antigenic specificity of 2F9. Western blot from resting platelets from normal and Glanzmann's thrombasthenia (GT) dogs were lysed and 40, 20 and 10 µg were separated on a 10% SDS-PAGE gel under nonreducing (Ox) and reducing (Red) conditions and then transferred onto a PVDF membrane which was probed with 2F9 and detected with a goat anti-murine HRP antibody and ECL. Note that no bands are observed in the GT platelet lanes indicating that 2F9 binds either GPIIb or GPIIIa. The prominent band at 124 under nonreducing conditions (and broad band ranging from 110 to 145) is most consistent with 2F9 binding GPIIb. The faint 108 band under reducing conditions likely represents a small amount of GPIIb adhered to GPIIIa despite the presence of EDTA in the reaction conditions. If 2F9 recognized GPIIIa, a band should be present at 100 kDa under both nonreducing and reducing conditions [37].

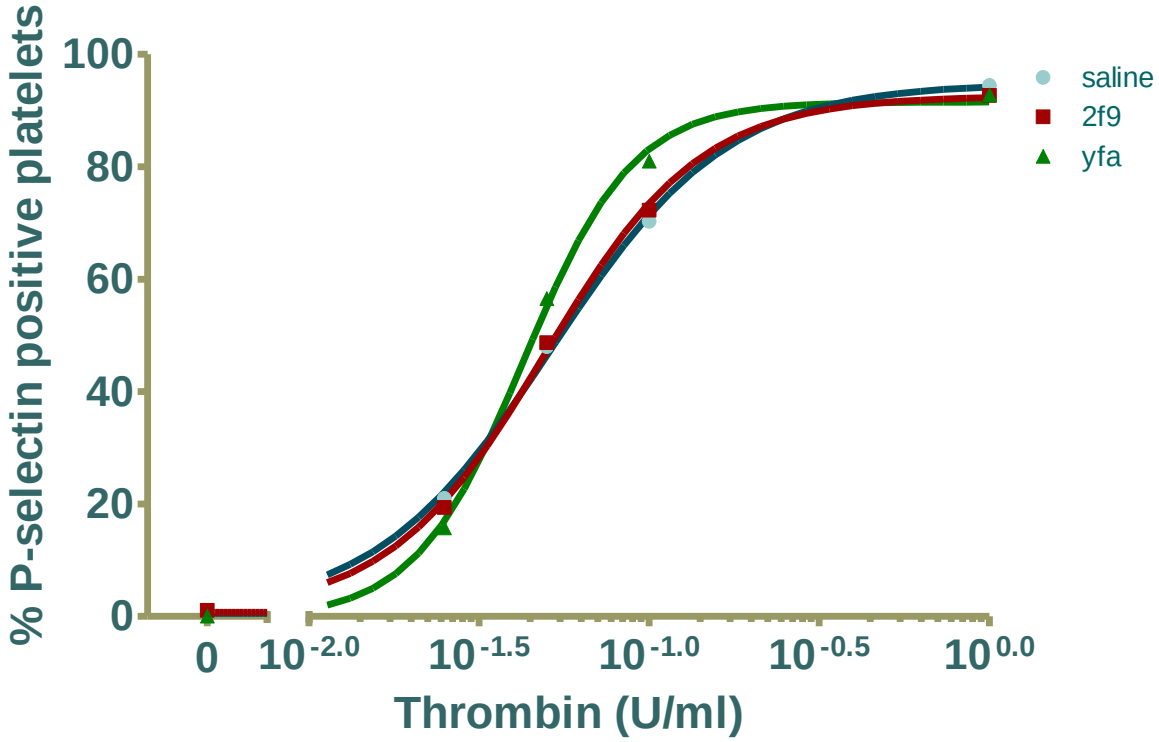


Figure 2. Exposure of canine platelets to 2F9 *ex vivo* does not alter their responsiveness to thrombin (expressed as percent of P-selectin positive platelets on the ordinate). Anticoagulated whole blood was incubated with 2 µg/ml 2F9 or αYFA isotype control or saline for 2 hours, followed by exposure to 0 to 1 U/ml of thrombin (abscissa – log concentration of thrombin U/ml). No significant differences were observed between the blood incubated with 2F9 (ED50 thrombin 0.049 U/ml), saline buffer (0.052 U/ml), or isotype control antibody (0.043 U/ml).

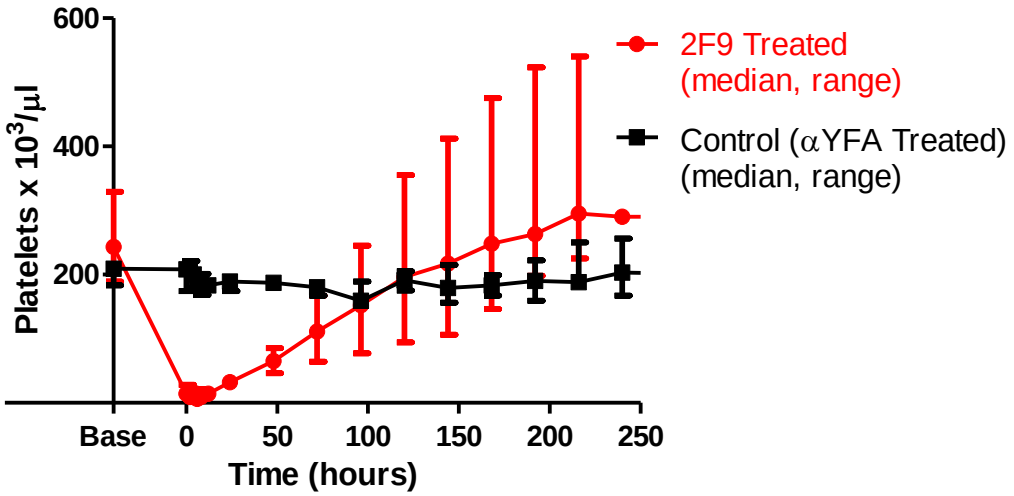
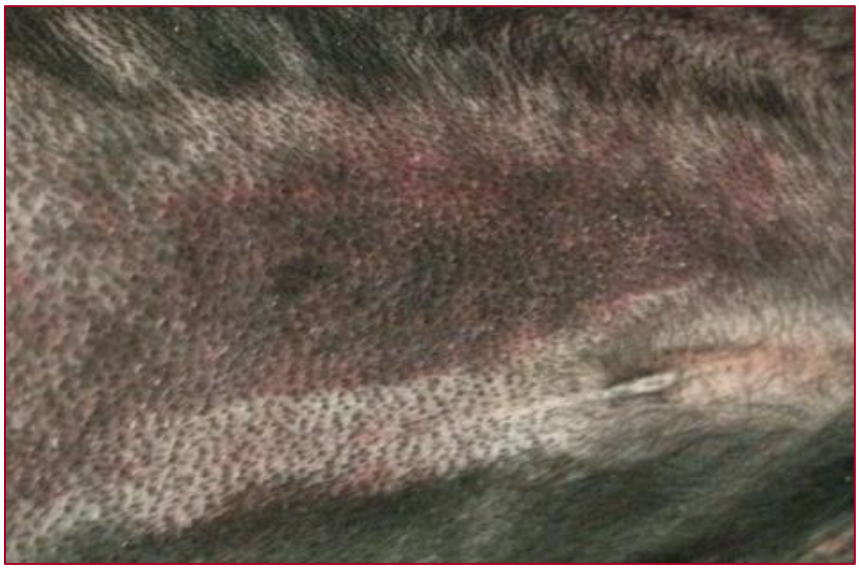


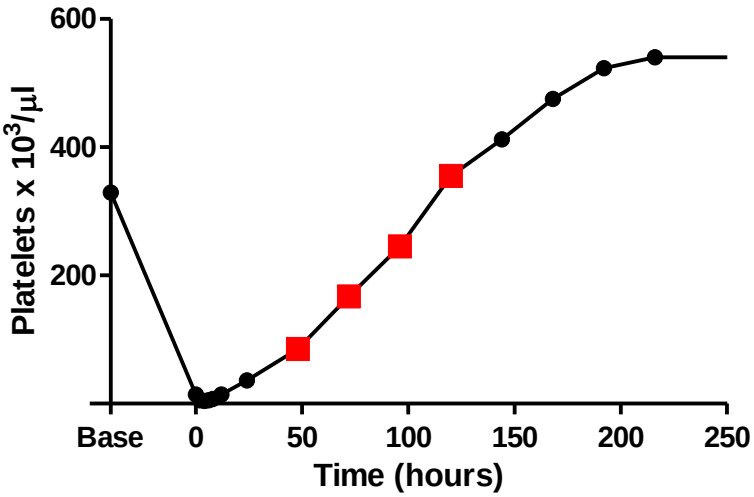
Figure 3. 2F9 induces a profound thrombocytopenia *in vivo*. Dogs received a median cumulative dose of 63 µg/kg 2F9 intravenously. Platelet counts were determined at the indicated times. Time zero occurred when platelet count first fell within the target platelet nadir (5,000-30,000 platelets/µl), a maximum of 2 hours after the last 2F9 dose, or was defined as 1 hour after control antibody administration. Injection of the isotype control antibody (αYFA) had no significant effect on the platelet count. Results are shown as median and range for 5 2F9-treated dogs and 3 control dogs.

Figure 4. Delayed ecchymosis formation during platelet count recovery. A. The photograph demonstrates a large abdominal ecchymosis (8.5 cm by 13 cm at its largest) in one 2F9-treated dog that began to form when platelet count had started to recover and continued to expand when the platelet count had exceeded baseline count. B. Red squares on the graph of platelet count over time demonstrate the period of active bleeding in this dog and in the second dog that developed a delayed ecchymosis (C).

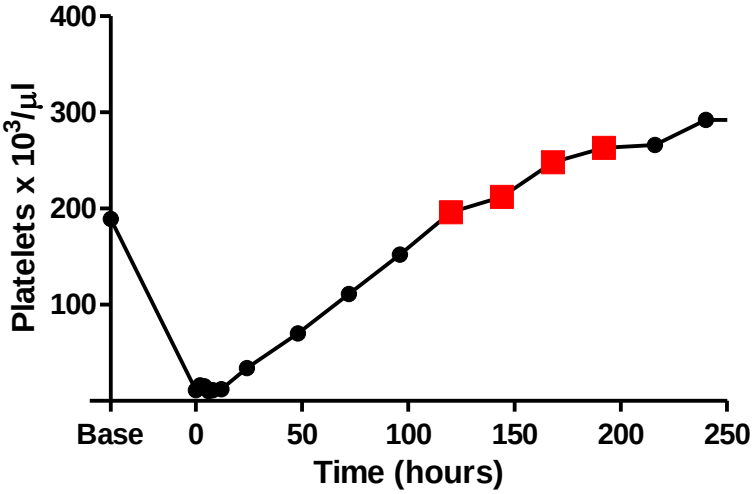
A.



B.


600
400
200
Platelets x 10^3/µl
Base
0
50
100
150
200
250
Time (hours)

C.


400
300
200
100
Platelets x 10³/µl
Base
0
50
100
150
200
250
Time (hours)

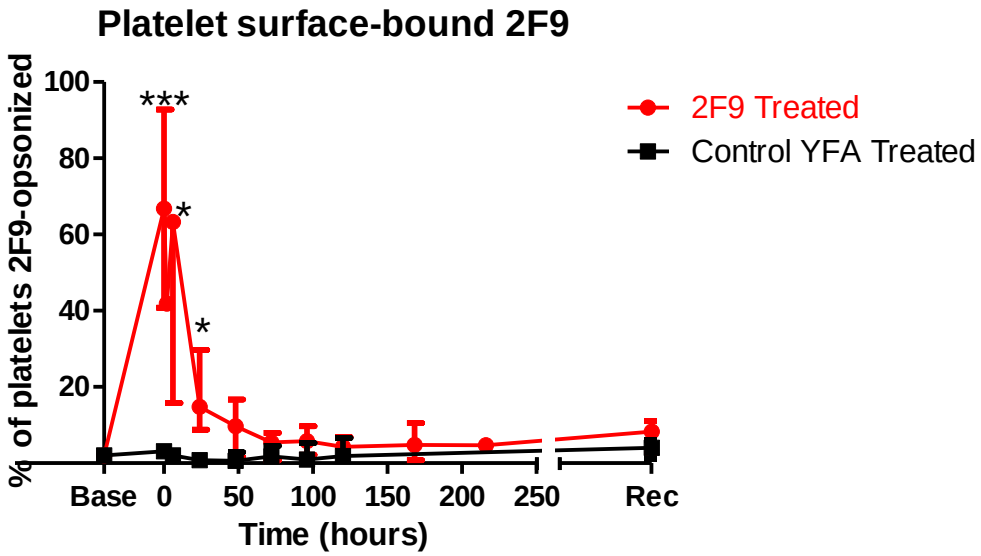
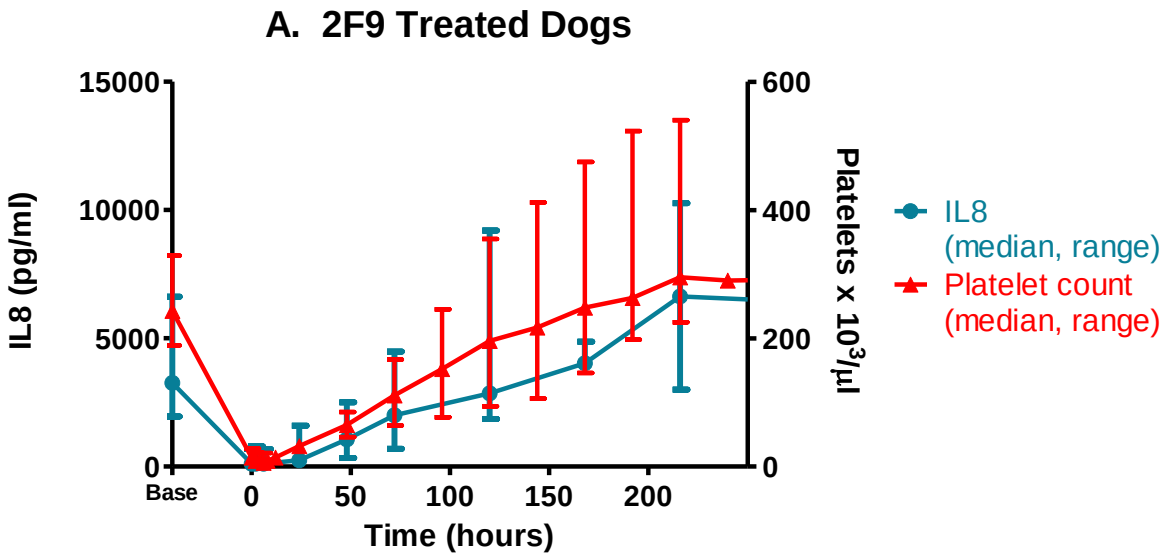
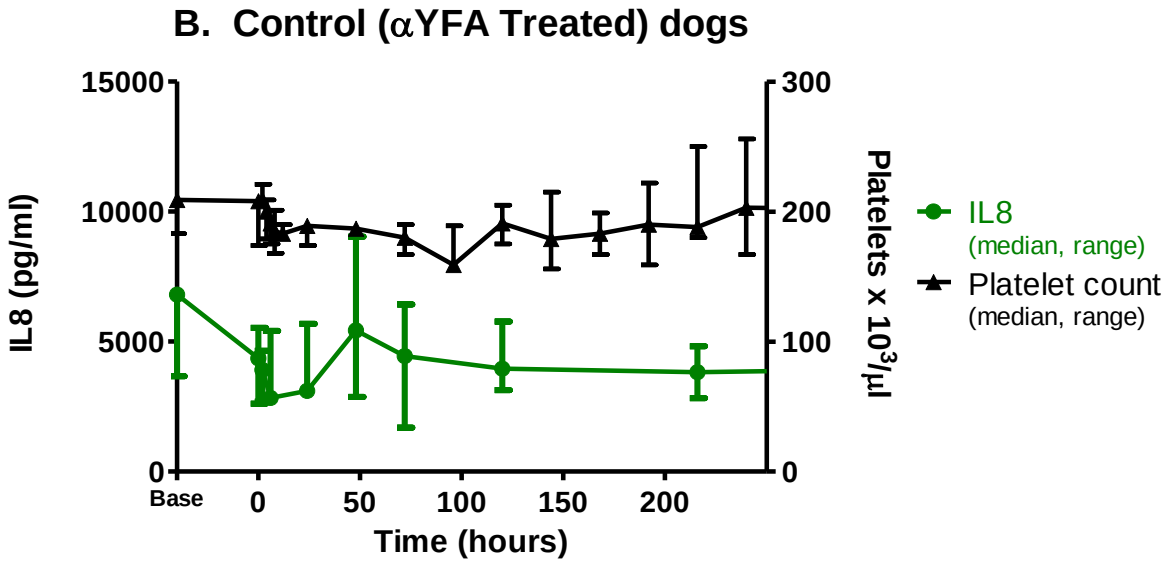
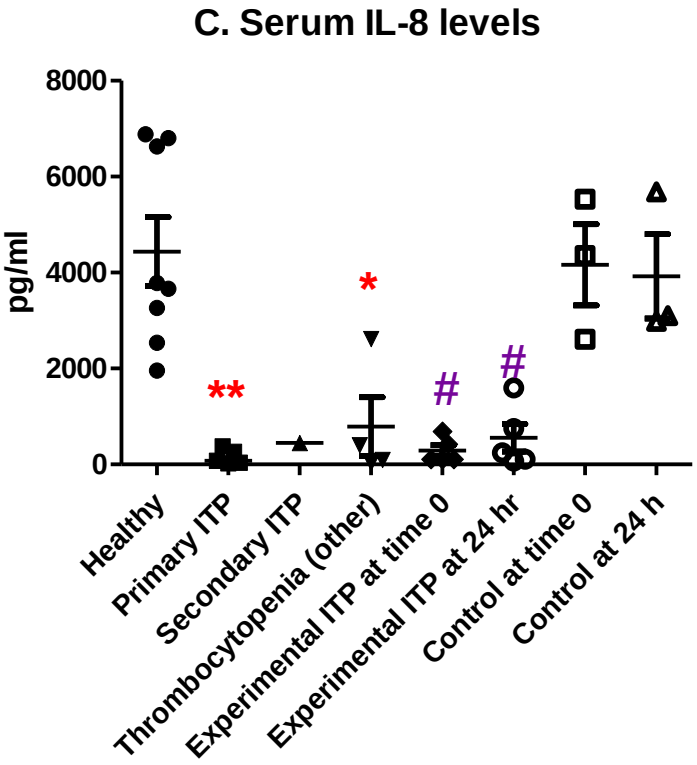


Figure 5. Platelets of 2F9-treated dogs are opsonized with 2F9 for an average of 24 hours. Median and range surface-bound 2F9 on platelets from isotype control (squares, n=3) or 2F9-treated dogs (circles, n=4) at the indicated times after final 2F9 dose. Base(line) is prior to antibody administration. Time zero is the time at which platelet count first fell into the target nadir range (5,000-30,000 platelets/µl) or 1 hour after control antibody administration. Rec(overy) is when platelet count returned to baseline or new bleeding stopped, whichever came later. 2F9 was detected via flow cytometry using a rat anti-mouse IgG2a antibody. ***P<0.001; *P<0.05 when comparing control and 2F9-treated dogs.

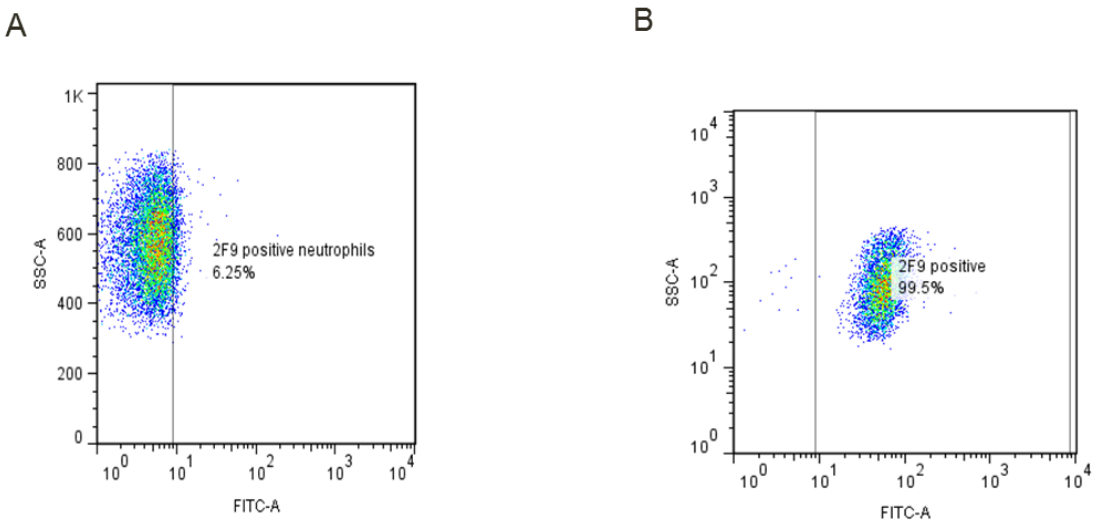
Figure 6. Serum IL-8 tracks with platelet count. Serum IL-8 (median, range) as measured by the Milliplex assay in 2F9-treated (A; n=5) and isotype control (αYFA) treated dogs (B; n=3) compared to platelet count. C. Serum IL-8 levels in dogs with naturally-occurring thrombocytopenia are decreased compared to healthy control dogs. **P=0.0016 healthy dogs (n=8) compared to primary ITP (n=5); *P=0.0162 healthy compared to thrombocytopenia other (n=4); #P=0.0357 experimental ITP at time 0 (n=5) compared to control at time 0 (n=3) and experimental at 24 (n=5) h compared to control at 24 h (n=3). Time zero is when platelet count first fell to target nadir of 5,000-30,000 platelets/μl or 1 hour after isotype control administration. Horizontal lines denote median values. Secondary ITP (n=1) was not considered in statistical analysis. Healthy dogs were not compared with control or experimental dogs since healthy dogs were these same dogs at baseline; instead control and experimental were compared to each other at matching time points.


A. 2F9 Treated Dogs
15000
10000
5000
0
IL8 (pg/ml)
600
400
200
0
Platelets x 10³/µl
Base
0
50
100
150
200
Time (hours)
IL8
(median, range)
Platelet count
(median, range)

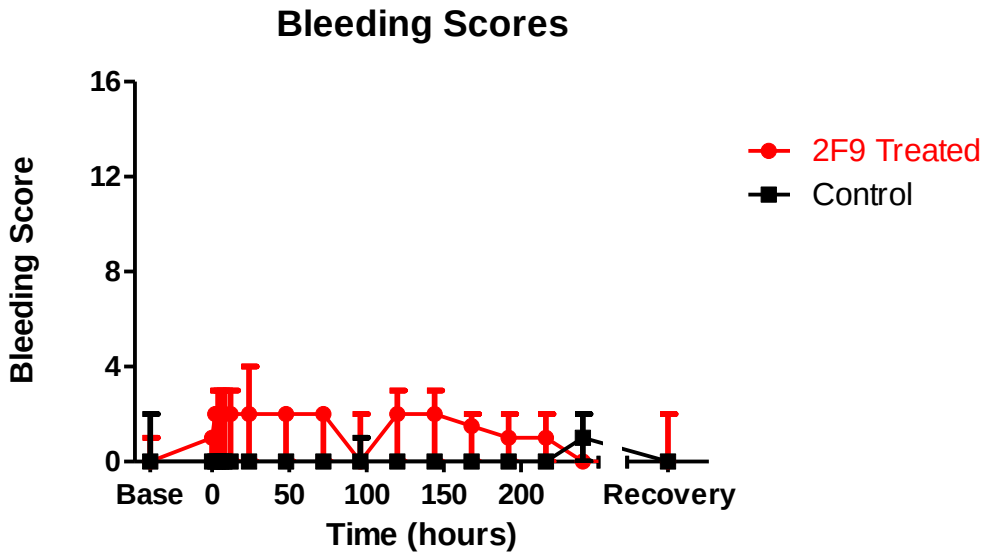

B. Control (αYFA Treated) dogs
15000
10000
5000
0
IL8 (pg/ml)
300
200
100
0
Platelets x 10³/µl
Base
0
50
100
150
200
Time (hours)
IL8
(median, range)
Platelet count
(median, range)


C. Serum IL-8 levels
pg/ml
8000
6000
4000
2000
0
**
*
#
#
Healthy
Primary ITP
Secondary ITP
Thrombocytopenia (other)
Experimental ITP at time 0
Experimental ITP at 24 hr
Control at time 0
Control at 24 h

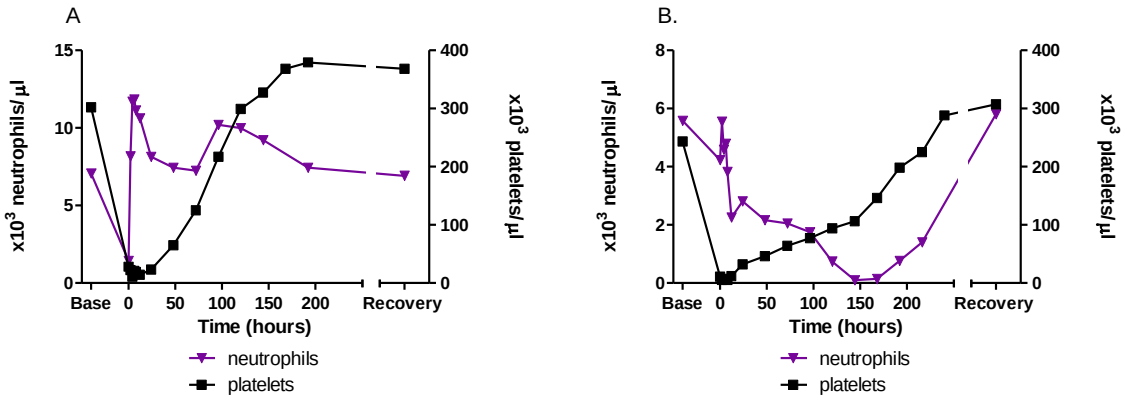
Supplemental Figures



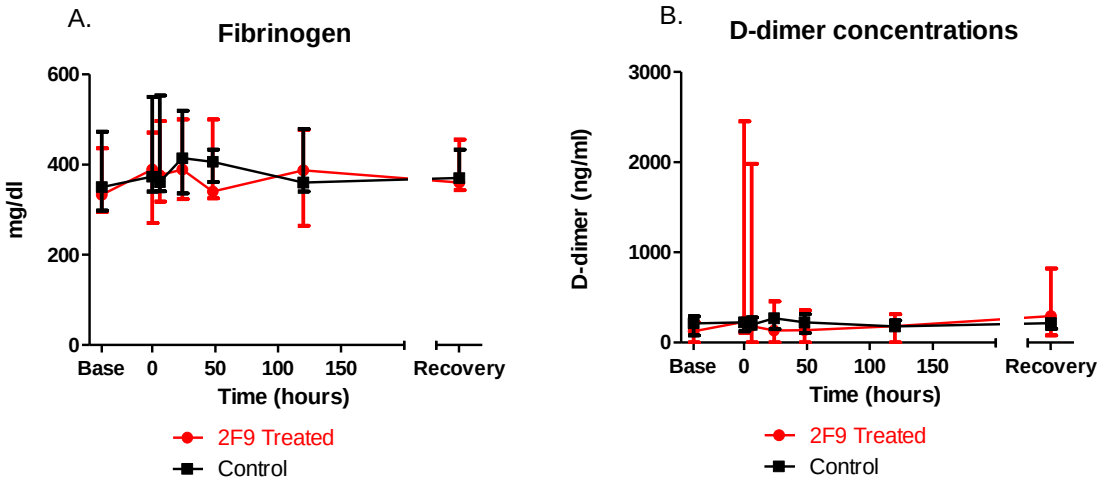
Supplemental Figure 1. 2F9 binding of canine neutrophils. A) 2F9 binding of neutrophils compared to canine platelets (B). At 4 µg/ml, 2F9 labeled 6.25% of canine neutrophils compared to 99.5% canine platelets. 100% of canine platelets are bound at as low as 0.4 µg/ml 2F9. The antibody interaction with neutrophils is believed to be nonspecific through the neutrophil Fc receptor (see results text). 2F9 positivity was determined with an isotype control antibody.



Supplemental Figure 2. Bleeding scores of 2F9-treated compared to control dogs. Maximum bleeding score was 16. Bleeding was variable but mild in 2F9-treated dogs. Data shown as median and range.

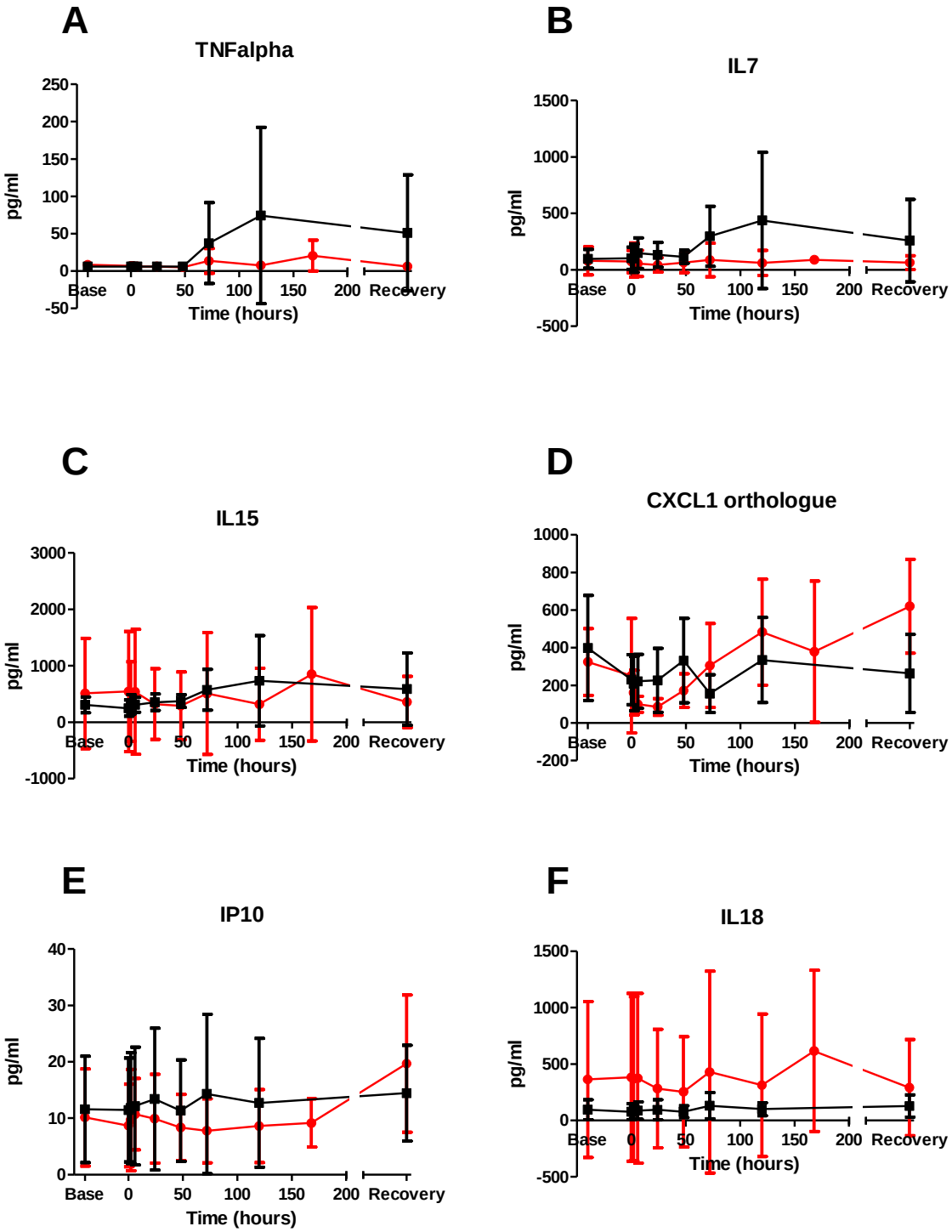


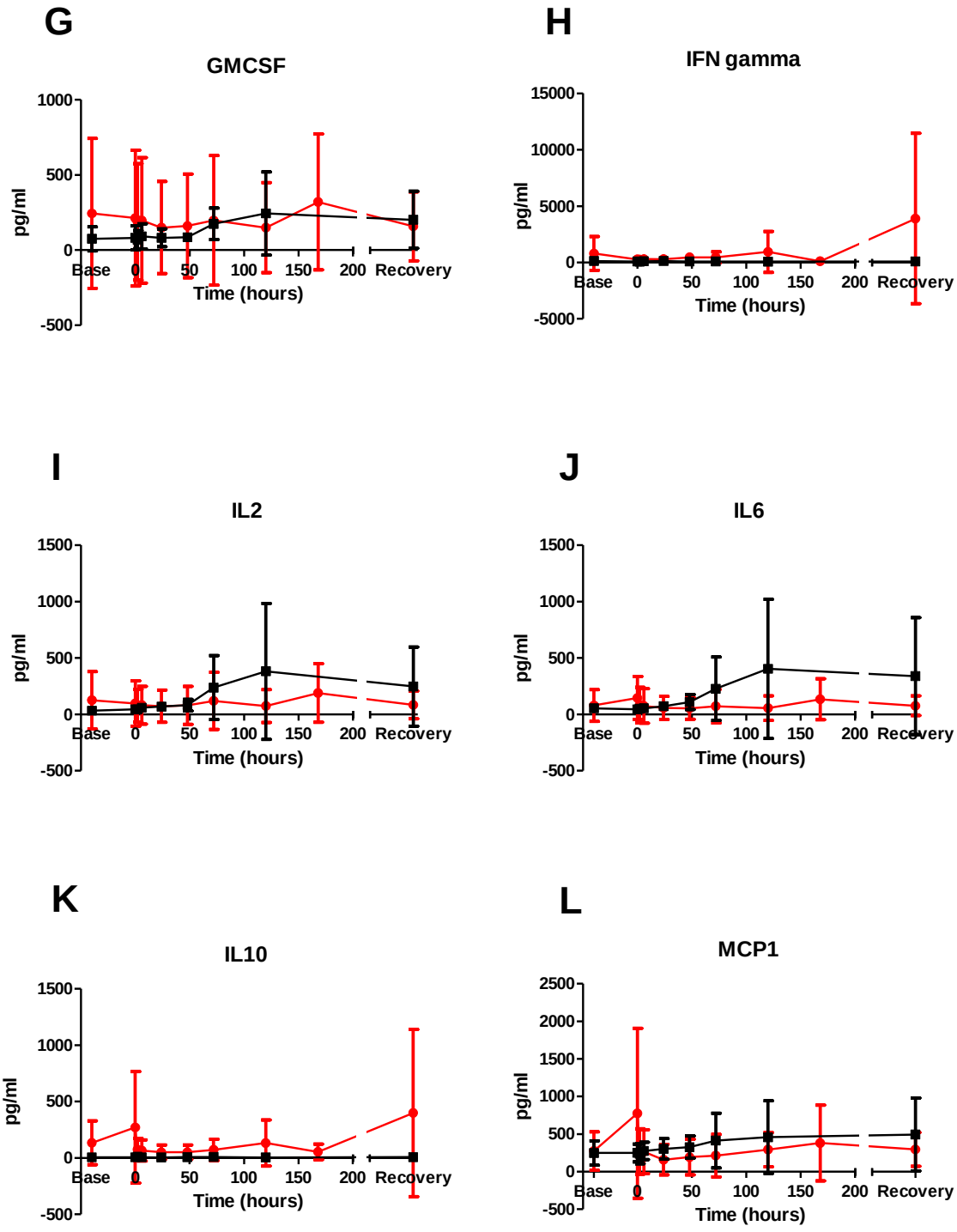
Supplemental Figure 3. Neutrophil counts in the two dogs that developed neutropenia. A. One dog developed very transient neutropenia immediately after 2F9 infusion. B. The second dog developed severe delayed neutropenia, likely due to activation of the reticuloendothelial system.



Supplemental Figure 4. Canine ITP model is not pro-thrombotic. A. Fibrinogen (P=0.268) and B. D-dimers (P=0.457) were not significantly different between 2F9-treated and control dogs. Data shown as median and range.

Supplemental Figure 5. Canine ITP model is not pro-inflammatory. In the measured chemokines and cytokines, over time there are no significant differences between 2F9-treated (●———●) (n=5) and control dogs (■———■) (n=3) (mean ± SD). Measured cytokines and their P-values comparing treated and control dogs are as follows A. TNFα (P=0.157) B. IL-7 (P=0.018, not significant with Bonferroni correction) C. IL-15 (P=0.903) D. CXCL-1 orthologue (P=0.345) E. IP-10 (P=0.589) F. IL-18 (P=0.069) G. GM-CSF (P=0.358) H. INF γ (P=0.412) I. IL-2 (P=0.603) J. IL-6 (P=0.330) K. IL-10 (P=0.086) L. MCP-1 (P=0.928).


A
TNFalpha
pg/ml
250
200
150
100
50
0
-50
Base 0 50 100 150 200 Recovery
Time (hours)
B
IL7
pg/ml
1500
1000
500
0
-500
Base 0 50 100 150 200 Recovery
Time (hours)
C
IL15
pg/ml
3000
2000
1000
0
-1000
Base 0 50 100 150 200 Recovery
Time (hours)
D
CXCL1 orthologue
pg/ml
1000
800
600
400
200
0
-200
Base 0 50 100 150 200 Recovery
Time (hours)
E
IP10
pg/ml
40
30
20
10
0
Base 0 50 100 150 200 Recovery
Time (hours)
F
IL18
pg/ml
1500
1000
500
0
-500
Base 0 50 100 150 200 Recovery
Time (hours)


G
GMCSF
pg/ml
1000
500
0
-500
Base
0
50
100
150
200
Recovery
Time (hours)
H
IFN gamma
pg/ml
15000
10000
5000
0
-5000
Base
0
50
100
150
200
Recovery
Time (hours)
I
IL2
pg/ml
1500
1000
500
0
-500
Base
0
50
100
150
200
Recovery
Time (hours)
J
IL6
pg/ml
1500
1000
500
0
-500
Base
0
50
100
150
200
Recovery
Time (hours)
K
IL10
pg/ml
1500
1000
500
0
-500
Base
0
50
100
150
200
Recovery
Time (hours)
L
MCP1
pg/ml
2500
2000
1500
1000
500
0
-500
Base
0
50
100
150
200
Recovery
Time (hours)

References

1. Nieswandt, B., et al., *Identification of critical antigen-specific mechanisms in the development of immune thrombocytopenic purpura in mice.* Blood, 2000. 96(7): p. 2520-7.

2. Bauer, M., et al., *A retrospective observational single-centre study on the burden of immune thrombocytopenia (ITP).* Onkologie, 2012. 35(6): p. 342-8.

3. Danese, M.D., et al., *Cost and mortality associated with hospitalizations in patients with immune thrombocytopenic purpura.* Am J Hematol, 2009. 84(10): p. 631-5.

4. Terrell, D.R., et al., *Prevalence of primary immune thrombocytopenia in Oklahoma.* Am J Hematol, 2012. 87(9): p. 848-52.

5. Norgaard, M., et al., *Long-term clinical outcomes of patients with primary chronic immune thrombocytopenia: a Danish population-based cohort study.* Blood, 2011. 117(13): p. 3514-20.

6. Portielje, J.E., et al., *Morbidity and mortality in adults with idiopathic thrombocytopenic purpura.* Blood, 2001. 97(9): p. 2549-54.

7. Saleh, M.N., M. Fisher, and K.M. Grotzinger, *Analysis of the impact and burden of illness of adult chronic ITP in the US.* Curr Med Res Opin, 2009. 25(12): p. 2961-9.

8. Kenet, G., et al., *Cone and platelet analyser (CPA): a new test for the prediction of bleeding among thrombocytopenic patients.* Br J Haematol, 1998. 101(2): p. 255-9.

9. Eldor, A., et al., *Prediction of haemorrhagic diathesis in thrombocytopenia by mean platelet volume.* Br Med J (Clin Res Ed), 1982. 285(6339): p. 397-400.

10. Psaila, B., et al., *Differences in platelet function in patients with acute myeloid leukemia and myelodysplasia compared to equally thrombocytopenic patients with immune thrombocytopenia.* J Thromb Haemost, 2011. 9(11): p. 2302-10.

11. Semple, J.W., *Animal models of immune thrombocytopenia.* Annals of Hematology, 2010. 89(Suppl 1): p. S34-S44.

12. Ware, J., *Dysfunctional platelet membrane receptors: from humans to mice.* Thromb Haemost, 2004. 92(3): p. 478-85.

13. Levin, J. and S. Ebbe, *Why are recently published platelet counts in normal mice so low?* Blood, 1994. 83(12): p. 3829-31.

14. McKenzie, S.E. and M.P. Reilly, *Heparin-induced thrombocytopenia and other immune thrombocytopenias: lessons from mouse models.* Semin Thromb Hemost, 2004. 30(5): p. 559-68.

15. Lewis, D.C. and K.M. Meyers, *Canine idiopathic thrombocytopenic purpura.* J Vet Intern Med, 1996. 10(4): p. 207-18.

16. Son, K.H., et al., *Inter-species hemorheologic differences in arterial and venous blood.* Clin Hemorheol Microcirc, 2010. 44(1): p. 27-33.

17. Suo, J., et al., *Hemodynamic shear stresses in mouse aortas: implications for atherogenesis.* Arterioscler Thromb Vasc Biol, 2007. 27(2): p. 346-51.

18. Schmidt-Nielsen, K. and P. Pennycuik, *Capillary density in mammals in relation to body size and oxygen consumption.* Am J Physiol, 1961. 200: p. 746-50.

19. Diehl, K.H., et al., *A good practice guide to the administration of substances and removal of blood, including routes and volumes.* J Appl Toxicol, 2001. 21(1): p. 15-23.

20. Kahn, M.L., et al., *Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin.* J Clin Invest, 1999. 103(6): p. 879-87.

21. Nakanishi-Matsui, M., et al., *PAR3 is a cofactor for PAR4 activation by thrombin.* Nature, 2000. 404(6778): p. 609-13.

22. Boudreaux, M.K., J.L. Catalfamo, and M. Klok, *Calcium-diacylglycerol guanine nucleotide exchange factor I gene mutations associated with loss of function in canine platelets.* Transl Res, 2007. 150(2): p. 81-92.

23. Beardsley, D.S. and M. Ertem, *Platelet autoantibodies in immune thrombocytopenic purpura.* Transfus Sci, 1998. 19(3): p. 237-44.

24. Lewis, D.C. and K.M. Meyers, *Studies of platelet-bound and serum platelet-bindable immunoglobulins in dogs with idiopathic thrombocytopenic purpura.* Exp Hematol, 1996. 24(6): p. 696-701.

25. Nieswandt, B., et al., *Targeting of platelet integrin alphaIIbbeta3 determines systemic reaction and bleeding in murine thrombocytopenia regulated by activating and inhibitory FcgammaR.* Int Immunol, 2003. 15(3): p. 341-9.

26. Newton, J.L., et al., *Fatigue in adult patients with primary immune thrombocytopenia.* Eur J Haematol, 2011. 86(5): p. 420-9.

27. Hosono, M., et al., *Kinetics of platelets in dogs with thrombocytopenia induced by antiglycoprotein IIb/IIIa receptor monoclonal antibody.* Nucl Med Biol, 1995. 22(1): p. 71-6.

28. Joshi, B.C. and N.C. Jain, *Experimental immunologic thrombocytopenia in dogs: a study of thrombocytopenia and megakaryocytopoiesis.* Res Vet Sci, 1977. 22(1): p. 11-17.

29. Tocantins, L.M. and H.L. Stewart, *Pathological anatomy of experimental thrombopenic purpura in the dog.* Am J Pathol, 1939. 15(1): p. 1-24 9.

30. Burstein, S.A., et al., *Canine megakaryocytopoiesis: analysis utilizing a monoclonal antibody to a 140-kd dog platelet protein.* Exp Hematol, 1991. 19(1): p. 47-52.

31. Smithberg, S.R., et al., *In vivo depletion of CD4+CD25+ regulatory T cells in cats.* J Immunol Methods, 2008. 329(1-2): p. 81-91.

32. Li, J., et al., *Expression and function of triggering receptor expressed on myeloid cells-1 (TREM-1) on canine neutrophils.* Dev Comp Immunol, 2011. 35(8): p. 872-80.

33. Brooks, M. and J. Catalfamo, *Buccal mucosa bleeding time is prolonged in canine models of primary hemostatic disorders.* Thromb Haemost, 1993. 70(5): p. 777-80.

34. Page, L.K., et al., *The immune thrombocytopenic purpura (ITP) bleeding score: assessment of bleeding in patients with ITP.* Br J Haematol, 2007. 138(2): p. 245-8.

35. Stokol, T., M.B. Brooks, and H.N. Erb, *Effect of citrate concentration on coagulation test results in dogs.* J Am Vet Med Assoc, 2000. 217(11): p. 1672-7.

36. Delgado, M.A., et al., *Peritoneal D-dimer concentration for assessing peritoneal fibrinolytic activity in horses with colic.* J Vet Intern Med, 2009. 23(4): p. 882-9.

37. Boudreaux, M.K. and J.L. Catalfamo, *Molecular and genetic basis for thrombasthenic thrombopathia in otterhounds.* Am J Vet Res, 2001. 62(11): p. 1797-804.

38. Phillips, D.R., et al., *The platelet membrane glycoprotein IIb-IIIa complex.* Blood, 1988. 71(4): p. 831-43.

39. Jennings, L.K. and D.R. Phillips, *Purification of glycoproteins IIb and III from human platelet plasma membranes and characterization of a calcium-dependent glycoprotein IIb-III complex.* J Biol Chem, 1982. 257(17): p. 10458-66.

40. Jung, S.M., et al., *Thrombasthenia with an abnormal platelet membrane glycoprotein IIb of different molecular weight.* Blood, 1988. 71(4): p. 915-22.

41. Abuelo, A., J.L. Catalfamo, and M.B. Brooks, *Platelet TMEM16F expression in canine Scott syndrome*, in *Platelets International*. 2012: Endicott College, Beverly, MA.

42. Boudreaux, M.K., V.S. Panangala, and C. Bourne, *A platelet activation-specific monoclonal antibody that recognizes a receptor-induced binding site on canine fibrinogen.* Vet Pathol, 1996. 33(4): p. 419-27.

43. D'Souza, S.E., et al., *The ligand binding site of the platelet integrin receptor GPIIb-IIIa is proximal to the second calcium binding domain of its alpha subunit.* J Biol Chem, 1990. 265(6): p. 3440-6.

44. Dodds, W.J., *Animal model: canine and equine immune-mediated thrombocytopenia, and idiopathic thrombocytopenic purpura.* Am J Pathol, 1977. 86(2): p. 489-91.

45. Wilkerson, M.J., et al., *Platelet size, platelet surface-associated IgG, and reticulated platelets in dogs with immune-mediated thrombocytopenia.* Vet Clin Pathol, 2001. 30(3): p. 141-149.

46. Wilkins, R.J., A.I. Hurvitz, and W.J. Dodds-Laffin, *Immunologically mediated thrombocytopenia in the dog.* J Am Vet Med Assoc, 1973. 163(3): p. 277-82.

47. Stasi, R., et al., *Idiopathic thrombocytopenic purpura: current concepts in pathophysiology and management.* Thromb Haemost, 2008. 99(1): p. 4-13.

48. Gear, A.R. and D. Camerini, *Platelet chemokines and chemokine receptors: linking hemostasis, inflammation, and host defense.* Microcirculation, 2003. 10(3-4): p. 335-50.

49. Court, W.S., et al., *Platelet surface-bound IgG in patients with immune and nonimmune thrombocytopenia.* Blood, 1987. 69(1): p. 278-83.

50. Cines, D.B., et al., *The ITP syndrome: pathogenic and clinical diversity.* Blood, 2009. 113(26): p. 6511-21.

51. Psaila, B., et al., *Intracranial hemorrhage (ICH) in children with immune thrombocytopenia (ITP): study of 40 cases.* Blood, 2009. 114(23): p. 4777-83.

52. Provan, D., et al., *International consensus report on the investigation and management of primary immune thrombocytopenia.* Blood, 2010. 115(2): p. 168-86.

53. Rozanski, E.A., et al., *Comparison of platelet count recovery with use of vincristine and prednisone or prednisone alone for treatment for severe immune-mediated thrombocytopenia in dogs.* J Am Vet Med Assoc, 2002. 220(4): p. 477-81.

54. O'Marra, S.K., A.M. Delaforcade, and S.P. Shaw, *Treatment and predictors of outcome in dogs with immune-mediated thrombocytopenia.* J Am Vet Med Assoc, 2011. 238(3): p. 346-52.

55. Jergens, A.E., et al., *Buccal mucosa bleeding times of healthy dogs and of dogs in various pathologic states, including thrombocytopenia, uremia, and von Willebrand's disease.* Am J Vet Res, 1987. 48(9): p. 1337-42.

56. Gimbrone, M.A., Jr., et al., *Preservation of vascular integrity in organs perfused in vitro with a platelet-rich medium.* Nature, 1969. 222(5188): p. 33-6.

57. Ho-Tin-Noe, B., M. Demers, and D.D. Wagner, *How platelets safeguard vascular integrity.* J Thromb Haemost, 2011. **9 Suppl 1**: p. 56-65.

58. Kitchens, C.S. and J.F. Pendergast, *Human thrombocytopenia is associated with structural abnormalities of the endothelium that are ameliorated by glucocorticosteroid administration.* Blood, 1986. 67(1): p. 203-6.

59. Panitsas, F.P., et al., *Adult chronic idiopathic thrombocytopenic purpura (ITP) is the manifestation of a type-1 polarized immune response.* Blood, 2004. 103(7): p. 2645-7.

60. Semple, J.W., et al., *Differences in serum cytokine levels in acute and chronic autoimmune thrombocytopenic purpura: relationship to platelet phenotype and antiplatelet T-cell reactivity.* Blood, 1996. 87(10): p. 4245-54.

61. Andersson, P.O., et al., *A transforming growth factor-beta1-mediated bystander immune suppression could be associated with remission of chronic idiopathic thrombocytopenic purpura.* Ann Hematol, 2000. 79(9): p. 507-13.

62. Lazarus, A.H., et al., *Comparison of platelet immunity in patients with SLE and with ITP.* Transfus Sci, 2000. 22(1-2): p. 19-27.

63. Emadi, S., et al., *IL-8 and its CXCR1 and CXCR2 receptors participate in the control of megakaryocytic proliferation, differentiation, and ploidy in myeloid metaplasia with myelofibrosis.* Blood, 2005. 105(2): p. 464-73.

64. Van Damme, J., et al., *The neutrophil-activating proteins interleukin 8 and beta-thromboglobulin: in vitro and in vivo comparison of NH2-terminally processed forms.* Eur J Immunol, 1990. 20(9): p. 2113-8.

65. Strieter, R.M., et al., *Disparate gene expression of chemotactic cytokines by human mononuclear phagocytes.* Biochem Biophys Res Commun, 1990. 166(2): p. 886-91.

66. Strieter, R.M., et al., *Human neutrophils exhibit disparate chemotactic factor gene expression.* Biochem Biophys Res Commun, 1990. 173(2): p. 725-30.

67. Gesser, B., et al., *Interleukin-8 induces its own production in CD4+ T lymphocytes: a process regulated by interleukin 10.* Biochem Biophys Res Commun, 1995. 210(3): p. 660-9.

68. Matsushima, K. and J.J. Oppenheim, *Interleukin 8 and MCAF: novel inflammatory cytokines inducible by IL 1 and TNF*. Cytokine, 1989. 1(1): p. 2-13.

69. Larsen, C.G., et al., *Production of interleukin-8 by human dermal fibroblasts and keratinocytes in response to interleukin-1 or tumour necrosis factor*. Immunology, 1989. 68(1): p. 31-6.

70. Kaplanski, G., et al., *Interleukin-1 induces interleukin-8 secretion from endothelial cells by a juxtacrine mechanism.* Blood, 1994. 84(12): p. 4242-8.

71. Gibbs, B.F., et al., *Human skin mast cells rapidly release preformed and newly generated TNF-alpha and IL-8 following stimulation with anti-IgE and other secretagogues.* Exp Dermatol, 2001. 10(5): p. 312-20.

72. Rodenburg, R.J., et al., *Superinduction of interleukin 8 mRNA in activated monocyte derived macrophages from rheumatoid arthritis patients.* Ann Rheum Dis, 1999. 58(10): p. 648-52.

73. von Hundelshuasen, P. and C. Weber, *Platelets as immune cells: bridging inflammation and cardiovascular disease.* Circ Res, 2007. 100: p. 27-40.

74. Su, S.B., N. Mukaida, and K. Matsushima, *Rapid secretion of intracellularly pre-stored interleukin-8 from rabbit platelets upon activation.* J Leukoc Biol, 1996. 59(3): p. 420-6.

75. Devi, S., et al., *Platelet recruitment to the inflamed glomerulus occurs via an alphaIIbbeta3/GPVI-dependent pathway.* Am J Pathol, 2010. 177(3): p. 1131-42.

76. Iannacone, M., et al., *Platelets prevent IFN-alpha/beta-induced lethal hemorrhage promoting CTL-dependent clearance of lymphocytic choriomeningitis virus.* Proc Natl Acad Sci U S A, 2008. 105(2): p. 629-34.

77. Zarbock, A., K. Singbartl, and K. Ley, *Complete reversal of acid-induced acute lung injury by blocking of platelet-neutrophil aggregation.* J Clin Invest, 2006. 116(12): p. 3211-9.

78. Huo, Y., et al., *Circulating activated platelets exacerbate atherosclerosis in mice deficient in apolipoprotein E.* Nat Med, 2003. 9(1): p. 61-7.

79. Kameyoshi, Y., et al., *Cytokine RANTES released by thrombin-stimulated platelets is a potent attractant for human eosinophils.* J Exp Med, 1992. 176(2): p. 587-92.

80. Dole, V.S., et al., *Activated platelets induce Weibel-Palade-body secretion and leukocyte rolling in vivo: role of P-selectin.* Blood, 2005. 106(7): p. 2334-9.

81. Hol, J., L. Wilhelmsen, and G. Haraldsen, *The murine IL-8 homologues KC, MIP-2, and LIX are found in endothelial cytoplasmic granules but not in Weibel-Palade bodies.* J Leukoc Biol, 2010. 87(3): p. 501-8.

82. Saitoh, T., et al., *Interleukin-10 gene polymorphism reflects the severity of chronic immune thrombocytopenia in Japanese patients.* Int J Lab Hematol, 2011. 33(5): p. 526-32.

83. Tesse, R., et al., *Association of interleukin-(IL)10 haplotypes and serum IL-10 levels in the progression of childhood immune thrombocytopenic purpura.* Gene, 2012. 505(1): p. 53-6.

84. McMillan, R., et al., *Suppression of in vitro megakaryocyte production by antiplatelet autoantibodies from adult patients with chronic ITP*. Blood, 2004. 103(4): p. 1364-9.

85. Goerge, T., et al., *Inflammation induces hemorrhage in thrombocytopenia.* Blood, 2008. 111(10): p. 4958-64.

ENDOTHELIAL ALTERATIONS IN A CANINE MODEL OF IMMUNE THROMBOCYTOPENIA

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Introduction

Platelets play a pivotal role in the formation of blood clots at the sites of vascular injury. However, petechial hemorrhages and capillary leakage also occur with thrombocytopenia in the absence of vascular injury. The fact that bleeding can occur without vascular injury has led to the idea that platelets support the vascular endothelium and maintain the structural integrity of intact blood vessels [1, 2]. The mechanisms by which platelets maintain vascular integrity and preserve the endothelial barrier function are unknown. Previous studies have shown that platelets can support vascular integrity even if they are defective in primary hemostatic capacity and cannot form a platelet plug [3]. As such, it is speculated that platelets support vascular integrity independently of their primary hemostatic function [2, 3].

Studies have shown that platelets support vascular integrity through release of vasoactive compounds [4]. Platelets produce many soluble vasoactive mediators that may mediate their vascular-stabilizing function. Which mediator, or combination of mediators, is the most important in the maintenance of endothelial integrity remains unknown. Sphingosine 1-phoshate (S1P), a lysosphingolipid, is one such platelet-derived mediator [5]. S1P is released upon platelet activation and plays an important role in preserving vascular integrity by maintaining interendothelial cell junctions [6-8]. S1P induces reorganization of the endothelial cytoskeleton into a strong cortical actin ring, and leads to the assembly and stabilization of adherens junctions, the predominant endothelial cell-cell junction and regulator of endothelial permeability [9-11]. S1P has been reported to reduce vascular leakage in murine and canine acute lung injury models, preventing associated pulmonary edema.[12] Though its established functions make S1P an excellent candidate effector of the platelet vascular stabilizing function, its relative importance in this role is undetermined. S1P has other cellular sources such as erythrocytes which may be more significant than platelets [13].

It is not understood why some patients with thrombocytopenia bleed in the absence of trauma while others do not. One possible explanation is that there are inter-individual differences in endothelial integrity that can be ascribed to inter-individual differences in the capacity of the remaining circulating platelets to maintain vascular integrity. Differences in plasma concentrations of platelet-derived vasoactive mediators like S1P could account for patient variations in endothelial integrity and subsequent variations in bleeding tendency.

While it is accepted that platelets support endothelial function**,** the ultrastructural impact of thrombocytopenia on endothelium is disputed. Kitchens and colleagues, in a widely referenced paper, reported capillary endothelial thinning in experimental thrombocytopenia in rabbits and spontaneous severe thrombocytopenia in people [14, 15]. However, other electron microscopic studies from a variety of thrombocytopenic animal models have not demonstrated ultrastructural changes in the microvascular endothelium [1, 16-18]. Whether erythrocytes extravasate in thrombocytopenic bleeding by paracellular or transcellular routes is also disputed in ultrastructural studies [17, 19].

We set out to assess the impact of thrombocytopenia on cutaneous endothelial ultrastructure in an induced canine model of immune thrombocytopenia (ITP). We hypothesized that thrombocytopenic bleeding is caused by ultrastructural alterations in the microvascular endothelium. We selected cutaneous endothelium as this was readily accessible for evaluation at multiple time points and since the skin is a common location for thrombocytopenic bleeding (petechiae and ecchymoses). In ITP model dogs we also examined plasma levels of the endothelial-stabilizing factor, S1P, to determine whether S1P can be related to endothelial ultrastructure. Similarly, we measured a circulating biomarker of endothelial cell damage**,** plasma von Willebrand factor (vWf), in these model dogs to evaluate vWf as an indicator of endothelial ultrastructural changes [20].

Materials and Methods

Animals

Eight healthy adult (median age 2 years old; range 1-4) intact male mixed breed dogs were used in this study (28.4 ± 5.6 kg). Research dogs were loaned from Francis Owen Blood Research Laboratory at the University of North Carolina at Chapel Hill or Laboratory Animal Resources at the North Carolina State College of Veterinary Medicine (NCSU) as described in Chapter 2. All protocols were approved by the Institutional Animal Care and Use Committee of North Carolina State University.

Induction of Thrombocytopenia

Thrombocytopenia was induced in 5 dogs with infusion of 2F9, a murine monoclonal antibody to canine GPIIb as previously described in Chapter 2. Three control dogs received isotype control murine IgG2a anti-yellow fever (αYFA or CRL-1689.1) antibody as detailed in Chapter 2.

Blood Sampling and Preparation

Blood was obtained from model dogs via indwelling central catheters that were placed at baseline under dexmedetomidine sedation as described in Chapter 2. Two ml of blood were

placed in an EDTA tube for platelet count (Kendall, Mansfield, MA). One ml of citrated blood (0.38% final concentration) was immediately placed on ice and spun at 4 °C at 3000 g for 10 minutes and the subsequent platelet poor plasma (PPP) was aliquoted and stored at -80°C for S1P measurement. Immediate cooling of blood for S1P measurement was necessary to avoid time and temperature-dependent leak of S1P from erythrocytes to the plasma [21]. The remainder of the citrated blood (3 ml) was centrifuged at room temperature at 2440 g for 15 minutes and supernatant spun again at 3500 g for 15 minutes to generate platelet free plasma (PFP) which was then aliquoted and immediately stored at -80°C for future von Willebrand factor analysis.

Blood was drawn at the following time points: baseline, time zero (when platelet count first fell into the target nadir range or 1 hour after control antibody administration), 2, 4, 6, 8, 12, 24 hours, and then every 24 hours until platelet count returned to baseline and new bleeding stopped, whichever came later (168-384 hours). This last time point was termed "recovery."

Bleeding Score

At the time of each blood draw, bleeding scores were assessed as described in Chapter 2.

Biopsy Procurement and Tissue Processing

Dogs were sedated for biopsy procurement at baseline (in conjunction with central intravenous catheter placement), time of initial platelet nadir (time zero), 24 hours after platelet nadir, on platelet count recovery (recovery), and at the time of bleed if one occurred. Sedation was achieved with intravenous dexmedetomidine dosed to effect (range 3.6-21.9 μg/kg) (Dexdomitor, Pfizer Animal Health, NY, NY). Cutaneous biopsies were performed in the region of the dorsal flank using two 8 mm biopsy punches (Miltex, York, PA) and wounds were closed with 3-0 PDS II (Ethicon, Inc., Somerville, NJ). In the dogs that developed pronounced cutaneous ecchymoses, skin biopsies were taken from the border of the ecchymoses and normal skin (time bleed).

Immediately following surgical collection, two 8 mm skin punch biopsy samples were processed for histopathology, transmission electron microscopy and future immunohistochemistry. The first punch biopsy was bisected and half was collected into 10% neutral buffered formalin for histopathology and the other half was sectioned into 1 mm thick columns and collected into fixative for routine transmission electron microscopy (2% paraformaldehyde/2.5 % glutaraldehyde in 0.15 M sodium phosphate buffer, pH 7.4). The second punch biopsy was also bisected and half was flash frozen over a 30 second interval in Optimal Cutting Temperature (OCT) compound (Fisher Scientific, Pittsburgh, PA) utilizing a liquid nitrogen bath and the other half was sectioned into 1 mm thick columns and collected into fixative for future immunoelectron microscopy (4% paraformaldehyde/0.15M sodium phosphate buffer, pH 7.4). Frozen OCT blocks were stored at -80 °C until use. Tissues for EM were stored at 4°C prior to processing.

For histopathology, half of an 8 mm skin punch biopsy sample, fixed in 10% neutral buffered formalin, was routinely embedded in paraffin and sectioned at 5 micrometers for staining with hematoxylin and eosin. Following aldehyde fixation the samples for TEM were washed in 0.15M sodium phosphate buffer followed by post-fixation in 1% osmium tetroxide/0.15M sodium phosphate buffer, pH 7.4, for 1 hour. Samples were dehydrated through a graded series of ethanols (30%, 50%, 75%, 95%, 100%, 100%), followed by 2 changes in propylene oxide. The biopsies were infiltrated and embedded in an epoxy resin mixture of 1 part Polybed 812 resin: 1 part Spurr's resin (Polysciences, Inc., Warrington, PA) and polymerized for 24 hours at 60˚C. One μm semithin transverse sections were cut with a diamond knife, mounted on slides, stained with 1% toluidine blue and viewed using a light microscope to select the region of interest for TEM. Ultrathin sections (70nm) were cut using a diamond knife, mounted on 200 mesh Formvar/carbon-coated copper grids and stained with 4% aqueous uranyl acetate and Reynolds' lead citrate.

Histology

Histological sections were randomized (Random.org) and evaluated by a board certified veterinary pathologist (KEL) who was blinded to specimen identity. Sections were evaluated

for lesions and scored for evidence of inflammatory cells, hemorrhage, edema, vessel thrombosis and endothelial cell hypertrophy. Scoring of each of these changes followed a standard lesions severity scale; 0 = no change, 1 = minimal change, 2 = mild change, 3 = moderate change and 4 = marked change.

Electron Microscopy

Tissues were examined using a LEO EM910 transmission electron microscope operating at 80kV (Carl Zeiss SMT, Inc., Peabody, MA) at magnifications of 5,000x, 10,000x, and 20,000x. All capillaries in a section were examined and the entire vessel photographed at magnifications of 5,000x and 10,000x. Digital images were acquired using a Gatan Orius SC1000 CCD Digital Camera with Digital Micrograph 3.11.0 (Gatan, Inc., Pleasanton, CA). The vessels were also screened at 20,000x and any unusual features were photographed at this magnification. Capillaries were photographed sequentially, as encountered on the grid. All capillaries that were encountered were photographed with a minimum requirement of 10 capillaries from each time point in each dog. If a minimum of ten capillaries was not observed in a given section, an additional block from that time point was prepared and all the capillaries on that grid were examined. Vessels were excluded if they crossed a grid bar or if the lumen was mostly closed which would make subsequent vessel thickness measurements challenging.

Initially all of one 2F9-treated dog's time points were examined to determine what time points would be the highest yield. As greater thinning was demonstrated at 24 hours than at the platelet nadir, in future dogs, only tissue from baseline, 24 hours, and bleed were examined. Subsequent to method development, the electron microscopist (DNL) was blinded to the time point or treatment group.

A board certified veterinary pathologist (REC) who was blinded to sample identity, evaluated all photomicrographs. Mean thickness of each capillary was determined using the 5,000x magnification electron micrographs. If the entire capillary was not captured in one micrograph, Gnu image manipulation program (GIMP) 2.8 was used to create a montage of micrographs to form a complete vessel. A grid with straight lines every 22.5 degrees like

spokes on a wheel was generated in GIMP and was randomly superimposed on the capillary as shown in Figure 1. The vessel thickness at each point where the grid crossed the vessel was measured. Perinuclear regions were excluded. Intersections between the lines and the first point at which they crossed the basal lamina were used for measurements. At each intersection, a line perpendicular to the basal lamina was drawn towards the lumen and its length measured. Up to 16 thickness measurements were taken of a given vessel and the mean vessel wall thickness was determined for each animal/time point.

Evaluation of capillaries for ultrastructural features was performed at magnifications of 5,000x, 10,000x, and 20,000x. Each capillary was examined for pinocytotic vesicles (0.07-0.08 μm), large vacuoles (>0.1 μm), and number of gaps between cells. Figure 2 demonstrates these terms. Scoring of pinocytotic vacuoles was performed as follows: 0-absent, 1-rare, 2-present multifocally, 3-present diffusely; grading of large vacuoles was 0-absent, 1-focal, 2-multiple, 3-numerous/many. Average number of gaps between cells was determined by averaging the number of gaps per each individual capillary in a given section.

Sphingosine 1-Phosphate (S1P) Measurement

PPP samples were analyzed at the University of Kentucky Gill Heart Institute for determination of plasma S1P levels. Lipids were extracted from plasma using acidified organic solvents, as previously described using 50 pmol of a C17-S1P internal standard [13, 22]. S1P levels were measured using ultra-fast liquid chromatography coupled with electrospray ionization tandem mass spectrometry as previously described [13].

Von Willebrand Factor Measurement

We assessed plasma von Willebrand factor (vWf) concentration as a marker of endothelial cell damage. Plasma (PFP) von Willebrand factor concentration [von Willebrand factor antigen (vWf:Ag)] was measured at the Comparative Coagulation Laboratory (Animal Health Diagnostic Laboratory, Cornell University) using an ELISA [23], configured with monoclonal anti-canine vWf antibodies. A canine plasma standard, with an assigned value of

100% vWf:Ag, was prepared at the Coagulation Laboratory as a pooled plasma collected from 20 healthy dogs and stored in single-use aliquots at -70^0 C.

Platelet Counts

Platelet counts were determined from EDTA whole blood by the Clinical Pathology Laboratory at NCSU using an Advia 120 (Siemens Healthcare Diagnostics, Inc., Norwood, MA) or manual hemocytometer counts if platelets were <10,000 platelets/µl as described in Chapter 2.

Statistical Analysis

For electron micrograph parameters (thickness, vesicle score, vacuole score, spaces between cells), the mean change in values from baseline to 24 hours was calculated for each treatment group, and a two-sample t-test was performed to test for differences in these outcomes by treatment group.

Univariate association between variables (bleeding score and vWf; bleeding score and S1P) was tested using a t-test for correlations with the appropriate degrees of freedom. Similarly, correlations between the changes in measured EM parameters and S1P, sedation dose, and vWf were also assessed. Bleeding scores were treated as ordinate variables as described by Mantel-Haenszel.

To test for differences in S1P and vWf across all time points by treatment group, repeated measures generalized linear modeling (analysis of variance) was performed, where time point and the variable of interest were entered into the model. The P-value for each parameter was evaluated and tested for statistical significance. To control for multiple testing, a Bonferroni correction (for each set of hypotheses) was used to determine the alpha level for statistical significance that insured a family-wise error rate of 0.05; a P-value <0.002 was considered significant.

Associations between two quantitative variables were assessed by linear regression. $P<0.05$ was significant.

A power calculation was performed to help guide future studies. The change in thickness was used for power calculation, using a t distribution with the appropriate degrees of freedom, assuming a balanced design and an alpha of 0.05.

All calculations were performed using Stata version 10 (Statacorp LP).

Results

Platelet Levels Induced by Antibody Infusion

Platelet counts are shown in Table 1. In 2F9-treated dogs, platelet nadir was 4,000 to 11,000 platelets/µl (compared to baseline counts of 183,000 to 329,000 platelets/µl) and nadir platelet counts were on average 3% of baseline counts. Mild variable mucocutaneous bleeding was noted in 2F9 treated dogs as described in Chapter 2. Platelet count in 2F9-treated dogs remained below 40,000 platelets/µl for 24 hours, after which point counts began to spontaneously recover. No changes in platelet count were observed in the dogs treated with control antibody.

Histological Observations

Table 2 demonstrates our light microscopic data. Minimal changes were seen in most specimens. Interestingly, one dog that developed a delayed ecchymosis as platelet count was recovering (dog 2, bleed) demonstrated marked hemorrhage, mild edema, and marked endothelial cell hypertrophy in a biopsy obtained from the ecchymosis at this time. No evidence of thrombosis was present. These lesions were resolved on the recovery biopsy 7 days later.

Ultrastructural Observations

Sections from the skin of 3 control dogs and 5 2F9-treated dogs were examined at baseline and after 24 hours of thrombocytopenia or 24 hours after control antibody administration.

Normal Dogs

Figure 3 demonstrates a normal cutaneous capillary sampled from a dog at baseline with abundant pinocytotic vesicles, no endothelial cell-cell gaps, and a tightly apposed cell junction. A basement membrane surrounds the vessel and supporting pericytes are present. The vessel is composed of nonfenestrated continuous endothelium as is characteristic of normal cutaneous vasculature [24]. In the normal dogs (baseline samples), capillary wall thickness in regions away from the endothelial nuclei varied from 0.62 to 0.95 μm.

There Is a Trend Towards Endothelial Thinning in Thrombocytopenic Dogs

Table 3 shows our ultrastructural data. There is a trend towards endothelial thinning (Figures 4 and 5) in 2F9-treated dogs from baseline to 24 hours of thrombocytopenia, however, this is not statistically different than the changes observed in control dogs ($P=0.60$). Variation in vessel thickness was high in all samples. The coefficient of variation for vessel thickness in all baseline samples was 0.52.

Endothelial Ultrastructural Changes in Thrombocytopenia

After 24 hours of thrombocytopenia, thrombocytopenic vessels have significantly decreased numbers of pinocytotic vesicles from baseline when compared to changes in time-matched controls ($P<0.0045$) (Table 3; Figures 5-6). Thrombocytopenic microvascular endothelium tends to have increased numbers of large vacuoles and increased gaps between endothelial cells compared to baseline endothelium (Table 3; Figure 6B-C). These alterations are consistent with changes that would increase vascular permeability. However, these findings were not statistically different compared to time-matched controls ($P=0.76$ for change in vacuoles; $P=0.77$ for change in spaces between cells).

Endothelial Ultrastructural Changes Were Observed in Dogs with Delayed Ecchymoses

Two dogs developed large delayed ecchymoses (one on the abdominal skin and one on the axillary skin) as their platelet counts were recovering (Figure 7A). Ultrastructural changes

were observed in their endothelial cells at the time of their delayed bleeds including pale swollen endothelial cells and gaps between adjacent endothelial cells (Figure 7C). In the dog that developed the large abdominal bruise, large inter-endothelial cell gaps were present after 24 hours of thrombocytopenia, 24 hours prior to the development of the bleed (Figure 7B). For comparison, a normal endothelial cell junction is pictured in Figure 7D. Compared to each dog's baseline capillaries, capillaries in these bleed biopsies had increases in the average number of spaces between endothelial cells and the number of large vacuoles and decreases in the number of pinocytotic vesicles (Table 4). One dog's mean vessel wall thickness at bleed was reduced from that of baseline; the other dog had a slight increase in thickness from baseline thickness (Table 4). Histopathologic evaluation of tissues showed no other explanation besides hemorrhage for the ecchymoses such as thrombosis or vasculitis (Table 2).

Thrombocytopenia Is Associated with a Reduction in Plasma S1P

Plasma S1P decreased over time in treated compared to control dogs ($P<0.001$) (Figure 8A). Plasma S1P had a linear association with platelet count in treated and control dogs over all time points ($R=0.28$, $P<0.0016$) (Figure 8B). Hematocrit was evaluated as a potential confounder. As has been previously reported in people, hematocrit was significantly correlated with plasma S1P ($R=0.51$, $P<0.0001$), [13] but was not significantly correlated with platelet count directly ($P=0.0764$). In a model with platelet count, S1P, and hematocrit, neither hematocrit nor platelet count is significantly associated with S1P, likely due to low power for higher dimension models in the current data.

There were no correlations between plasma S1P and endothelial thickness or the evaluated endothelial ultrastructural parameters. Similarly, there was no significant correlation between plasma S1P and bleeding score ($P=0.104$).

Plasma Von Willebrand Factor Increases Over Time in Thrombocytopenic and Control Dogs

In both 2F9-treated dogs that became thrombocytopenic and control dogs, plasma vWf increased similarly over time (difference between treatment groups P=0.845; Figure 9). Given that similar increases occurred in the control group as the thrombocytopenic group, vWf increases can more readily be attributed to vWf acting as an acute phase reactant than as a marker of vascular damage [25]. Repeated biopsies and infusion of antibody in both groups might have generated mild inflammation and resulted in the induction of vWf as an acute phase reactant.

There were no correlations between plasma vWf and endothelial thickness, the evaluated endothelial ultrastructural parameters, or bleeding score.

Discussion

Thrombocytopenic Endothelial Ultrastructural Alterations

In a canine model of ITP we observed changes in the endothelial ultrastructure of cutaneous vessels. Most notably, pinocytotic vesicles decreased significantly in the thrombocytopenic endothelium. The exact mechanism of vesicle decrease is unknown but vesicle reduction is observed in several other examples of endothelial damage including methotrexate toxicity, hypoxia, and snake bite envenomation [26-28]. Pinocytotic vesicles provide a reserve of plasma membrane and can be translocated to the endothelial cell surface by exocytosis when there is a demand for increased membrane either due to cellular distention or membrane damage [26]. This is believed to be the reason for vesicle decrease following snake bite envenomation [26]. Formation of pinocytotic vesicles is an energy dependent process [11]. An alternate explanation for their observed decrease is an endothelial metabolic derangement and a perturbation in overall endothelial cell health. The exact mechanism for platelet metabolic support of endothelium is unknown, but many platelet-derived growth factors such as vascular endothelial growth factor (VEGF) promote endothelial cell survival [29, 30]. Reduction of these factors in the face of thrombocytopenia likely result in altered endothelial

metabolism. VEGF can also directly induce vesicle formation in endothelial cells [31]. Regardless of the exact mechanism, the observed vesicle decrease is clear evidence of a thrombocytopenia-induced endothelial cell abnormality.

Thrombocytopenic endothelium also demonstrated some lesions that would result in increased vascular permeability including endothelial cell thinning, increased frequency of gaps between endothelial cells, and increased number of large vacuoles. However, these changes were not statistically significant. The large vacuoles likely represent part of the vesiculo-vacuolar organelle (VVO), an endothelial cell permeability structure that provides a major route of extravasation of macromolecules at sites of augmented vascular permeability [32]. VVOs are a series of interconnecting vacuoles (on average 100 nm in diameter) that provide a transcellular pathway from vascular lumen to ablumen [32].

Our observed trend towards endothelial thinning in thrombocytopenia is consistent with that described by Kitchens and colleagues in thrombocytopenic rabbits and humans [14]. The thinner appearance of endothelial cells could be due to loss of endothelial cells leading to fewer endothelial cells available to cover a fixed luminal surface area or due to increased blood flow leading to increased mean circumference of the of the microcirculation [14]. Endothelial thinning occurs in snake bite envenomation because of basement membrane damage that makes capillaries more susceptible to distention [26]. At the time of baseline biopsies, dog required higher doses of dexmedetomidine sedative than they did at 24 hours because they were less adapted to the study and more anxious. Dexmedetomidine is an α_2-agonist that can cause peripheral vasoconstriction [33]; increased doses at baseline might have resulted in decreased proximal blood flow, decreased vascular distention, and increased endothelial thickness. We did not find any correlation between dexmedetomidine dose and endothelial thickness or any other measured endothelial parameters (not shown). Most likely the endothelial thinning reflected damage to the vessels that resulted in fewer endothelial cells or less endothelial membrane to support the same lumen surface area.

Despite the overall trend towards endothelial thinning that we observed, there was tremendous variability in vessel thickness even within a given dog and time point (coefficient of variation for baseline vessel thickness was 0.52). Furthermore, at 24 hours, some

thrombocytopenic dogs had an increase in endothelial thickness while some control dogs demonstrated endothelial thinning. Variations in endothelial thickness found in both control and thrombocytopenic dogs could be accounted for by the fact that we performed immersion rather than perfusion fixation so vessels would have been variably distended at the time of sampling. Endothelial thinning may not be the most important lesion induced by thrombocytopenia. Indeed, if endothelial thinning had not been previously described by other investigators, we would not have predicted it to occur. The other thrombocytopenic trends we observed—increases in intercellular spaces and increases in large vacuoles—are more aligned with what we would expect based on our knowledge of how endothelial permeability is controlled. Pro-integrity factors like S1P stabilize endothelial cell junctions [10] while pro-permeability factors disrupt junctions and promote VVO transport [30, 32].

The breaches in the endothelial walls and widened endothelial junctions observed in the dogs that developed delayed ecchymoses are noteworthy (Figure 7). There is controversial data regarding the integrity of endothelial junctions in the face of thrombocytopenia. Kitchens and colleagues observed normal endothelial junctions and predicted that increased vesicular transport accounts for alterations in endothelial permeability during thrombocytopenia [34]. Van Horn and Johnson demonstrated erythrocyte passage through endothelial cells rather than between them in thrombocytopenic guinea pigs [17]. Others have reported normal endothelial junctions in thrombocytopenic guinea pigs, rats, and mice [18]. Similar to our observations in these two dogs, Gore and colleagues observed widened intercellular junctions sometimes containing erythrocytes in thrombocytopenic guinea pigs [35]. Dale and Hurley observed gaps in the endothelial intercellular junctions of thrombocytopenic rats in areas that were exposed to venous constriction and erythrocytes escaped via these open intercellular junctions [19]. Our findings, in conjunction with the studies of Dale and Hurley and Gore and colleagues, suggest that in thrombocytopenic bleeding, erythrocytes extravasate through altered endothelial cell junctions or damaged endothelial cell walls. The junctions of thrombocytopenic dogs that did not develop delayed bleeds appeared normal. Further studies are needed to resolve this controversy.

The dogs that developed the delayed ecchymoses were also interesting in that their platelet counts should have been adequate at the time of their bleeds to achieve primary hemostasis (85,000 platelets/µl in one dog and 196,000 platelets/µL in the other dog). Given the endothelial abnormalities we observed in biopsies taken from those sites of bleeding (Figure 7), we speculate that the delayed bruising may be a result of endothelial destabilization that may have taken time to peak following the platelet nadir.

Our ultrastructural studies have several limitations. We were evaluating structural lesions when the endothelial-stabilizing effect of platelets may be more of a functional one. Rodent models often use Evans Blue dye leakage to assess vascular integrity, however because canine skin is so much thicker this would not be a reliable marker in dogs nor could infusion of this toxic dye be employed in survival studies. In the future, we plan to conduct functional studies utilizing the ITP dog model such as monitoring lymphatic volume by cannulating lymphatics as an assessment of the effect of thrombocytopenia on vascular permeability. Alternatively, we could infuse dogs with labeled albumin to assess the vessel permeability in thrombocytopenia. Functional studies will also enable us to observe the sequelae of what are likely very transient alterations in endothelial ultrastructure. Additionally, ultrastructural evaluation of the endothelium may not be the ideal mechanism to assess endothelial integrity as there appears to be tremendous variation in thickness and the ultrastructural features we evaluated among healthy animals.

A major limitation of our study was our small sample size. Given the variation in endothelial ultrastructural features that we observed even within a given dog and time point, a far greater number of dogs would be needed to identify statistically significant changes in thickness or ultrastructural features other than pinocytotic vesicles. A calculation using our observed changes in thickness determined that at least 142 dogs would be needed to have 80% power. However, our data are suggestive and warrant further studies involving larger numbers of animals. Additionally, our study may have been limited by the short duration of the severe thrombocytopenia. By 24 hours, platelet counts had started to recover (>30,000 platelet/µl in 4 of 5 of the 2F9-treated dogs) which may be above the threshold for maintenance of vascular integrity. In murine models of thrombocytopenic hemorrhage, only

five percent of the normal platelet count was needed to achieve the vascular protective effect of platelets [2, 3]. It may be that longer duration of severe thrombocytopenia in our dogs is necessary to accentuate relevant lesions. We plan to achieve this in future studies with repeated 2F9 administration.

Recent work by Goerge and colleagues suggests one possible explanation for the relatively mild bleeding and the overall minor changes in endothelial ultrastructure observed in the model dogs [3]. This group demonstrated that thrombocytopenic hemorrhage only occurs in the face of inflammation [3]. In order for bleeding to occur, leukocyte-mediated vessel damage may be necessary in combination with the vascular destabilizing effects of thrombocytopenia [36]. Ultrastructural studies have not been performed to look at the combined effects of inflammation and hemorrhage. As our dogs had minimal to no cutaneous inflammation (Table 2), they may have lacked a second "hit" necessary to create sufficient vascular damage for significant bleeding to occur. In the future, we plan to repeat the same studies in conjunction with the induction of local inflammation to determine the combined effects of inflammation and thrombocytopenia on endothelial ultrastructure.

Role of S1P in Mediating the Vascular-Stabilizing Effect of Platelets

With the goal of identifying a mechanism by which platelets maintain vascular integrity, we measured plasma S1P over time in treated and control dogs. Platelet count correlated significantly with plasma S1P and plasma S1P was lower in treated than control dogs (Figure 8). To our knowledge this platelet count-plasma S1P correlation has not been previously reported. In murine models of thrombocytopenia, plasma S1P and platelet count are correlated early in the time course, but after the first four hours this relationship is lost (Susan Smyth, personal communication, September 25, 2012). Presumably in mice another source of S1P, potentially erythrocytes, buffers the platelet-related decrease after this point.

Our observed relationship between S1P and platelet count are not surprising given that S1P is most abundantly stored in platelets compared with in other blood cells (6 times more S1P per platelet than per red cell in humans) [37, 38]. However, because of the large circulating red cell mass, erythrocytes are thought to provide a plasma S1P buffering capacity

and determine plasma levels of S1P [13]. Recent studies in people have shown that erythrocytes actually buffer plasma S1P by sequestering and releasing it [13]. In humans, anemia is associated with a reduction in plasma S1P [13]. We also found a correlation between hematocrit and S1P in the study dogs, however, the platelet-S1P relationship was independent of hematocrit.

Nonetheless, we did not find any correlations between plasma S1P and endothelial ultrastructure or bleeding score. That overall circulating plasma S1P does correlate with endothelial ultrastructure does not exclude the possibility that platelet-derived S1P may have important local effects on preserving the vasculature. In the future, local S1P concentrations could be assessed by immunostaining our endothelial biopsies.

S1P is also just one of many candidate platelet vasoactive mediators. We attempted to measure plasma levels of other potential mediators including angiopoietin-1 and endostatin [39-42], but were limited by a lack of available canine-reactive assays. Future studies in human and canine ITP patients should investigate these and other mediators.

Von Willebrand Factor as a Marker of Endothelial Damage

We investigated the applicability of plasma vWf as a biomarker of thrombocytopenia-induced endothelial damage. vWf is synthesized and stored in endothelial cells; high circulating vWf levels reflect endothelial damage or endothelial dysfunction [25]. We selected vWf because of its endothelial specificity: it can be released from endothelial cell Wiebel-Palade bodies upon endothelial cell damage [25]. Although human platelets contain vWf, most of plasma vWf is derived from endothelial cells [25]. Since canine platelets contain almost no vWf [23], plasma vWf is an even more endothelial-specific marker in canines.

However, as plasma vWf increased over time in both our control and treated dogs, we highlighted one of the major limitations of vWf: although it origin is specific, causes for its increase are not. vWf can be released from endothelial cells when they are damaged, but it can also be released from endothelial cells as an acute phase reactant [25, 43]. Since vWf increased over time in both treated and control dogs, we presume its increase reflected some

degree of inflammation experienced by all groups of dogs secondary to antibody administration or repeated biopsies or a combination thereof. The redundancy in causes of vWf increase may explain why we did not see a correlation between vWf levels and endothelial alterations or bleeding score.

Unfortunately, specific soluble biomarkers of endothelial damage are lacking. Other potential biomarkers of endothelial damage such as soluble vascular cell adhesion molecular (VCAM) and intercellular adhesion molecule (ICAM) can be influenced by non-endothelial factors [44]. Circulating endothelial cells, endothelial microparticles, and endothelial progenitor cells represent other, more specific ways to assess endothelial dysfunction that could be investigated as markers of thrombocytopenic endothelial damage [44].

Conclusion

In summary, although greater power is needed, our present study suggests that there is an association between thrombocytopenia and ultrastructural endothelial abnormalities in a canine model of ITP. The significant decrease in pinocytotic vesicles in thrombocytopenic endothelium shows that endothelial damage occurs in our ITP model and warrants further study. Variability in the degree of alterations of vascular ultrastructure may account for differences in bleeding phenotype in immune thrombocytopenia. Further investigations are necessary to assess functional effects of thrombocytopenia on vascular integrity, to identify an ideal endothelial damage biomarker or cell marker, and to determine the mechanism by which platelets maintain vascular integrity. S1P remains a candidate effector of platelets' vascular stabilizing function and its local concentrations should be assessed in regions of normal and disrupted thrombocytopenic endothelium. Ultimately, measurement of markers of endothelial dysfunction or platelet vascular stabilizing mediators could enable us to predict which ITP patients are at risk of bleeding and tailor patient management accordingly. Furthermore, better understanding of the mechanism of thrombocytopenic bleeding will allow for generation of new therapeutics to prevent and treat bleeding in thrombocytopenia.

Table 1. Platelet counts ($x10^3/\mu l$) in 2F9 and control antibody-treated dogs. Counts are shown as mean ± SD. The platelet counts for the two dogs that experienced large delayed ecchymoses are both listed (bleed). Time zero is when the platelet count first fell into the target platelet range of 5,000-30,000 platelets/µl or 1 hour after control antibody infusion.

	Treated (n=5)	Control (n=3)
Baseline	235.2 ± 61.0	200.7 ± 15.3
Time 0 h	15.6 ± 2.1	197.7 ± 20.6
Time 24 h	31.4 ± 5.1	184.0 ± 8. 7
Bleed	85; 196	N/A
Recovery	366.2 ± 101.2	208.0 ± 41.7

Table 2. Histologic evaluation of cutaneous biopsies. Time zero was time of platelet nadir or 1 hour after isotype control antibody infusion. L-lymphocytes; P-plasma cells; N-neutrophils; E-eosinophils. Severity was scored as follows: 0 = no change, 1 = minimal change, 2 = mild change, 3 = moderate change, and 4 = marked change.

Dog	Time	Hemorrhage	Edema	Inflammation	Thrombosis	Endothelial Hypertrophy	Other
Treated Dogs							
1	Baseline	0	0	0	0	0	Perifollicular and follicular lymphocytes, focal
	0	0	0	1 L>P	0	0	
	24	0	0	0	0	0	
	Recovery	0	1	2 L>P, E, rare N	0	0	Focal lympho-histiocytic exocytosis
2	Baseline	0	0	1 L>P	0	0	
	0	0	0	1 L>P	0	0	
	24	0	1	0	0	0	
	Bleed	4	2	1 L>P, rare N	0	4	Endothelial hypertrophy; dilated lymphatics
	Recovery	0	0	1 L>P	0	0	

Table 2 Continued

3	Baseline	0	0	1 L>P, rare N	0	0	
	0	1	1	1 L>P	0	0	Focal edema; very minimal inflammatory cells appreciated in superficial dermis
	24	0	0	1 L>P	0	0	
	Recovery	0	0	0	0	0	
4	Baseline	0	0	0	0	0	
	0	0	0	0	0	0	
	24	0	0	0	0	0	
	Recovery	0	0	0	0	0	
5	Baseline	0	0	0	0	0	
	0	0	0	0	0	0	
	24	0	0	0	0	0	
	Bleed	1	1 (very low)	0	0	0	Focal area of irregular collagen distribution in deep dermis
	Recovery	0	0	0	0	0	
Control Dogs							
1	Baseline	0	0	0	0	0	
	0	0	0	0	0	0	
	24	0	0	0	0	0	
	Recovery	0	0	0	0	0	
2	Baseline	0	0	0	0	0	
	0	0	0	0	0	0	
	24	0	0	0	0	0	
	Recovery	0	0	0	0	0	1 focus mild perifollicular fibrosis

Table 2 Continued

3	Baseline	0	0	0	0	0	
	0	0	0	0	0	0	
	24	1	0	0	0	0	Focal hemorrhage at surface
	Recovery	0	0	0	0	0	

Table 3. Ultrastructural features of endothelium in healthy dogs and dogs after 24 hours of severe thrombocytopenia. See methods for vesicle and vacuole scoring system. Results are mean ± S.D. **P<0.0045 compared to change in time-matched controls.

	Treated (n=5)			Control (n=3)		
	Baseline	**24 hours**	**Difference**	**Baseline**	**24 hours**	**Difference**
Vessel thickness	0.78±0.42	0.71±0.38	-0.08±0.57	0.76±0.39	0.75±0.38	-0.01±0.55
Pinocytotic vesicle score	2.41±0.48	2.16±0.53	-0.25±0.71**	2.28±0.57	2.59±0.51	0.31±0.76
Large vacuole score	0.71±0.77	0.83±0.86	0.13±1.16	0.84±0.78	0.91±0.79	0.07±1.11
Number of spaces between cells	0.69±0.7	0.84±0.79	0.15±1.06	0.73±0.6	0.81±0.69	0.08±0.92

Table 4. Endothelial ultrastructural features at the time of large delayed ecchymosis formation in two dogs. Results are mean ± S.D.

	Dog 1		Dog 2	
	Baseline	Bleed	Baseline	Bleed
Vessel thickness	0.85±0.56	0.68±0.43	0.77±0.38	0.80±0.45
Pinocytotic vesicle score	2.08±0.49	1.75±0.58	2.76±0.44	2.23±0.53
Large vacuole score	1±1	1.13±0.89	0.29±0.47	0.45±0.67
Number of spaces between cells	0.69±0.75	1.31±0.79	0.71±0.69	1.1±0.97

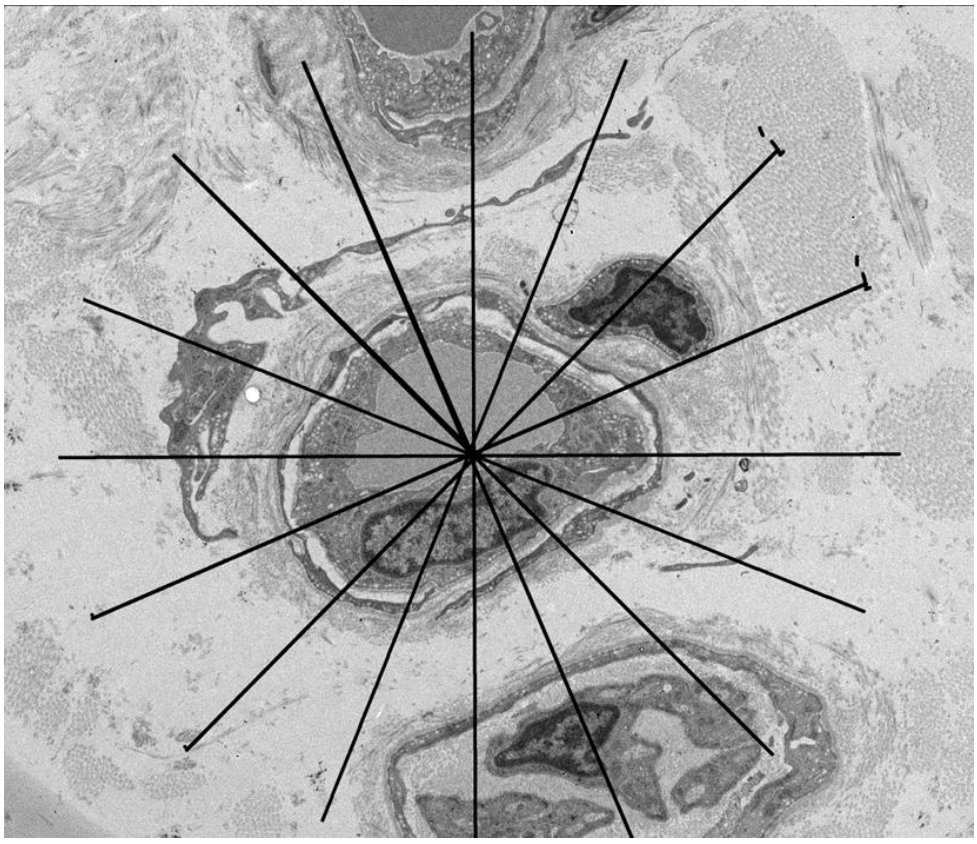
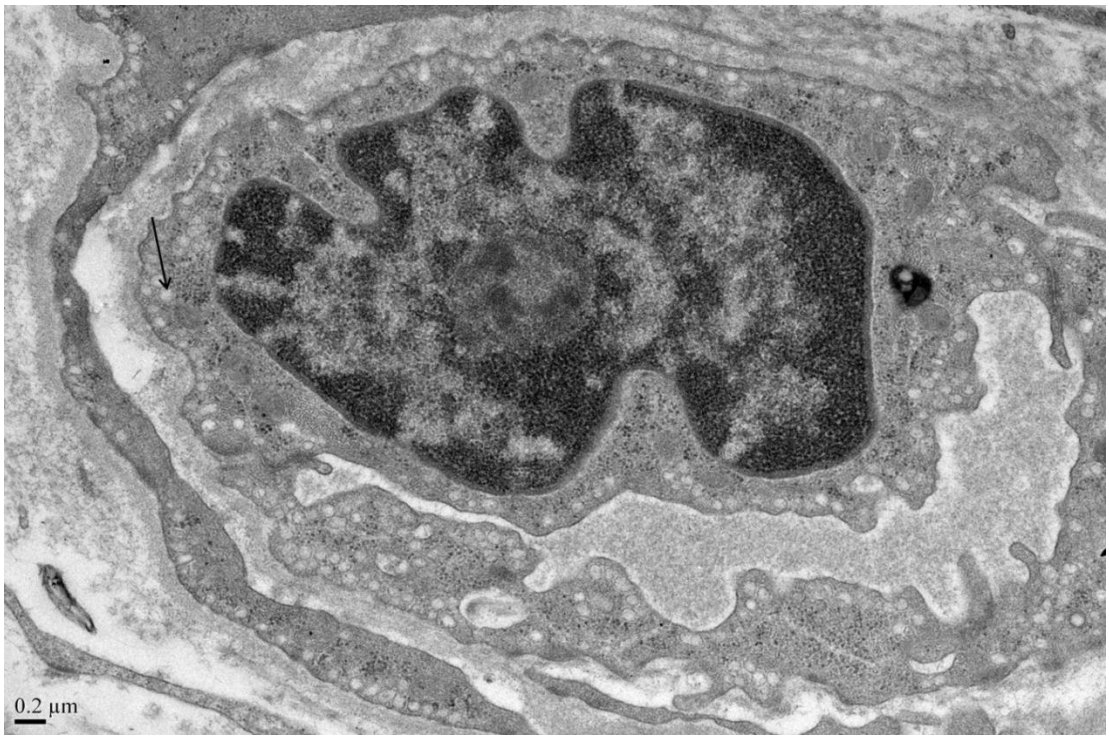


Figure 1. Demonstration of determination of endothelial cell thickness. A grid was superimposed on endothelial cells and endothelial cell thickness measured each place the grid crossed the vessel. Average thickness was calculated from each individual measurement. 5,000x.

Figure 2. Definition of ultrastructural features. A. Pinocytotic vesicles are 0.07-0.08 µm in diameter (arrow). 20,000x; B. Space between cells (arrow). 20,000x. C. Large vacuoles are >0.1 µm (arrows). 20,000x.

A.



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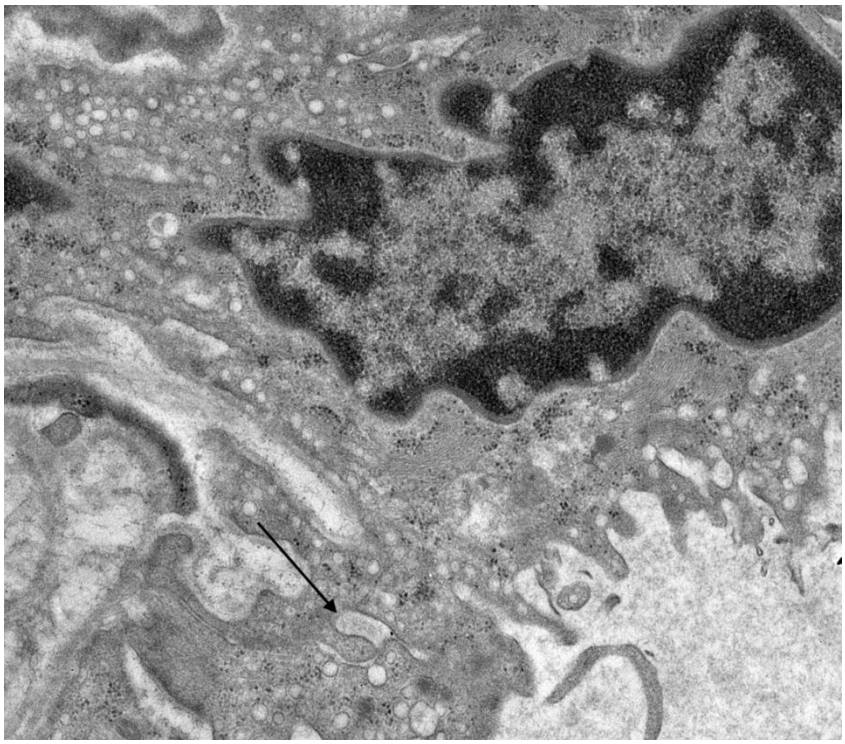

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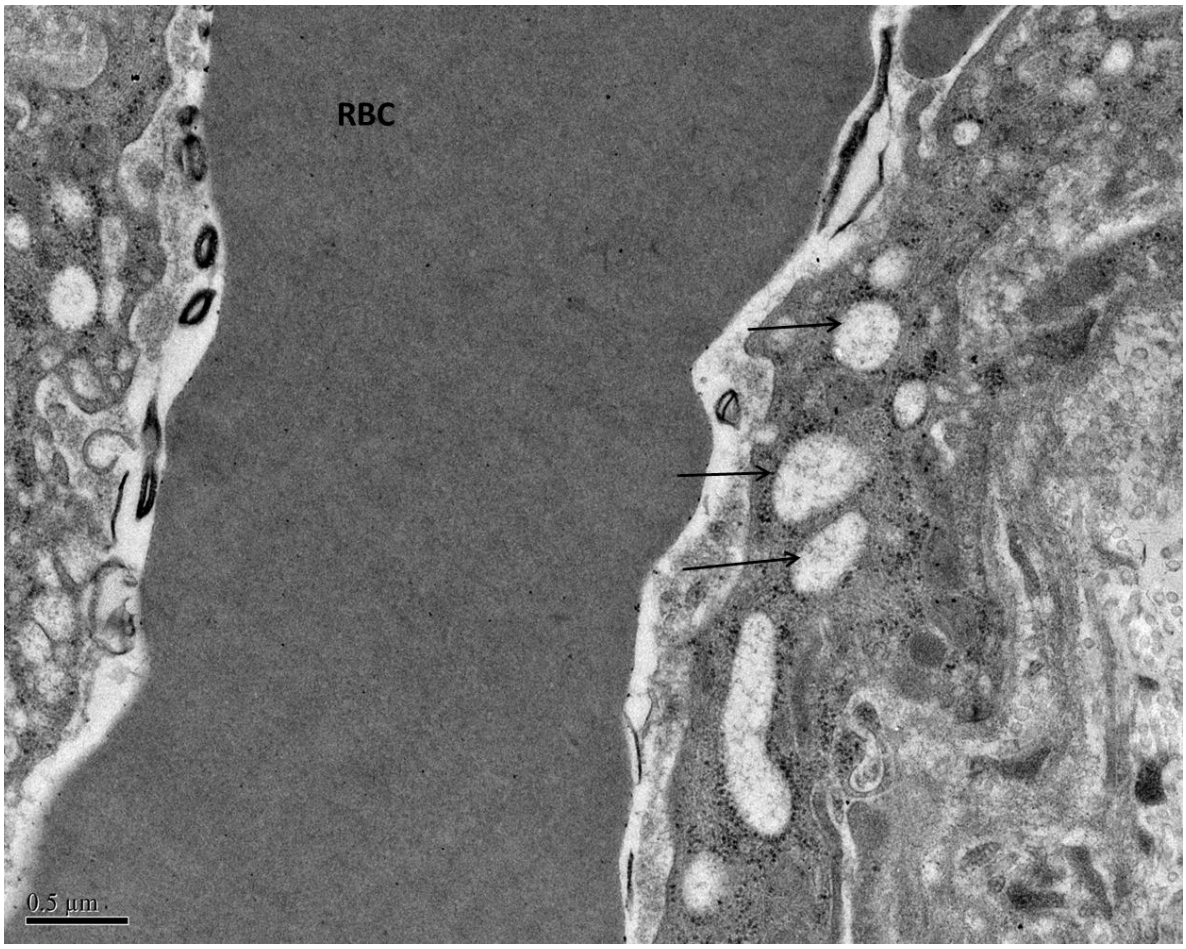


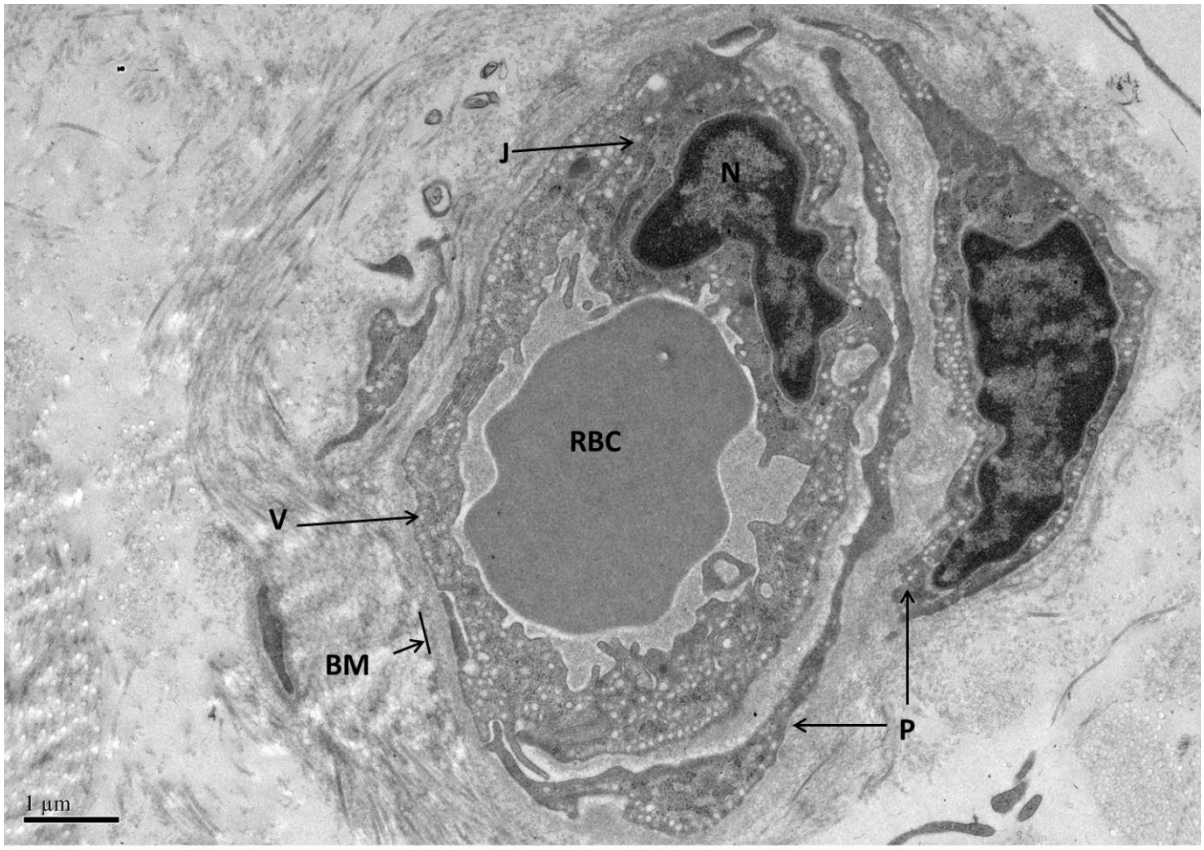


Figure 3. Normal cutaneous capillary. The vessel is enveloped by the basement membrane (BM). Two pericytes are visible (P). Pinocytotic vesicles are numerous (V). There are no spaces between endothelial cells and a well-apposed junction (J) is present connecting two adjacent endothelial cells adjacent to the nucleus (N). A red blood cell (RBC) is present in the vessel lumen. 10,000x.

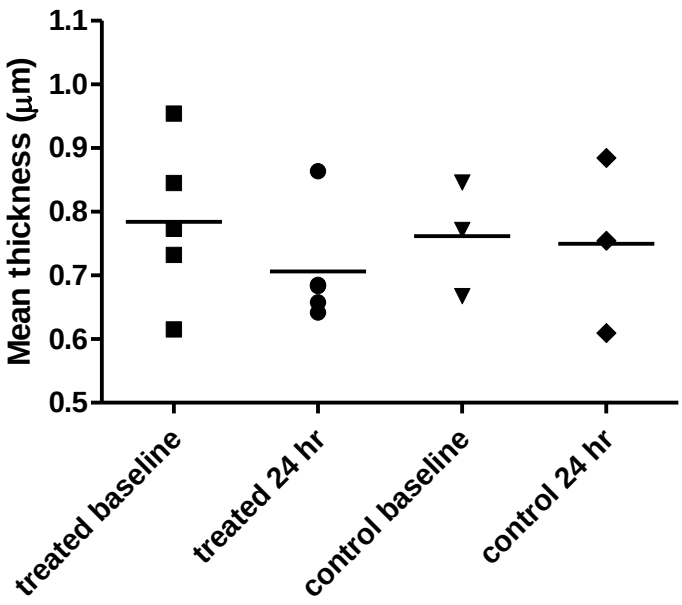


Figure 4. Effect of thrombocytopenia on vascular wall thickness. There is a non-statistically significant trend towards endothelial wall thinning in thrombocytopenic dogs after 24 hours of thrombocytopenia (n=5) compared to time-matched controls (n=3). Symbols represent the mean value for all capillaries evaluated in each dog at that time point. Bars represent overall treatment group mean.

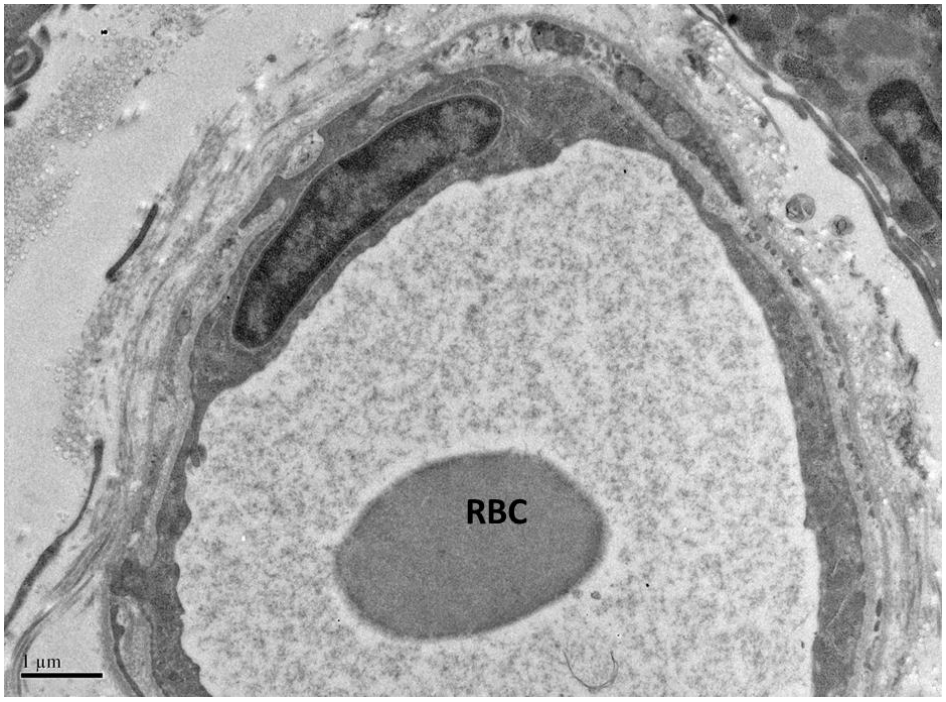
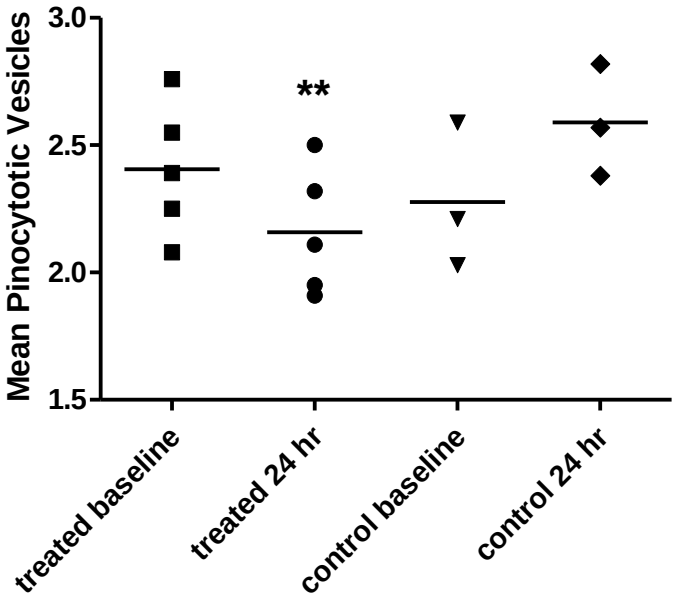


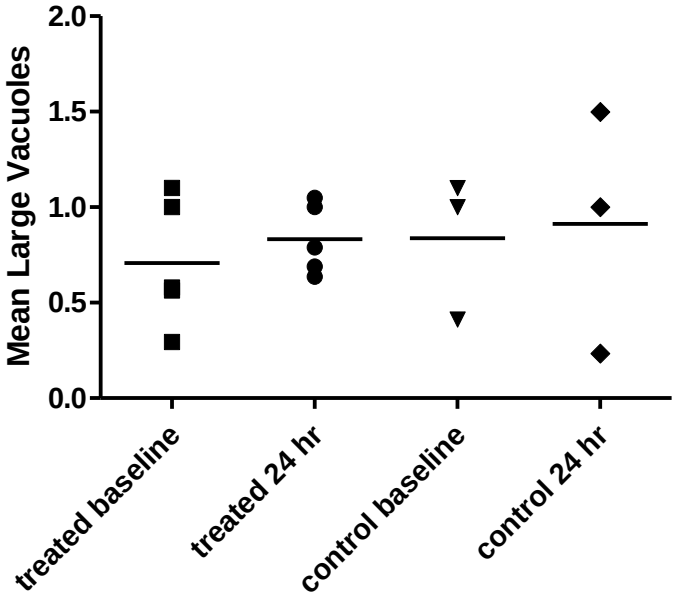
Figure 5. Representative micrograph demonstrating the endothelial thinning and reduction in number of pinocytotic vesicles observed in thrombocytopenic dogs. Note that vesicles are almost absent compared to the normal vessel shown in Figure 2. A RBC is shown in the vessel lumen. 10,000x.

Figure 6. Effect of thrombocytopenia on endothelial ultrastructure. A) Number of endothelial pinocytotic vesicle score, (B) large vacuole score, and (C) number of spaces between cells. **There is a significant decrease in number pinocytotic vesicles after 24 hours of thrombocytopenia compared to time-matched controls ($P<0.0045$). See text for vacuole and vesicle scoring system. Symbols represent the mean value for all capillaries evaluated in each dog at that time point. Bars represent overall treatment group mean. n=5 2F9 treated dogs; n=3 control dogs.

A.


Mean Pinocytotic Vesicles
3.0
2.5
2.0
1.5
**
treated baseline
treated 24 hr
control baseline
control 24 hr

B.


Mean Large Vacuoles
2.0
1.5
1.0
0.5
0.0
treated baseline
treated 24 hr
control baseline
control 24 hr

C.

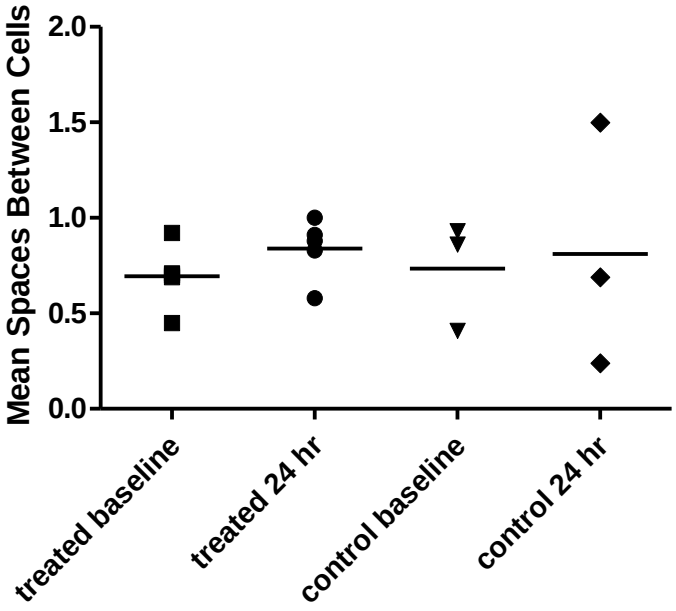
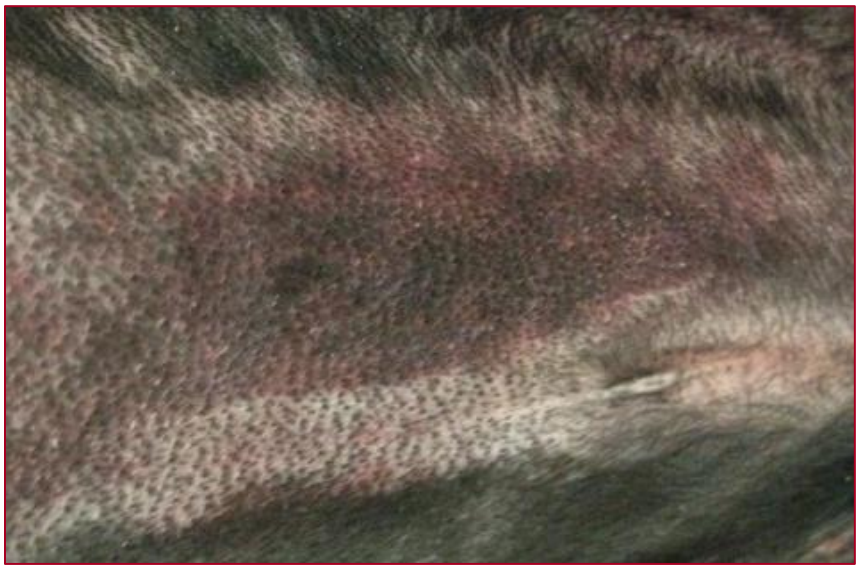
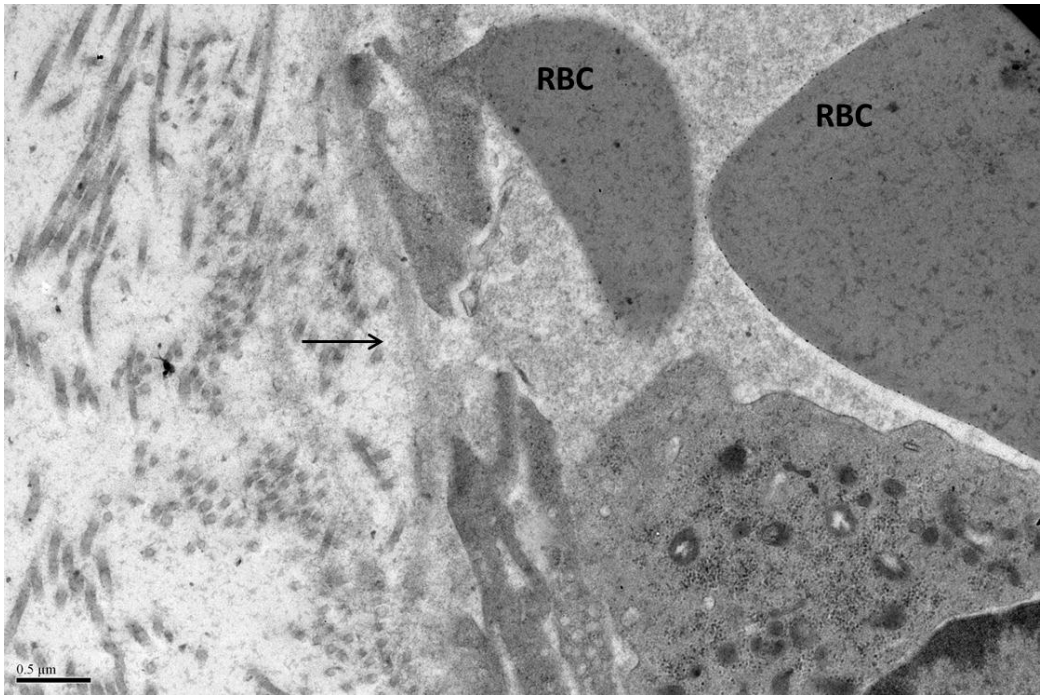

Mean Spaces Between Cells
2.0
1.5
1.0
0.5
0.0
treated baseline
treated 24 hr
control baseline
control 24 hr

Figure 7. Delayed ecchymoses occurred in two dogs during the period of platelet count recovery. A. Photograph of the large abdominal bruise (8.5 x 13 cm) that formed at 48 hours in one dog when platelet count had already recovered to 85,000 platelets/µl. B. Electron micrograph from the dog in A after 24 hours of thrombocytopenia, 24 hours prior to the development of the bruise. A large gap between adjacent endothelial cells is marked with an arrow. RBCs are shown in lumen. 20,000x. C. Pale, swollen endothelial cells consistent with necrotic cells and inter-endothelial cell gaps (arrows) present in an electron micrograph from the bruised region of another dog that developed a delayed ecchymosis. 20,000x. D. For comparison, normal closely apposed adjacent endothelial cells connected by an intact junction (arrow) in a dog at baseline are shown. A RBC is present in the vessel lumen. 20,000x.

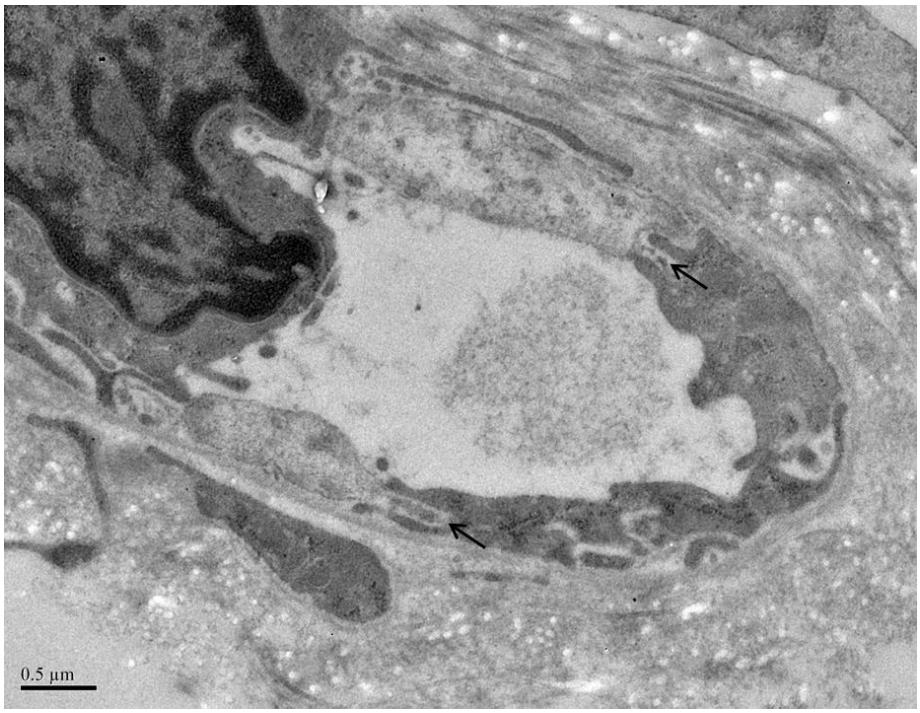
A.



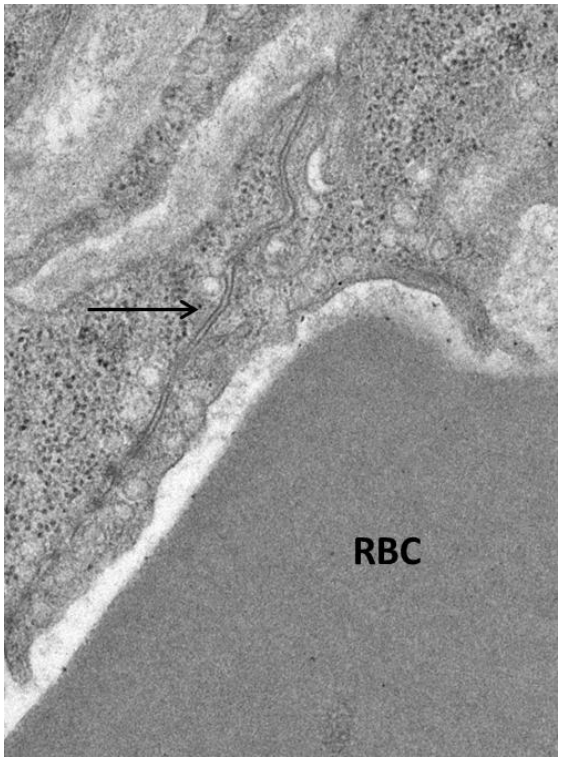
B.



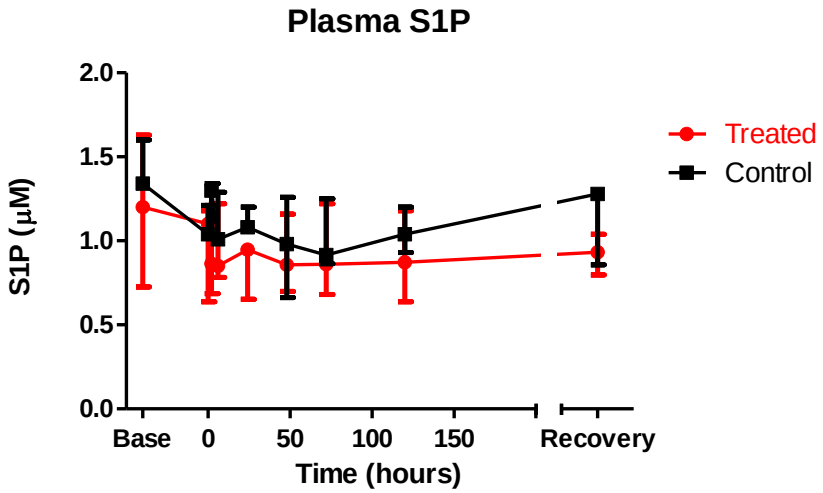
C.



D.



A.



B.

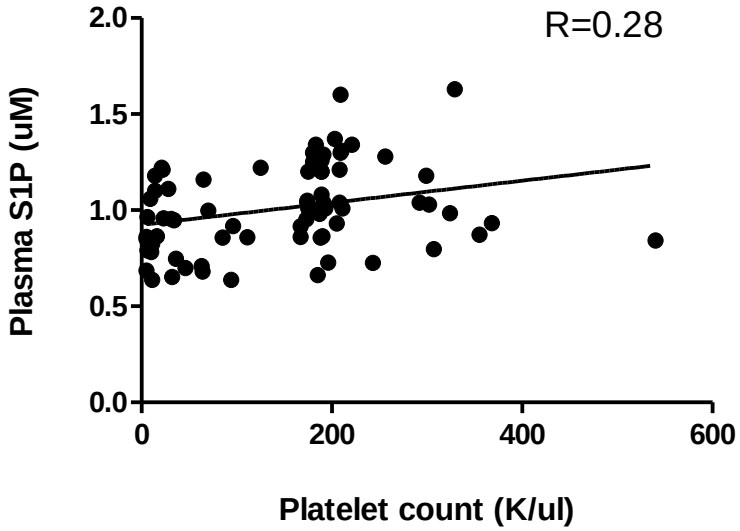


Figure 8. Relationship between platelet count and plasma S1P levels. A. Total plasma S1P (median, range) over time in 2F9-treated and control dogs. S1P decreases significantly over time in treated compared to control dogs (P<0.001). Time zero is when platelet count fell into target nadir range (5-30K platelets/µl) or 1 hour after control antibody administration. B. Total plasma S1P plotted against platelet count in all dogs over all time points. R=0.28 (P<0.016) by linear regression analysis.

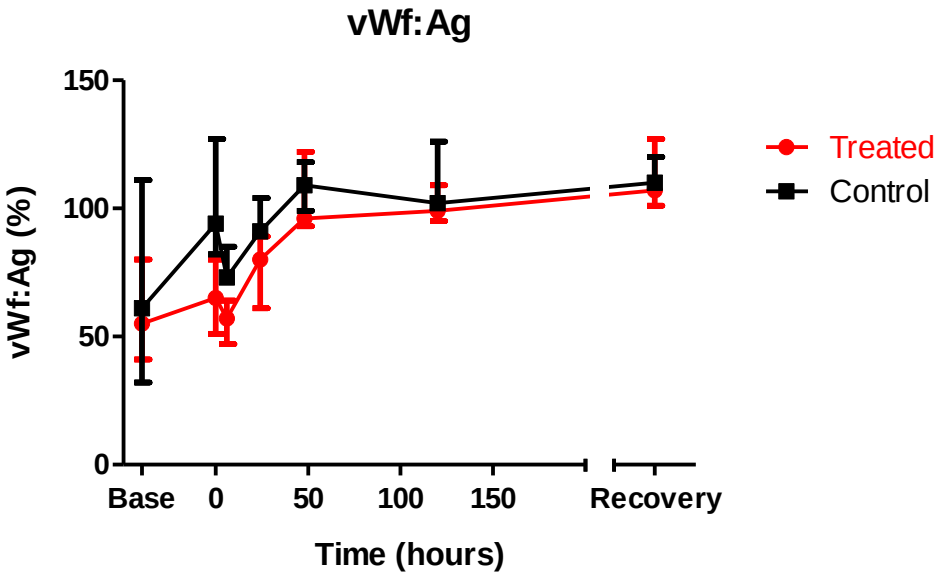


Figure 9. Alterations of von Willebrand factor over time in thrombocytopenic and control dogs. Similar increases in vWf in both groups of dogs likely reflect vWf's induction as an acute phase reactant rather than elevation as a marker of vascular damage. Plasma vWf:Ag concentration is reported as percent compared to a normal canine standard (median, range).

References

1. Shepro, D., H.E. Sweetman, and H.B. Hechtman, *Experimental thrombocytopenia and capillary ultrastructure.* Blood, 1980. **56**(5): p. 937-9.

2. Ho-Tin-Noe, B., M. Demers, and D.D. Wagner, *How platelets safeguard vascular integrity.* J Thromb Haemost, 2011. **9 Suppl 1**: p. 56-65.

3. Goerge, T., et al., *Inflammation induces hemorrhage in thrombocytopenia.* Blood, 2008. **111**(10): p. 4958-64.

4. Ho-Tin-Noe, B., et al., *Platelet granule secretion continuously prevents intratumor hemorrhage.* Cancer Res, 2008. **68**(16): p. 6851-8.

5. Takuwa, N., et al., *Tumor-suppressive sphingosine-1-phosphate receptor-2 counteracting tumor-promoting sphingosine-1-phosphate receptor-1 and sphingosine kinase 1—Jekyll Hidden behind Hyde.* Am J Cancer Res, 2011. **1**(4): p. 460-81.

6. Camerer, E., et al., *Sphingosine-1-phosphate in the plasma compartment regulates basal and inflammation-induced vascular leak in mice.* J Clin Invest, 2009. **119**(7): p. 1871-9.

7. Schaphorst, K.L., et al., *Role of sphingosine-1 phosphate in the enhancement of endothelial barrier integrity by platelet-released products.* Am J Physiol Lung Cell Mol Physiol, 2003. **285**(1): p. L258-67.

8. Polzin, A., et al., *Sphingosine-1-phosphate is thromboxane-dependently released from platelets and modulates chemotaxis of human monocytes,* in *American Heart Association*. 2010: Chicago, IL.

9. Dejana, E., *Endothelial cell-cell junctions: happy together.* Nat Rev Mol Cell Biol, 2004. **5**(4): p. 261-70.

10. McVerry, B.J. and J.G. Garcia, *In vitro and in vivo modulation of vascular barrier integrity by sphingosine 1-phosphate: mechanistic insights.* Cell Signal, 2005. **17**(2): p. 131-9.

11. Chavez, A., M. Smith, and D. Mehta, *New insights into the regulation of vascular permeability.* Int Rev Cell Mol Biol, 2011. **290**: p. 205-48.

12. McVerry, B.J., et al., *Sphingosine 1-phosphate reduces vascular leak in murine and canine models of acute lung injury.* Am J Respir Crit Care Med, 2004. **170**(9): p. 987-93.

13. Selim, S., et al., *Plasma levels of sphingosine 1-phosphate are strongly correlated with haematocrit, but variably restored by red blood cell transfusions.* Clin Sci (Lond), 2011. **121**(12): p. 565-72.

14. Kitchens, C.S. and J.F. Pendergast, *Human thrombocytopenia is associated with structural abnormalities of the endothelium that are ameliorated by glucocorticosteroid administration.* Blood, 1986. **67**(1): p. 203-6.

15. Kitchens, C.S., *Amelioration of endothelial abnormalities by prednisone in experimental thrombocytopenia in the rabbit.* J Clin Invest, 1977. **60**(5): p. 1129-34.

16. Van Horn, D.L. and S.A. Johnson, *The escape of carbon from intact capillaires in experimental thrombocytopenia.* J Lab Clin Med, 1968. **71**(2): p. 301-311.

17. Van Horn, D.L. and S.A. Johnson, *The mechanism of thrombocytopenic bleeding.* Am J Clin Pathol, 1966. **46**(2): p. 204-13.

18. Miles, R.G. and J.V. Hurley, *The effect of thrombocytopenia on the ultrastructure and reaction to injury of vascular endothelium.* Microvasc Res, 1983. **26**(3): p. 273-90.

19. Dale, C. and J.V. Hurley, *An electron-microscope study of the mechanism of bleeding in experimental thrombocytopenia.* J Pathol, 1977. **121**(4): p. 193-204.

20. Davidson, S.J., et al., *Endothelial cell damage in heparin-induced thrombocytopenia.* Blood Coagul Fibrinolysis, 2007. **18**(4): p. 317-20.

21. Hanel, P., P. Andreani, and M.H. Graler, *Erythrocytes store and release sphingosine 1-phosphate in blood.* FASEB J, 2007. **21**(4): p. 1202-9.

22. Mathews, T.P., et al., *Discovery, biological evaluation, and structure-activity relationship of amidine based sphingosine kinase inhibitors.* J Med Chem, 2010. **53**(7): p. 2766-78.

23. Catalfamo, J.L., et al., *Canine platelets are virtually devoid of von Willebrand factor.* Blood, 1991. **78**: p. 261a.

24. Aird, W.C., *Phenotypic heterogeneity of the endothelium: I. Structure, function, and mechanisms.* Circ Res, 2007. **100**(2): p. 158-73.

25. Lip, G.Y. and A. Blann, *von Willebrand factor: a marker of endothelial dysfunction in vascular disorders?* Cardiovasc Res, 1997. **34**(2): p. 255-65.

26. Gutierrez, J.M., et al., *Blood flow is required for rapid endothelial cell damage induced by a snake venom hemorrhagic metalloproteinase.* Microvasc Res, 2006. **71**(1): p. 55-63.

27. Fitzl, G., et al., *Protective effects of Gingko biloba extract EGb 761 on myocardium of experimentally diabetic rats. I: ultrastructural and biochemical investigation on cardiomyocytes.* Exp Toxicol Pathol, 1999. **51**(3): p. 189-98.

28. Fuskevag, O.M., et al., *Microvascular perturbations in rats receiving the maximum tolerated dose of methotrexate or its major metabolite 7-hydroxymethotrexate.* Ultrastruct Pathol, 2000. **24**(5): p. 325-32.

29. Mohle, R., et al., *Constitutive production and thrombin-induced release of vascular endothelial growth factor by human megakaryocytes and platelets.* Proc Natl Acad Sci U S A, 1997. **94**(2): p. 663-8.

30. Nachman, R.L. and S. Rafii, *Platelets, petechiae, and preservation of the vascular wall.* N Engl J Med, 2008. **359**(12): p. 1261-70.

31. Chen, J., et al., *VEGF-induced mobilization of caveolae and increase in permeability of endothelial cells.* Am J Physiol Cell Physiol, 2002. **282**(5): p. C1053-63.

32. Dvorak, A.M. and D. Feng, *The vesiculo-vacuolar organelle (VVO). A new endothelial cell permeability organelle.* J Histochem Cytochem, 2001. **49**(4): p. 419-32.

33. Talke, P., et al., *Effect of alpha2B-adrenoceptor polymorphism on peripheral vasoconstriction in healthy volunteers.* Anesthesiology, 2005. **102**(3): p. 536-42.

34. Kitchens, C.S. and L. Weiss, *Ultrastructural changes of endothelium associated with thrombocytopenia.* Blood, 1975. **46**(4): p. 567-78.

35. Gore, I., M. Takada, and J. Austin, *Ultrastructural basis of experimental thrombocytopenic purpura.* Arch Pathol, 1970. **90**(3): p. 197-205.

36. Ho-Tin-Noe, B., et al., *Innate immune cells induce hemorrhage in tumors during thrombocytopenia.* Am J Pathol, 2009. **175**(4): p. 1699-708.

37. Bode, C., et al., *Erythrocytes serve as a reservoir for cellular and extracellular sphingosine 1-phosphate.* J Cell Biochem. **109**(6): p. 1232-43.

38. Yatomi, Y., et al., *Sphingosine 1-phosphate, a bioactive sphingolipid abundantly stored in platelets, is a normal constituent of human plasma and serum.* J Biochem, 1997. **121**(5): p. 969-73.

39. Brankin, B., et al., *Endostatin modulates VEGF-mediated barrier dysfunction in the retinal microvascular endothelium.* Exp Eye Res, 2005. **81**(1): p. 22-31.

40. Li, J.J., et al., *Thrombin induces the release of angiopoietin-1 from platelets.* Thromb Haemost, 2001. **85**(2): p. 204-6.

41. Gamble, J.R., et al., *Angiopoietin-1 is an antipermeability and anti-inflammatory agent in vitro and targets cell junctions.* Circ Res, 2000. **87**(7): p. 603-7.

42. Italiano, J.E., Jr., et al., *Angiogenesis is regulated by a novel mechanism: pro- and antiangiogenic proteins are organized into separate platelet alpha granules and differentially released.* Blood, 2008. **111**(3): p. 1227-33.

43. Pottinger, B.E., et al., *von Willebrand factor is an acute phase reactant in man.* Thromb Res, 1989. **53**(4): p. 387-94.

44. Burger, D. and R.M. Touyz, *Cellular biomarkers of endothelial health: microparticles, endothelial progenitor cells, and circulating endothelial cells.* J Am Soc Hypertens, 2012. **6**(2): p. 85-99.

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ALTERATIONS IN PLATELET HEMOSTATIC FUNCTION IN A CANINE MODEL OF ITP

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Introduction

Although severe thrombocytopenia (below 30,000 platelet/µl) is a risk factor for bleeding, there is great variability in bleeding signs in thrombocytopenic patients. Some patients suffer life-threatening hemorrhage with platelet counts greater than 50,000/µl, while others with platelet counts lower than 5,000/µl do not bleed [1-4]. In both canine and human patients with immune thrombocytopenia (ITP), bleeding risk is challenging to predict, which potentially leads to over-treatment of patients at low risk. Therapy for ITP is not benign. In many patients, the morbidity and mortality of treatment-related side effects exceed the problems of ITP itself, making it ideal to avoid treatment in patients who are not at risk of bleeding [5, 6]. Paradoxically, recent studies have highlighted the risk of thrombosis in ITP, and it is unknown how to identify at-risk patients who may actually require anti-thrombotic

therapy [4, 7, 8]. Given the variability of bleeding in ITP, we hypothesized that in ITP, changes in platelet hemostatic function may occur in addition to changes in platelet numbers. A better understanding of platelet function in ITP is necessary to enable targeted patient management.

In response to vascular injury, platelets accumulate rapidly to form a platelet plug [9]. Platelets can be activated by soluble agonists like thrombin and when adherent, by components of the extracellular matrix such as collagen [9]. Platelet activation results in shape change, granule release including P-selectin from alpha granules, and activation of the fibrinogen receptor allowing for platelet aggregation [9]. Platelet P-selectin surface expression is often used as a sensitive and specific indicator of platelet activation state [10, 11]. One subpopulation of highly procoagulant platelets, termed coated-platelets, develops after dual stimulation with thrombin and collagen or thrombin and convulxin, an agonist for the collagen receptor glycoprotein VI [12, 13].

Coated-platelets retain high levels of alpha granule-derived procoagulant proteins on their surface (e.g., fibrinogen, factor V, thrombospondin), express surface phosphatidylserine, and support formation of the active prothrombinase complex, thereby setting the stage for secondary hemostasis [12]. Because they are “coated” with procoagulant proteins, these platelets have a greater ability to promote thrombin generation [12]. The exact physiological significance of coated-platelets is unknown, though they are speculated to be significant contributors to thrombotic processes [12]. Increased coated-platelet levels have been correlated with thrombotic outcomes and decreased ability to form coated-platelets is associated with hemorrhagic outcomes [13-15].

Little is known about platelet function in thrombocytopenia because thrombocytopenia interferes with most common methods of assessing platelet function such as aggregometry, making it impossible to differentiate effects of reduced platelet function from those of thrombocytopenia [1]. Flow cytometry can be performed at very low platelet counts and allows for individual platelet function to be analyzed separately from platelet number [1, 16].

Many studies have shown that autoantibodies present in ITP serum can enhance or inhibit normal platelet function, depending on the antibody [17, 18]. However, these studies have not investigated the function of ITP patients' own platelets [19]. Of the few flow cytometric studies that have been performed directly on ITP patients' platelets, results have been confusing and contradictory. Some studies report increased *ex vivo* reactivity of ITP platelets to agonists [1, 20], whereas others report decreased reactivity [21].

There are also conflicting descriptions of the degree of circulating (unstimulated) platelet activation state in ITP. Some investigators have described increased platelet activation in ITP, while others have not detected platelet activation [1, 19, 22-25]. Even amongst the studies that report increased platelet activation in ITP, the relevance of platelet activation state to bleeding risk is disputed [1, 19]. Traditionally, the larger, younger platelets of ITP patients have been viewed as more functional than normal platelets and this has been thought to account for why ITP patients seem to bleed less than patients with thrombocytopenia due to bone marrow failure [1, 20, 26, 27]. However, these assumptions have never been validated and do not explain why some ITP patients do bleed severely. Inter-individual differences in hemostatic platelet function may explain the variability of bleeding phenotypes in ITP, but the sparse and contradictory current literature demonstrates the need for further investigation of the relationship between platelet hemostatic function and thrombocytopenic bleeding.

Accordingly, in this exploratory study, we serially evaluated platelet hemostatic function in a novel canine model of ITP. Using flow cytometry assays, platelet surface expression of P-selectin, the thrombin concentration required to induce half-maximal P-selectin expression (ED50 thrombin), and coated-platelet production were measured to assess circulating platelet activation state (resting P-selectin) and reactivity (ED50 thrombin and coated-platelets) in model dogs. In addition, platelet function was compared to a clinical bleeding score to determine if there was a relationship between platelet function and bleeding tendency.

Materials and Methods:

Animals

Eight healthy adult (median 2 years old; range 1-4) intact male mixed breed dogs were used in this study (28.4 ± 5.6 kg). Research dogs were loaned from Francis Owen Blood Research Laboratory at the University of North Carolina at Chapel Hill or Laboratory Animal Resources at the North Carolina State College of Veterinary Medicine (NCSU) as described in Chapter 2. All protocols were approved by the Institutional Animal Care and Use Committee of North Carolina State University.

Thrombocytopenia Induction

As previously described in Chapter 2, dogs (n=5) were infused with a murine monoclonal anti-GPIIb antibody (2F9) in order to model ITP and generate predictable severe thrombocytopenia (target nadir 5,000 to 30,000 platelets/µl). Control dogs (n=3) were infused with an IgG2a isotype control antibody (anti-yellow fever antibody; αYFA or CRL-1689.1). Due to technical issues with the flow cytometer, one treated dog was excluded from further analysis.

Blood Sampling and Preparation

Blood was obtained via 19 g indwelling catheters that were placed under dexmedetomidine sedation as described in Chapter 2. Catheters were first flushed with 6 ml of 0.9% sodium chloride. A 5 ml catheter purge sample was then collected into 1 ml of 0.9% sodium chloride and set aside. Blood (2 ml) was collected into a plain syringe and transferred immediately to EDTA tube (Kendall, Mansfield, MA) for platelet count and into a syringe (2ml) containing 0.2 ml acid citrate dextrose (ACD) for platelet rich plasma preparation. The initial 6 ml purge sample was then returned to the dog and the catheter flushed with 6 ml of 0.9% sodium chloride. To generate platelet rich plasma (PRP) for flow cytometry reactions, the ACD blood was combined with an equal volume of buffered saline glucose citrate (BSGC; 129 mM NaCl, 14 mM Na_3citrate, 11 mM glucose, 10 mM NaH_2PO_4, pH 7.3) and centrifuged at

275g for 5 minutes. The supernatant PRP was harvested and used within 4 hours in flow cytometry reactions. If the indwelling catheter was not patent at the sampling time, blood was obtained via careful cephalic vein venipuncture through a 21 gauge butterfly needle (Terumo, Elkton, MD).

Blood was drawn at the following time points: baseline, time zero (when platelet count first fell into the target nadir range of 5-30K platelets/µl or 1 hour after control antibody administration), 6, 24 hours, and then every 24 hours until platelet count recovered and new bleeding stopped, whichever came later (168-384 hours). This last time point was termed "recovery."

Assessment of Platelet Activation in Response to Thrombin (ED50 Thrombin)

For P-selectin studies, activation reactions were carried out in 5mL polystyrene tubes (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA) in a 100 µL (total) assay volume containing 1 µl PRP in a reaction mixture containing HEPES saline (10 mM HEPES, 150 mM NaCl, pH 7.5) and 0.4mM gly-proarg-pro-NH2 (Sigma-Aldrich, St. Louis, MO). Platelets were stimulated with varying concentrations of thrombin (none, 1, 0.1, 0. 05, 0. 025 U/ml final concentration; bovine thrombin, King Pharmaceuticals, Bristol, TN), for 15 minutes at room temperature. Fluorescent conjugated antibodies were then added directly to the reaction tubes to identify platelets based on constitutive membrane GPIb/IX (142.2 alexa 488, kind gift of David Wilcox, Milwaukee, WI, 0.13 µg/100 µL reaction) and to detect surface expression of P-selectin (CD62P-phycoerythrin [PE], 8 mL/100 µL reaction; clone AC1.2, BD Biosciences). Additional unstimulated control reaction tubes were also labeled with the appropriate isotype controls (BD Biosciences). After labeling for 20 minutes in the dark, samples were fixed for 15 minutes by the addition of $1/10^{th}$ the volume of 4% paraformaldehyde. After fixation, samples were quench diluted with 0.5 ml of HEPES saline.

Within 1 hour, samples were collected and analyzed with a FACSCalibur cytometer and CELLQUEST software (Becton Dickinson), using logarithmic gain settings for light scatter and fluorescence. After initial optimization and compensation, the same settings were used throughout the study. Samples were gated to collect events in the characteristic forward

(FSC) and side (SSC) location for platelets; due to severe thrombocytopenia in some of these samples, a minimum of 2,000 events were collected in the platelet gate by collecting the entire contents of each tube. In samples with more platelets, 10,000 platelet events were collected. For analysis, platelets were first discriminated based on their 142.2-alexa 488 fluorescence (Figure 1A), then based on their characteristic FSC-SSC properties (Figure 1B), and finally all 142.2-positive events with platelet light scatter properties were assessed for FL2 positivity to identify the proportion of platelets binding CD62P-PE, denoting P-selectin expression (Figure 1C). Activated platelets were distinguished from resting platelets by use of an isotype control antibody.

The percentage of P-selectin positive platelets was determined for each thrombin dose. A dose-response curve was plotted and the concentration of thrombin inducing half-maximal activation (ED50 thrombin) was calculated with GraphPad PRISM 5 (San Diego, CA).

To determine if PRP preparation activated platelets, in each dog at baseline whole blood resting P-selectin expression was compared to resting PRP P-selectin expression. No difference between whole blood and PRP resting P-selectin was detected (data not shown).

All flow data analysis was performed with FlowJo (Tree Star, Inc., Ashland, OR).

Determination of Coated-Platelet Levels

As previously described, coated-platelet activation assays were performed in 100 µl total volume with 1 µl PRP and the following reagents (final concentrations): 1 mg/ml BSA, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 µg/ml biotin-fibrinogen (gift of George Dale, University of Oklahoma) 500 ng/ml convulxin (Pentapharm, Basel, Switzerland), 0.5 U/ml bovine thrombin (Sigma), 0.4 mM gly-pro-arg-pro-NH_2, 150 mM NaCl, and 10 mM HEPES, pH 7.5 [13]. Following incubation with thrombin and convulxin at 37°C for 5 minutes, platelets were stained with CD61-FITC to identify membrane GPIIbIIIa (clone Y2/51, Dako Cytomation, Carpenteria, CA) and phycoerythrin-streptavidin (PE-SA, Molecular Probes, Eugene, OR) was added to label surface bound biotinylated fibrinogen, a marker of coated-platelets. After incubation for an additional 5 minutes, the reaction was quenched with 1% (final concentration) formalin. The percentage of CD61 positive events (platelets) with bound

biotin-fibrinogen was quantitated by flow cytometry (CyAn, Beckman Coulter, Fullerton, CA) and reported as the percent of coated-platelets.

Assessment of the Effect of 2F9 on Platelet Activation and Reactivity

To determine the effect of 2F9 on platelet activation state or reactivity, flow cytometry assays were performed to assess baseline and thrombin-induced platelet P-selectin externalization and coated-platelet levels after blood was incubated with 2F9. Whole blood from a healthy research dog was collected via cephalic venipuncture into a syringe containing ACD (1 vol ACD: 9 vol blood). The whole blood was partitioned and incubated with 2F9 (2 µg/ml) or αYFA (2 µg/ml) or HEPES saline for 1 hour at room temperature. The concentration of 2F9 selected for these experiments was based on the dose of 2F9 that induced thrombocytopenia in the model dogs: about 150 µg/kg, which using an 80 ml/kg canine blood volume translates to 2 µg/ml [28]. Platelet-rich plasma (PRP) was generated from the whole blood as described above and measurement of platelet P-selectin, thrombin ED50, and coated-platelet levels was performed as above.

Platelet Counts

Platelet counts were determined from EDTA whole blood by the Clinical Pathology Laboratory at NCSU using an Advia 120 (Siemens Healthcare Diagnostics, Inc., Norwood, MA) or manual hemocytometer counts if platelets were <10,000 platelets/µl as described in Chapter 2.

Bleeding Score

At the time of each blood draw, bleeding score using a bleeding scale that quantifies bleeding at 8 anatomic sties was assessed as described in Chapter 2.

Statistical Analysis

Platelet activation state (baseline P-selectin positivity), ED50, and coated-platelet levels were compared over time to each dog's baseline values. To test for differences in platelet activation state and reactivity across all time points by treatment group (2F9 or control

antibody), repeated measures generalized linear modeling (analysis of variance or ANOVA) was performed, where time point and the variable of interest were entered into the model. For all variables, the ANOVA models were checked for Gaussian residuals and equality of variance. The P-value for each parameter was evaluated and tested for significance. To control for multiple testing, a Bonferroni correction (for each set of hypotheses) was used to determine the alpha level for statistical significance that insured a family-wise error rate of 0.05, meaning that a comparison was considered to be statistically significant if $P<0.002$. To compare platelet reactivity in treatment groups at an individual time point, a two-tailed t test was used and significance was set at $P<0.05$.

To identify candidate bleeding predictors, univariate association between bleeding scores and platelet activation state and reactivity was testing using a t-test for correlations and appropriate degrees of freedom. Bleeding scores were treated as ordinate variables as described by Mantel-Haenszel. Again, a Bonferroni correction was applied as above so $P<0.002$ was considered significant.

All calculations were performed using Stata version 10 (Statacorp LP) and GraphPad PRISM 5.0. Graphs were drawn with GraphPad PRISM 5.0.

Results

Platelet Counts

Severe thrombocytopenia (median 12,500 platelets/µl; range 11,000-14,000 platelets/µl) was induced in the 2F9-treated dogs within two hours after the final 2F9 administration (time zero) and remained below 40,000 platelets/µl for 24 hours after which time platelet counts gradually returned to baseline (Figure 2). Control platelet counts remained unchanged.

Resting P-Selectin Expression

With the exception of one time point in one dog, all non-stimulated (no agonist added) platelets were ≤6% P-selectin positive. When normalized to baseline values, there was no difference in resting P-selectin expression over time in 2F9 treated dogs compared to control

dogs (P=0.114; data not shown). Resting P-selectin expression and bleeding score were not inversely correlated (P=0.095).

Longitudinal Changes in Thrombin ED50 in ITP Model Dogs

ED50 was first assessed at 6 hours following baseline. Figure 3A shows a representative experiment of the thrombin dose-response curve a platelets from a 2F9-treated dog at baseline, 6 hours, and recovery. At 6 hours, a shift to the right in the dose response curve is noted, indicating that more thrombin is required for activation of the platelets (ED50, or dose of thrombin required to activate 50% of platelets, was 0.0382 U/ml at baseline and 0.0614 U/ml at 6 hours). At recovery, the response curve is shifted to the left of baseline, indicating that less thrombin is required for activation of the platelets at recovery (ED50 was 0.0266 U/ml).

Overall, in 2F9-treated dogs the thrombin concentration required for half-maximal P-selectin expression (ED50) was nearly twofold higher during the platelet nadir (t=6 hours) than at baseline (median 1.6 times baseline, range 1.3-2.3) (Figure 3B). This increase in ED50 means that platelets were less reactive to thrombin stimulation at the platelet nadir and a similar increase in ED50 was not observed in control dogs. Over time, ED50s gradually returned to and below baseline ED50. At recovery, ED50 for both treated and control dogs was decreased compared to baseline, meaning platelets were more reactive at recovery than at baseline. Over the course of the study, there was no statistically significant difference in ED50s between treated and control dogs, likely due to small sample size (P=0.847).

Longitudinal Changes in Coated-Platelet Levels in ITP Model Dogs

Similar the observed ED50 pattern, in 2F9-treated dogs, coated-platelet levels decreased to nearly half of baseline at platelet nadir (time 0 and 6 hours) (Figure 4). Decreased coated-platelet levels indicate decreased platelet reactivity and procoagulant potential. At 6 hours, coated-platelet levels in treated dogs were 52.4% baseline (range 19.6-61.5%) compared to 109.3% (108.1-114.7%) baseline in control dogs (P<0.0068). As platelet counts recovered in treated dogs, coated-platelet levels returned to and exceeded baseline. Recovery coated-

platelet levels in control dogs were not greater than baseline but the difference between treatment groups was not significant. Over the course of the study, there was no statistically significant difference in coated-platelet levels between treated and control dogs, likely due to small sample size (P=0.669).

Platelet Reactivity in Relationship to Bleeding

Overall, dogs demonstrated variable but clinically mild bleeding with bleeding scores ranging from 0 to 4 out of a possible 16. Bleeding scores were not correlated with platelet reactivity (P=0.211 for coated-platelets and P=0.335 for ED50).

Interestingly, two dogs developed large delayed cutaneous ecchymoses as their platelet counts were recovering. As shown in Figure 5, their platelet reactivity was equal to or greater than their baseline at the time that new bleeding began. In other words at the time of ecchymosis development, thrombin ED50 was that of baseline or lower and coated-platelet levels were those of baseline or higher. Similarly, circulating (*in vivo*) P-selectin expression at the time the bleeds began were the same or higher than baseline (not shown). Platelet hemostatic function should have been adequate to achieve primary hemostasis.

Ex Vivo Effect of 2F9 on Platelet Reactivity

To determine if exposure of canine blood to 2F9 in vitro altered capacity of the platelets to activate in response to thrombin or thrombin and convulxin, blood was incubated with about the same concentration as was given to model dogs (2 µg/ml) when the dose was converted to a concentration using an 80 ml/kg blood volume. Resting P-selectin expression was the same in all treatment groups (saline: 0.829%, 2F9: 1.08%, YFA: 0.908%). No alteration in ED50 thrombin was observed (Figure 6). Coated-platelet levels were the same in blood incubated with (30.0%) and without 2F9 (29.5%).

Discussion

ITP patients have variable bleeding symptoms despite similarly low platelet counts [29]. Although bleeding is more likely at platelet counts below 30,000 platelets/µl, some patients with higher platelet counts bleed significantly, while many with platelet counts below 5,000

platelets/µl do not bleed at all [1, 2]. Platelet number alone is an insufficient predictor of bleeding tendency [30, 31]. First-line therapy for ITP includes immunosuppression, which reduces patient quality of life and can result in fatal secondary infections and hematological malignancies [6, 32, 33]. Ideally, ITP treatment should be tailored to the patient's bleeding risk. Currently we are unable to accurately predict bleeding risk in ITP. Understanding platelet function in ITP may enable better prediction of bleeding risk and lead to improved clinical management of ITP patients [1].

Methodology has been a major limiting factor in understanding platelet function in ITP as many tests of platelet function are impacted by platelet count. Light aggregometry, for example, the gold standard for platelet function evaluation, cannot be used if platelet count is less than 100,000/µL [34]. We employed flow cytometry to evaluate platelet circulating activation state (P-selectin expression) and reactivity to thrombin alone (ED50 thrombin) or thrombin and convulxin combined (coated-platelet levels) as flow cytometry can evaluate the function of individual platelets and is reliable even in the presence of substantial thrombocytopenia [1, 34]. To our knowledge, this is the first report of coated-platelets in ITP. Of note, coated-platelets not only reflect platelet reactivity, but also the potential of platelets to contribute to secondary hemostasis and thrombin generation.

In a canine induced model of ITP, using two different measures of platelet reactivity (coated-platelet levels and ED50 thrombin for P-selectin expression), we observed a reduction in platelet reactivity during the platelet nadir (0-6 hours). The mechanism by which this reduction in platelet reactivity during the platelet nadir occurs is unknown. Conceivably, the more reactive population of platelets is preferentially cleared in this ITP model. Platelets that are more activatable may express more fibrinogen receptor on their surface making them more susceptible to clearance by the anti-GPIIb antibody. Platelet surface expression of GPIIbIIIa increases with thrombin stimulation so more activated or activatable platelets likely have more GPIIbIIIa on their surface [35]. Alternatively, of the remaining circulating platelets, those that are more reactive may be being actively used in regions of injured vasculature. Subsequently, those platelets that were available for sampling would reflect a more refractory population of platelets that was not as biologically useful in platelet plug

formation. Based on our *ex vivo* studies, it seems unlikely that 2F9 directly alters platelet reactivity (Figure 6) meaning that the presence of 2F9 did not directly cause this nadir reduction in platelet reactivity.

One possible consideration is that the reduction in platelet reactivity at platelet nadir was an assay artifact due to the thrombocytopenia. Reduced concentrations of platelets could lead to decreased ability of agonist-activated platelets to activate neighboring platelets by ADP release, resulting in the appearance of decreased responsiveness to agonist stimulation. However, other investigators using the same assays that we employed have not observed any impact of severe thrombocytopenia (2,000 platelets/μl) on thrombin ED50 or coated-platelet levels (George Dale, personal communication, December 21, 2012).

Whether this relative reduction in the reactive population of platelets reflects what occurs early in the course of ITP will need to be assessed by performing longitudinal studies in ITP patients beginning early in their diagnosis. However, such an early time point may not be feasible to capture clinically.

We did not find a relationship between platelet reactivity and bleeding outcome. Bleeding score did not correlate with platelet reactivity, which was not unexpected given the relatively mild bleeding observed in all dogs. Probably more telling is the fact that dogs displayed very mild bleeding signs despite severe thrombocytopenia coupled with platelet dysfunction (decreased reactivity) at the time of platelet nadir. This suggests that the markedly decreased platelet reactivity did not allow for significant bleeding. Also revealing is that two dogs developed large ecchymoses (one 8.5 by 13 cm) late in the study time course when platelet count was recovering and when platelet reactivity as assessed by both assays was the same or greater than of the dogs' baseline platelet reactivity (Figure 5). At least in these dogs, reduced platelet hemostatic function was not the cause of bleeding. Given the hyperfunctionality of their platelets, we did examine the dogs with delayed ecchymoses for evidence of thrombus formation via plasma D-dimer concentrations and histology of lesions and found no evidence to support that their bruises were due to thrombi rather than bleeds (data not shown). We have shown (Chapter 3) that endothelial alterations were present in

these dogs during their delayed bleeds, suggesting that a defect in platelet vascular stabilizing function rather than platelet hemostatic function may be at play.

Our inability to find a clear relationship between platelet hemostatic function and bleeding are consistent with other recent studies. Psaila and colleagues found that ITP patients with bleeding had lower platelet reactivity than those with no bleeding [1]. This implies that platelet reactivity protects against bleeding. However, the same study also found the opposite to be true in thrombocytopenic patients with acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS). In these patients, platelet reactivity was higher in those that bled than in those that did not [1]. As in our study, Panzer and colleagues were also unable to find the expected inverse correlation between circulating platelet activation state (P-selectin expression) and bleeding symptoms [19]. Whether circulating P-selectin expression (*in vivo* activation state) or *ex vivo* reactivity to agonists is more demonstrative of *in vivo* hemostatic function is unknown. Thus, the physiologic role of platelet activation state and reactivity in determining bleeding tendency remains uncertain.

Some of the conflicting reports in the literature regarding platelet function in ITP probably reflect the diversity in ITP autoantibodies. Some autoantibodies in ITP can bind to receptors essential for adhesion or aggregation, impairing platelet function while others bind to sites that induce platelet activation [36-40]. Indeed, one of the main challenges in assessing platelet function in ITP is that ITP is a diverse syndrome and thus diversity in platelet function likely exists between patients that will prevent investigators from making any overarching conclusions about platelet function in ITP [41]. Our study is unique in that we were able to examine longitudinal changes in platelet reactivity in ITP in the context of an antibody that is neither activating nor inhibitory (Figure 6). In this more uniform setting, platelet reactivity does not appear to inform bleeding risk.

Interestingly, we observed a general, though not statistically significant, trend towards increasing platelet reactivity during platelet recovery in the ITP model dogs. In 2F9-treated dogs, but not the control dogs, coated-platelet levels rose as platelets rebounded and were slightly higher than baseline at recovery (Figure 4). We also observed a trend of declining ED50 thrombin during the recovery period in treated dogs, but no difference

between treatment groups at the recovery time point (Figure 3). These findings may be important given the recent reports of increased risk of venous and arterial thromboembolism in ITP patients [4, 7, 8, 42].

The most likely explanation for the increased platelet reactivity as platelets were recovering was that these platelets represent young platelets. As early as 1969, young platelets have been reported to be hyperfunctional [26, 27, 43, 44]. Inversely, a previous study in dogs showed that older platelets have an impaired reactivity to thrombin as assessed by P-selectin expression (increased thrombin ED50) [45]. Young platelets are also more capable of coated-platelet generation than older platelets [46]. Since ITP is a disease of increased platelet turnover, ITP platelets are traditionally viewed as larger, younger, and more functional than normal platelets and this has been thought to account for why ITP patients seem to bleed less than patients with thrombocytopenia due to bone marrow failure [1, 27, 47]. Our observed increased platelet reactivity, especially coated-platelet potential, during platelet recovery is consistent with these traditional views and could explain the thrombotic tendency observed in some ITP patients. Given the prothrombotic potential of coated-platelets and previous reports of increased coated-platelets in ischemic stroke patients and transient ischemic attacks, future longitudinal studies of coated-platelets should be performed in patients with spontaneous ITP [14, 15]. Anti-coagulation during platelet count recovery may be necessary in some ITP patients.

The major limitation of this pilot study is the small sample size. However, this study was designed to be a hypothesis-generating study. Our results, combined with the conflicting data in the literature, suggest that platelet function may not be the prime determinant of bleeding outcome in ITP. Our study also proposes an interesting potential role for increased platelet reactivity and especially increased coated-platelet synthesis in the recovery phase of ITP that may provide an explanation for pro-thrombotic complications in ITP patients. Platelet function may even be more important for understanding thrombosis risk than bleeding risk in ITP. Other platelet functions such as maintenance of vascular integrity may determine bleeding risk. Further studies of platelet function are necessary to better understand platelet function in ITP and thrombocytopenia in general. If a relationship

between platelet function and bleeding or thrombosis risk could be established, our management of ITP patients would be greatly improved.

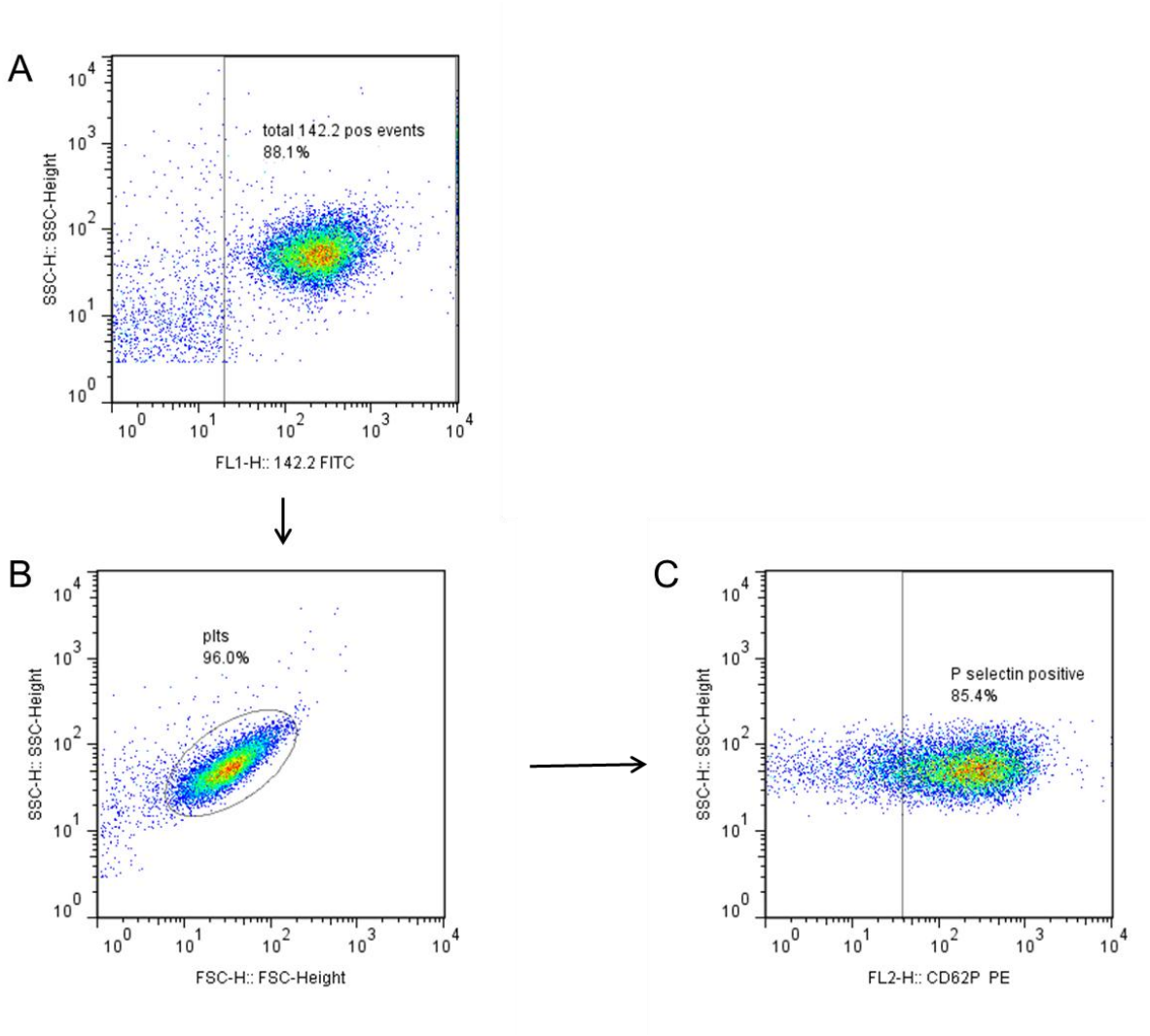


Figure 1. Flow cytometric analyses of platelet surface P-selectin expression. Dot plots show platelet rich plasma stimulated with thrombin (0.1 U/ml) and double labeled to detect platelet membrane GPIb/IX (142.2 alexa 488) and P-selectin (CD62P). A) Platelets are first gated based on positive labeling with 142.2-Alexa 488. B) In the 142.2 positive population, a gate is constructed around the characteristic forward and side scatter of platelets (plts). C) The platelet population (plts) is then further separated based on CD62P-phycoerythrin (PE) labeling. In this example, 85.4% of platelets are expressing P-selectin on their surface (CD62P-PE positive). Thresholds for 142.2 positive and CD62P positive were determined by isotype controls.

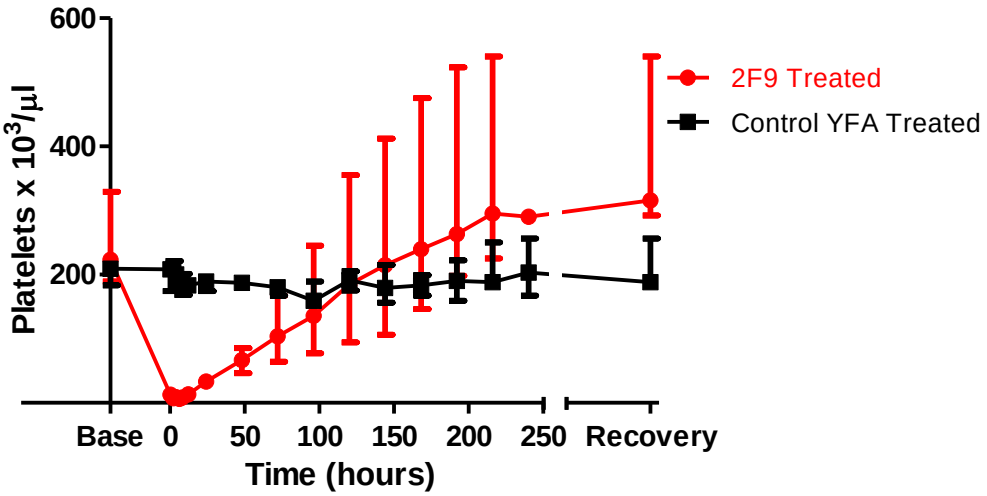
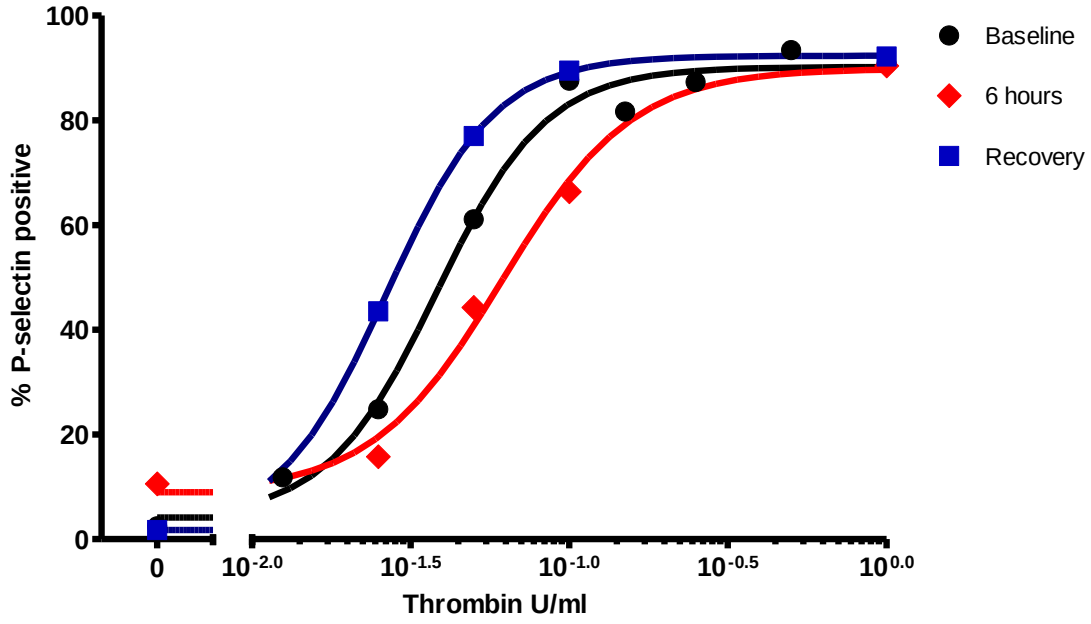


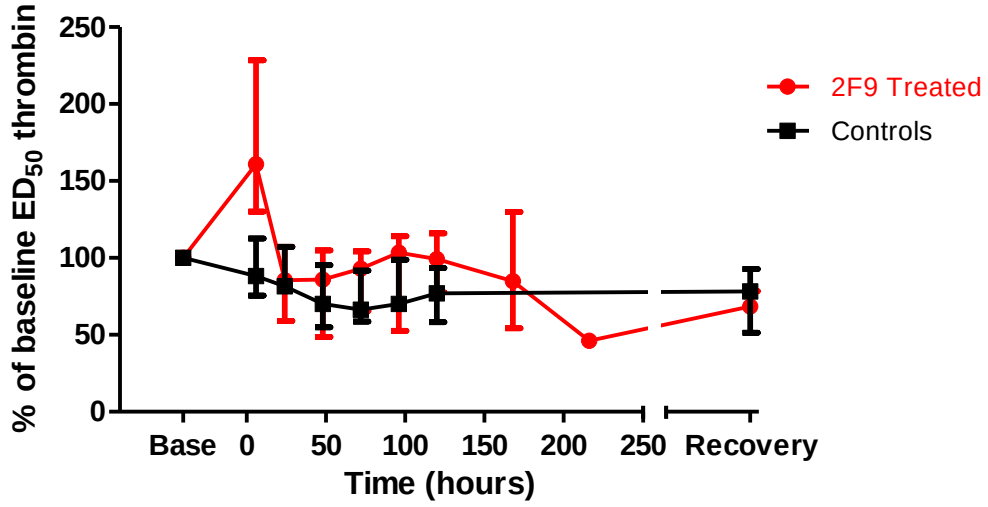
Figure 2. Platelet counts in 2F9-treated and control dogs. Time zero occurred when platelet count first fell within the target platelet nadir (5,000-30,000 platelets/µl), a maximum of 2 hours after the last 2F9 dose, or was defined as 1 hour after control antibody administration. Recovery occurred when platelet count returned to baseline or when new bleeding stopped, whichever came later (168-384 hours). Injection of the isotype control antibody (αYFA) had no significant effect on the platelet count. Results are shown as median and range for 4 2F9-treated dogs and 3 control dogs.

Figure 3. Thrombin ED50 alterations in ITP model dogs. Thrombin ED50 is the thrombin concentration required to induce half-maximal P-selectin expression. A. Dose-response curve of platelets exposed to thrombin in one 2F9-treated dog at baseline (●———●), during platelet nadir (6 hours, ♦——♦), and at recovery (■———■). The percentage of activated platelets is shown against the log concentration of thrombin (units/ml). ED50 at baseline was 0.0382 U/ml. The dose-response curve is shifted to the right at platelet nadir (less-reactive; ED50 0.0614 U/ml) and to the left on recovery (more reactive; ED 0.0266 U/ml). B. ED50 thrombin expressed as a percentage of baseline ED50 in 2F9-treated (n=4) compared to control dogs (n=3). Time zero is when platelet count first fell into target nadir range (5-30,000 platelets/µl) or 1 hour after control antibody infusion. Following baseline assessment, ED50 was first assessed after 6 hours of thrombocytopenia. Results are shown as median and range.

A.


100
80
60
40
20
0
% P-selectin positive
0
10-2.0
10-1.5
10-1.0
10-0.5
100.0
Thrombin U/ml
Baseline
6 hours
Recovery

B.


250
200
150
100
50
0
% of baseline ED50 thrombin
Base
0
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100
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200
250
Recovery
Time (hours)
2F9 Treated
Controls

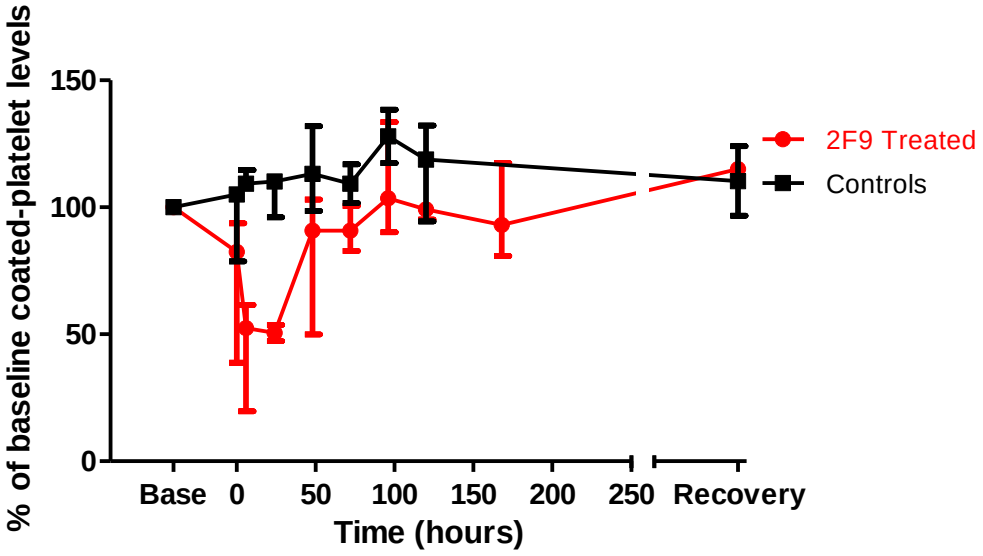

Figure 4. Coated-platelet levels expressed as a percentage of baseline coated-platelets in 2F9-treated (n=4) compared to control dogs (n=3). A significant decreased in coated-platelet levels in ITP model dogs compared to control dogs was observed after 6 hours of thrombocytopenia ($P<0.0068$). Results are shown as median and range.

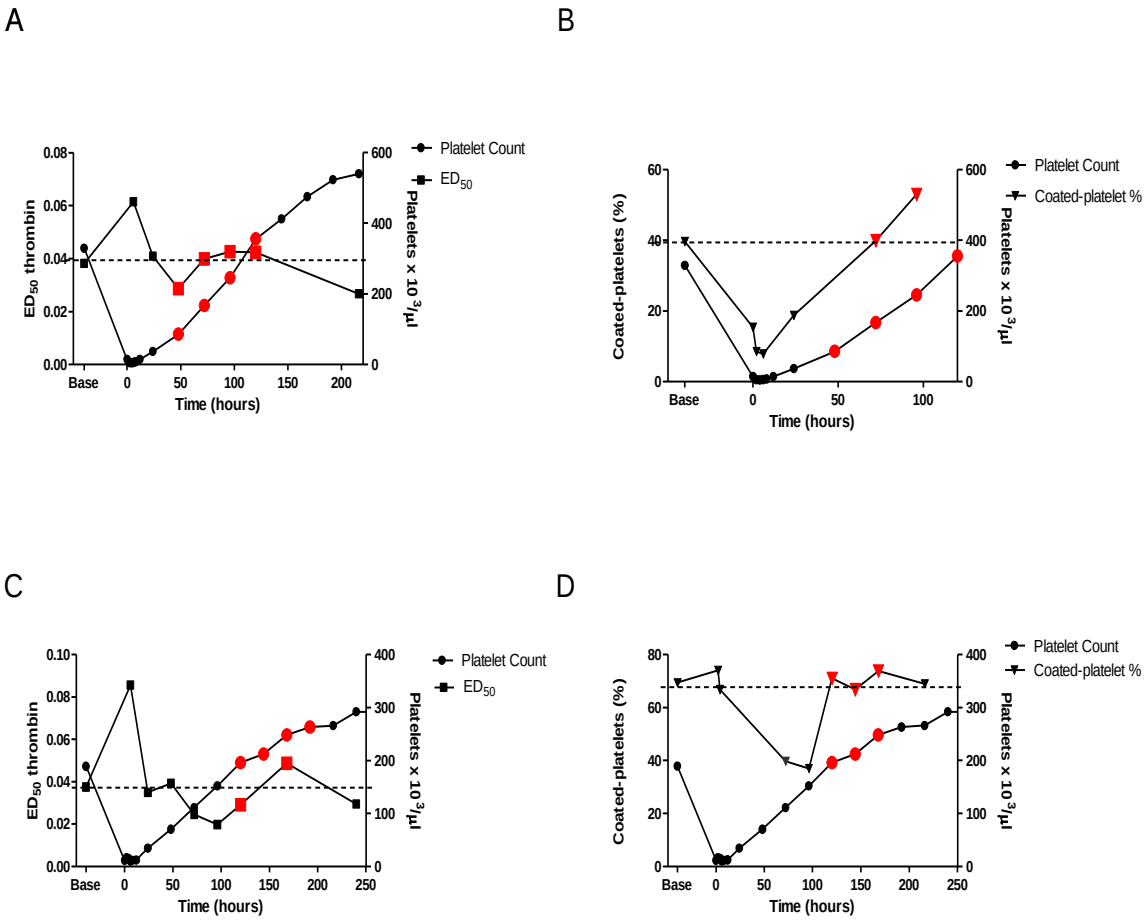

Figure 5. Platelet reactivity in two dogs that developed large delayed ecchymoses. Red symbols show time at which new bleeding was occurring. A. ED50 thrombin for P-selectin expression and platelet count in one dog. Dashed line indicates baseline ED50 in that dog. B. Coated-platelet levels and platelet count in the same dog. Dashed line indicates baseline coated-platelets in that dog. C. ED50 thrombin and platelet count in the second dog. D. Coated-platelet levels and platelet count in the second dog.

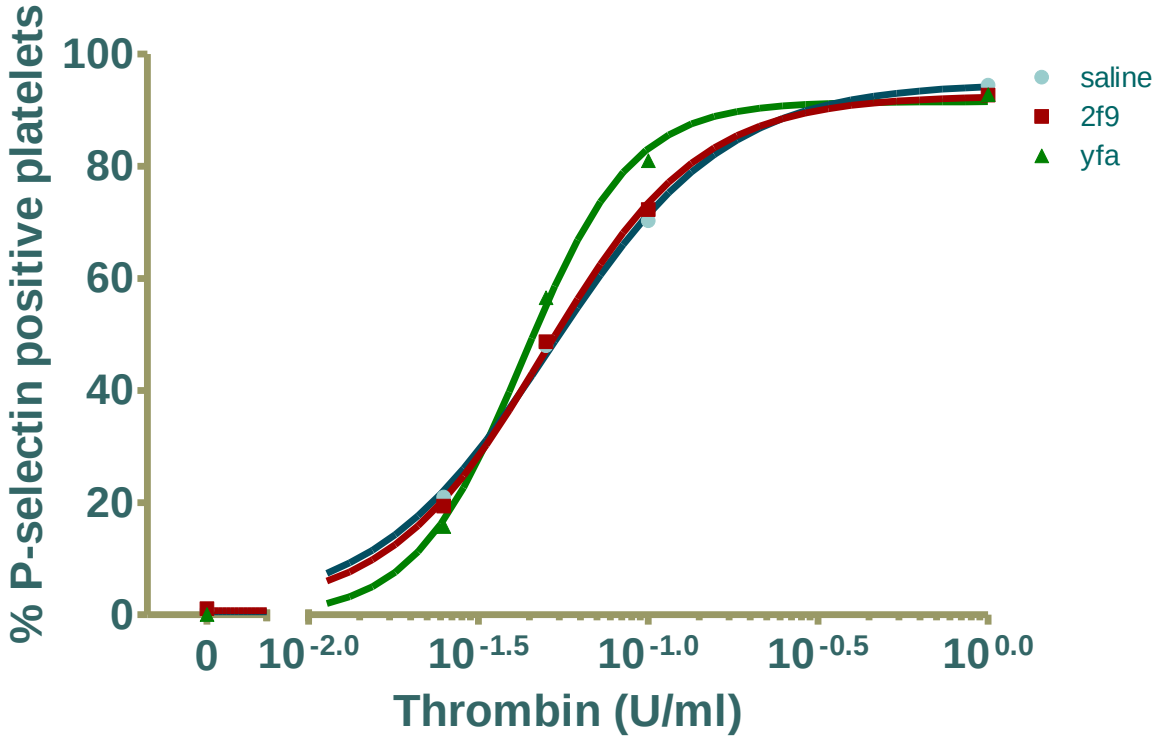

Figure 6. Exposure of canine platelets to 2F9 *ex vivo* does not alter their responsiveness to thrombin (expressed as percent of P-selectin positive platelets on the ordinate). Anticoagulated whole blood was incubated with 2 μg/ml 2F9 or αYFA isotype control or saline for 2 hours, followed by exposure to 0 to 1 U/ml of thrombin (abscissa – log concentration of thrombin U/ml). No differences were observed between the blood incubated with 2F9 (thrombin ED50 0.049 U/ml), saline buffer (0.052 U/ml), or isotype control antibody (0.043 U/ml).

References

1. Psaila, B., et al., *Differences in platelet function in patients with acute myeloid leukemia and myelodysplasia compared to equally thrombocytopenic patients with immune thrombocytopenia.* J Thromb Haemost, 2011. **9**(11): p. 2302-10.

2. Provan, D., et al., *International consensus report on the investigation and management of primary immune thrombocytopenia.* Blood, 2010. **115**(2): p. 168-86.

3. Rozanski, E.A., et al., *Comparison of platelet count recovery with use of vincristine and prednisone or prednisone alone for treatment for severe immune-mediated thrombocytopenia in dogs.* J Am Vet Med Assoc, 2002. **220**(4): p. 477-81.

4. O'Marra, S.K., S.P. Shaw, and A.M. Delaforcade, *Investigating hypercoagulability during treatment for immune-mediated thrombocytopenia: a pilot study.* J Vet Emerg Crit Care (San Antonio), 2012. **22**(1): p. 126-30.

5. Gernsheimer, T., *Epidemiology and pathophysiology of immune thrombocytopenic purpura.* Eur J Haematol Suppl, 2008(69): p. 3-8.

6. Portielje, J.E., et al., *Morbidity and mortality in adults with idiopathic thrombocytopenic purpura.* Blood, 2001. **97**(9): p. 2549-54.

7. Norgaard, M., et al., *Risk of arterial thrombosis in patients with primary chronic immune thrombocytopenia: a Danish population-based cohort study.* Br J Haematol, 2012. **159**(1): p. 109-11.

8. Severinsen, M.T., et al., *Risk of venous thromboembolism in patients with primary chronic immune thrombocytopenia: a Danish population-based cohort study.* Br J Haematol, 2011. **152**(3): p. 360-2.

9. Jobe, S.M., et al., *Critical role for the mitochondrial permeability transition pore and cyclophilin D in platelet activation and thrombosis.* Blood, 2008. **111**(3): p. 1257-65.

10. Yeo, E.L., et al., *Characterization of canine platelet P-selectin (CD 62) and its utility in flow cytometry platelet studies.* Comp Biochem Physiol B, 1993. **105**(3-4): p. 625-36.

11. Shattil, S.J., M. Cunningham, and J.A. Hoxie, *Detection of activated platelets in whole blood using activation-dependent monoclonal antibodies and flow cytometry.* Blood, 1987. **70**(1): p. 307-15.

12. Dale, G.L., *Coated-platelets: an emerging component of the procoagulant response.* J Thromb Haemost, 2005. **3**(10): p. 2185-92.

13. Brooks, M.B., et al., *Scott syndrome dogs have impaired coated-platelet formation and calcein-release but normal mitochondrial depolarization.* J Thromb Haemost, 2007. **5**(9): p. 1972-4.

14. Prodan, C.I., A.S. Vincent, and G.L. Dale, *Coated-platelet levels are elevated in patients with transient ischemic attack.* Transl Res, 2011. **158**(1): p. 71-5.

15. Prodan, C.I., et al., *Higher coated-platelet levels are associated with stroke recurrence following nonlacunar brain infarction.* J Cereb Blood Flow Metab, 2012.

16. Panzer, S., et al., *Flow cytometric evaluation of platelet activation in chronic autoimmune thrombocytopenia.* Pediatr Blood Cancer, 2006. **47**(5 Suppl): p. 694-6.

17. Yanabu, M., et al., *Influences of antiplatelet autoantibodies on platelet function in immune thrombocytopenic purpura.* Eur J Haematol, 1991. **46**(2): p. 101-6.

18. Olsson, A., et al., *Serum from patients with chronic idiopathic thrombocytopenic purpura frequently affect the platelet function.* Thromb Res, 2002. **107**(3-4): p. 135-9.

19. Panzer, S., et al., *Platelet function to estimate the bleeding risk in autoimmune thrombocytopenia.* Eur J Clin Invest, 2007. **37**(10): p. 814-9.

20. Rinder, H.M., et al., *Differences in platelet alpha-granule release between normals and immune thrombocytopenic patients and between young and old platelets.* Thromb Haemost, 1998. **80**(3): p. 457-62.

21. Panzer, S., et al., *Agonist-inducible platelet activation in chronic idiopathic autoimmune thrombocytopenia.* Eur J Haematol, 2007. **79**(3): p. 198-204.

22. Haznedaroglu, I.C., et al., *Thrombopoietin, interleukin-6, and P-selectin at diagnosis and during post-steroid recovery period of patients with autoimmune thrombocytopenic purpura.* Ann Hematol, 1998. **77**(4): p. 165-70.

23. Cahill, M.R., et al., *Protein A immunoadsorption in chronic refractory ITP reverses increased platelet activation but fails to achieve sustained clinical benefit.* Br J Haematol, 1998. **100**(2): p. 358-64.

24. Semple, J.W., et al., *Differences in serum cytokine levels in acute and chronic autoimmune thrombocytopenic purpura: relationship to platelet phenotype and antiplatelet T-cell reactivity.* Blood, 1996. **87**(10): p. 4245-54.

25. Chong, B.H., et al., *Plasma P-selectin is increased in thrombotic consumptive platelet disorders.* Blood, 1994. **83**(6): p. 1535-41.

26. Karpatkin, S., *Heterogeneity of human platelets. II. Functional evidence suggestive of young and old platelets.* J Clin Invest, 1969. **48**(6): p. 1083-7.

27. Karpatkin, S., *Heterogeneity of human platelets. VI. Correlation of platelet function with platelet volume.* Blood, 1978. **51**(2): p. 307-16.

28. Diehl, K.H., et al., *A good practice guide to the administration of substances and removal of blood, including routes and volumes.* J Appl Toxicol, 2001. **21**(1): p. 15-23.

29. Nieswandt, B., *How do platelets prevent bleeding?* Blood, 2008. **111**(10): p. 4835.

30. Kenet, G., et al., *Cone and platelet analyser (CPA): a new test for the prediction of bleeding among thrombocytopenic patients.* Br J Haematol, 1998. **101**(2): p. 255-9.

31. Eldor, A., et al., *Prediction of haemorrhagic diathesis in thrombocytopenia by mean platelet volume.* Br Med J (Clin Res Ed), 1982. **285**(6339): p. 397-400.

32. Norgaard, M., et al., *Long-term clinical outcomes of patients with primary chronic immune thrombocytopenia: a Danish population-based cohort study.* Blood, 2011. **117**(13): p. 3514-20.

33. Guidry, J.A., et al., *Corticosteroid side-effects and risk for bleeding in immune thrombocytopenic purpura: patient and hematologist perspectives.* Eur J Haematol, 2009. **83**(3): p. 175-82.

34. Misgav, M., et al., *Differential roles of fibrinogen and von Willebrand factor on clot formation and platelet adhesion in reconstituted and immune thrombocytopenia.* Anesth Analg, 2011. **112**(5): p. 1034-40.

35. Savage, B., et al., *Thrombin-induced increase in surface expression of epitopes on platelet membrane glycoprotein IIb/IIIa complex and GMP-140 is a function of platelet age.* Blood, 1989. **74**(3): p. 1007-14.

36. Ahn, Y.S., et al., *Vascular dementia in patients with immune thrombocytopenic purpura.* Thromb Res, 2002. **107**(6): p. 337-44.

37. Kristensen, A.T., D.J. Weiss, and J.S. Klausner, *Platelet dysfunction associated with immune-mediated thrombocytopenia in dogs.* J Vet Intern Med, 1994. **8**(5): p. 323-7.

38. Niessner, H., et al., *Acquired thrombasthenia due to GPIIb/IIIa-specific platelet autoantibodies.* Blood, 1986. **68**(2): p. 571-6.

39. Yanabu, M., et al., *Platelet activation induced by an antiplatelet autoantibody against CD9 antigen and its inhibition by another autoantibody in immune thrombocytopenic purpura.* Br J Haematol, 1993. **84**(4): p. 694-701.

40. Gardiner, E.E., et al., *Compromised ITAM-based platelet receptor function in a patient with immune thrombocytopenic purpura.* J Thromb Haemost, 2008. **6**(7): p. 1175-82.

41. Cines, D.B., et al., *The ITP syndrome: pathogenic and clinical diversity.* Blood, 2009. **113**(26): p. 6511-21.

42. Sarpatwari, A., et al., *Thromboembolic events among adult patients with primary immune thrombocytopenia in the United Kingdom General Practice Research Database.* Haematologica, 2010. **95**(7): p. 1167-75.

43. Thompson, C.B., et al., *Platelet size and age determine platelet function independently.* Blood, 1984. **63**(6): p. 1372-5.

44. Blajchman, M.A., et al., *Hemostatic function, survival, and membrane glycoprotein changes in young versus old rabbit platelets.* J Clin Invest, 1981. **68**(5): p. 1289-94.

45. Peng, J., et al., *Alteration of platelet function in dogs mediated by interleukin-6.* Blood, 1994. **83**(2): p. 398-403.

46. Alberio, L., et al., *Surface expression and functional characterization of alpha-granule factor V in human platelets: effects of ionophore A23187, thrombin, collagen, and convulxin.* Blood, 2000. **95**(5): p. 1694-702.

47. Rand, M.L. and J.A. Dean, *Platelet function in autoimmune (idiopathic) thrombocytopenic purpura.* Acta Paediatr Suppl, 1998. **424**: p. 57-60.

SUMMARY AND FUTURE DIRECTIONS

As demonstrated by our literature review (see Chapter 1), there are a plethora of rodent models of immune thrombocytopenia (ITP) that have been integral to our current understanding of ITP [1]. However, the translatability of rodent models to human disease is limited by the facts that rodents do not develop spontaneous ITP and that significant differences exist between murine and human platelet counts, volume, and signaling [1, 2]. Although dogs develop spontaneous ITP that is similar to human ITP, no induced canine models of ITP were available until this study.

An induced canine model has several advantages over naturally-occurring disease including 1) the ability to study the disease in the absence of co-existing diseases or treatment effects and 2) the ability to manipulate model dogs more than could be done with client-owned dogs with spontaneous disease. Accordingly, the primary goal of this dissertation was to develop an induced canine model of ITP. By infusing an antibody to a component of the platelet fibrinogen receptor, a common autoantibody target in ITP, we created a repeatable dose-dependent mechanism of inducing severe thrombocytopenia in dogs [3].

As demonstrated in Chapter 2, this novel induced canine model of ITP is very analogous to the spontaneous disease in humans and canines. The model ITP dogs had a similar clinical and laboratory presentation to both canines and humans with spontaneous disease. As in the spontaneous disease, severe thrombocytopenia was accompanied by variable mucocutaneous bleeding in the absence of other clinical signs [4]. The cytokine profile was non-inflammatory and paralleled that of the dogs we evaluated with spontaneous disease. The type-1 immune response observed in human ITP was not documented in dogs with induced or spontaneous disease, but larger numbers of dogs must be evaluated to confirm its absence [5, 6]. In contrast, current murine models utilizing antibodies to the fibrinogen receptor demonstrate acute systemic reactions and hypothermia that are inconsistent with the spontaneous disease [7].

Unexpectedly, our model revealed canine platelets to be a major source of serum interleukin-8 (IL-8), as this cytokine faithfully tracked with platelet count in model dogs. This led us to investigate serum IL-8 levels in dogs with ITP and other causes of thrombocytopenia and we determined that serum IL-8 levels were significantly reduced in these patients compared to healthy controls. Similarly, our investigations in human ITP patients showed a direct correlation between serum IL-8 and platelet count. Although identifying platelets as a major IL-8 source does not necessarily relate to the pathogenesis of ITP, it demonstrates a novel putative mechanism of platelet-neutrophil cross-talk that warrants further investigation. Platelet recruitment of neutrophils to tissues has both beneficial and deleterious effects in a variety of diseases and knowledge of how to manipulate this interaction could have invaluable therapeutic implications [8]. Such a finding would not have been possible in rodents as they lack IL-8 [9].

Ultimately, we were interested in understanding why canine and human patients with ITP demonstrate variable bleeding symptoms despite similar platelet counts. We hypothesized that differences in platelet vascular stabilizing function or platelet hemostatic function in patients' remaining circulating platelets could explain variations in bleeding phenotype. We examined the relative role of each platelet function in bleeding outcome in the context of our canine ITP model.

Investigations in Chapter 3 are suggestive of a relationship between thrombocytopenic bleeding and altered endothelial ultrastructure. We found a significant decrease in endothelial pinocytotic vesicles in thrombocytopenic dogs, likely reflecting endothelial membrane damage [10]. Furthermore, the dogs that developed the most significant cutaneous bruising had widened endothelial junctions and gaps between endothelial cells. These findings implicate that loss of the platelet vascular stabilizing function in thrombocytopenia leads to endothelial disruption, which, if severe enough, results in bleeding.

It remains unknown which platelet-derived mediators effect platelets' endothelial maintenance function. Although our studies confirmed the importance of platelets as a source of plasma sphingosine 1-phosphate (S1P), they did not provide evidence for the importance

of circulating plasma S1P in maintaining normal endothelial ultrastructure or its relationship to thrombocytopenic bleeding. Plasma S1P needs to be measured in greater numbers of clinical cases and related to bleeding scores in these patients. Similarly, other candidate mediators of the platelet vascular stabilizing function such as angiopoietin-1 and endostatin should be measured in model dogs and ITP patients and evaluated for correlations with bleeding [8, 11-13]. Local concentrations of S1P also need to be measured and compared to alterations in endothelial ultrastructure since local S1P levels may be more important in vascular maintenance than overall circulating S1P levels. As platelet contributions to vascular stabilization may occur more at the local level, identification of the key mediator(s) involved in this process may be challenging but is essential to truly understand thrombocytopenic bleeding. One approach to evaluate candidate mediators would be to infuse them locally or systemically to evaluate their impact on endothelial ultrastructure and bleeding in ITP model dogs.

In the future, we plan to evaluate the combined effect of inflammation and thrombocytopenia on endothelial ultrastructure. Thrombocytopenic bleeding may be facilitated by disruption of endothelial integrity by inflammation combined with reduced platelet vascular stabilizing function. It has been proposed that inflammation is necessary for thrombocytopenic bleeding to occur in mice [14]. Lack of inflammation may also explain why our model dogs developed only mild bleeding. We can capitalize on our knowledge that our model is non-inflammatory by assessing how bleeding phenotype differs when inflammation is induced locally or systemically in model dogs.

Our studies of platelet hemostatic function in Chapter 4 failed to demonstrate a relationship between platelet reactivity (ED50 thrombin and coated-platelet levels) and bleeding phenotype. Despite severe thrombocytopenia coupled with reduced platelet reactivity, dogs developed minimal bleeding. When dogs did develop large cutaneous ecchymoses, their platelet reactivity was the same or greater than that of baseline, suggesting their platelet hemostatic function was sufficient to achieve primary hemostasis. Further investigations of platelet reactivity are necessary in larger numbers of model dogs and in dogs with more extreme variation in bleeding.

Interestingly, we noted increased platelet reactivity, especially increased coated-platelet levels, in dogs during platelet count recovery. This may reveal a mechanism for the thromboembolic complications that have been recently reported in some human and canine ITP patients [15-17]. Platelet hemostatic function may be more informative for predicting thromboembolic risk than bleeding risk. To our knowledge, this is the first study of coated-platelets in ITP. Similar longitudinal studies of coated-platelets are warranted in human and canine patients with spontaneous disease to determine if increased coated-platelet formation in ITP explains the observed thromboembolic complications.

In summary, we have developed the only large animal model of ITP. It has demonstrated great potential for understanding mechanisms of variable bleeding in ITP. The model also provides a tool for testing novel ITP therapeutics in a species that is much closer in size and physiology to humans than rodents [18]. Our studies set the stage for investigation of mechanisms of bleeding in ITP and suggest that platelet vascular stabilizing function may be more important than platelet hemostatic function in determining bleeding outcome. Future identification of specific bleeding predictors will enable tailored treatment of only those patients at risk for bleeding and ultimately the development of novel therapeutics for thrombocytopenic hemorrhage. Further understanding of mechanisms of bleeding in ITP will lead to better treatment of ITP in both human and veterinary patients.

References

1. Semple, J.W., *Animal models of immune thrombocytopenia.* Annals of Hematology, 2010. **89**(Suppl 1): p. S34-S44.

2. Ware, J., *Dysfunctional platelet membrane receptors: from humans to mice.* Thromb Haemost, 2004. **92**(3): p. 478-85.

3. Beardsley, D.S. and M. Ertem, *Platelet autoantibodies in immune thrombocytopenic purpura.* Transfus Sci, 1998. **19**(3): p. 237-44.

4. Newton, J.L., et al., *Fatigue in adult patients with primary immune thrombocytopenia.* Eur J Haematol, 2011. **86**(5): p. 420-9.

5. Panitsas, F.P., et al., *Adult chronic idiopathic thrombocytopenic purpura (ITP) is the manifestation of a type-1 polarized immune response.* Blood, 2004. **103**(7): p. 2645-7.

6. Semple, J.W., et al., *Differences in serum cytokine levels in acute and chronic autoimmune thrombocytopenic purpura: relationship to platelet phenotype and antiplatelet T-cell reactivity.* Blood, 1996. **87**(10): p. 4245-54.

7. Nieswandt, B., et al., *Identification of critical antigen-specific mechanisms in the development of immune thrombocytopenic purpura in mice.* Blood, 2000. **96**(7): p. 2520-7.

8. Ho-Tin-Noe, B., M. Demers, and D.D. Wagner, *How platelets safeguard vascular integrity.* J Thromb Haemost, 2011. **9 Suppl 1**: p. 56-65.

9. Hol, J., L. Wilhelmsen, and G. Haraldsen, *The murine IL-8 homologues KC, MIP-2, and LIX are found in endothelial cytoplasmic granules but not in Weibel-Palade bodies.* J Leukoc Biol, 2010. **87**(3): p. 501-8.

10. Gutierrez, J.M., et al., *Blood flow is required for rapid endothelial cell damage induced by a snake venom hemorrhagic metalloproteinase.* Microvasc Res, 2006. **71**(1): p. 55-63.

11. Thurston, G., et al., *Angiopoietin-1 protects the adult vasculature against plasma leakage.* Nat Med, 2000. **6**(4): p. 460-3.

12. Brankin, B., et al., *Endostatin modulates VEGF-mediated barrier dysfunction in the retinal microvascular endothelium.* Exp Eye Res, 2005. **81**(1): p. 22-31.

13. Italiano, J.E., Jr., et al., *Angiogenesis is regulated by a novel mechanism: pro- and antiangiogenic proteins are organized into separate platelet alpha granules and differentially released.* Blood, 2008. **111**(3): p. 1227-33.

14. Goerge, T., et al., *Inflammation induces hemorrhage in thrombocytopenia.* Blood, 2008. **111**(10): p. 4958-64.

15. Severinsen, M.T., et al., *Risk of venous thromboembolism in patients with primary chronic immune thrombocytopenia: a Danish population-based cohort study.* Br J Haematol, 2011. **152**(3): p. 360-2.

16. O'Marra, S.K., S.P. Shaw, and A.M. Delaforcade, *Investigating hypercoagulability during treatment for immune-mediated thrombocytopenia: a pilot study.* J Vet Emerg Crit Care (San Antonio), 2012. **22**(1): p. 126-30.

17. Norgaard, M., et al., *Risk of arterial thrombosis in patients with primary chronic immune thrombocytopenia: a Danish population-based cohort study.* Br J Haematol, 2012. **159**(1): p. 109-11.

18. Schmidt-Nielsen, K. and P. Pennycuik, *Capillary density in mammals in relation to body size and oxygen consumption.* Am J Physiol, 1961. **200**: p. 746-50.