

## ABSTRACT

PRITCHARD, JESSICA CHRISTINE. B Cell Activating Factor Characterization and Use as a Biomarker in Dogs with Primary Immune-Mediated Thrombocytopenia. (Under the direction of Dr. Adam Birkenheuer).

A canine biomarker to identify and differentiate active primary immune mediated thrombocytopenia (ITP) from ITP that is in remission would provide a valuable diagnostic tool to modulate therapy and reduce patient exposure to potentially harmful immunosuppressive medications. Treatment for ITP in dogs attempts to balance high rates of disease mortality and relapse with the risks of extended systemic immunosuppression required to manage the disease. Currently, return of the platelet count to within the reference interval is used to indicate treatment success, however this finding does not guarantee cessation of the aberrant immune response. It is impossible for clinicians to know if the ITP is controlled until therapy is withdrawn. This lack of an effective monitoring tool results in either disease relapse after therapy withdrawal or extended and potentially unnecessary immunosuppression leading to life-threatening complications such as infections. An ITP biomarker that is elevated in active disease and that decreases as the aberrant immune response is controlled would aid in ITP diagnosis and treatment. B cell activating factor (BAFF) is one such possible biomarker.

BAFF is a cytokine of the tumor necrosis factor superfamily with important roles in B cell maturation, survival, and class-switching. In humans, excessive BAFF rescues self-reactive B cells and has been associated with the development of autoimmune disease, including ITP. Serum and plasma BAFF concentrations in humans with ITP are significantly higher than healthy humans and humans with ITP in remission. In dogs, only recombinant BAFF produced in *E. coli* has been previously described, and native BAFF protein

production has yet to be documented from canine tissue. Nothing is known of BAFF in dogs with ITP.

Herein, we describe BAFF transcription and production in the canine macrophage cell line O3O-D by the use of RT-qPCR and Western blotting, respectively. Additionally, it is shown that BAFF transcription is significantly increased in dogs with ITP compared to dogs with other causes of thrombocytopenia and healthy controls. The BAFF regulatory splice variant,  $\Delta$ BAFF, is not different between dogs with ITP and healthy dogs. Finally, a portion of the BAFF promoter region failed to identify any polymorphisms that may explain higher BAFF transcription in dogs with ITP compared to healthy controls.

© Copyright 2017 by Jessica Pritchard

All Rights Reserved

B Cell Activating Factor Characterization and Use as a Biomarker in Dogs with Primary  
Immune-Mediated Thrombocytopenia

by  
Jessica Christine Pritchard

A thesis submitted to the Graduate Faculty of  
North Carolina State University  
in partial fulfillment of the  
requirements for the degree of  
Master of Science

Comparative Biomedical Sciences

Raleigh, North Carolina

2017

APPROVED BY:

---

Dr. Adam Birkenheuer  
Committee Chair

---

Dr. Michael Wood

---

Dr. Katharine Lunn

**DEDICATION**

To my family.

## **BIOGRAPHY**

Jessica Pritchard attended Wake Forest University and was a Biology major and Journalism minor. She later attended veterinary school at the University of Pennsylvania. Upon graduation, she completed a small animal rotating internship and then Small Animal Internal Medicine residency at North Carolina State University. She is currently a clinical instructor in Small Animal Internal Medicine at the University of Wisconsin-Madison.

## ACKNOWLEDGMENTS

Thank you to all who made this work possible; your dedication to teaching and scientific inquiry are inspiring. A special debt of gratitude is owed to my advisor Adam Birkenheuer and my committee members Katharine Lunn and Michael Wood. For the countless hours of assistance and encouragement in the lab, thank you to Henry Marr. Thank you to Michael Levy for the canine macrophage cell line O3O-D used in this thesis. Additionally, the aid of the faculty clinicians, house officers, and technicians in the NCSU-VTH is greatly appreciated. Finally, thank you to all the families who generously agreed to allow their dogs' blood to be used for these studies.

## TABLE OF CONTENTS

LIST OF TABLES.....	vi
LIST OF FIGURES.....	vii
CHAPTER ONE: COMPARISON OF THE PATHOGENESIS OF PERIPHERAL PLATELET DESTRUCTION IN IMMUNE THROMBOCYTOPENIA OF HUMANS AND DOGS.....	1
Introduction.....	1
Pathogenesis of platelet destruction in human ITP.....	4
The pathogenesis of peripheral platelet destruction in dogs with ITP.....	19
Conclusions.....	20
References.....	22
CHAPTER TWO: CHARACTERIZATION OF B CELL ACTIVATING FACTOR IN A CANINE MACROPHAGE CELL LINE AND HEALTHY DOGS.....	30
Introduction.....	30
Materials and Methods.....	31
Results.....	36
Discussion.....	40
References.....	43
CHAPTER THREE: B CELL ACTIVATING FACTOR mRNA EXPRESSION IN DOGS WITH PRIMARY IMMUNE-MEDIATED THROMBOCYTOPENIA.....	46
Introduction.....	46
Materials and Methods.....	48
Results.....	51
Discussion.....	54
References.....	58
CHAPTER FOUR: B CELL ACTIVATING FACTOR PROMOTER POLYMORPHISMS IN DOGS WITH PRIMARY IMMUNE-MEDIATED THROMBOCYTOPENIA.....	61
Introduction.....	61
Materials and Methods.....	63
Results.....	67
Discussion.....	69
References.....	72
CHAPTER FIVE: $\Delta$ BAFF TRANSCRIPTION IS NOT INCREASED IN DOGS WITH PRIMARY ITP COMPARED WITH HEALTHY DOGS.....	75
Introduction.....	75
Materials and Methods.....	76
Results.....	79
Discussion.....	80
References.....	83

**LIST OF TABLES**

## CHAPTER THREE

Table 1	Individual cycle threshold values for RT-qPCR for BAFF and $\beta$ -actin.....	53
---------	--	----

## LIST OF FIGURES

### CHAPTER TWO

- Figure 1. Western blot of O3O-D lysates and canine splenic tissue.....37
- Figure 2. Western blot of commercially available recombinant canine BAFF fragment.....38
- Figure 3. Agarose gel electrophoresis of RT-PCR product identifying deltaBAFF.....39

### CHAPTER THREE

- Figure 1. Relative quantitative BAFF mRNA expression in dogs with ITP compared with other causes of thrombocytopenia and healthy controls.....52

### CHAPTER FOUR

- Figure 1. Genomic sequence of the predicted canine BAFF promoter....64
- Figure 2. Representative agarose gel electrophoresis of CPR1 PCR product....65
- Figure 3. Presence of -742CA insertion in dogs with ITP versus healthy controls.....67
- Figure 4. Mean BAFF CT values and -742CA genotype in dogs with ITP.....68

### CHAPTER FIVE

- Figure 1. Relative quantitative deltaBAFF mRNA expression in dogs with ITP compared to healthy controls.....79

## **CHAPTER ONE: COMPARISON OF THE PATHOGENESIS OF PERIPHERAL PLATELET DESTRUCTION IN IMMUNE THROMBOCYTOPENIA OF HUMANS AND DOGS**

### **1.1 Introduction**

Immune thrombocytopenia (ITP) is an acquired autoimmune disorder in which patients are severely thrombocytopenic in the absence of known, identifiable causes of decreased platelets. As a clinical entity in humans, ITP was first described in 1025 (Stasi & Newland, 2011), at which point it was diagnosed via the presence of purpuric hemorrhages in otherwise healthy individuals. It wasn't until the middle of the 19<sup>th</sup> century that platelets were discovered and not until the 1880s that a connection between the described clinical entity of ITP and platelets was made (Stasi & Newland, 2011).

Throughout the early 1900s, autoimmunity and platelet destruction versus decreased production were postulated as the causes of ITP (Stasi & Newland, 2011). By 1951, an experiment by two hematology fellows, known as the Harrington-Hollingsworth Experiment, provided substantial evidence of peripheral destruction of platelets as the driving factor for thrombocytopenia in ITP (Stasi & Newland, 2011). It is now commonly recognized that in addition to auto-antibody mediated peripheral platelet destruction by macrophages, platelet destruction mediated by T cells, a lack of megakaryocyte production within the bone marrow, and megakaryocyte-directed bone marrow inhibition and destruction all play a role in ITP in humans (McKenzie, Guo, Freedman, & Semple, 2013). Recently, with the advent

of thrombopoietin (TPO) agonists, there has been renewed interest in decreased platelet production as an additional factor in ITP.

Treatment of ITP in humans is evolving as knowledge of the immune responses driving thrombocytopenia expands. The American Society of Hematology last released guidelines on the treatment of ITP in 2011. Initial treatments suggested for patients with a platelet count  $<30 \times 10^9/L$  include long courses of corticosteroids, with the possible addition of intravenous immunoglobulin (Neunert et al., 2011). Patients that fail or relapse after their initial corticosteroid therapy are treated with either splenectomy, TPO agonists, or B cell depletion via the anti-CD20 antibody rituximab (Neunert et al., 2011). For further refractory patients, additional immunosuppressive drugs are often used in combination with corticosteroids or TPO agonists including vinca alkaloids, cyclosporine, and mycophenolate (Cuker & Neunert, 2016). However, these second-line therapies often have unacceptable side effects, including hepatotoxicity, nephrotoxicity, and secondary infections (Cuker & Neunert, 2016).

ITP has been recognized as a clinical entity in dogs since the 1950s (Magrane, Magrane, & Ross, 1959) and dogs have been suggested as an animal model of the disease in humans (Dodds, 1977; LeVine et al., 2014). Any breed of dog can be afflicted with ITP but certain breeds are overrepresented including Old English Sheepdogs, Cocker Spaniels, and Poodles (Lewis & Meyers, 1996; O'Marra, Delaforcade, & Shaw, 2011; Putsche & Kohn, 2008). As in humans, treatment of dogs with ITP focuses on use of immunosuppressive doses of corticosteroids with the frequent addition of a second immunosuppressive drug, such as

azathioprine or cyclosporine, to attenuate the adverse immune response driving the platelet destruction (O'Marra et al., 2011). However, several retrospective studies of ITP in dogs and individual case reports list complications attributed to various therapies such as leukopenia, urinary tract infections, skin abscesses, signs of hypercortisolemia, and pancreatitis (Hsu, Snead, Davies, & Carr, 2012; O'Marra et al., 2011; Putsche & Kohn, 2008). Corticosteroids can be particularly damaging long term as their continual use has been associated with increased risk of thrombosis, iatrogenic hyperadrenocorticism, secondary infection, bone demineralization, and gastric ulceration (Behrend & Kemppainen, 1997; Costa et al., 2010; Fry, McSporran, Ellis, & Harvey, 2009; Hsu et al., 2012; Jacoby, Owings, Ortega, Gosselin, & Feldman, 2001; Laurensen, Hopper, Herrera, & Johnson, 2010; Rose, Dunn, Allegret, & Bedard, 2011).

In humans and dogs, a better understanding of the aberrant immune response driving platelet destruction would allow for more precise, individualized treatment and monitoring than the current methods of inhibiting large arms of the immune system, leaving patients (both human and canine) vulnerable to harmful adverse events. Herein, we will focus on the pathophysiology of the destruction of peripheral platelets, what is known in humans and in dogs with regard to the roles of APCs, B cells and T cells in driving immune imbalance and thrombocytopenia, and areas for potential impact and discovery.

## 1.2 Pathogenesis of Platelet Destruction in Human ITP

### 1.2.1 T cells in ITP: Tregs, Th22 cells, and Th17 cells

The maintenance of tolerance to self-antigen is both one of the vital functions of the immune system and an aspect of immunity that is disrupted in autoimmune disease. For T cells this tolerance is maintained both centrally and peripherally. Centrally, in the thymus T cells are positively selected to recognize self MHC molecules and negatively selected to remove those that recognize self-antigen. In the periphery, this maintenance of tolerance involves the need for two signals for T cell activation by APCs. Additionally, a class of regulatory T cells (Tregs) have been noted to exist (Jonuleit & Schmitt, 2003). These  $CD4^+CD25^+FoxP3^+$  T cells contribute to the maintenance of tolerance and prevention of autoimmunity (Gratz, Rosenblum, & Abbas, 2013). Two classes of Tregs exist: naturally occurring and induced (Jonuleit & Schmitt, 2003).

Naturally occurring Tregs have properties allowing them to be both suppressive to other T-helper type cells and anergic, and in humans represent approximately 5-10% of all peripheral  $CD4^+$  T cells (Jonuleit & Schmitt, 2003). Early studies in mice showed that removal of the thymus, thus preventing the development of  $CD4^+CD25^+$  cells, resulted in autoimmune disorders (Suri-Payer, Amar, Thornton, & Shevach, 1998). Isolation of Tregs and incubation with  $CD25^-$  T cells later demonstrated their ability to suppress that population once their own T cell receptor (TCR) was activated (Shevach, 2002). Induced Tregs are formed in the periphery from a  $CD4^+CD25^-$  population in response to the local environment. Unlike naturally occurring Tregs, inducible Tregs are contact independent (Jonuleit &

Schmitt, 2003). TGF- $\beta$  or IL-10 exposure primarily from dendritic cells as APCs induces their differentiation to CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> cells (Chen et al., 2003; Jonuleit & Schmitt, 2003).

Because of their purported regulatory abilities, Tregs have been evaluated in many autoimmune and inflammatory diseases. In 2006, the first study evaluating Tregs in patients with ITP found that the percentage of Tregs as identified by flow cytometry was significantly lower in patients with active ITP not in remission compared with both patients with ITP in remission and healthy subjects (B. Liu et al., 2007). In this study, there was no difference in the percentage of Tregs in ITP patients in remission and healthy controls (B. Liu et al., 2007). However, other studies have shown there was no difference noted in the percentage of Tregs detected by flow cytometry in the peripheral blood of patients with chronic refractory ITP compared to healthy controls (J. Yu et al., 2008), suggesting that the shift in Treg percentages may not be universal in all stages of ITP.

If the percentage of Tregs is reduced in ITP, the question of what leads to that reduction remains. Tregs are known to be induced partially by the cytokine profile of their environment (Jonuleit & Schmitt, 2003), and in turn help with modulation of dendritic cell survival and function (Catani et al., 2013). However, the dendritic cell population also contributes to Treg generation. Dendritic cells produce indoleamine 2,3-dioxygenase 1 (IDO1), an immunoregulatory enzyme involved in tryptophan metabolism. The upregulation of IDO1 production can lead to changes in immune cell populations secondary to tryptophan deprivation (Hill et al., 2007). Additionally, it was shown by Hill et al. that IDO production

by dendritic cells led to an expansion of Treg populations (Hill et al., 2007). Given these findings, Catani et al. evaluated IDO expression in mature dendritic cells from acute and chronic ITP patients and healthy controls. They found reduced expression of IDO1 expression in mature dendritic cells of patients with ITP compared with controls, although no difference between groups of ITP patients (acute versus chronic disease) (Catani et al., 2013). Additionally, dendritic cells taken from ITP patients could not stimulate Treg expansion *in vitro* to the same degree as dendritic cells from control patients (Catani et al., 2013). Mirroring previous studies as well, Tregs from those ITP patients had a significantly lower suppressive ability *in vitro* on CD4<sup>+</sup>CD25<sup>-</sup> cells than Tregs from control patients (Catani et al., 2013).

The possibility that not only Treg numbers, but also function, may be affected in ITP has been raised as well. Tregs constitutively express TNF receptor II (TNFR2), which when bound to TNF- $\alpha$  suppresses their usual functions (Yazdanbakhsh, 2016). Previous studies had shown increased TNF- $\alpha$  concentrations in patients with ITP, but Zhong et al. showed that CD4<sup>+</sup> cells specifically made more TNF- $\alpha$ , and that Tregs from patients in ITP were more sensitive to the inhibitory effects of TNF- $\alpha$  secondary to increased TNFR2 on their surface (Zhong, Bussel, & Yazdanbakhsh, 2015). Liu et al. demonstrated that Tregs isolated from splenic tissue of ITP patients had limited suppressive capabilities *in vitro* when compared with Tregs isolated from splenic tissue of patients with hereditary splenocytosis, thus indicating a possible functional abnormality as well as deficiency of Treg numbers in ITP patients (B. Liu et al., 2007). This was duplicated by Yu et al. in which a failure of Tregs

from patients with chronic ITP to suppress CD4<sup>+</sup>CD25<sup>-</sup> T cell proliferation in vitro was demonstrated (J. Yu et al., 2008).

Opposing the role of Tregs is a subset of T-cells known as Th17 cells. Th17 cells secrete IL-17 and other cytokines leading to inflammation and possible autoimmune disease (H. Park et al., 2005). IL-17 most commonly refers to the cytokine IL-17A, as six family members (A-F) exist (Kolls & Lindén, 2004). IL-17 is known for its potent inflammatory properties including induction of G-CSF, chemokines, IL-6, TNF, and matrix metalloproteinases (Zenobia & Hajishengallis, 2015). Interestingly, both Th17 and Tregs share a common precursor and are induced by TGF- $\beta$ , and it is the presence or absence of other cytokines such as ROR- $\gamma$ -t, IL-1 $\beta$ , IL-23, and others that determine if the CD4<sup>+</sup> cells differentiate to become Tregs or Th17 cells (Manel, Unutmaz, & Littman, 2008).

The role of Th17 cells in ITP was initially debated, but has since become largely accepted as both a possible mediator of pathogenesis and potential therapeutic target (Ye et al., 2015). One group evaluated the percentages of both Tregs and Th17 cells and the Treg/Th17 ratios in patients with newly diagnosed ITP that required treatment and those that were observed with no treatment needed, compared to healthy adults (Ji et al., 2012). While the percentage of Th17 cells was significantly higher in those ITP patients who required treatment and healthy controls, and ITP patients who did not require treatment and healthy controls, there was no significant difference in Th17 cell percentage in patients with ITP who did or did not require treatment (Ji et al., 2012). Similar to prior studies, the patients with ITP requiring treatment had a significantly lower percentage of Tregs compared to those with ITP

not requiring treatment and healthy adults (Ji et al., 2012). These percentages resulted in a significantly lower Treg/Th17 ratio in ITP patients not receiving treatment compared to healthy controls, ITP patients receiving treatment compared to those who did not receive treatment, and ITP patients receiving treatment compared to controls (Ji et al., 2012). In patients receiving treatment, a small number were evaluated for response and it was noted that the responders had significantly higher Treg percentages, and thus a higher Treg/Th17 ratio, at diagnosis than non-responders (Ji et al., 2012).

These findings are similar to both previous and later studies evaluating Th17 cell populations by flow cytometry in patients with chronic or active ITP compared with controls, where the percentage of Th17 cells was markedly increased in patients with ITP (Ye et al., 2015; Zhang et al., 2009). The genesis of the shift toward a Th17 signature and away from Tregs is postulated to be partially mediated by increased IL-23. IL-23, a member of the IL-12 family of cytokines, is crucial to the development of Th17 cells from a common progenitor cell with Tregs. The Th17/IL-23 pathway is known to be important in several other autoimmune diseases (Lubberts, 2008) therefore, it stood to reason that the Th17 shift in ITP may also be secondary to increased IL-23. Ye et al. compared cytokine mRNA expression, plasma cytokine concentration, and percentage of Th17 cells in patients with chronic and newly diagnosed ITP and healthy controls. They found that IL-23 mRNA was increased, as were plasma levels of IL-17 and IL-23 and percentages of Th17 cells in ITP patients compared to controls (Ye et al., 2015). A small percentage of the patients enrolled in the study were followed throughout treatment, and with control of their disease, both plasma IL-

23 and IL-17 levels fell (Ye et al., 2015). These findings mirrored another study evaluating the IL-12 family of cytokines, specifically IL-12, IL-23, IL-27, IFN- $\gamma$ , IL-4 and IL-17A in patients with chronic ITP who had not received steroids for at least one month (Q. Li, L. Zhang, et al., 2015). They found patients with untreated ITP had significantly higher IL-12, IL-23, IL-27, IFN- $\gamma$ , and IL-17A, and significantly lower IL-35 and IL-4 than controls (Q. Li, L. Zhang, et al., 2015). Moreover, when the ITP patients were treated with dexamethasone and exhibited a complete response, their cytokine levels normalized, and were not significantly different from controls (Q. Li, L. Zhang, et al., 2015).

Importantly, these cytokine findings also reconfirmed previous work showing a Th1/Th2 imbalance in ITP, with a polarization toward Th1 cells (producing IL-2) and away from Th2 cells (producing IL-4, IL-5) (Panitsas et al., 2004). Furthermore, when humans with active, untreated ITP received dexamethasone and had a response in platelet count, their Th1/Th2 ratios normalized as determined by chemokine receptor mRNA expression (Z. Liu et al., 2016).

Another CD4<sup>+</sup> T cell lineage, Th22 cells, have been found to correlate with Th17 cell populations in ITP and may also contribute to the pathogenesis of disease. Th22 cells produce IL-22 and TNF- $\alpha$ , but not IL-17 or IFN- $\gamma$ . IL-22 is a pro-inflammatory cytokine with in the IL-10 family that has been linked to several autoimmune diseases (Cao et al., 2011). Patients with newly-diagnosed ITP had significantly more Th22 cells identified by flow cytometry and significantly higher plasma IL-22 concentrations by ELISA than healthy controls (Hu et al., 2012). Additionally, this same group found ITP patients had higher

percentages of Th17 and Th1 cells than the healthy population, and that there was a positive correlation between IL-22 plasma concentrations and percentage of Th22 cells in patients with ITP (Hu et al., 2012).

The cytokine IL-10 is a point of conflicting information. IL-10 was found to be higher in some patients with active ITP compared with controls in an earlier study evaluating Tregs (B. Liu et al., 2007). This was important because it is known that IL-10 helps with differentiation of Tregs (Chen et al., 2003; Jonuleit & Schmitt, 2003). The source of IL-10 was unknown at that time, although it had been previously reported that IL-10 was both decreased in patients with active ITP and increased in patients with chronic ITP (Panitsas et al., 2004). Further work to clarify the concentrations and role of IL-10 in other autoimmune diseases led to the discovery of a subset of regulatory B cells, B10 cells, that produce IL-10 in a regulatory fashion. Investigating the prevalence of these IL-10 producing cells in humans with newly diagnosed ITP showed a positive correlation between B10 cell percentages and Treg percentages at diagnosis (Hua et al., 2014). Additionally, the overall percentage of B10 cells was increased in ITP patients, both before and after treatment, compared to healthy control humans (Hua et al., 2014). The results suggested that there may be decreased or inhibited secretion of IL-10 from these cells given previous work showing conflicting levels of IL-10 in patients with ITP (Hua et al., 2014). These conclusions had been mirrored in previous work by the same group showing higher numbers of B10 cells in patients with ITP, higher IL-10 mRNA in those cells, but lower IL-10 concentrations in patient plasma (Hua et al., 2012). These conclusions were also later documented in Treg production of IL-10, where

a greater percentage of Tregs from patients with active ITP were IL-10<sup>+</sup> via intracellular staining, but supernatant of those cultured cells contained less IL-10 than healthy controls or ITP in remission (F. Li et al., 2015). This led to the conclusion that IL-10 secretion appears impaired in Tregs as in B10 cells, and may additionally be partially responsible for the pathogenesis of ITP (F. Li et al., 2015).

### 1.2.2 B cells in ITP: Bregs, B10 cells, and B cell activating factor

While the involvement of various T cell subsets is a relatively new discovery related to the pathogenesis of ITP, the involvement of B cells in platelet destruction has been well-established since the 1970s (McMillan et al., 1971). It is accepted that in the majority of patients with ITP, B cells produce antibodies against platelet glycoproteins (most commonly GPIIb/IIIa and GPIb/IX). These antibodies are bound by the Fc receptors of macrophages in the reticuloendothelial system and result in platelet destruction. Thus, what drives the production of the autoantibodies and allows the proliferation and survival of self-reactive B cells is an active area of research and discovery in ITP. This problem has been addressed from several angles, often mirroring the investigation into the role of T cells in ITP: failure of regulatory B cells, rescue or proliferation of self-reactive B cells, and aberrant cell-signaling such as through Toll-Like Receptors (TLRs).

Similar to T cells, there exist subsets of B cells with immune regulatory mechanisms: Bregs (CD19<sup>+</sup>CD24<sup>hi</sup>CD38<sup>hi</sup>). Studies in mice have shown that several subsets of Bregs produce IL-10, which helps drive the differentiation of immature T cells toward Tregs and

away from Th1 and Th17 cells (Carter, Rosser, & Mauri, 2012). In human patients with ITP, given what is known about the shift toward a Th1/Th17 environment, it is natural to explore the connection between these two cell lines. One group evaluated the ability of Bregs from chronic, untreated ITP patients to express IL-10 and inhibit monocyte TNF- $\alpha$  (X. Li et al., 2012). They found not only did untreated ITP patients with  $<50 \times 10^9/L$  platelets have fewer CD19<sup>+</sup>CD24<sup>hi</sup>CD38<sup>hi</sup> cells, but also that those CD19<sup>+</sup>CD24<sup>hi</sup>CD38<sup>hi</sup> cells that were present expressed significantly less IL-10 after stimulation than in healthy controls and were less able to inhibit monocytes than those of healthy controls (X. Li et al., 2012). However, as discussed previously, Hua et al. found instead a higher number of CD19<sup>+</sup> IL-10 producing cells in newly diagnosed ITP patients compared to control patients (Hua et al., 2014). In Hua's study, of those patients in that cohort that were followed, those with no response to therapy began with a significantly lower number of CD19<sup>+</sup> IL-10 producing cells than the partial responders or those experiencing complete remission (Hua et al., 2014). It is important to note, however, that the function of the Breg cells was not assessed in that study, and the selection of Breg cells differed between the two groups, with one using the stricter inclusion criteria of CD19<sup>+</sup>CD24<sup>hi</sup>CD38<sup>hi</sup>. Impaired function of Bregs classified as CD19<sup>+</sup>CD24<sup>hi</sup>CD38<sup>hi</sup> has been shown in SLE compared to healthy controls, despite absolute numbers of those cells not differing significantly between those groups (Blair et al., 2010). Taken together these studies suggest that IL-10 production capabilities by Breg cells may influence ITP outcome, and like Tregs, both changes in number and functional capacity are likely important in Bregs in ITP.

Complicating studies of Bregs in immune disease, multiple lineages of Bregs appear to exist, and a single discriminatory marker analogous to the Treg-FoxP3 connection has yet to be found (Mauri & Menon, 2015). B10 cells are identified solely by their ability to produce IL-10, typically via intracellular staining (Kalampokis, Yoshizaki, & Tedder, 2013), although various studies have defined them differently as noted previously. Several mechanisms of IL-10 induction exist including CD40 ligation or exposure to lipopolysaccharide or CpG oligonucleotides (Kalampokis et al., 2013). Additionally, Bregs may represent a transient state of B cells, such that any B cell may exert regulatory capacity given an environment that demands it, rather than a final state toward which B cell subsets mature (Mauri & Menon, 2015).

What drives either a deficiency of B10 cells, or functional impairment of B10 cells, in autoimmune disease is unknown. Recently, a cytokine involved in B cell maturation, differentiation and survival, B cell activating factor (BAFF), has been implicated as contributing to a Breg imbalance in autoimmune disease in mice (Ma et al., 2017). Both lupus-prone and experimental allergic encephalomyelitis (EAE) mice were evaluated for proportions of B10 cells and the effects of BAFF blockade (Ma et al., 2017). In inflammatory states, BAFF selected IL-10(-) cells preferentially over IL-10(+) cells due to a higher number of BAFF receptors on the IL-10(-) cells, while in the absence of inflammation, BAFF stimulation led to the expansion of IL-10(+) B cells (Ma et al., 2017).

Antigen presenting cells, T cells and peripheral blood mononuclear cells produce BAFF both constitutively and in response to other cytokines such as interferons, IL-10 and

G-CSF (Huard et al., 2004; Nardelli et al., 2001). Once produced, BAFF initially exerts its effects by binding to a BAFF receptor ligand located on B cells. Bound BAFF ultimately leads to cell growth and inhibition of apoptotic signals resulting in prolonged B-cell survival (Patke, Mecklenbrauker, Erdjument-Bromage, Tempst, & Tarakhovsky, 2006; Woodland et al., 2008).

*In vivo* models using BAFF transgenic mice demonstrated that high concentrations of BAFF are associated with increased numbers of mature B cells, circulating immune-complexes, effector T cells (F. Mackay et al., 1999) and the development of an autoimmune lupus-like disease (Khare et al., 2000). Further investigation has shown that numerous autoimmune diseases in people have been associated with elevated serum BAFF concentrations, increased autoantibody production, and expanded low-affinity self-reactive B cell populations (I. R. Mackay, Groom, & Mackay, 2002; Pers et al., 2005; Thien et al., 2004). The link between BAFF and some autoimmune diseases such as systemic lupus erythematosus (SLE) is so strong that monoclonal antibodies against BAFF, which lower the level of the cytokine within the blood, are used as treatments (Navarra et al., 2011).

Several independent studies have demonstrated increased levels of BAFF in humans with active ITP compared with humans with secondary ITP, healthy controls, or patients with ITP in remission (Emmerich et al., 2007; Thomas, Machin, Mackie, & Scully, 2011; Zhu et al., 2009). What drives the BAFF increase in autoimmune disease is unknown, but several groups have implicated Toll-like receptors (TLRs) in ITP specifically (Yang, Xu, et al., 2011; H. Yu et al., 2011). TLRs play a key role in activating B cells, often via dendritic cells

(DCs), and increased levels of TLRs in ITP have been noted in microarray studies (Sood, Wong, Jeng, & Zehnder, 2006; Yang, Xu, et al., 2011), however, the role of TLR upregulation was unknown. One group found that *in vitro* DCs stimulated with a TLR7 agonist produced large amounts of BAFF, and in patients with ITP, there was a positive correlation between concentrations of anti-GP antibody and serum BAFF, and between serum BAFF and relative TLR7 mRNA expression (H. Yu et al., 2011). This suggested a synergy between TLR7 from DCs and increased serum BAFF in ITP.

These findings were mirrored in an additional study in which TLR7 expression was found to be increased in a mouse model of ITP (Yang, Xu, et al., 2011). When the ITP mice were treated with a TLR7 silencing lentivirus their platelet counts increased, and when they were treated with the TLR7 agonist imiquimod their platelet counts further decreased (Yang, Xu, et al., 2011). Additionally, platelet-associated IgG (PAIgG) was noted to increase after TLR7 agonist administration and decrease with lentiviral silencing (Yang, Xu, et al., 2011). Importantly, there was no difference in platelet count or PAIgG in any of the control groups of mice with TLR7 silencing or TLR agonist administration. Correlating these TLR7 findings to BAFF, splenic mononuclear cells from the ITP mice were cultured *in vitro* and found to have significantly higher supernatant BAFF than controls (Yang, Xu, et al., 2011), which was further increased with imiquimod. However, TLR7 silencing did not change the concentrations of BAFF in ITP mice or controls (Yang, Xu, et al., 2011). Overall, this supported a role for TLR7 in driving increased BAFF production via APCs in ITP.

More recently, TLR expression was investigated in a human cohort of patients with newly diagnosed and refractory ITP (Hua et al., 2016). Splenic B cells and peripheral B cells were evaluated for mRNA expression of TLRs and downstream cytokines. However, only IL-10 and IL-1 $\beta$  were found to be significantly upregulated in both splenic and peripheral B cells, and not TLR7 (Hua et al., 2016). It is unknown if the APCs in those patients may have had upregulation of TLR7 expression, similar to the prior mouse studies (Yang, Xu, et al., 2011).

### 1.2.3 Antigen Presenting Cells in ITP

Antigen presenting cells (APCs) are the first line of defense against many pathogens, and have an important role in the maintenance of self-tolerance. In the case of the pathophysiology of ITP, they have important roles not only in direct platelet destruction but also as the facilitators between the innate and adaptive immune responses. Thus, antigen presenting cells have a vital role in the pathogenesis of ITP through mediating direct destruction of platelets, production of cytokines implicated in self-reactive B cell survival, and shifts in T cell populations.

Macrophages in the spleen are normally responsible for removal of aged platelets from filtered blood, and thus when anti-GP antibodies opsonize platelets these macrophages work quickly to destroy them (McKenzie et al., 2013). With regards to B cells, the previously mentioned studies show a change in TLR7 in mouse models of ITP, and changes in several downstream TLR cytokines in ITP in humans (Hua et al., 2016; Yang, Xu, et al., 2011), suggesting that perhaps TLR activation by DCs leads to production of cytokines such as

BAFF that promote self-reactive B cell proliferation, or changes in regulatory B cell cytokines like IL-10.

The links between shifts of T cell populations and antigen presenting cells are numerous. The DC regulatory molecule IDO1 helps to ensure adequate populations of Tregs, and as referenced previously has been shown to be decreased in DC populations in patients with ITP (Catani et al., 2013). Additionally, it has been shown that monocytes can drive T cell differentiation.

Monocytes, the precursors to macrophages and dendritic cells found in the peripheral blood, have three distinct lineages based on two surface markers CD16 and CD14: classical ( $CD14^{hi}CD16^{-}$ ), intermediate ( $CD14^{++}CD16^{+}$ ), and nonclassical ( $CD14^{+}CD16^{++}$ ) (Zhong et al., 2012; Ziegler-Heitbrock, 2015; Ziegler-Heitbrock et al., 2010).  $CD14^{hi}CD16^{-}$  cells, the most common lineage accounting for up to 90% of monocytes, act primarily as phagocytic cells (Ziegler-Heitbrock et al., 2010). The nonclassical cells have higher intracellular proinflammatory TNF and lower anti-inflammatory IL-10 compared to classical monocytes, and appear to present certain antigens in a more effective manner than classical monocytes (Ziegler-Heitbrock, 2007). During inflammation the cell populations shift from the classical to intermediate and then nonclassical cell types (Ziegler-Heitbrock et al., 2010).

In humans with chronic ITP being treated with various thrombopoietin agonists,  $CD16^{+}$  monocytes were higher in patients with very low platelet counts ( $<50 \times 10^9/L$ ) compared to those with higher platelet counts ( $>50 \times 10^9/L$ ) and controls (Zhong et al., 2012). Those patients with higher  $CD16^{+}$  cells were also found to have higher numbers of

CD4<sup>+</sup>IFN- $\gamma$ <sup>+</sup> T cells (indicating a Th1 response), and lower Treg and CD4<sup>+</sup>IL-17<sup>+</sup> T cells (a reduced Th17 response) (Zhong et al., 2012). When CD16<sup>+</sup> cells were depleted in blood from those ITP patients and controls, an increase in Treg frequency and CD4<sup>+</sup>IL-17<sup>+</sup> frequency was seen, indicating that the CD16<sup>+</sup> cells could drive T cell polarization (Zhong et al., 2012). Importantly, in co-culture experiments with isolated T cells and CD16<sup>+</sup> cells from the ITP patients, blocking IL-12 secreted from CD16<sup>+</sup> cells nearly abolished the ability of those cells to induce Th1 polarization (Zhong et al., 2012). Taken together this indicates that CD16<sup>+</sup> cells, via IL-12, can contribute to the decrease in Tregs and increase in a Th1-type response in ITP patients. Importantly, CD16<sup>+</sup> cells in nonhuman primates have increased TLR7 expression, which as discussed previously is known to activate B cells and contribute to the production of cytokines like BAFF that are increased in ITP patients and to drive IL-12 production contributing to T cell imbalance (Yang, Wang, et al., 2011).

As previously discussed, antigen presenting cells also produce IL-23, which influences T cell population development. While IL-12 appears to shift polarization of T cells away from IL-17<sup>+</sup> cells, IL-23 has been shown to expand Th17 responses in ITP (Ye et al., 2015). In patients with both acute and chronic ITP relative gene expression of IL-12 and IL-23 and absolute concentrations of plasma IL-12 and IL-23 were higher in ITP patients PBMCs than controls (Q. Li, Yang, Xia, Xia, & Zhang, 2015). The authors of that study postulated that a positive feedback loop exists, with the IL-12 leading to production of Th1 cells, which in turn produce IFN- $\gamma$ , which then primes additional APCs for IL-12 production.

### 1.3 The pathogenesis of peripheral platelet destruction in dogs with ITP

In comparison to humans, little is known regarding the exact pathogenesis of ITP in dogs. Several groups have identified platelet-associated IgG in dogs suspected of having ITP, although in veterinary medicine PAIgG has not frequently been used in the diagnosis or monitoring of disease due to low availability of the assay and its inability to distinguish primary (autoimmune) ITP from secondary (triggered by other diseases) ITP (Dircks, Schuberth, & Mischke, 2009). One group has evaluated changes in Treg frequencies in dogs with ITP and chronic enteropathy (Volkman et al., 2014). Using flow cytometry Volkman et al. compared Treg frequencies in dogs with clinical ITP, dogs with ITP in remission, and healthy controls, finding that the clinically affected dogs had fewer Tregs in peripheral circulation than either control group. While this study was limited by small group sizes (7 dogs total with ITP and 13 healthy controls), it did establish a link between humans and dogs in the pathogenesis of spontaneous ITP.

One study evaluating experimentally-induced and naturally occurring ITP in dogs with cytokine/chemokine analysis found that lower serum IL-10 concentrations were directly correlated with severity of bleeding (LeVine et al., 2014). While the outcome of that study was not to evaluate cytokine profiles of naturally occurring ITP in dogs, given what is known about IL-10 producing cells and the pathophysiology of ITP in humans, this finding warrants further investigation.

## 1.4 Conclusions

ITP has several similarities in both humans and dogs (LeVine et al., 2014), however much more is known of the pathogenesis of ITP in humans. Advances in the knowledge of the etiology of ITP in humans in recent years have led to reliance on new treatments such as TPO agonists (Cuker & Neunert, 2016; Neunert et al., 2011). At present, the knowledge gaps in terms of the pathogenesis of ITP in dogs are large: do dogs share the same effector cells beyond B cells, including nonclassical monocytes and various T cell subsets? Does decreased platelet production play a role in thrombocytopenia? Does megakaryocyte destruction play a role? Is there a Th cell polarization that occurs?

Based on one study it does seem possible that Tregs are also affected in dogs with ITP (Volkman et al., 2014), but little is known of other groups of CD4<sup>+</sup> T cells or the change in polarization with disease. Th1, Th2, Treg and Th17 cytokine expression has been previously evaluated via RT-qPCR in dogs with inflammatory bowel disease and sterile meningitis (E. S. Park, Uchida, & Nakayama, 2013; Schmitz, Garden, Werling, & Allenspach, 2012). Th17 cells have recently been characterized with flow cytometry in peripheral blood of healthy dogs and with immunofluorescence in tissues of dogs with inflammatory diseases, representing an important step in establishing a protocol to evaluate the cells in other diseases such as ITP (Kol, Walker, Nordstrom, & Borjesson, 2016).

While little is known of newly discovered T cell subsets in dogs, even less is certain regarding B regulatory cells, B cell survival cytokines, and monocyte lineages in dogs. Canine total monocyte numbers have been previously evaluated primarily in neoplasia,

although in a recent study a subset of intermediate CD14<sup>+</sup>CD16<sup>+</sup> monocytes was found in dogs with osteosarcoma (Tuohy, Lascelles, Griffith, & Fogle, 2016). Recently IgD<sup>hi</sup> B cells that produce IL-10 similarly to certain groups of Breg cells described previously in humans and mice were noted in dogs with visceral leishmaniasis (Schaut et al., 2016). Another group investigating changes in mucosal immunoglobulins in dogs with IBD evaluated BAFF in attempts to explain a decreased concentration of duodenal IgA in biopsy samples from affected dogs (Maeda et al., 2013). However, no group has evaluated BAFF in dogs with other inflammatory or immune-mediated diseases in dogs. Given recent advances in the knowledge of BAFF in other human immune-mediated diseases such as systemic lupus erythematosus and its use as a therapeutic target for that disease (Coquery et al., 2014; Navarra et al., 2011; Pers et al., 2005), we elected to evaluate BAFF in dogs with ITP as a potential diagnostic biomarker. We hypothesize that BAFF will be a sensitive and specific biomarker for the diagnosis of ITP, able to aid in treatment duration decisions and monitoring for the risk of relapse.

## References

- Behrend, E. N., & Kemppainen, R. J. (1997). Glucocorticoid therapy. Pharmacology, indications, and complications. *Vet Clin North Am Small Anim Pract*, 27(2), 187-213.
- Blair, P. A., Noreña, L. Y., Flores-Borja, F., Rawlings, D. J., Isenberg, D. A., Ehrenstein, M. R., & Mauri, C. (2010). CD19(+)CD24(hi)CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic Lupus Erythematosus patients. *Immunity*, 32(1), 129-140. doi:10.1016/j.immuni.2009.11.009
- Cao, J., Chen, C., Zeng, L., Li, L., Li, X., Li, Z., & Xu, K. (2011). Elevated plasma IL-22 levels correlated with Th1 and Th22 cells in patients with immune thrombocytopenia. *Clinical Immunology*, 141(1), 121-123.
- Carter, N. A., Rosser, E. C., & Mauri, C. (2012). Interleukin-10 produced by B cells is crucial for the suppression of Th17/Th1 responses, induction of T regulatory type 1 cells and reduction of collagen-induced arthritis. *Arthritis Res Ther*, 14(1), R32. doi:10.1186/ar3736
- Catani, L., Sollazzo, D., Trabanelli, S., Curti, A., Evangelisti, C., Polverelli, N., . . . Lemoli, R. M. (2013). Decreased expression of indoleamine 2,3-dioxygenase 1 in dendritic cells contributes to impaired regulatory T cell development in immune thrombocytopenia. *Ann Hematol*, 92(1), 67-78. doi:10.1007/s00277-012-1556-5
- Chen, W., Jin, W., Hardegen, N., Lei, K. J., Li, L., Marinos, N., . . . Wahl, S. M. (2003). Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med*, 198(12), 1875-1886. doi:10.1084/jem.20030152
- Coquery, C. M., Wade, N. S., Loo, W. M., Kinchen, J. M., Cox, K. M., Jiang, C., . . . Erickson, L. D. (2014). Neutrophils contribute to excess serum BAFF levels and promote CD4+ T cell and B cell responses in lupus-prone mice. *PLoS One*, 9(7), e102284. doi:10.1371/journal.pone.0102284
- Costa, L. A., Lopes, B. F., Lanis, A. B., De Oliveira, D. C., Giannotti, J. G., & Costa, F. S. (2010). Bone demineralization in the lumbar spine of dogs submitted to prednisone therapy. *J Vet Pharmacol Ther*, 33(6), 583-586. doi:10.1111/j.1365-2885.2010.01174.x
- Cuker, A., & Neunert, C. E. (2016). How I treat refractory immune thrombocytopenia. *Blood*, 128(12), 1547-1554. doi:10.1182/blood-2016-03-603365

- Dircks, B. H., Schuberth, H. J., & Mischke, R. (2009). 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*, 235(8), 960-966. doi:10.2460/javma.235.8.960
- Dodds, W. J. (1977). Animal model: canine and equine immune-mediated thrombocytopenia, and idiopathic thrombocytopenic purpura. *Am J Pathol*, 86(2), 489-491.
- Emmerich, F., Bal, G., Barakat, A., Milz, J., Muhle, C., Martinez-Gamboa, L., . . . Salama, A. (2007). High-level serum B-cell activating factor and promoter polymorphisms in patients with idiopathic thrombocytopenic purpura. *Br J Haematol*, 136(2), 309-314. doi:10.1111/j.1365-2141.2006.06431.x
- Fry, D. R., McSporran, K. D., Ellis, J. T., & Harvey, C. (2009). Protozoal hepatitis associated with immunosuppressive therapy in a dog. *J Vet Intern Med*, 23(2), 366-368. doi:10.1111/j.1939-1676.2008.0263.x
- Gratz, I. K., Rosenblum, M. D., & Abbas, A. K. (2013). The life of regulatory T cells. *Ann N Y Acad Sci*, 1283, 8-12. doi:10.1111/nyas.12011
- Hill, M., Tanguy-Royer, S., Royer, P., Chauveau, C., Asghar, K., Tesson, L., . . . Anegon, I. (2007). IDO expands human CD4+CD25high regulatory T cells by promoting maturation of LPS-treated dendritic cells. *Eur J Immunol*, 37(11), 3054-3062. doi:10.1002/eji.200636704
- Hsu, K., Snead, E., Davies, J., & Carr, A. (2012). Iatrogenic hyperadrenocorticism, calcinosis cutis, and myocardial infarction in a dog treated for IMT. *J Am Anim Hosp Assoc*, 48(3), 209-215. doi:10.5326/jaaha-ms-5740
- Hu, Y., Li, H., Zhang, L., Shan, B., Xu, X., Liu, X., . . . Hou, M. (2012). Elevated profiles of Th22 cells and correlations with Th17 cells in patients with immune thrombocytopenia. *Hum Immunol*, 73(6), 629-635. doi:10.1016/j.humimm.2012.04.015
- Hua, F., Ji, L., Zhan, Y., Li, F., Zou, S., Chen, L., . . . Cheng, Y. (2014). Aberrant frequency of IL-10-producing B cells and its association with Treg/Th17 in adult primary immune thrombocytopenia patients. *Biomed Res Int*, 2014, 571302. doi:10.1155/2014/571302
- Hua, F., Ji, L., Zhan, Y., Li, F., Zou, S., Wang, X., . . . Cheng, Y. (2012). Pulsed high-dose dexamethasone improves interleukin 10 secretion by CD5+ B cells in patients with primary immune thrombocytopenia. *J Clin Immunol*, 32(6), 1233-1242. doi:10.1007/s10875-012-9714-z

- Hua, F., Li, Y., Zhao, X., Zhang, D., Zhan, Y., Ji, L., . . . Cheng, Y. (2016). The expression profile of toll-like receptor signaling molecules in CD19(+) B cells from patients with primary immune thrombocytopenia. *Immunol Lett*, *176*, 28-35. doi:10.1016/j.imlet.2016.05.008
- Huard, B., Arlettaz, L., Ambrose, C., Kindler, V., Mauri, D., Roosnek, E., . . . French, L. E. (2004). BAFF production by antigen-presenting cells provides T cell co-stimulation. *Int Immunol*, *16*(3), 467-475.
- Jacoby, R. C., Owings, J. T., Ortega, T., Gosselin, R., & Feldman, E. C. (2001). Biochemical basis for the hypercoagulable state seen in Cushing syndrome; discussion 1006-7. *Arch Surg*, *136*(9), 1003-1006.
- Ji, L., Zhan, Y., Hua, F., Li, F., Zou, S., Wang, W., . . . Cheng, Y. (2012). The ratio of Treg/Th17 cells correlates with the disease activity of primary immune thrombocytopenia. *PLoS One*, *7*(12), e50909. doi:10.1371/journal.pone.0050909
- Jonuleit, H., & Schmitt, E. (2003). The regulatory T cell family: distinct subsets and their interrelations. *J Immunol*, *171*(12), 6323-6327.
- Kalampokis, I., Yoshizaki, A., & Tedder, T. F. (2013). IL-10-producing regulatory B cells (B10 cells) in autoimmune disease. *Arthritis Res Ther*, *15 Suppl 1*, S1. doi:10.1186/ar3907
- Khare, S. D., Sarosi, I., Xia, X. Z., McCabe, S., Miner, K., Solovyev, I., . . . Hsu, H. (2000). Severe B cell hyperplasia and autoimmune disease in TALL-1 transgenic mice. *Proc Natl Acad Sci U S A*, *97*(7), 3370-3375. doi:10.1073/pnas.050580697
- Kol, A., Walker, N. J., Nordstrom, M., & Borjesson, D. L. (2016). Th17 Pathway As a Target for Multipotent Stromal Cell Therapy in Dogs: Implications for Translational Research. *PLoS One*, *11*(2), e0148568. doi:10.1371/journal.pone.0148568
- Kolls, J. K., & Lindén, A. (2004). Interleukin-17 family members and inflammation. *Immunity*, *21*(4), 467-476. doi:10.1016/j.immuni.2004.08.018
- Laurenson, M. P., Hopper, K., Herrera, M. A., & Johnson, E. G. (2010). Concurrent diseases and conditions in dogs with splenic vein thrombosis. *J Vet Intern Med*, *24*(6), 1298-1304. doi:10.1111/j.1939-1676.2010.0593.x
- LeVine, D. N., Birkenheuer, A. J., Brooks, M. B., Nordone, S. K., Bellinger, D. A., Jones, S. L., . . . Key, N. S. (2014). A novel canine model of immune thrombocytopenia: has immune thrombocytopenia (ITP) gone to the dogs? *Br J Haematol*, *167*(1), 110-120. doi:10.1111/bjh.13005

- Lewis, D. C., & Meyers, K. M. (1996). Canine idiopathic thrombocytopenic purpura. *J Vet Intern Med*, *10*(4), 207-218.
- Li, F., Ji, L., Wang, W., Hua, F., Zhan, Y., Zou, S., . . . Cheng, Y. (2015). Insufficient secretion of IL-10 by Tregs compromised its control on over-activated CD4+ T effector cells in newly diagnosed adult immune thrombocytopenia patients. *Immunol Res*, *61*(3), 269-280. doi:10.1007/s12026-014-8620-2
- Li, Q., Yang, M., Xia, R., Xia, L., & Zhang, L. (2015). Elevated expression of IL-12 and IL-23 in patients with primary immune thrombocytopenia. *Platelets*, *26*(5), 453-458. doi:10.3109/09537104.2014.934217
- Li, Q., Zhang, L., Xia, R., Zeng, Q., Wang, Y., Xia, L., & Yang, M. (2015). Plasma Levels of Interleukin 12 Family Cytokines and Their Relevant Cytokines in Adult Patients with Chronic Immune Thrombocytopenia before and after High-Dose Dexamethasone Treatment. *Med Princ Pract*, *24*(5), 458-464. doi:10.1159/000433472
- Li, X., Zhong, H., Bao, W., Boulad, N., Evangelista, J., Haider, M. A., . . . Yazdanbakhsh, K. (2012). Defective regulatory B-cell compartment in patients with immune thrombocytopenia. *Blood*, *120*(16), 3318-3325. doi:10.1182/blood-2012-05-432575
- Liu, B., Zhao, H., Poon, M. C., Han, Z., Gu, D., Xu, M., . . . Han, Z. C. (2007). Abnormality of CD4(+)CD25(+) regulatory T cells in idiopathic thrombocytopenic purpura. *Eur J Haematol*, *78*(2), 139-143. doi:10.1111/j.1600-0609.2006.00780.x
- Liu, Z., Wang, M., Zhou, S., Ma, J., Shi, Y., Peng, J., . . . Guo, C. (2016). Pulsed high-dose dexamethasone modulates Th1-/Th2-chemokine imbalance in immune thrombocytopenia. *J Transl Med*, *14*(1), 301. doi:10.1186/s12967-016-1064-9
- Lubberts, E. (2008). IL-17/Th17 targeting: on the road to prevent chronic destructive arthritis? *Cytokine*, *41*(2), 84-91. doi:10.1016/j.cyto.2007.09.014
- Ma, N., Zhang, Y., Liu, Q., Wang, Z., Liu, X., Zhu, G., . . . Wang, R. (2017). B cell activating factor (BAFF) selects IL-10(-)B cells over IL-10(+)B cells during inflammatory responses. *Mol Immunol*, *85*, 18-26. doi:10.1016/j.molimm.2017.02.002
- Mackay, F., Woodcock, S. A., Lawton, P., Ambrose, C., Baetscher, M., Schneider, P., . . . Browning, J. L. (1999). Mice transgenic for BAFF develop lymphocytic disorders along with autoimmune manifestations. *J Exp Med*, *190*(11), 1697-1710.
- Mackay, I. R., Groom, J., & Mackay, C. R. (2002). Levels of BAFF in serum in primary biliary cirrhosis and autoimmune diabetes. *Autoimmunity*, *35*(8), 551-553.

- Maeda, S., Ohno, K., Uchida, K., Nakashima, K., Fukushima, K., Tsukamoto, A., . . . Tsujimoto, H. (2013). Decreased immunoglobulin A concentrations in feces, duodenum, and peripheral blood mononuclear cells of dogs with inflammatory bowel disease. *J Vet Intern Med*, 27(1), 47-55. doi:10.1111/jvim.12023
- Magrane, H. J., Magrane, W. G., & Ross, J. R. (1959). Idiopathic thrombocytopenic purpura in a dog—a case report. *J Am Vet Med Assoc*, 135, 520-522.
- Manel, N., Unutmaz, D., & Littman, D. R. (2008). The differentiation of human T(H)-17 cells requires transforming growth factor-beta and induction of the nuclear receptor RORgammat. *Nat Immunol*, 9(6), 641-649. doi:10.1038/ni.1610
- Mauri, C., & Menon, M. (2015). The expanding family of regulatory B cells. *Int Immunol*, 27(10), 479-486. doi:10.1093/intimm/dxv038
- McKenzie, C. G., Guo, L., Freedman, J., & Semple, J. W. (2013). Cellular immune dysfunction in immune thrombocytopenia (ITP). *Br J Haematol*, 163(1), 10-23. doi:10.1111/bjh.12480
- McMillan, R., Smith, R. S., Longmire, R. L., Yelenosky, R., Reid, R. T., & Craddock, C. G. (1971). Immunoglobulins associated with human platelets. *Blood*, 37(3), 316-322.
- Nardelli, B., Belvedere, O., Roschke, V., Moore, P. A., Olsen, H. S., Migone, T. S., . . . Hilbert, D. M. (2001). Synthesis and release of B-lymphocyte stimulator from myeloid cells. *Blood*, 97(1), 198-204.
- Navarra, S. V., Guzman, R. M., Gallacher, A. E., Hall, S., Levy, R. A., Jimenez, R. E., . . . Group, B.-S. (2011). Efficacy and safety of belimumab in patients with active systemic lupus erythematosus: a randomised, placebo-controlled, phase 3 trial. *Lancet*, 377(9767), 721-731. doi:10.1016/s0140-6736(10)61354-2
- Neunert, C., Lim, W., Crowther, M., Cohen, A., Solberg, L., Jr., & Crowther, M. A. (2011). The American Society of Hematology 2011 evidence-based practice guideline for immune thrombocytopenia. *Blood*, 117(16), 4190-4207. doi:10.1182/blood-2010-08-302984
- O'Marra, S. K., Delaforcade, A. M., & Shaw, S. P. (2011). Treatment and predictors of outcome in dogs with immune-mediated thrombocytopenia. *J Am Vet Med Assoc*, 238(3), 346-352. doi:10.2460/javma.238.3.346
- Panitsas, F. P., Theodoropoulou, M., Kouraklis, A., Karakantza, M., Theodorou, G. L., Zoumbos, N. C., . . . Mouzaki, A. (2004). Adult chronic idiopathic thrombocytopenic purpura (ITP) is the manifestation of a type-1 polarized immune response. *Blood*, 103(7), 2645-2647. doi:10.1182/blood-2003-07-2268

- Park, E. S., Uchida, K., & Nakayama, H. (2013). Th1-, Th2-, and Th17-related cytokine and chemokine receptor mRNA and protein expression in the brain tissues, T cells, and macrophages of dogs with necrotizing and granulomatous meningoencephalitis. *Vet Pathol*, *50*(6), 1127-1134. doi:10.1177/0300985813488957
- Park, H., Li, Z., Yang, X. O., Chang, S. H., Nurieva, R., Wang, Y. H., . . . Dong, C. (2005). A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol*, *6*(11), 1133-1141. doi:10.1038/ni1261
- Patke, A., Mecklenbrauker, I., Erdjument-Bromage, H., Tempst, P., & Tarakhovsky, A. (2006). BAFF controls B cell metabolic fitness through a PKC beta- and Akt-dependent mechanism. *J Exp Med*, *203*(11), 2551-2562. doi:10.1084/jem.20060990
- Pers, J. O., Daridon, C., Devauchelle, V., Jousse, S., Saraux, A., Jamin, C., & Youinou, P. (2005). BAFF overexpression is associated with autoantibody production in autoimmune diseases. *Ann N Y Acad Sci*, *1050*, 34-39. doi:10.1196/annals.1313.004
- Putsche, J. C., & Kohn, B. (2008). Primary immune-mediated thrombocytopenia in 30 dogs (1997-2003). *J Am Anim Hosp Assoc*, *44*(5), 250-257. doi:10.5326/0440250
- Rose, L. J., Dunn, M. E., Allegret, V., & Bedard, C. (2011). Effect of prednisone administration on coagulation variables in healthy Beagle dogs. *Vet Clin Pathol*, *40*(4), 426-434. doi:10.1111/j.1939-165X.2011.00364.x
- Schaut, R. G., Lamb, I. M., Toepp, A. J., Scott, B., Mendes-Aguiar, C. O., Coutinho, J. F., . . . Petersen, C. A. (2016). Regulatory IgDhi B Cells Suppress T Cell Function via IL-10 and PD-L1 during Progressive Visceral Leishmaniasis. *J Immunol*, *196*(10), 4100-4109. doi:10.4049/jimmunol.1502678
- Schmitz, S., Garden, O. A., Werling, D., & Allenspach, K. (2012). Gene expression of selected signature cytokines of T cell subsets in duodenal tissues of dogs with and without inflammatory bowel disease. *Vet Immunol Immunopathol*, *146*(1), 87-91. doi:10.1016/j.vetimm.2012.01.013
- Shevach, E. M. (2002). CD4+ CD25+ suppressor T cells: more questions than answers. *Nat Rev Immunol*, *2*(6), 389-400. doi:10.1038/nri821
- Sood, R., Wong, W., Jeng, M., & Zehnder, J. L. (2006). Gene expression profile of idiopathic thrombocytopenic purpura (ITP). *Pediatr Blood Cancer*, *47*(5 Suppl), 675-677. doi:10.1002/pbc.20981
- Stasi, R., & Newland, A. C. (2011). ITP: a historical perspective. *Br J Haematol*, *153*(4), 437-450. doi:10.1111/j.1365-2141.2010.08562.x

- Suri-Payer, E., Amar, A. Z., Thornton, A. M., & Shevach, E. M. (1998). CD4+CD25+ T cells inhibit both the induction and effector function of autoreactive T cells and represent a unique lineage of immunoregulatory cells. *J Immunol*, *160*(3), 1212-1218.
- Thien, M., Phan, T. G., Gardam, S., Amesbury, M., Basten, A., Mackay, F., & Brink, R. (2004). Excess BAFF rescues self-reactive B cells from peripheral deletion and allows them to enter forbidden follicular and marginal zone niches. *Immunity*, *20*(6), 785-798. doi:10.1016/j.immuni.2004.05.010
- Thomas, M. R., Machin, S. J., Mackie, I., & Scully, M. A. (2011). B cell activating factor is elevated in acute idiopathic thrombotic thrombocytopenic purpura. *Br J Haematol*, *155*(5), 620-622. doi:10.1111/j.1365-2141.2011.08730.x
- Tuohy, J. L., Lascelles, B. D., Griffith, E. H., & Fogle, J. E. (2016). Association of Canine Osteosarcoma and Monocyte Phenotype and Chemotactic Function. *J Vet Intern Med*, *30*(4), 1167-1178. doi:10.1111/jvim.13983
- Volkman, M., Hepworth, M. R., Ebner, F., Rausch, S., Kohn, B., & Hartmann, S. (2014). Frequencies of regulatory T cells in the peripheral blood of dogs with primary immune-mediated thrombocytopenia and chronic enteropathy: A pilot study. *Vet J*, *202*(3), 630-633. doi:10.1016/j.tvjl.2014.10.012
- Woodland, R. T., Fox, C. J., Schmidt, M. R., Hammerman, P. S., Opferman, J. T., Korsmeyer, S. J., . . . Thompson, C. B. (2008). Multiple signaling pathways promote B lymphocyte stimulator dependent B-cell growth and survival. *Blood*, *111*(2), 750-760. doi:10.1182/blood-2007-03-077222
- Yang, Q., Wang, B., Yu, H., Zhu, Y., Wang, X., Jiang, H., . . . Hou, M. (2011). TLR7 promotes Th1 polarization in immune thrombocytopenia. *Thromb Res*, *128*(3), 237-242. doi:10.1016/j.thromres.2011.02.024
- Yang, Q., Xu, S., Li, X., Wang, B., Wang, X., Ma, D., . . . Hou, M. (2011). Pathway of Toll-like receptor 7/B cell activating factor/B cell activating factor receptor plays a role in immune thrombocytopenia in vivo. *PLoS One*, *6*(7), e22708. doi:10.1371/journal.pone.0022708
- Yazdanbakhsh, K. (2016). Imbalanced immune homeostasis in immune thrombocytopenia. *Semin Hematol*, *53 Suppl 1*, S16-19. doi:10.1053/j.seminhematol.2016.04.006
- Ye, X., Zhang, L., Wang, H., Chen, Y., Zhang, W., Zhu, R., . . . Qian, B. (2015). The role of IL-23/Th17 pathway in patients with primary immune thrombocytopenia. *PLoS One*, *10*(1), e0117704. doi:10.1371/journal.pone.0117704

- Yu, H., Liu, Y., Han, J., Yang, Z., Sheng, W., Dai, H., . . . Hou, M. (2011). TLR7 regulates dendritic cell-dependent B-cell responses through BlyS in immune thrombocytopenic purpura. *Eur J Haematol*, *86*(1), 67-74. doi:10.1111/j.1600-0609.2010.01534.x
- Yu, J., Heck, S., Patel, V., Levan, J., Yu, Y., Bussel, J. B., & Yazdanbakhsh, K. (2008). Defective circulating CD25 regulatory T cells in patients with chronic immune thrombocytopenic purpura. *Blood*, *112*(4), 1325-1328. doi:10.1182/blood-2008-01-135335
- Zenobia, C., & Hajishengallis, G. (2015). Basic biology and role of interleukin-17 in immunity and inflammation. *Periodontol 2000*, *69*(1), 142-159. doi:10.1111/prd.12083
- Zhang, J., Ma, D., Zhu, X., Qu, X., Ji, C., & Hou, M. (2009). Elevated profile of Th17, Th1 and Tc1 cells in patients with immune thrombocytopenic purpura. *Haematologica*, *94*(9), 1326-1329. doi:10.3324/haematol.2009.007823
- Zhong, H., Bao, W., Li, X., Miller, A., Seery, C., Haq, N., . . . Yazdanbakhsh, K. (2012). CD16+ monocytes control T-cell subset development in immune thrombocytopenia. *Blood*, *120*(16), 3326-3335. doi:10.1182/blood-2012-06-434605
- Zhong, H., Bussel, J., & Yazdanbakhsh, K. (2015). In vitro TNF blockade enhances ex vivo expansion of regulatory T cells in patients with immune thrombocytopenia. *Br J Haematol*, *168*(2), 274-283. doi:10.1111/bjh.13126
- Zhu, X. J., Shi, Y., Sun, J. Z., Shan, N. N., Peng, J., Guo, C. S., . . . Hou, M. (2009). High-dose dexamethasone inhibits BAFF expression in patients with immune thrombocytopenia. *J Clin Immunol*, *29*(5), 603-610. doi:10.1007/s10875-009-9303-y
- Ziegler-Heitbrock, L. (2007). The CD14+ CD16+ blood monocytes: their role in infection and inflammation. *J Leukoc Biol*, *81*(3), 584-592. doi:10.1189/jlb.0806510
- Ziegler-Heitbrock, L. (2015). Blood Monocytes and Their Subsets: Established Features and Open Questions. *Front Immunol*, *6*, 423. doi:10.3389/fimmu.2015.00423
- Ziegler-Heitbrock, L., Ancuta, P., Crowe, S., Dalod, M., Grau, V., Hart, D. N., . . . Lutz, M. B. (2010). Nomenclature of monocytes and dendritic cells in blood. *Blood*, *116*(16), e74-80. doi:10.1182/blood-2010-02-258558

## CHAPTER TWO: CHARACTERIZATION OF B CELL ACTIVATING FACTOR IN A CANINE MACROPHAGE CELL LINE AND HEALTHY DOGS

### 2.1 Introduction

B cell activating factor (BAFF, also described as tumor necrosis factor superfamily member 13b, and B lymphocyte stimulator) is a cytokine of the tumor necrosis factor superfamily (TNFSF). While BAFF production has been documented in a variety of cells and tissues, the primary sources are T cells, dendritic cells, and macrophages (Kim, Seo, & Kim, 2011; Moore et al., 1999; Schneider et al., 1999). These cells produce BAFF in response to interferons, IL-10 and toll-like receptor stimulation (Huard et al., 2004; Nardelli et al., 2001). Once produced, BAFF binds to one of three receptors, BAFF-R, transmembrane activator and CAML interactor (TACI), or B cell maturation antigen (BCMA) and contributes to B cell maturation, T cell independent class switching, and plasma cell survival (Bossen & Schneider, 2006). Additionally, an alternatively spliced version of BAFF, termed  $\Delta$ BAFF, is present in humans and mice and acts as a negative regulator of BAFF (Gavin, Ait-Azzouzene, Ware, & Nemazee, 2003; Gavin et al., 2005).

After the discovery of BAFF, studies utilizing BAFF-transgenic mice noted not only the development of more B cells and larger lymphoid organs, but also the development of a disease similar to systemic lupus erythematosus (SLE) (Mackay et al., 1999; Thien et al., 2004). Since then, the link between increased BAFF and autoimmune disease has been strengthened through associations found in many diseases including SLE, Sjogren's syndrome, rheumatoid arthritis, and immune thrombocytopenia (Emmerich et al., 2007; Pers

et al., 2005). The link between BAFF and autoimmune disease is so strong that monoclonal antibodies against BAFF have been approved for use to treat SLE (Navarra et al., 2011).

BAFF has been previously described in dogs and noted to have a similar structure and function in stimulating B cell survival (Shen et al., 2011). However, this work dealt primarily with recombinant BAFF produced in *E. coli* rather than BAFF isolated from canine tissues. Despite the clear connection between autoimmune disease and BAFF in humans, there are few published studies evaluating BAFF levels in dogs with analogous autoimmune diseases, and only one commercial assay to quantify BAFF exists (Maeda et al., 2014; Maeda et al., 2013). There has been no characterization of canine  $\Delta$ BAFF. Further characterization of BAFF in canine tissues would facilitate future study of this cytokine.

Given that BAFF is produced by both human and murine macrophages (Kim et al., 2011; Munari et al., 2014), we selected a canine macrophage cell line (030-D) to evaluate for BAFF and  $\Delta$ BAFF. Here we describe BAFF and  $\Delta$ BAFF production in 030-D cells through RT-qPCR, Western blotting, and validation of a commercially available canine BAFF ELISA, as well as identification of native canine BAFF via Western blotting with splenic tissue lysates.

## **2.2 Materials and Methods**

### **2.2.1 RNA extraction, cDNA synthesis, and RT-PCR of 030-D cells**

030-D cells were cultured at 37°C with 5% CO<sub>2</sub> in RPMI with glutamine with 10% FBS for 5 days or until post-confluency. Total RNA extraction was performed using TRIzol

LS (ThermoFisher Scientific, Grand Island, NY) as per the manufacturer's instructions.

Purified RNA was quantified via NanoDrop Spectrophotometry. For reverse transcription to synthesize cDNA, 100ng of RNA was combined with distilled water to a volume of 19.25µl, 11µl 25mM MgCl<sub>2</sub>, 5µl 10x RT buffer, 10µl 2.5mM dNTPs, 2.5µl 50uM random hexamers, 1µl 20U/ul RNase inhibitor, and 1.25µl 50U/ul reverse transcriptase as part of a commercial kit (TaqMan Gold RT-PCR, ThermoFisher Scientific, Grand Island, NY). Samples were held at 25°C for 10 minutes, followed by 48°C for 1 hour, and 95°C for 5 min.

Intron-spanning primers were manually designed using a putative BAFF homologue (NM\_001161710.2) in the canine genome to amplify a 129 base pair region of the mRNA and consisted of Forward (Baff F1: 5'CCTCAGCTCTGAACGGAATC3') and Reverse (Baff R1: 5'GCAATCAGTTGCAAGCAGTC3'). Each 50µl reaction consisted of 1µl cDNA, 25µl Power SYBR Green Master Mix (ThermoFisher Scientific, Grand Island, NY), 0.5µl 50uM Fwd primer, 0.5µl 50uM Rev primer, and 25µl water. Reaction times included an initial 3min at 95C, followed by 45 cycles of 10sec at 95C, 20sec at 58C, and 30sec at 72C. Amplicons were visualized on an agarose gel and bidirectionally sequenced (Genewiz, South Plainfield, NJ), with the sequence constructs submitted to a basic local alignment search tool (BLAST) to confirm 100% homology with the expected product from NM\_001161710.2.

### **2.2.2 030-D Cell lysis and Immunoblotting**

030-D cells cultured as described above were harvested for immunoblotting both with and without the use of extraction buffers. Initially, cells were harvested by removal of culture media, washing with ice cold phosphate buffered saline (PBS), and addition of 0.5ml per

$5 \times 10^6$  cells/60mm dish/75cm<sup>2</sup> flask) RIPA Lysis and Extraction Buffer (ThermoFisher Scientific, Grand Island, NY) or 5x Extraction Buffer (abcam, Cambridge, Mass.). Adherent cells were removed from the plate using a cold plastic scraper and transferred into a precooled microcentrifuge tube, which was then agitated constantly for 30 min at 4°C, followed by centrifuging at 12,000 rpm for 20 min at 4°C. The resultant supernatant was decanted and stored at -80°C until analysis.

Without the use of extraction buffers, cells were harvested by removal of culture media followed by washing with ice-cold PBS, manual scraping of cells and collection in a precooled microcentrifuge tube, centrifugation and washing with PBS three times, resuspension in ice-cold PBS, and sonication for 30 seconds three times, three freeze-thaw cycles, or both, followed by centrifugation at 3,000rpm for 15min at 4°C.

Whole lysate protein was quantified with the use of a commercially available kit (QuantiPro BCA Assay, Sigma Aldrich). The protocol for this assay was modified in that the standard was prepared at the following concentrations: 2mg/ml, 1.5mg/ml, 1mg/ml, 0.75mg/ml, 0.5mg/ml, 0.25mg/ml, 0.125mg/ml, and 0.025mg/ml, 25µl of each standard or sample was used in duplicate, 200µl of working reagent (250µl Cu<sup>++</sup>, 6.25ml QA, 6.25ml QB) was added to each well, and the plate was incubated at 37C for 30 minutes. Optical density (OD) was measured at a wavelength of 562nm with a microplate reader.

Polyacrylamide gel electrophoresis was performed on each of the whole cell lysate preparations using 4-12% Bis-Tris gradient gels under denaturing conditions. Proteins were transferred to a PVDF membrane and after blocking were probed with an anti-human BAFF

primary antibody (EMD Millipore, Germany) and an HRP-labeled appropriate secondary antibody. Chemiluminescence was detected using a commercially available chemiluminescent HRP substrate (Immobilon Western, Millipore, Billerica, Mass.). A commercially available recombinant canine BAFF fragment (Kingfisher Biotech, St. Paul, Minn.) was used as a positive control.

### **2.2.3 ELISA quantification of 030-D BAFF protein**

A commercially available canine BAFF ELISA (amsbio, Lake Forest, Calif.) was evaluated for its ability to detect BAFF in each of the three cell lysate preparations as described above as well as 030-D protein extractions in RIPA Lysis and Extraction Buffer (ThermoFisher Scientific, Grand Island, NY) and 5x Extraction Buffer (abcam, Cambridge, Mass.). The ELISA was initially performed as per the manufacturer's instructions, with the use of a Balance solution in the cell lysate preparations.

To evaluate the influence of the extraction buffers on the ELISA capture antibody the kit standard solution was diluted from a concentration of 5000pg/ml to 2500pg/ml, 1000pg/ml, 500pg/ml, 250pg/ml, and 0pg/ml (extraction buffer only) with each extraction buffer in addition to being run as described in the kit instructions. The ELISA was also performed once with an extra wash step between the incubation with samples/standards and application of the secondary antibody, to assess the affect this may have on interference from the extraction buffers.

For each of the three extraction buffer-free protein extraction methods, each sample was run neat, diluted 1:2 with PBS (frozen, sonicated, and both lysates), or extraction buffer,

and diluted 1:4 with PBS (frozen, sonicated, and both lysates), or extraction buffer.

Additionally, each dilution of the physically disrupted cells was run in duplicate, with one set boiled for 5min prior to application. A commercially available recombinant canine BAFF fragment (Kingfisher Biotech, St. Paul, Minn.) was used as an additional positive control. Optical density (OD) of the samples was measured at a wavelength of 462nm with a microplate reader, as per the kit's instructions.

#### **2.2.4 RT-PCR for identification of $\Delta$ BAFF in 030-D cells**

Amino acid and mRNA alignments of human and murine  $\Delta$ BAFF (acc. nos. GenBank: AY302751.1 and GenBank: AY290823) showed each missing nearly completely homologous (49/57nt) nucleotide sequences, although different predicted exons (exon 3 in humans and exon 4 in mice). The missing amino acid sequence was aligned with the reported exon 3 of canine BAFF (NM\_001161710.2). Primers were designed manually to flank exon three, resulting in a BAFF product of 257bp and  $\Delta$ BAFF product of 200bp in the same reaction, and consisted of Forward (Baff F1: 5'CCTCAGCTCTGAACGGAATC3') and Reverse (Baffe4PR: 5'GTAACCGGCTTCTTTTACAAGT3'). Each 50 $\mu$ l reaction consisted of 1 $\mu$ l previously synthesized 030-D cDNA, 25 $\mu$ l Power SYBR Green Master Mix (ThermoFisher Scientific, Grand Island, NY), 0.5 $\mu$ l 50uM Fwd primer, 0.5 $\mu$ l 50uM Rev primer, and 25 $\mu$ l water. Reaction times included an initial 5min at 95C, followed by 45 cycles of 15sec at 95C, 30sec at a gradient from 55-62C, and 60sec and 72C. Amplicons were visualized on an agarose gel, extracted from the gel and purified via a commercial kit per the manufacturer's instructions (QIAQuick, Valencia, Calif.), and bidirectionally

sequenced (Genewiz, South Plainfield, NJ), with the sequence constructs submitted to a basic local alignment search tool (BLAST) to confirm 100% homology with the expected products.

### **2.2.5 Splenic cell lysis and immunoblotting**

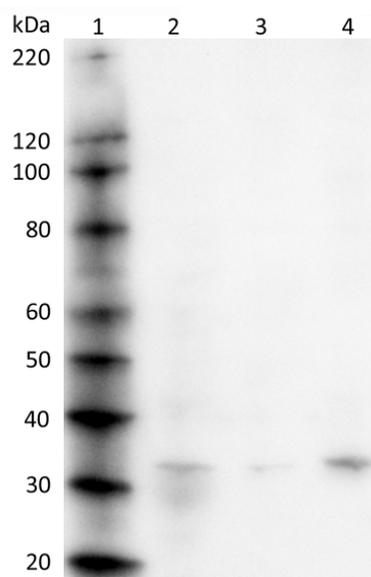
Splenic tissue was obtained from a recently deceased, previously healthy adult terrier mix dog and frozen at -80C until processing. For protein extraction, 0.2g of splenic tissue was washed with ice cold PBS until the PBS remained clear, minced and homogenized in 1.5ml cold PBS with a glass homogenizer, followed by three cycles of sonication and centrifugation as described previously for the 030-D cells. The resultant supernatant was decanted and used in immunoblotting as described above to assess the presence of BAFF in canine splenic tissue.

## **2.3 Results**

### **2.3.1 Production of BAFF mRNA by 030-D cells**

The canine macrophage cell line 030-D expresses BAFF mRNA as indicated by PCR amplification of a 129bp segment of the BAFF mRNA sequence. The identity of this amplicon was confirmed via agarose gel electrophoresis and bidirectional sequencing.

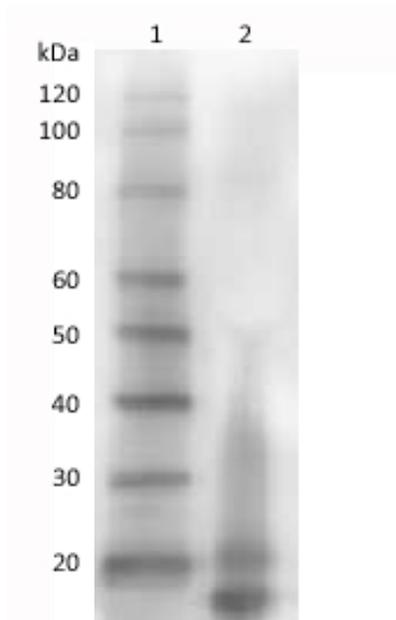
### 2.3.2 Production of BAFF protein by 030-D cells



**Figure 1. Western blot of 030-D cell lysates and canine splenic tissue.** Magic Mark XP Standard (lane 1) shows bands in lanes 2 (spleen), 3 (RIPA extracted 030-D), and 4 (Extraction Buffer extracted 030-D) at approximately 32kDa. This is consistent with the reported size of canine full length BAFF.

The canine macrophage cell line 030-D expresses BAFF protein as indicated by Western blotting and detection of an appropriately sized 32kDa product for various extraction methods with 030-D cells (Figure 1). The 17kDa commercially available recombinant canine BAFF fragment was also detected using this antibody pair (Figure 2).

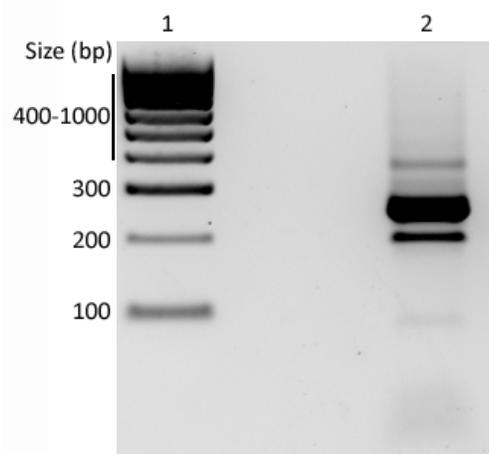
### 2.3.3 Failure of a commercially available canine BAFF ELISA to detect BAFF in 030-D cell lysate



**Figure 2. Western blot of commercially available recombinant canine BAFF fragment.** The Magic Mark XP Standard (lane 1) shows a band of approximately 17 kDa in the Kingfisher recombinant canine BAFF (lane 2) consistent with the product's reported size.

A commercially available canine BAFF ELISA (amsbio, Cambridge, Mass.) failed to detect canine BAFF in any of the 030-D lysates positive for BAFF protein via Western blotting or the purchased recombinant canine BAFF protein. Boiling the lysates failed to enhance detection, as did 1:2 and 1:4 dilutions. Protein extraction from 030-D cells was attempted with RIPA lysis and extraction buffer (ThermoFisher Scientific, Grand Island, NY) and 5x Extraction buffer (abcam, Cambridge, Mass.) as well, but each buffer was noted to interfere with the ELISA standard detection.

### 2.3.4 Production of $\Delta$ BAFF mRNA by 030-D cells



**Figure 3. Agarose gel electrophoresis of RT-PCR product identifying deltaBAFF.** RT-PCR utilizing BAFF primers spanning the Exon 1-2 junction (fwd) and Exon 4 (rev) resulted in two products (Lane 2) of the expected 257bp and 200bp sizes corresponding with BAFF and deltaBAFF respectively.

The canine macrophage cell line 030-D expresses  $\Delta$ BAFF mRNA as indicated by PCR amplification of a 200bp segment of the BAFF mRNA sequence using the previously described primer pair. The identity of this amplicon was confirmed via agarose gel electrophoresis (Figure 3) and bidirectional sequencing showing a missing homologous section of mRNA corresponding with exon 3 in the dog and human.

### 2.3.5 Presence of BAFF protein in healthy canine splenic tissue

Canine BAFF protein was detected in splenic tissue from a healthy animal as assessed by Western blotting and detection of an appropriately sized 32kDa product (Figure 1). The 030-D lysates were used as positive controls for the assay.

## 2.4 Discussion

Herein we document production of BAFF and  $\Delta$ BAFF mRNA and BAFF protein by a canine macrophage cell line (030-D), as well as BAFF presence in splenic tissue from a healthy dog. Currently, it is known that BAFF production is increased in many disease states in humans (Emmerich et al., 2007; Pers et al., 2005; Zhu et al., 2009). BAFF in dogs has been investigated only in the etiology of selective mucosal IgA deficiency in intestinal biopsies and as a candidate gene in Nova Scotia Duck Tolling Retrievers with hypoadrenocorticism (Hughes, Bannasch, Kellett, & Oberbauer, 2011; Maeda et al., 2013). In both humans and dogs, little is known about what drives or suppresses BAFF production outside the bounds of homeostasis. This cell line could provide valuable information regarding the production of canine BAFF in response to certain cytokines, infectious agents, and other physiologic states (Coquery et al., 2014; Yang et al., 2011). Additionally, the development of these assays provides a reliable method to quantify BAFF transcription in various disease states in dogs.

This study failed to validate a commercially available canine BAFF ELISA, and thus it cannot be recommended as a tool to measure canine BAFF protein. Lysates of 030-D cells, which had been noted to contain BAFF protein based on the Western blotting described above, were not detected by the ELISA. To explore reasons for the lack of detection alternative extraction buffers were used, physical disruption rather than chemical disruption of the cells was used, the cell lysate samples were boiled to attempt to eliminate the possibility of post-translational folding shielding the epitope, additional wash steps were

added to remove extraneous debris, and canine recombinant BAFF protein was used as an additional positive control.

Ideally, the standards within the kit, the lysates from the 030-D cells, and the purchased recombinant canine BAFF would be analyzed via mass spectrometry to confirm the presence of BAFF protein. Other veterinary ELISAs that suffered similar validation problems have been found lacking the protein of interest in the provided standards (Floras et al., 2014). Other possibilities include that the BAFF produced by the 030-D cells exists in a form that shields the detection or capture epitopes. It is known that secreted human BAFF can exist in many forms including homotrimers, heterotrimers, and 60-mers (Mackay & Schneider, 2009). If canine BAFF also exists in these forms it is possible the quaternary structure may preclude epitope binding in this ELISA.

Ultimately, while the commercially available ELISA tested here was not validated for the measurement of canine BAFF, the ability to detect canine BAFF via Western blotting in 030-D cell lysates makes the possibility of the development of a new canine-specific BAFF ELISA possible. Alternatively, quantitative Western blotting could be used to determine the amount of canine BAFF present in tissues and sample going forward (Eaton et al., 2014), or alternative protein quantification methods such as mass spectrometry could be used.

Developing an assay to quantify BAFF protein concentration reliably will be important as it is known that mRNA levels do not always correlate well with protein levels (Gygi, Rochon, Franza, & Aebersold, 1999). However, the assays to identify BAFF mRNA described here can in the future be used on canine samples both in health, to establish a

reference range, and in disease states similar to those in humans with abnormal BAFF transcription noted. Additionally, the canine macrophage cell line 030-D could be used to gain a better understanding of the processes driving canine BAFF production in health and disease.

## References

- Bossen, C., & Schneider, P. (2006). BAFF, APRIL and their receptors: structure, function and signaling. *Semin Immunol*, *18*(5), 263-275. doi:10.1016/j.smim.2006.04.006
- Coquery, C. M., Wade, N. S., Loo, W. M., Kinchen, J. M., Cox, K. M., Jiang, C., . . . Erickson, L. D. (2014). Neutrophils contribute to excess serum BAFF levels and promote CD4+ T cell and B cell responses in lupus-prone mice. *PLoS One*, *9*(7), e102284. doi:10.1371/journal.pone.0102284
- Eaton, S. L., Hurtado, M. L., Oldknow, K. J., Graham, L. C., Marchant, T. W., Gillingwater, T. H., . . . Wishart, T. M. (2014). A Guide to Modern Quantitative Fluorescent Western Blotting with Troubleshooting Strategies. (93), e52099. doi:doi:10.3791/52099
- Emmerich, F., Bal, G., Barakat, A., Milz, J., Muhle, C., Martinez-Gamboa, L., . . . Salama, A. (2007). High-level serum B-cell activating factor and promoter polymorphisms in patients with idiopathic thrombocytopenic purpura. *Br J Haematol*, *136*(2), 309-314. doi:10.1111/j.1365-2141.2006.06431.x
- Floras, A. N., Holowaychuk, M. K., Hodgins, D. C., Marr, H. S., Birkenheuer, A., Sharif, S., . . . Bienzle, D. (2014). Investigation of a commercial ELISA for the detection of canine procalcitonin. *J Vet Intern Med*, *28*(2), 599-602. doi:10.1111/jvim.12309
- Gavin, A. L., Ait-Azzouzene, D., Ware, C. F., & Nemazee, D. (2003). DeltaBAFF, an alternate splice isoform that regulates receptor binding and biopresentation of the B cell survival cytokine, BAFF. *J Biol Chem*, *278*(40), 38220-38228. doi:10.1074/jbc.M306852200
- Gavin, A. L., Duong, B., Skog, P., Ait-Azzouzene, D., Greaves, D. R., Scott, M. L., & Nemazee, D. (2005). deltaBAFF, a splice isoform of BAFF, opposes full-length BAFF activity in vivo in transgenic mouse models. *J Immunol*, *175*(1), 319-328.
- Gygi, S. P., Rochon, Y., Franza, B. R., & Aebersold, R. (1999). Correlation between protein and mRNA abundance in yeast. *Mol Cell Biol*, *19*(3), 1720-1730.
- Huard, B., Arlettaz, L., Ambrose, C., Kindler, V., Mauri, D., Roosnek, E., . . . French, L. E. (2004). BAFF production by antigen-presenting cells provides T cell co-stimulation. *Int Immunol*, *16*(3), 467-475.
- Hughes, A. M., Bannasch, D. L., Kellett, K., & Oberbauer, A. M. (2011). Examination of candidate genes for hypoadrenocorticism in Nova Scotia Duck Tolling Retrievers. *Vet J*, *187*(2), 212-216. doi:10.1016/j.tvjl.2009.10.012

- Kim, H. A., Seo, G. Y., & Kim, P. H. (2011). Macrophage-derived BAFF induces AID expression through the p38MAPK/CREB and JNK/AP-1 pathways. *J Leukoc Biol*, 89(3), 393-398. doi:10.1189/jlb.1209787
- Mackay, F., & Schneider, P. (2009). Cracking the BAFF code. *Nat Rev Immunol*, 9(7), 491-502. doi:10.1038/nri2572
- Mackay, F., Woodcock, S. A., Lawton, P., Ambrose, C., Baetscher, M., Schneider, P., . . . Browning, J. L. (1999). Mice transgenic for BAFF develop lymphocytic disorders along with autoimmune manifestations. *J Exp Med*, 190(11), 1697-1710.
- Maeda, S., Ohno, K., Fujiwara-Igarashi, A., Tomiyasu, H., Fujino, Y., & Tsujimoto, H. (2014). Methylation of TNFRSF13B and TNFRSF13C in duodenal mucosa in canine inflammatory bowel disease and its association with decreased mucosal IgA expression. *Vet Immunol Immunopathol*, 160(1-2), 97-106. doi:10.1016/j.vetimm.2014.04.005
- Maeda, S., Ohno, K., Uchida, K., Nakashima, K., Fukushima, K., Tsukamoto, A., . . . Tsujimoto, H. (2013). Decreased immunoglobulin A concentrations in feces, duodenum, and peripheral blood mononuclear cells of dogs with inflammatory bowel disease. *J Vet Intern Med*, 27(1), 47-55. doi:10.1111/jvim.12023
- Moore, P. A., Belvedere, O., Orr, A., Pieri, K., LaFleur, D. W., Feng, P., . . . Hilbert, D. M. (1999). BLyS: member of the tumor necrosis factor family and B lymphocyte stimulator. *Science*, 285(5425), 260-263.
- Munari, F., Fassan, M., Capitani, N., Codolo, G., Vila-Caballer, M., Pizzi, M., . . . de Bernard, M. (2014). Cytokine BAFF released by Helicobacter pylori-infected macrophages triggers the Th17 response in human chronic gastritis. *J Immunol*, 193(11), 5584-5594. doi:10.4049/jimmunol.1302865
- Nardelli, B., Belvedere, O., Roschke, V., Moore, P. A., Olsen, H. S., Migone, T. S., . . . Hilbert, D. M. (2001). Synthesis and release of B-lymphocyte stimulator from myeloid cells. *Blood*, 97(1), 198-204.
- Navarra, S. V., Guzman, R. M., Gallacher, A. E., Hall, S., Levy, R. A., Jimenez, R. E., . . . Group, B.-S. (2011). Efficacy and safety of belimumab in patients with active systemic lupus erythematosus: a randomised, placebo-controlled, phase 3 trial. *Lancet*, 377(9767), 721-731. doi:10.1016/S0140-6736(10)61354-2
- Pers, J. O., Daridon, C., Devauchelle, V., Jousse, S., Saraux, A., Jamin, C., & Youinou, P. (2005). BAFF overexpression is associated with autoantibody production in autoimmune diseases. *Ann N Y Acad Sci*, 1050, 34-39. doi:10.1196/annals.1313.004

- Schneider, P., MacKay, F., Steiner, V., Hofmann, K., Bodmer, J. L., Holler, N., . . . Tschopp, J. (1999). BAFF, a novel ligand of the tumor necrosis factor family, stimulates B cell growth. *J Exp Med*, *189*(11), 1747-1756.
- Shen, Y., Wang, S., Ai, H., Min, C., Chen, Y., & Zhang, S. (2011). Molecular structure, bioinformatics analysis, expression and bioactivity of BAFF (TNF13B) in dog (*Canis familiaris*). *Vet Immunol Immunopathol*, *142*(1-2), 133-139. doi:10.1016/j.vetimm.2011.04.014
- Thien, M., Phan, T. G., Gardam, S., Amesbury, M., Basten, A., Mackay, F., & Brink, R. (2004). Excess BAFF rescues self-reactive B cells from peripheral deletion and allows them to enter forbidden follicular and marginal zone niches. *Immunity*, *20*(6), 785-798. doi:10.1016/j.immuni.2004.05.010
- Yang, Q., Xu, S., Li, X., Wang, B., Wang, X., Ma, D., . . . Hou, M. (2011). Pathway of Toll-like receptor 7/B cell activating factor/B cell activating factor receptor plays a role in immune thrombocytopenia in vivo. *PLoS One*, *6*(7), e22708. doi:10.1371/journal.pone.0022708
- Zhu, X. J., Shi, Y., Peng, J., Guo, C. S., Shan, N. N., Qin, P., . . . Hou, M. (2009). The effects of BAFF and BAFF-R-Fc fusion protein in immune thrombocytopenia. *Blood*, *114*(26), 5362-5367. doi:10.1182/blood-2009-05-217513

## **CHAPTER THREE: B CELL ACTIVATING FACTOR mRNA EXPRESSION IN DOGS WITH PRIMARY IMMUNE-MEDIATED THROMBOCYTOPENIA**

### **3.1 Introduction**

Primary immune-mediated thrombocytopenia (ITP) is a disease in dogs in which B cells produce antibodies directed against platelet surface self-antigens leading to platelet destruction and resultant clinical bleeding. Dogs with ITP experience high rates of mortality (10-30%), and disease relapse (9-40%) (Lewis & Meyers, 1996; O'Marra, Delaforcade, & Shaw, 2011; Putsche & Kohn, 2008). Treatment requires the use of immunosuppressive doses of corticosteroids with the frequent addition of a second immunosuppressive drug to attenuate the adverse immune response driving the platelet destruction (O'Marra et al., 2011). Several retrospective studies of ITP and individual case reports list complications attributed to various therapies such as leukopenia, urinary tract infections, skin abscesses, hepatopathy, and pancreatitis (Hsu, Snead, Davies, & Carr, 2012; O'Marra et al., 2011; Putsche & Kohn, 2008; Wallisch & Trepanier, 2015). Corticosteroids can be particularly damaging long term as their continual use has been associated with increased risk of thrombosis, iatrogenic hyperadrenocorticism, secondary infection, bone demineralization, and gastric ulceration (Behrend & Kemppainen, 1997; Costa et al., 2010; Fry, McSparran, Ellis, & Harvey, 2009; Hsu et al., 2012; Huang, Yang, Liang, Lien, & Chen, 1999; Jacoby, Owings, Ortega, Gosselin, & Feldman, 2001; Laurensen, Hopper, Herrera, & Johnson, 2010; Rose, Dunn, Allegret, & Bedard, 2011).

Normalization of platelet numbers is the primary biomarker used to assess control of the immune response driving platelet destruction, and as platelet counts rise, immunosuppressive drug dosages are decreased slowly over time. Due to the risk of relapse and lack of definitive methods to demonstrate immunosuppression, treatment duration often lasts months to over a year (O'Marra et al., 2011). This inexact method results in prolonged treatment of some dogs in which shorter durations of treatment may have been effective and inadequate duration of treatments and resultant relapse in other dogs, demonstrating a critical need for a precise biomarker of active ITP in dogs.

In humans with ITP a cytokine, B cell activating factor (BAFF), is increased during active disease and decreases as the aberrant immune response is controlled. BAFF, a member of the tumor necrosis factor family of cytokines, plays a crucial role in B cell growth, survival, and class switching (Schneider et al., 1999). Antigen presenting cells, T cells and peripheral blood mononuclear cells produce BAFF both constitutively and in response to other cytokines such as interferons, IL-10 and G-CSF (Huard et al., 2004; Nardelli et al., 2001). Several independent studies have demonstrated increased concentrations of BAFF in humans with active ITP compared with humans with secondary ITP, healthy controls, or patients with ITP in remission (Emmerich et al., 2007; Thomas, Machin, Mackie, & Scully, 2011; Zhu, Shi, Peng, et al., 2009). These characteristics of BAFF may make it an ideal biomarker to aid in the diagnosis and treatment of ITP in dogs.

Only a few published reports exist evaluating BAFF in dogs (Maeda et al., 2014; Maeda et al., 2013; Shen et al., 2011), and none have been published evaluating BAFF in

canine ITP. The objective of this study was to quantify and compare the transcription of BAFF in the blood of dogs with untreated ITP, dogs with secondary thrombocytopenia, and healthy control dogs. We hypothesized that BAFF expression as demonstrated by RT-qPCR would be elevated in dogs with untreated ITP compared with dogs with secondary thrombocytopenia and healthy dogs. Here we demonstrate that BAFF mRNA is significantly and specifically elevated in dogs with ITP compared with secondary thrombocytopenia and healthy control dogs.

## **3.2 Materials and Methods**

### **3.2.1 Study Population**

EDTA-anticoagulated whole blood was collected from 10 client-owned dogs that were presented to the Veterinary Hospital at the North Carolina State University College of Veterinary Medicine (NCSU-CVM) from November 2014 to October 2015 and diagnosed with primary ITP. Dogs were eligible for inclusion with a clinician diagnosis of ITP, initial platelet count less than 50,000 cells/ $\mu$ l, and response to treatment for ITP in surviving dogs (Putsche & Kohn, 2008). Exclusion criteria included administration of immunosuppressive medications or medications known to be associated with ITP prior to sample collection for this study, identification of neoplasia on abdominal imaging, thoracic radiographs, or necropsy, or vector-borne disease testing results consistent with infections known to cause thrombocytopenia.

Blood from dogs with secondary causes of thrombocytopenia were drawn from a prior study on sepsis, SIRS, and neoplasia (Cruse, 2009). Healthy control dogs were drawn from a population of dogs presenting to NCSU-CVM during the same time period for wellness exams with normal platelet counts and previous control dogs from the aforementioned SIRS study. The NCSU Animal Care and Use Committee approved the study.

### **3.2.2 RNA Isolation & cDNA Synthesis**

EDTA-anticoagulated whole blood was collected within 24 hours of ITP dog admission, preserved with ZR RNA Buffer (Zymo Research, Irvine, Calif.), and stored at -80°C. Total RNA was later isolated via a ZR Whole Blood Total RNA kit (Zymo Research, Irvine, Calif.) according to the manufacturer's instructions. Purified RNA was quantified by NanoDrop Spectrophotometry. For each sample, 100ng of RNA was reverse transcribed into cDNA using a commercially available kit (TaqMan Gold RT-PCR Kit, ThermoFisher Scientific). Each 50µl reaction consisted of 100ng RNA combined with distilled water to a volume of 19.25µl, 11µl 25mM MgCl<sub>2</sub>, 5µl 10x RT buffer, 10µl 2.5mM dNTPs, 2.5µl 50uM random hexamers, 1µl 20U/ul RNase inhibitor, 1.25µl 50U/ul reverse transcriptase. Samples were held at 25°C for 5 minutes, followed by 48°C for 1 hour, and 95°C for 5 min.

### **3.2.3 Quantitative RT-PCR for BAFF**

Using a putative BAFF homologue (NM\_001161710.2) in the canine genome intron-spanning primers to amplify a 129 base pair region of the mRNA were manually designed and consisted of Forward (Baff F1: 5'CCTCAGCTCTGAACGGAATC3') and Reverse (Baff

R1 5'GCAATCAGTTGCAAGCAGTC3'). After reverse transcription, RT-qPCR was performed to evaluate expression of BAFF using  $\beta$ -actin as a reference gene. Each 50 $\mu$ l reaction consisted of 1 $\mu$ l cDNA, 25 $\mu$ l Power SYBR Green Master Mix (ThermoFisher Scientific, Grand Island, NY), 0.5 $\mu$ l 50uM Fwd primer, 0.5 $\mu$ l 50uM Rev primer, and 23 $\mu$ l water. Thermal cycling conditions (MJ Mini, Bio-rad Laboratories, Inc. Hercules, CA) for the BAFF PCR consisted of an initial denaturing for 3min at 95C, followed by 45 amplification cycles of 95C for 10 seconds, annealing at 58C for 20 seconds, and 30 seconds of extension at 72C.

For the  $\beta$ -actin PCR each 50  $\mu$ L reaction contained 25  $\mu$ L of 2X SYBR Green PCR Master Mix, 0.5 $\mu$ l of each 50uM primer ( $\beta$ -actin forward 5'-GACCCTGAAGTACCCCATGAG-3' and  $\beta$ -actin reverse 5'-TTGTAGAAGGTGTGGTGCCAGAT-3'), 1  $\mu$ L of cDNA template, and 23 $\mu$ l water. Thermal cycling conditions (MJ Mini, Bio-Rad Laboratories, Inc., Hercules, CA) consisted of an initial denaturation step at 95C for 3 minutes followed by 45 amplification cycles (95C for 10 seconds, 64.4°C for 20 seconds and 72°C for 20 seconds).

These assays do not amplify canine genomic DNA and under optimized reaction conditions have an amplification efficiency of 100%. Melt curve analysis was performed to confirmed expected products.

#### 3.2.4 Statistics

Preliminary data was used to perform an a priori power analysis with a balanced ANOVA (f-test) calculator (G\*Power 3.1). Based on these calculations samples from a total

of 30 dogs (10 in each group) would be necessary for a power of 0.85 with  $\alpha$  error probability of 0.05 and an effect size of 0.65 for the three groups (dogs with active primary ITP, dogs with secondary thrombocytopenia, and healthy control patients). Data from RT-qPCR was analyzed by comparison of the  $\Delta C_T$  values obtained from the two control populations and the  $\Delta C_T$  values from dogs with primary ITP. The fold change in gene expression relative to the control subjects was calculated as  $2^{(-\Delta\Delta C_T)}$ , where  $\Delta\Delta C_T = \text{mean } \Delta C_T \text{ control group} - \Delta C_T \text{ ITP group}$ , as previously described (Schmittgen & Livak, 2008). Statistical analysis was performed with the use of commercially available software (GraphPad Prism, La Jolla, Calif.). The difference between groups of nonparametric data was compared with a Kruskal-Wallis test. Statistical significance was assigned a p value of  $\leq 0.05$ .

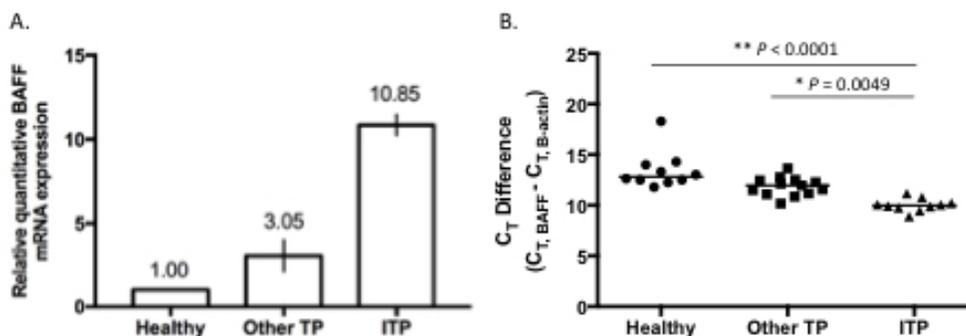
### 3.3 Results

#### 3.3.1 Study populations

EDTA-anticoagulated whole blood was collected from 10 dogs with ITP. The breeds represented were two each of Maltese and Labrador retrievers, and one each of the following: West Highland white terrier, American cocker spaniel, German shepherd dog, miniature poodle, shih tzu, and Jack Russell terrier. Ages of the dogs with ITP ranged from 2 years to 12.5 years (median: 8.20 years), with 6 male castrated dogs, 1 intact male, and 3 spayed females. In this group, 9/10 dogs (90%) survived to discharge, although three were subsequently euthanized due to complications related to treatment of their disease.

The dogs with secondary causes of thrombocytopenia comprised 10 dogs with SIRS and 3 dogs with neoplasia, with an age range of 1.92 years to 14.5 years (median: 6.00 years), and sex distribution of 8 male castrated, 3 female spayed, 1 male intact and 1 female intact. The most commonly represented breed was Labrador retriever (n=2), with one each of the following: Basset hound, beagle, Belgian tervuren, border collie, bull terrier, collie, corgi, great Dane, great Pyrenees, Rottweiler, and Shetland sheepdog.

The 10 healthy control dogs ranged in age from 0.5 years to 9.92 years (median: 5.29 years) and was comprised of 6 spayed females, 2 castrated males, and 2 intact males. The most common breeds in the control group were golden retriever, Italian greyhound, and Portuguese water dog with 2 each, and one each of Jack Russell terrier, hound, boxer dog, and Shiba Inu. There was no statistically significant difference in age between any of the groups ( $p=0.1490$ ).



**Figure 1. Relative quantitative BAFF mRNA expression in dogs with ITP compared with other causes of thrombocytopenia and healthy controls.** (A) The ratios of BAFF mRNA in dogs with ITP compared to other causes of thrombocytopenia and healthy dogs are 3.05 and 10.85, respectively. (B) Individual cycle threshold differences for BAFF and B-actin in each of the three groups. Average cycle threshold difference was significantly different between dogs with ITP and healthy dogs, and dogs with other causes of thrombocytopenia and dogs with ITP.

**Table 1.** Individual cycle threshold values for RT-qPCR for BAFF and  $\beta$ -actin. The average difference between the values for each group is used to calculate the relative quantity of BAFF compared to healthy animals.

	$C_T$ $\beta$ -actin	$C_T$ BAFF	$\Delta C_T$	Average $\Delta C_T$ BAFF – $\beta$ -actin	$-\Delta\Delta C_T$ $-(\Delta C_T - \Delta C_{T, \text{Healthy}})$	RQ BAFF Rel. to Healthy
ITP (n=10)	24.42	35.17	10.75	10.03 ( $\pm 0.64$ )	3.44	10.85
	22.61	31.54	8.9			
	24.02	33.91	9.89			
	24.24	34.31	10.07			
	24.77	34.82	10.05			
	25.16	34.92	9.76			
	25.13	34.59	9.46			
	25.37	35.59	10.22			
	24.14	34.07	9.93			
	26.46	37.63	11.17			
Other TP (n=13)	24.92	36.01	11.09	11.86 ( $\pm 0.93$ )	1.61	3.05
	19.59	33.25	13.66			
	20.27	32.23	11.96			
	19.22	30.74	11.52			
	21.07	32.25	11.18			
	22.35	33.21	10.86			
	20.54	33.33	12.79			
	21.2	32.78	11.58			
	18.85	29.02	10.17			
	19.54	32.01	12.47			
Healthy (n=10)	27.07	39.35	12.28	13.47 ( $\pm 1.87$ )	0	1
	21.24	33.77	12.53			
	22.05	34.19	12.14			
	24.26	36.06	11.8			
	28.07	42.4	14.3			
	23.73	42.02	18.3			
	23.61	36.24	12.63			
	23.84	36.11	12.27			
	23.1	35.62	12.52			
	21.98	35.98	14			
21.03	33.53	12.5				
21.69	34.72	13.03				
21.71	35.04	13.33				

### 3.3.2 BAFF mRNA is increased in dogs with ITP compared to those with secondary thrombocytopenia and healthy controls

Use of the  $\Delta\Delta C_T$  method to analyze RT-qPCR showed BAFF mRNA levels 10.85 times higher in the ITP group compared to healthy dogs, and 3.05 times higher than dogs

with secondary causes of thrombocytopenia (Figure 1, Table 1). There was a significant difference in BAFF mRNA concentration between ITP and healthy controls ( $p < 0.0001$ ) and between ITP and secondary thrombocytopenia ( $p = 0.0049$ ). There was not a significant difference in BAFF mRNA levels between healthy control dogs and dogs with secondary causes of thrombocytopenia ( $p = 0.1691$ ).

### 3.4 Discussion

These results demonstrate that BAFF transcription is significantly higher in dogs with ITP compared with dogs with other causes of thrombocytopenia and healthy dogs. This is the first study in dogs evaluating BAFF transcription in disease, and offers evidence that BAFF may be a biomarker for primary ITP in dogs compared with other causes of thrombocytopenia.

Dogs with ITP are at significant risk of both mortality and relapse (Lewis & Meyers, 1996; O'Marra et al., 2011; Putsche & Kohn, 2008), and for surviving dogs and owners the financial and emotional burdens of long treatment durations can be taxing. Currently, platelet count is the most commonly used biomarker to assess disease status in dogs with ITP, however this imprecise measurement of the immune response driving platelet destruction leads to treatment durations too long for some dogs and too short for others. In the past, attempts have been made to use the presence of platelet associated IgG (PAIgG) as a marker of ITP, however this diagnostic aid has a number of shortcomings. In humans measurement of PAIgG is rarely used in the diagnosis or monitoring of ITP as while the finding of

antibodies on platelets is moderately sensitive, a number of other conditions can lead to elevations in PAIgG including sepsis, disseminated intravascular coagulation, and viral infection (Beardsley & Ertem, 1998), making it a low specificity test. Additionally, PAIgG levels are also affected by several inherent factors including the age of the platelets (Beardsley & Ertem, 1998). In veterinary medicine PAIgG has not frequently been used due to low availability of the assay and its inability to distinguish primary (autoimmune) ITP from other causes such as infection and neoplasia (Dircks, Schuberth, & Mischke, 2009).

In humans with ITP, serum and plasma BAFF levels and BAFF transcription have been noted to be elevated during active disease (Emmerich et al., 2007; Thomas et al., 2011; Zhu, Shi, Peng, et al., 2009). While BAFF is not used as a biomarker for the diagnosis of ITP in humans, other diseases such as SLE in humans have shown favorable responses to BAFF blockade with monoclonal antibodies (Navarra et al., 2011). Currently in humans it is unknown what drives the elevated BAFF response in ITP and other autoimmune diseases. One group has shown TLR7 activation in a mouse model of ITP to decrease platelet counts, increase PAIgG, and increase serum BAFF levels (Yang et al., 2011). Given these findings, BAFF or BAFF receptor (BAFF-R) blockade in humans may be a possible future treatment, and one monoclonal BAFF-Ab, belimumab, has been used in a clinical trial for ITP treatment (ClinicalTrials.gov identifier: NCT01440361). This strong association of BAFF and ITP in humans coupled with the findings of higher BAFF mRNA in dogs with active ITP in this study make it a promising candidate biomarker for distinguishing primary from secondary disease in dogs.

It is estimated that 80% of ITP cases in humans are primary, with the most common secondary causes being SLE, antiphospholipid syndrome, chronic lymphocytic leukemia, Hepatitis C, and miscellaneous systemic infections (Cines, Bussel, Liebman, & Luning Prak, 2009). Importantly, in humans secondary causes of thrombocytopenia such as HIV are distinctly different from primary ITP in terms of BAFF levels (Thomas et al., 2011). One limitation of this study is that while a thrombocytopenic control group was included, it did not consist primarily of dogs with diseases commonly confused with ITP. In the future, dogs with cancers such as lymphoma, and vector-borne diseases such as Babesiosis and Leishmaniasis, should be evaluated as thrombocytopenic controls for BAFF transcription comparison.

Additionally, little is known about BAFF in dogs, and whether levels of the cytokine vary with age, breed, or sex has never been investigated. In this study, healthy control dogs and secondary thrombocytopenic dogs were not age, breed and sex-matched. In mice, estrogen strongly influences levels of BAFF in models of SLE, and splenic cells from female mice have higher BAFF levels than males (Panchanathan & Choubey, 2013). Serum BAFF levels in humans were also inversely correlated with age in various studies (Jin et al., 2008). In the future, comparing BAFF transcription and quantifying serum BAFF in dogs of varying breeds, ages and sex would be helpful to ascertain the importance of such stringently matched controls.

Additional limitations of this study include a relatively small number of dogs and lack of a patient-side assay. Although the numbers included were sufficient for adequate power, it

is important to see these data corroborated over a large group of animals. Additionally, an ideal biomarker would be easy to measure quickly in hospital, and measuring transcription levels currently lacks both ease and patient-side characteristics.

In the future, evaluation of serum or plasma BAFF would have the potential for the development of a rapid, patient-side test. Initial efforts to use human ELISAs for these have been unsuccessful, although Western blotting has confirmed the presence of BAFF protein in splenic cells of dogs. Additionally, the next steps for this method of BAFF measurement would be following BAFF levels during treatment of dogs with ITP. In humans, treatment with dexamethasone causes a rapid drop in detectable BAFF (Zhu, Shi, Sun, et al., 2009). Evaluation of BAFF levels during treatment and remission in dogs could help confirm its use as a biomarker to monitor for attenuation of the immune response driving platelet destruction, or as a monitoring tool for relapse during treatment. Finally, given the association with BAFF and ITP in dogs, the evaluation of BAFF in other B cell-driven disease states in dogs such as lymphoma and other immune-mediated disorders is warranted.

## References

- Beardsley, D. S., & Ertem, M. (1998). Platelet autoantibodies in immune thrombocytopenic purpura. *Transfus Sci*, 19(3), 237-244.
- Behrend, E. N., & Kemppainen, R. J. (1997). Glucocorticoid therapy. Pharmacology, indications, and complications. *Vet Clin North Am Small Anim Pract*, 27(2), 187-213.
- Cines, D. B., Bussel, J. B., Liebman, H. A., & Luning Prak, E. T. (2009). The ITP syndrome: pathogenic and clinical diversity. *Blood*, 113(26), 6511-6521. doi:10.1182/blood-2009-01-129155
- Costa, L. A., Lopes, B. F., Lanis, A. B., De Oliveira, D. C., Giannotti, J. G., & Costa, F. S. (2010). Bone demineralization in the lumbar spine of dogs submitted to prednisone therapy. *J Vet Pharmacol Ther*, 33(6), 583-586. doi:10.1111/j.1365-2885.2010.01174.x
- Cruse, A., JA Yoder, SK Nordone, HS Marr, and AJ Birkenheuer. (2009). Evaluation of potential biomarkers for canine sepsis. In Research Abstract Program of the 2009 ACVIM Forum & Canadian Veterinary Medical Association Convention. *Journal of Veterinary Internal Medicine*, 23(3), 673-786. doi:10.1111/j.1939-1676.2009.0316.x
- Dircks, B. H., Schuberth, H. J., & Mischke, R. (2009). 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*, 235(8), 960-966. doi:10.2460/javma.235.8.960
- Emmerich, F., Bal, G., Barakat, A., Milz, J., Muhle, C., Martinez-Gamboa, L., . . . Salama, A. (2007). High-level serum B-cell activating factor and promoter polymorphisms in patients with idiopathic thrombocytopenic purpura. *Br J Haematol*, 136(2), 309-314. doi:10.1111/j.1365-2141.2006.06431.x
- Fry, D. R., McSporran, K. D., Ellis, J. T., & Harvey, C. (2009). Protozoal hepatitis associated with immunosuppressive therapy in a dog. *J Vet Intern Med*, 23(2), 366-368. doi:10.1111/j.1939-1676.2008.0263.x
- Hsu, K., Snead, E., Davies, J., & Carr, A. (2012). Iatrogenic hyperadrenocorticism, calcinosis cutis, and myocardial infarction in a dog treated for IMT. *J Am Anim Hosp Assoc*, 48(3), 209-215. doi:10.5326/jaaha-ms-5740
- Huang, H. P., Yang, H. L., Liang, S. L., Lien, Y. H., & Chen, K. Y. (1999). Iatrogenic hyperadrenocorticism in 28 dogs. *J Am Anim Hosp Assoc*, 35(3), 200-207.

- Huard, B., Arlettaz, L., Ambrose, C., Kindler, V., Mauri, D., Roosnek, E., . . . French, L. E. (2004). BAFF production by antigen-presenting cells provides T cell co-stimulation. *Int Immunol*, *16*(3), 467-475.
- Jacoby, R. C., Owings, J. T., Ortega, T., Gosselin, R., & Feldman, E. C. (2001). Biochemical basis for the hypercoagulable state seen in Cushing syndrome; discussion 1006-7. *Arch Surg*, *136*(9), 1003-1006.
- Jin, R., Kaneko, H., Suzuki, H., Arai, T., Teramoto, T., Fukao, T., & Kondo, N. (2008). Age-related changes in BAFF and APRIL profiles and upregulation of BAFF and APRIL expression in patients with primary antibody deficiency. *Int J Mol Med*, *21*(2), 233-238.
- Laurenson, M. P., Hopper, K., Herrera, M. A., & Johnson, E. G. (2010). Concurrent diseases and conditions in dogs with splenic vein thrombosis. *J Vet Intern Med*, *24*(6), 1298-1304. doi:10.1111/j.1939-1676.2010.0593.x
- Lewis, D. C., & Meyers, K. M. (1996). Canine idiopathic thrombocytopenic purpura. *J Vet Intern Med*, *10*(4), 207-218.
- Maeda, S., Ohno, K., Fujiwara-Igarashi, A., Tomiyasu, H., Fujino, Y., & Tsujimoto, H. (2014). Methylation of TNFRSF13B and TNFRSF13C in duodenal mucosa in canine inflammatory bowel disease and its association with decreased mucosal IgA expression. *Vet Immunol Immunopathol*, *160*(1-2), 97-106. doi:10.1016/j.vetimm.2014.04.005
- Maeda, S., Ohno, K., Uchida, K., Nakashima, K., Fukushima, K., Tsukamoto, A., . . . Tsujimoto, H. (2013). Decreased immunoglobulin A concentrations in feces, duodenum, and peripheral blood mononuclear cells of dogs with inflammatory bowel disease. *J Vet Intern Med*, *27*(1), 47-55. doi:10.1111/jvim.12023
- Nardelli, B., Belvedere, O., Roschke, V., Moore, P. A., Olsen, H. S., Migone, T. S., . . . Hilbert, D. M. (2001). Synthesis and release of B-lymphocyte stimulator from myeloid cells. *Blood*, *97*(1), 198-204.
- Navarra, S. V., Guzman, R. M., Gallacher, A. E., Hall, S., Levy, R. A., Jimenez, R. E., . . . Group, B.-S. (2011). Efficacy and safety of belimumab in patients with active systemic lupus erythematosus: a randomised, placebo-controlled, phase 3 trial. *Lancet*, *377*(9767), 721-731. doi:10.1016/S0140-6736(10)61354-2
- O'Marra, S. K., Delaforcade, A. M., & Shaw, S. P. (2011). Treatment and predictors of outcome in dogs with immune-mediated thrombocytopenia. *J Am Vet Med Assoc*, *238*(3), 346-352. doi:10.2460/javma.238.3.346

- Panchanathan, R., & Choubey, D. (2013). Murine BAFF expression is up-regulated by estrogen and interferons: implications for sex bias in the development of autoimmunity. *Mol Immunol*, *53*(1-2), 15-23. doi:10.1016/j.molimm.2012.06.013
- Putsche, J. C., & Kohn, B. (2008). Primary immune-mediated thrombocytopenia in 30 dogs (1997-2003). *J Am Anim Hosp Assoc*, *44*(5), 250-257. doi:10.5326/0440250
- Rose, L. J., Dunn, M. E., Allegret, V., & Bedard, C. (2011). Effect of prednisone administration on coagulation variables in healthy Beagle dogs. *Vet Clin Pathol*, *40*(4), 426-434. doi:10.1111/j.1939-165X.2011.00364.x
- Schmittgen, T. D., & Livak, K. J. (2008). Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc*, *3*(6), 1101-1108.
- Schneider, P., MacKay, F., Steiner, V., Hofmann, K., Bodmer, J. L., Holler, N., . . . Tschopp, J. (1999). BAFF, a novel ligand of the tumor necrosis factor family, stimulates B cell growth. *J Exp Med*, *189*(11), 1747-1756.
- Shen, Y., Wang, S., Ai, H., Min, C., Chen, Y., & Zhang, S. (2011). Molecular structure, bioinformatics analysis, expression and bioactivity of BAFF (TNF13B) in dog (*Canis familiaris*). *Vet Immunol Immunopathol*, *142*(1-2), 133-139. doi:10.1016/j.vetimm.2011.04.014
- Thomas, M. R., Machin, S. J., Mackie, I., & Scully, M. A. (2011). B cell activating factor is elevated in acute idiopathic thrombotic thrombocytopenic purpura. *Br J Haematol*, *155*(5), 620-622. doi:10.1111/j.1365-2141.2011.08730.x
- Wallisch, K., & Trepanier, L. A. (2015). Incidence, timing, and risk factors of azathioprine hepatotoxicosis in dogs. *J Vet Intern Med*, *29*(2), 513-518. doi:10.1111/jvim.12543
- Yang, Q., Xu, S., Li, X., Wang, B., Wang, X., Ma, D., . . . Hou, M. (2011). Pathway of Toll-like receptor 7/B cell activating factor/B cell activating factor receptor plays a role in immune thrombocytopenia in vivo. *PLoS One*, *6*(7), e22708. doi:10.1371/journal.pone.0022708
- Zhu, X. J., Shi, Y., Peng, J., Guo, C. S., Shan, N. N., Qin, P., . . . Hou, M. (2009). The effects of BAFF and BAFF-R-Fc fusion protein in immune thrombocytopenia. *Blood*, *114*(26), 5362-5367. doi:10.1182/blood-2009-05-217513
- Zhu, X. J., Shi, Y., Sun, J. Z., Shan, N. N., Peng, J., Guo, C. S., . . . Hou, M. (2009). High-dose dexamethasone inhibits BAFF expression in patients with immune thrombocytopenia. *J Clin Immunol*, *29*(5), 603-610. doi:10.1007/s10875-009-9303-y

## CHAPTER FOUR: B CELL ACTIVATING FACTOR PROMOTER POLYMORPHISMS IN DOGS WITH PRIMARY IMMUNE-MEDIATED THROMBOCYTOPENIA

### 4.1 Introduction

Immune-mediated thrombocytopenia (ITP) is a disease in which platelet membrane-directed antibodies result in platelet destruction and clinical bleeding. In humans, approximately 60% of patients have antibodies directed specifically against platelet surface glycoproteins (GP) IIb/IIIa and GPIb/IX (Warner, Moore, Warkentin, Santos, & Kelton, 1999). While the exact mechanism for the production of these antibodies in ITP is unknown, several studies have implicated various cytokine imbalances that may promote or perpetuate a loss of self-tolerance (Panitsas et al., 2004; Rocha et al., 2011). Additionally, single nucleotide polymorphisms (SNPs) in the regulatory and promoter regions of inflammatory cytokines that have been associated with chronic ITP in children and adults have been identified (Foster et al., 2001; Rocha et al., 2010). Current therapy for ITP in humans consists of broad-spectrum immune suppression with the use of corticosteroids, intravenous immunoglobulins (IVIg) and anti-D Ig (Rodeghiero & Ruggeri, 2014). A more precise knowledge of the pathogenesis of ITP and associated cytokines could allow for the development of more specific treatment strategies.

B cell activating factor (BAFF, also described as TNFSF13B, and B-lymphocyte stimulator (BLyS)) is a TNFSF cytokine that is elevated in humans with active ITP compared to healthy humans and those with other causes of thrombocytopenia (Emmerich et al., 2007; Thomas, Machin, Mackie, & Scully, 2011; Zhu et al., 2009). Increased BAFF has also been

noted in other autoimmune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and Sjögren's syndrome (Pers et al., 2005). In health, BAFF contributes to B cell maturation, plasma cell longevity, and T cell-independent class switching (Mackay & Schneider, 2009; Schneider et al., 1999). Additional investigation into the pathogenesis of BAFF elevation in humans with ITP and other autoimmune diseases such as SLE and RA has led to the discovery of a promoter SNP, -871 C>T, that is associated with the presence of disease and in some cases with higher concentrations of BAFF (Abdel-Hamid & Al-Lithy, 2011; de Almeida & Petzl-Erler, 2013; Emmerich et al., 2007; Kawasaki, Tsuchiya, Fukazawa, Hashimoto, & Tokunaga, 2002; Zayed et al., 2013).

ITP in dogs is similar to the disease in humans (LeVine et al., 2014) and our previous work has shown that BAFF mRNA is significantly increased in dogs with ITP compared to dogs with other causes of thrombocytopenia and healthy control dogs. As with humans however, both the etiologies of ITP in dogs and of BAFF elevation in dogs with ITP are unknown. Previous candidate gene studies for autoimmune disease in dogs have not evaluated BAFF (Friedenberg et al., 2015), and it is unknown if there are SNPs in the promoter region of canine BAFF, similar to those found in humans.

The primary objective of this pilot study was to compare the incidence of polymorphisms in a selected portion of the BAFF promoter region in dogs with ITP and healthy dogs. The initial promoter region selected for evaluation contained a transcription factor binding site and a conserved site in which polymorphism is associated with autoimmune disease in humans including ITP (Abdel-Hamid & Al-Lithy, 2011; de Almeida

& Petzl-Erlner, 2013; Emmerich et al., 2007; Kawasaki et al., 2002; Zayed et al., 2013). We hypothesize that significantly more dogs with ITP will have polymorphisms identified in the BAFF promoter region compared to healthy dogs.

## **4.2 Materials and Methods**

### **4.2.1 Study Population**

Whole blood was collected from 11 client-owned dogs that were presented to the Veterinary Hospital at the North Carolina State University College of Veterinary Medicine (NCSU-CVM) and diagnosed with ITP. Dogs were eligible for inclusion with a clinician diagnosis of ITP, initial platelet count less than 70,000 cells/ $\mu$ l, and response to treatment for ITP in surviving dogs. Whole blood from 9 healthy control dogs was collected from a population of dogs presented to NCSU for wellness examinations and free from a known history of hematologic autoimmune diseases.

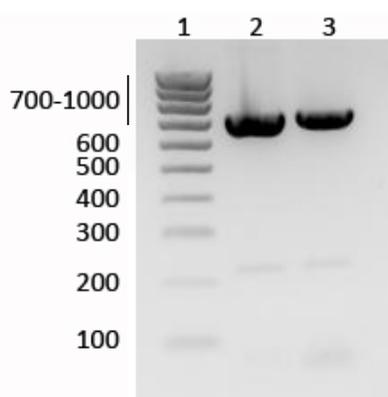
### **4.2.2 Identification of Promoter Region and Selection of Regions of Interest**

A promoter region for canine BAFF has not previously been described, therefore a combination of proposed locations in humans (-1320) (Kawasaki et al., 2002) and an arbitrary number approximately 10% the size of the genomic sequence of BAFF were used to



and commercial software identified binding sites for two transcription factors (GPMiner, Taiwan). Additionally, a sequence analogous to the human -871 C<T region was found within this region. The promoter sequence containing both a possible transcription factor binding site and similar sequence to that with a SNP in the human BAFF promoter region was then selected for initial PCR amplification and sequencing.

#### 4.2.3 Genomic PCR and Sequencing



**Figure 2. Representative agarose gel electrophoresis of CPR1 PCR product.** A 1kb ladder (lane 1) shows an appropriate band for each control dog at approximately 700bp (lanes 2 and 3). Given the smaller, approximately 225bp product also present, the 700bp product was cut from gel for sequencing.

Primers flanking the 703bp region designated Canine Promoter Region 1 (CPR1) were manually designed and consisted of BAFFPromFwd 5'-GACAGAGCTAGCGCTCCAGT-3' and BAFFPromRev 5'-GCACTGAACTGTACTCAGCTGG-3' (Figure 1). DNA was extracted with the use of a commercial kit (Quick-gDNA MiniPrep) according to the manufacturer's instructions (Zymo Research, Irvine, Calif.) and PCR was performed. Each reaction consisted of 35ng DNA,

0.5µl Fwd primer, 0.5µl Rev primer, TaKaRa LA Taq per the manufacturer's instructions, and water to 50µl. Reaction times included an initial 5min at 94C, followed by 45 cycles of 10sec at 94C, 20sec at 55C, and 30sec at 68C, with a final 5min extension at 72C. Amplicons were visualized on an agarose gel (Figure 2) and bidirectionally sequenced (Genewiz, South Plainfield, NJ). When multiple products were visualized on the agarose gel, the product with a size corresponding to CPR1 was cut from the gel, purified with a kit according to the manufacturer's instructions (QIAquick Gel Extraction Kit, Qiagen, Germantown, MD), and that product was submitted for sequencing.

Chromatograms were evaluated manually and contigs were assembled using a commercially available software package (BioEdit Sequence Alignment Editor; North Carolina State University, Raleigh, NC). Sequences of the 703bp region were trimmed by approximately 60bp at each end to account for sequencing inaccuracy, and compared between samples using a commercially available software package (BioEdit Sequence Alignment Editor; North Carolina State University, Raleigh, NC).

#### **4.2.4 RT-qPCR to Evaluate BAFF mRNA Expression**

For dogs with ITP with whole blood samples available at diagnosis and before treatment, RNA was extracted, cDNA synthesized, and RT-qPCR for BAFF was performed as previously described (See: Chapter 3).

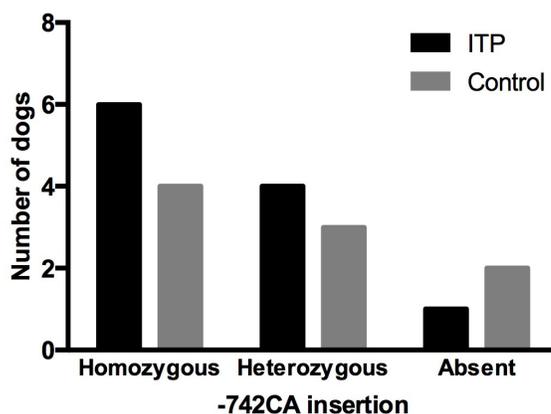
#### **4.2.5 Statistical analysis**

Statistical analysis was performed with the use of a commercially available software program (GraphPad Prism, La Jolla, Calif.). Genotypic frequencies were compared using  $\chi^2$

testing. Association between genotypic frequencies and BAFF mRNA level was evaluated with a Mann-Whitney U test. Power was calculated through the use of a commercially available software (G\*Power 3.1, Denmark).

### 4.3 Results

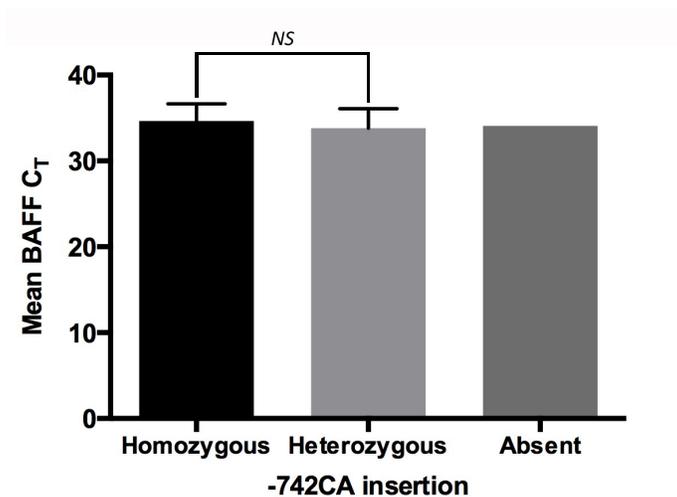
#### 4.3.1 Comparison of amplified regions of CPR1



**Figure 3. Presence of -742CA insertion in dogs with ITP versus healthy controls.** There was no significant difference in the genotypic distribution between groups of dogs.

The amplified regions of CPR1 were compared for each dog. The only commonly identified polymorphism was the insertion of a cysteine and adenine at position 2259 (-742CA) of the purported promoter region. Of the dogs with ITP, 6/11 (54.5%) were homozygous for -742CA, 4/11 (36.4%) were heterozygous, and 1/11 (9.1%) was missing -742CA on both chromosomes. Of the control dogs, 4/9 (44.4%) were homozygous for -742CA, 3/9 (33.3%) were heterozygous, and 2/9 (22.2%) lacked -742CA on both chromosomes (Figure 3). There was no significant difference between the groups and allelic distributions ( $p=0.7107$ ).

#### 4.3.2 Lack of Association between -742CA Genotype and BAFF mRNA level in canine ITP



**Figure 4. Mean BAFF mRNA CT values and -742CA genotype in dogs with ITP.** Standard deviation is indicated with bars for genotypes with more than one dog evaluated. There was no significant difference between mean BAFF mRNA CT between homozygous and heterozygous dogs.

Of the 11 dogs in the ITP group, 10 had whole blood samples available from diagnosis for measurement of BAFF mRNA (Figure 4). Average  $C_T$  values were compared between groups that had at least three values (homozygous and heterozygous -742CA) and there was no significant difference between mean BAFF mRNA  $C_T$  values between homozygous and heterozygous -742CA dogs with ITP at diagnosis ( $p=0.7143$ ).

#### 4.4 Discussion

This pilot study identified a polymorphism within a portion of the estimated promoter region for canine BAFF: -742CA. Initial evaluation showed no significant difference in the distribution of genotypes between dogs with ITP and healthy control dogs. Given that this pilot study was underpowered to detect a true difference between groups, the initial data was used to generate information about an appropriately powered study to evaluate if a difference in -742CA genotype frequency exists between dogs with ITP and healthy dogs. To ensure a power of 0.95 with an effect size of 0.49 and  $\alpha$  of .05 a total sample size of 65 dogs would be needed.

Evaluation of the -742CA region with several open source human promoter region evaluation programs (WWW Signal Scan, Minnesota; BDGP Neural Network Promoter Prediction, Berkeley, Calif.) identified possible promoter elements within that sequence. In humans with ITP the BAFF promoter polymorphism -871 C>T is at the location of a myeloid zinc finger binding site and was first noted to be associated with disease activity and serum BAFF concentration soon after BAFF's association with ITP was established (Emmerich et al., 2007). Humans with the homozygous (TT) polymorphism appeared to be more frequent in the ITP group versus normal population, and within the group of ITP patients, those with the TT allele had higher serum BAFF levels (Emmerich et al., 2007). Subsequent studies have found disparate results, identifying different alleles as being associated with higher BAFF concentrations and ITP. One group implicated the same homozygous (TT) polymorphism (Abdel-Hamid & Al-Lithy, 2011) as the initial work, while others noted the

homozygous (CC) polymorphism to be associated with higher BAFF expression by flow cytometry in healthy patients (de Almeida & Petzl-Erler, 2013), and others documented an association of the T allele with disease but did not note a significant difference in BAFF concentration with genotype (Liu et al., 2010).

The same -871 C>T polymorphism has also been investigated in humans with SLE and RA with mixed results (Eilertsen, Van Ghelue, Strand, & Nossent, 2011; Kawasaki et al., 2002; Zayed et al., 2013). Initial studies showed an association, although not significant, with the homozygous (TT) genotype and characteristics of SLE, and higher BAFF mRNA in peripheral blood monocytes with the presence of a -871T allele (Kawasaki et al., 2002). These findings were replicated in different populations, with a significant association between the homozygous (TT) genotype and SLE (Zayed et al., 2013). However, subsequent analyses showed increased serum BAFF concentrations to be associated with inflammation in SLE and not a specific promoter genotype, and in fact found no association between SLE and the -871 C>T polymorphism (Eilertsen et al., 2011).

In our pilot study, there did not appear to be an association between BAFF mRNA in dogs with newly diagnosed ITP and the -742CA genotype, however given the nature of the pilot study this evaluation was underpowered to detect an association if one existed. To ensure a power of 0.95 with an effect size of 0.40 and  $\alpha$  of .05 a total sample size of 102 dogs would be needed given our pilot data. Additionally, given that some studies have shown a poor correlation between mRNA and serum BAFF levels and no correlation between mRNA and the -871 C>T polymorphism (Gottenberg et al., 2006) it would be important to also

measure BAFF protein in future studies. Given that the knowledge of what drives ITP in dogs is limited, that it has been shown the BAFF mRNA is elevated in dogs with ITP, and that BAFF promoter polymorphisms in humans may be associated with increased BAFF concentrations in disease, this would be worth pursuing.

As with all pilot studies, the conclusions reached are limited by the small sample size and power. However, this work has identified a region of interest for future studies of the BAFF promoter region in dogs. Additionally, this study examined only a small portion of the possible canine BAFF regulatory region, so that not all possible promoter polymorphisms in canine BAFF have been identified. Future studies evaluating the remainder of the BAFF promoter in a similar protocol to this study would be worthwhile to identify other possible regions of variation between dogs with and without ITP and possible association with increased BAFF mRNA.

## References

- Abdel-Hamid, S. M., & Al-Lithy, H. N. (2011). B Cell Activating Factor Gene Polymorphisms in Patients With Risk of Idiopathic Thrombocytopenic Purpura. *The American Journal of the Medical Sciences*, *342*(1), 9-14. doi:10.1097/MAJ.0b013e31820e7f05
- de Almeida, E. R., & Petzl-Erler, M. L. (2013). Expression of genes involved in susceptibility to multifactorial autoimmune diseases: estimating genotype effects. *Int J Immunogenet*, *40*(3), 178-185. doi:10.1111/j.1744-313X.2012.01152.x
- Eilertsen, G. O., Van Ghelue, M., Strand, H., & Nossent, J. C. (2011). Increased levels of BAFF in patients with systemic lupus erythematosus are associated with acute-phase reactants, independent of BAFF genetics: a case-control study. *Rheumatology (Oxford)*, *50*(12), 2197-2205. doi:10.1093/rheumatology/ker282
- Emmerich, F., Bal, G., Barakat, A., Milz, J., Muhle, C., Martinez-Gamboa, L., . . . Salama, A. (2007). High-level serum B-cell activating factor and promoter polymorphisms in patients with idiopathic thrombocytopenic purpura. *Br J Haematol*, *136*(2), 309-314. doi:10.1111/j.1365-2141.2006.06431.x
- Foster, C. B., Zhu, S., Erichsen, H. C., Lehrnbecher, T., Hart, E. S., Choi, E., . . . Chanock, S. J. (2001). Polymorphisms in inflammatory cytokines and Fcγ receptors in childhood chronic immune thrombocytopenic purpura: a pilot study. *Br J Haematol*, *113*(3), 596-599.
- Friedenberg, S. G., Buhrman, G., Chdid, L., Olby, N. J., Olivry, T., Guillaumin, J., . . . Meurs, K. M. (2015). Evaluation of a DLA-79 allele associated with multiple immune-mediated diseases in dogs. *Immunogenetics*. doi:10.1007/s00251-015-0894-6
- Gottenberg, J. E., Sellam, J., Ittah, M., Lavie, F., Proust, A., Zouali, H., . . . Miceli-Richard, C. (2006). No evidence for an association between the -871 T/C promoter polymorphism in the B-cell-activating factor gene and primary Sjogren's syndrome. *Arthritis Res Ther*, *8*(1), R30. doi:10.1186/ar1884
- Kawasaki, A., Tsuchiya, N., Fukazawa, T., Hashimoto, H., & Tokunaga, K. (2002). Analysis on the association of human BLYS (BAFF, TNFSF13B) polymorphisms with systemic lupus erythematosus and rheumatoid arthritis. *Genes Immun*, *3*(7), 424-429. doi:10.1038/sj.gene.6363923
- LeVine, D. N., Birkenheuer, A. J., Brooks, M. B., Nordone, S. K., Bellinger, D. A., Jones, S. L., . . . Key, N. S. (2014). A novel canine model of immune thrombocytopenia: has immune thrombocytopenia (ITP) gone to the dogs? *Br J Haematol*, *167*(1), 110-120. doi:10.1111/bjh.13005

- Liu, J. Q., Yang, L. H., Chen, J. F., Chen, X. H., Liu, X. E., Chang, L. X., & Gao, Y. (2010). [Expression and gene polymorphisms of B cell activating factor in patients with idiopathic thrombocytopenic purpura.]. *Zhonghua Xue Ye Xue Za Zhi*, *31*(9), 586-589.
- Mackay, F., & Schneider, P. (2009). Cracking the BAFF code. *Nat Rev Immunol*, *9*(7), 491-502. doi:10.1038/nri2572
- Panitsas, F. P., Theodoropoulou, M., Kouraklis, A., Karakantza, M., Theodorou, G. L., Zoumbos, N. C., . . . Mouzaki, A. (2004). Adult chronic idiopathic thrombocytopenic purpura (ITP) is the manifestation of a type-1 polarized immune response. *Blood*, *103*(7), 2645-2647. doi:10.1182/blood-2003-07-2268
- Pers, J. O., Daridon, C., Devauchelle, V., Jousse, S., Saraux, A., Jamin, C., & Youinou, P. (2005). BAFF overexpression is associated with autoantibody production in autoimmune diseases. *Ann N Y Acad Sci*, *1050*, 34-39. doi:10.1196/annals.1313.004
- Rocha, A. M., De Souza, C., Rocha, G. A., De Melo, F. F., Saraiva, I. S., Clementino, N. C., . . . Queiroz, D. M. (2010). IL1RN VNTR and IL2-330 polymorphic genes are independently associated with chronic immune thrombocytopenia. *Br J Haematol*, *150*(6), 679-684. doi:10.1111/j.1365-2141.2010.08318.x
- Rocha, A. M., Souza, C., Rocha, G. A., de Melo, F. F., Clementino, N. C., Marino, M. C., . . . Queiroz, D. M. (2011). The levels of IL-17A and of the cytokines involved in Th17 cell commitment are increased in patients with chronic immune thrombocytopenia. *Haematologica*, *96*(10), 1560-1564. doi:10.3324/haematol.2011.046417
- Rodeghiero, F., & Ruggeri, M. (2014). ITP and international guidelines: what do we know, what do we need? *Presse Med*, *43*(4 Pt 2), e61-67. doi:10.1016/j.lpm.2014.02.004
- Schneider, P., MacKay, F., Steiner, V., Hofmann, K., Bodmer, J. L., Holler, N., . . . Tschopp, J. (1999). BAFF, a novel ligand of the tumor necrosis factor family, stimulates B cell growth. *J Exp Med*, *189*(11), 1747-1756.
- Thomas, M. R., Machin, S. J., Mackie, I., & Scully, M. A. (2011). B cell activating factor is elevated in acute idiopathic thrombotic thrombocytopenic purpura. *Br J Haematol*, *155*(5), 620-622. doi:10.1111/j.1365-2141.2011.08730.x
- Warner, M. N., Moore, J. C., Warkentin, T. E., Santos, A. V., & Kelton, J. G. (1999). A prospective study of protein-specific assays used to investigate idiopathic thrombocytopenic purpura. *Br J Haematol*, *104*(3), 442-447. doi:10.1046/j.1365-2141.1999.01218.x

- Zayed, R. A., Sheba, H. F., Abo Elazaem, M. A., Elsaadany, Z. A., Elmessery, L. O., Mahmoud, J. A., . . . Abdou, F. R. (2013). B-cell activating factor promoter polymorphisms in egyptian patients with systemic lupus erythematosus. *Ann Clin Lab Sci*, 43(3), 289-294.
- Zhu, X. J., Shi, Y., Peng, J., Guo, C. S., Shan, N. N., Qin, P., . . . Hou, M. (2009). The effects of BAFF and BAFF-R-Fc fusion protein in immune thrombocytopenia. *Blood*, 114(26), 5362-5367. doi:10.1182/blood-2009-05-217513

## **CHAPTER FIVE: $\Delta$ BAFF TRANSCRIPTION IS NOT INCREASED IN DOGS WITH PRIMARY ITP COMPARED WITH HEALTHY DOGS**

### **5.1 Introduction**

B cell activating factor (BAFF) transcription is increased in canine immune thrombocytopenia (ITP), as demonstrated in Chapter 3 of this manuscript. It remains unknown what drives increased BAFF transcription in dogs with ITP and if increased BAFF transcription is a major contributor to the pathogenesis of the disease. However, if elevated BAFF does drive autoimmunity, then decreasing BAFF transcription could serve to attenuate the dogs' aberrant immune response. A splice variant of BAFF excluding exon 3 in humans and exon 4 in mice, termed  $\Delta$ BAFF is a negative regulator of BAFF in vitro and in vivo (Gavin, Ait-Azzouzene, Ware, & Nemazee, 2003; Gavin et al., 2005).  $\Delta$ BAFF is thought to exert its regulatory effects via BAFF mRNA consumption, prevention of cleavage of membrane-bound BAFF, and the formation of heterotrimers with soluble BAFF (Gavin et al., 2003; Gavin et al., 2005; Roescher et al., 2014).  $\Delta$ BAFF has been identified in the canine macrophage cell line 030-D as described in Chapter 2 of this manuscript, and similarly to humans is lacking exon 3.

Given  $\Delta$ BAFF's negative regulatory role it has been evaluated in autoimmune diseases characterized by excess BAFF such as Sjögren's syndrome (SS), through the use of targeted mRNA splicing, resulting in the removal of exons missing in  $\Delta$ BAFF (Roescher et al., 2014). In this work it was found that by inducing exon skipping in a mouse model of SS, salivary epithelial cell BAFF concentrations were decreased (Roescher et al., 2014). No

published studies have evaluated  $\Delta$ BAFF transcription in ITP, in humans or in mouse or dog models of the disease.

The specific aims of this work were to evaluate dogs for evidence of  $\Delta$ BAFF transcription via RT-qPCR and to compare  $\Delta$ BAFF transcription between dogs with ITP and healthy dogs. Given its regulatory role in humans and mice, we hypothesized that  $\Delta$ BAFF transcription would be elevated in dogs with ITP compared to healthy dogs. Alternatively, if a lack of  $\Delta$ BAFF was driving BAFF transcription in dogs with ITP then there may be a less  $\Delta$ BAFF transcription in dogs with ITP compared with healthy dogs.

## **5.2 Materials and Methods**

### **5.2.1 Patient Population**

Dogs with ITP were drawn from a population of dogs described previously in Chapter 3 of this manuscript. Briefly, EDTA-anticoagulated whole blood was collected from 11 client-owned dogs that were presented to the Veterinary Hospital at the North Carolina State University College of Veterinary Medicine (NCSU-CVM) from and diagnosed with ITP. Dogs were eligible for inclusion with a clinician diagnosis of ITP, initial platelet count less than 50,000 cells/ $\mu$ l and response to treatment for ITP in surviving dogs (Putsche & Kohn, 2008). Exclusion criteria included administration of immunosuppressive medications or medications known to be associated with ITP prior to sample collection for this study, identification of neoplasia on abdominal imaging, thoracic radiographs, or necropsy, or

vector-borne disease testing results consistent with infections known to cause thrombocytopenia.

Healthy control dogs (n=9) were drawn from a population of dogs presenting to NCSU-CVM for wellness exams with normal platelet counts and previous control dogs from other studies. The NCSU Animal Care and Use Committee approved the study.

### **5.2.2 RNA Isolation & cDNA Synthesis**

EDTA-anticoagulated whole blood was collected within 24 hours of ITP dog admission, preserved with ZR RNA Buffer (Zymo Research, Irvine, Calif.), and stored at -80°C. Total RNA was later isolated using a ZR Whole Blood Total RNA kit (Zymo Research, Irvine, Calif.) as per the manufacturer's instructions. Purified RNA was quantified with NanoDrop Spectrophotometry. For each sample, 100ng of RNA was reverse transcribed into cDNA. Each 50µl reaction consisted of 100ng RNA combined with distilled water to a volume of 19.25µl, 11µl 25mM MgCl<sub>2</sub>, 5µl 10x RT buffer, 10µl dNTPs, 2.5µl random hexamers, 1µl RNase inhibitor, 1.25µl reverse transcriptase. Samples were held at 25°C for 5 minutes, followed by 48°C for 1 hour, and 95°C for 5 min.

### **5.2.3 Quantitative RT-PCR for ΔBAFF**

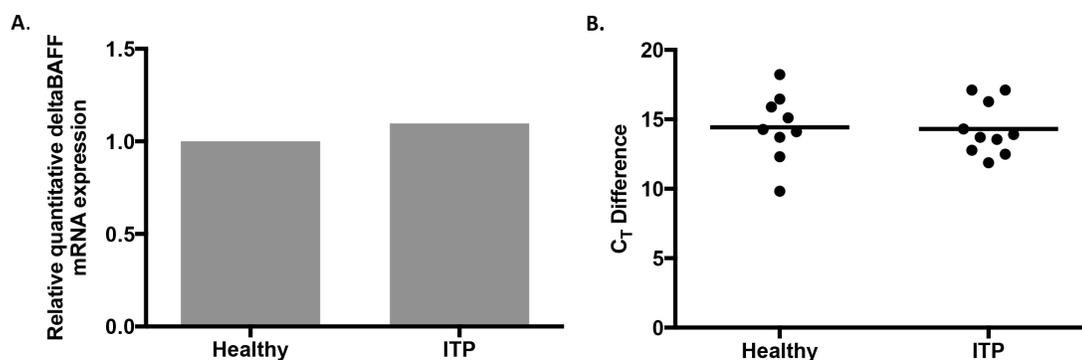
Primer pairs to specifically amplify canine ΔBAFF were manually designed and consisted of Forward (BaffEx2to4Fwd: 5'CCGGAAGAAACAGGAGCTTAC3') and Reverse (BaffE5PR: 5'GGGTAGTGTTTCAGGCATA3'). Using cDNA synthesized for BAFF evaluation as described previously, RT-qPCR was performed to evaluate expression of ΔBAFF using β-actin as a reference gene. Each 50µl reaction consisted of 1µl cDNA, 25µl

Power SYBR Green Master Mix (ThermoFisher Scientific, Grand Island, NY), 0.5 $\mu$ l Fwd primer, 0.5 $\mu$ l Rev primer, and 23 $\mu$ l water. Reaction times for the  $\Delta$ BAFF PCR included an initial 3min at 95C, followed by 45 cycles of 95C for 10 seconds, annealing at 62C for 30 seconds, and 30 seconds of extension at 72C.

Reaction times for the  $\beta$ -actin PCR included an initial 3min at 95C, followed by 45 cycles of 95C for 10 seconds, annealing at 64.4C for 20 seconds, and 20 seconds of extension at 72C. These assays were optimized, do not amplify canine genomic DNA and under optimized reaction conditions have amplification efficiencies of 99.9% and 100%, respectively. Amplicons were visualized on an agarose gel and melt curve analysis performed to confirm expected products.

#### 5.2.4 Statistics

Data from RT-qPCR was analyzed by comparison of the  $\Delta C_T$  values obtained from the control population and the  $\Delta C_T$  values from dogs with primary ITP. The fold change in gene expression relative to the control subjects was calculated as  $2^{(-\Delta\Delta C_T)}$ , where  $\Delta\Delta C_T = \text{mean } \Delta C_T \text{ control group} - \Delta C_T \text{ ITP group}$ , as previously described for BAFF,  $\beta$ -actin and  $\Delta$ BAFF (Schmittgen & Livak, 2008). A Mann-Whitney Test was used to compare the  $\Delta C_T$  values in dogs with ITP and healthy controls. Statistical analysis was performed with the use of commercially available software (Prism). Statistical significance was assigned a p value of  $\leq 0.05$ .



**Figure 1. Relative quantitative deltaBAFF mRNA expression in dogs with ITP compared to healthy controls.** (A) The ratio of deltaBAFF mRNA in ITP dogs compared with healthy dogs was 1.097. (B) Individual cycle threshold difference values for the dogs in each group.

## 5.3 Results

### 5.3.1 Clinically healthy dogs and dogs with ITP produce $\Delta$ BAFF

All but one dog in the ITP group, and all dogs in the control group, had evidence of  $\Delta$ BAFF transcription as demonstrated by amplification with RT-qPCR. Cycle threshold values for dogs in the healthy group had a mean of 39.23 (range: 37.15-44.22), cycle threshold values for dogs in the ITP group had a mean of 38.56 (range: 36.42-41.06).

### 5.3.2 $\Delta$ BAFF mRNA is similar in dogs with ITP and normal platelet counts

Use of the  $\Delta\Delta C_T$  method to analyze RT-qPCR showed  $\Delta$ BAFF mRNA levels to be nearly equivalent in the ITP group compared to healthy dogs (Figure 1). There was not a significant difference in  $\Delta$ BAFF mRNA levels between ITP and healthy controls ( $p=0.76$ ).

#### 5.4 Discussion

This study demonstrated  $\Delta$ BAFF mRNA production in the majority of a group of dogs and a lack of difference in relative quantitative  $\Delta$ BAFF mRNA expression between dogs with ITP and healthy dogs. While it has been shown that both humans and mice express  $\Delta$ BAFF, this is the first documentation of a  $\Delta$ BAFF homologue in dogs (Gavin et al., 2003; Gavin et al., 2005). All of the control dogs whole blood samples had measurable  $\Delta$ BAFF mRNA, while 10/11 dogs with ITP had measurable  $\Delta$ BAFF. It is unknown if humans, mice, or dogs express  $\Delta$ BAFF constitutively, although based on previous work no tested human lacked measurable  $\Delta$ BAFF mRNA (Lopez De Padilla et al., 2013). The lack of measurable  $\Delta$ BAFF mRNA in one dog in the ITP group may have been secondary to cDNA degradation or a low copy number. Alternatively,  $\Delta$ BAFF may not be constitutively expressed in dogs as it is in other species.

There was no significant difference in relative  $\Delta$ BAFF mRNA in dogs with ITP and healthy controls. These findings begin to address the question of what drives the BAFF mRNA increase in dogs with ITP compared to dogs with other causes of thrombocytopenia and healthy controls. The purported role of  $\Delta$ BAFF is negative regulation of BAFF through three mechanisms: 1) consumption of mRNA via alternative splicing, 2) blockage of BAFF cleavage at the cell membrane, and 3) trimerization with BAFF (Gavin et al., 2003; Gavin et al., 2005). It has been shown that ITP in humans and dogs is accompanied by increased BAFF transcription (Emmerich et al., 2007; Zhou et al., 2009; Zhu et al., 2009). Given those two findings, it would be expected that animals with aberrantly high BAFF would have a

concurrent increase in  $\Delta$ BAFF to counter the BAFF effects, and indeed this was found in humans with SLE and inflammatory myopathies (Lopez De Padilla et al., 2013). That dogs do not have changes in  $\Delta$ BAFF mRNA concentrations with ITP may point to a lack of  $\Delta$ BAFF, rather than increased BAFF, as the driving problem. This would be an important difference from other BAFF-driven autoimmune diseases in humans that have been evaluated for both BAFF and  $\Delta$ BAFF thus far (Lopez De Padilla et al., 2013). Currently, no other study has evaluated  $\Delta$ BAFF in ITP in any animal, so it is unknown if this finding would be true in humans and mice models as well.

This study was limited by a several factors including a small sample size. In future having more dogs available, both normal and affected with ITP, would help to address the issue of if  $\Delta$ BAFF is or is not constitutively expressed. Additionally, the use of more cDNA in the PCR reactions could help to identify samples with very low or single copy numbers of  $\Delta$ BAFF. Despite the relatively high cycle threshold values for the reactions reported here, the use of a melt curve analysis helped to ensure that the expected products were formed.

Another important limitation of this study is the assumption that the PCR target is  $\Delta$ BAFF. Ideally, our purported  $\Delta$ BAFF would be produced and its effect on BAFF binding BAFF-R and being shed from cells would be analyzed in canine cells as in prior species (Gavin et al., 2005). The molecule which we propose is the canine homologue of  $\Delta$ BAFF is missing a very similar sequence to human and mouse  $\Delta$ BAFF (exon 3 in dogs and humans, exon 4 in mice), but this does not guarantee an equivalent function. Indeed, there are numerous splice variants of BAFF that exist in humans and mice, many of which are

nonfunctional (Bossen & Schneider, 2006; Smirnova, Andrade-Oliveira, & Gerbase-DeLima, 2008). Our group has identified several additional canine BAFF splice variants, missing full or partial exons, and with alternate start sites as well (unpublished data). A reasonable next step to address this issue would be to assess the functional capacity of purported canine  $\Delta$ BAFF.

Overall, this study identified canine  $\Delta$ BAFF in most dogs in a population of dogs with and without ITP. There was no difference in  $\Delta$ BAFF transcription between the two groups of dogs, which could indicate that the purported canine  $\Delta$ BAFF is not in fact  $\Delta$ BAFF, that a lack of  $\Delta$ BAFF is driving BAFF increase in dogs with ITP, or that  $\Delta$ BAFF in dogs has a different function than in humans. Next steps include determining the function of possible canine  $\Delta$ BAFF and evaluating its presence in larger groups of dogs. This work could provide important insights into the pathogenesis of ITP and the role of BAFF in dogs in health and disease.

## References

- Bossen, C., & Schneider, P. (2006). BAFF, APRIL and their receptors: structure, function and signaling. *Semin Immunol*, *18*(5), 263-275. doi:10.1016/j.smim.2006.04.006
- Emmerich, F., Bal, G., Barakat, A., Milz, J., Muhle, C., Martinez-Gamboa, L., . . . Salama, A. (2007). High-level serum B-cell activating factor and promoter polymorphisms in patients with idiopathic thrombocytopenic purpura. *Br J Haematol*, *136*(2), 309-314. doi:10.1111/j.1365-2141.2006.06431.x
- Gavin, A. L., Ait-Azzouzene, D., Ware, C. F., & Nemazee, D. (2003). DeltaBAFF, an alternate splice isoform that regulates receptor binding and biopresentation of the B cell survival cytokine, BAFF. *J Biol Chem*, *278*(40), 38220-38228. doi:10.1074/jbc.M306852200
- Gavin, A. L., Duong, B., Skog, P., Ait-Azzouzene, D., Greaves, D. R., Scott, M. L., & Nemazee, D. (2005). deltaBAFF, a splice isoform of BAFF, opposes full-length BAFF activity in vivo in transgenic mouse models. *J Immunol*, *175*(1), 319-328.
- Lopez De Padilla, C. M., McNallan, K. T., Crowson, C. S., Bilgic, H., Bram, R. J., Hein, M. S., . . . Reed, A. M. (2013). BAFF expression correlates with idiopathic inflammatory myopathy disease activity measures and autoantibodies. *J Rheumatol*, *40*(3), 294-302. doi:10.3899/jrheum.120555
- Putsche, J. C., & Kohn, B. (2008). Primary immune-mediated thrombocytopenia in 30 dogs (1997-2003). *J Am Anim Hosp Assoc*, *44*(5), 250-257. doi:10.5326/0440250
- Roescher, N., Vosters, J. L., Alsaleh, G., Dreyfus, P., Jacques, S., Chiochia, G., . . . Gottenberg, J. E. (2014). Targeting the splicing of mRNA in autoimmune diseases: BAFF inhibition in Sjogren's syndrome as a proof of concept. *Mol Ther*, *22*(4), 821-827. doi:10.1038/mt.2013.275
- Schmittgen, T. D., & Livak, K. J. (2008). Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc*, *3*(6), 1101-1108.
- Smirnova, A. S., Andrade-Oliveira, V., & Gerbase-DeLima, M. (2008). Identification of new splice variants of the genes BAFF and BCMA. *Mol Immunol*, *45*(4), 1179-1183. doi:10.1016/j.molimm.2007.07.028
- Zhou, Z., Chen, Z., Li, H., Chen, X., Xu, J., Gu, D., . . . Yang, R. (2009). BAFF and BAFF-R of peripheral blood and spleen mononuclear cells in idiopathic thrombocytopenic purpura. *Autoimmunity*, *42*(2), 112-119. doi:10.1080/08916930802397848

Zhu, X. J., Shi, Y., Peng, J., Guo, C. S., Shan, N. N., Qin, P., . . . Hou, M. (2009). The effects of BAFF and BAFF-R-Fc fusion protein in immune thrombocytopenia. *Blood*, *114*(26), 5362-5367. doi:10.1182/blood-2009-05-217513