SUSPECTED PRECURSOR-TARGETED IMMUNE-MEDIATED ANEMIA IN DOGS

By

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ABSTRACT

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Immune-mediated hemolytic anemia (IMHA) results in regenerative anemia and is the most well studied immune-mediated anemia in dogs. However, the underlying cause of IMHA remains unknown, and mortality remains a problem. In a different condition, dogs with suspected precursor-targeted immune-mediated anemia (PIMA) present with nonregenerative anemia and ineffective erythropoiesis, which have occasionally been associated with phagocytosis of erythroid precursors (rubriphagocytosis) or myelofibrosis. The pathogenesis of PIMA has not yet been determined, but roles for immunoglobulin and/or complement have been suspected. Additional involvement of apoptosis-like mechanisms with phosphatidylserine (PS) exposure is possible in both canine IMHA and PIMA based on previous studies in people and dogs. Our central hypothesis is that PIMA is part of a spectrum of immune-mediated anemias including IMHA, in which IgG, with or without the contribution of phosphatidylserine (PS), target and promote phagocytosis of different stages of erythroid cells, simultaneously or independently. The rationale was that better characterizing the pathogenesis of PIMA and its association with IMHA will ultimately help establish diagnostic criteria, identify appropriate therapeutic strategies based on knowledge of the pathogenesis of the diseases, and raise veterinary awareness of PIMA. This dissertation first describes a retrospective study of dogs with PIMA with the main goal of helping characterize canine PIMA and facilitate its recognition and diagnosis. Then it describes the development of flow cytometric assays for RBC and erythroid precursor IgG, including a Percoll gradient

separation for erythroid populations. These methods were used for isolation of erythroid populations and their assessment for IgG and PS in IMHA and PIMA dogs, healthy dogs, and sick dogs with no evidence of IMHA or PIMA. We found that IMHA dogs had significantly higher IgG and PS when compared to healthy and non-IMHA dogs. Additionally, we showed that a subset of PIMA dogs had increased IgG-positive erythroid precursors when compared to healthy and non-IMHA dogs, and erythroid precursors from most tested PIMA dogs had more PS-positive erythroid precursors than healthy dogs; however, no sick dogs without PIMA were tested for comparison. These findings suggest a role for IgG in canine PIMA and for PS in canine IMHA and PIMA. Finally, we demonstrated the expression of DEA1.1 on canine erythroid precursors from rubriblasts through metarubricytes.

This dissertation is dedicated to all dogs, for their example of loyalty, cheerfulness, forgiveness, and unconditional love, and for making the world a better place.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

KEY TO ABBREVIATIONS

AIHA: Autoimmune hemolytic anemia

AIMF: Autoimmune myelofibrosis

BFU-E: Burst-forming units-erythroid

BSA: Bovine serum albumin

BzATP: 2′(3′)-O-(4-Benzoylbenzoyl)adenosine 5′-triphosphate triethylammonium

C3: Third component of complement

C4: Fourth component of complement

Ca^{2+}: Calcium

CBC: Complete blood count

CD: Cluster of differentiation

CFU-E: Colony-forming units-erythroid

CR1: Complement receptor 1

CR2: Complement receptor 2

CV: Coefficient of variation

DAT: Direct antiglobulin test

DCPAH: Diagnostic Center for Population and Animal Health

DEA: Dog erythrocyte antigen

EDTA: Ethylenediaminetetraacetic acid

EGTA: Ethylene glycol tetraacetic acid

ELISA: Enzyme-linked immunosorbent assay

EPO: Erythropoietin

EPOR: EPO receptor

FDP: Fibrin degradation products

FITC: Fluorescein isothiocyanate

FSC: Forward scatter

GSH: Reduced glutathione

Hct: Hematocrit

IFN: Interferon

IMHA: Immune-mediated hemolytic anemia

IMN: Immune-mediated neutropenia

IMT: Immune-mediated thrombocytopenia

M:E: Myeloid to erythroid ratio

MAC: Membrane attack complex

MCV: Median corpuscular volume

MDA: Malondialdehyde

MdFI: Median fluorescence intensity

MF: Myelofibrosis

MFI: Mean fluorescence intensity

mRNA: Messenger ribonucleic acid

MSU VMC: Michigan State University Veterinary Medical Center

NIMA: Nonregenerative immune-mediated anemia

nRBCs: Nucleated red blood cells

NRIMHA: Nonregenerative IMHA

PBS: Phosphate-buffered saline

PE: Phycoerythrin

PIMA: Precursor-targeted immune-mediated anemia

PRCA: Pure red cell aplasia

PS: Phosphatidylserine

RBCs: Red blood cells

RI: Reference interval

ROS: Reactive oxygen species

RP: Rubriphagocytosis

SCF: Stem cell factor

SD: Standard deviation

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SSC: Side scatter

TGF-β: Tumor growth factor β

TNF-R: TNF receptor

TNF: Tumor necrosis factor

WBC: White blood cells

WRI: Within reference interval

CHAPTER 1

Introduction and Literature Review

BACKGROUND

Overview of erythropoiesis

In normal erythropoiesis, stem cells commit to the erythroid lineage, developing into erythroid progenitors that are represented by burst-forming units-erythroid (BFU-E) and colony-forming units-erythroid (CFU-E), and subsequently into morphologically recognizable erythroid precursors from rubriblasts through prorubricytes, basophilic rubricytes, polychromatophilic rubricytes, and metarubricytes. After this stage, erythroid cells extrude their nucleus, leave the bone marrow, and are called reticulocytes, which then mature into red blood cells (RBCs) in the circulation. During this process, erythroid maturation is pyramidal, represented by fewer of the more immature forms, and progressively higher numbers of the more mature stages.

Several cytokines and growth factors are involved in erythropoiesis, with the primary proerythropoietic stimulus coming from erythropoietin (EPO), a hormone produced mainly in the kidneys. EPO interacts with the EPO receptor (EPOR) on the surface of erythroid precursors, stimulating their survival and differentiation. EPOR is expressed in stem cells and progenitor cells, and decreases gradually as erythroid maturation progresses (Olver 2010). Hypoxia can up-regulate EPO up to 1,000 fold, stimulating increased erythropoiesis (Olver 2010). Other proerythropoietic factors include: 1) IL-3, which acts on stem cells and progenitors to induce proliferation and self-renewal, and primes progenitor cells to more vigorously respond to EPO, and 2) stem cell factor (SCF, or c-kit ligand), which enhances the activity of EPO towards proliferation and differentiation of erythroid progenitors and precursors.

During erythroid differentiation, several molecules including transcription factors, growth factor receptors, and proteins that are important for erythroid cell structure and/or

function are expressed on the cell membrane (Testa 2004; Chen et al., 2009). Some of these markers are expressed in early but not late stage erythroid precursors, or vice versa, while others are expressed in all stages throughout development and including mature erythrocytes (Chen et al., 2009; Okumura et al., 1992; Spivak 2005).

Some of these membrane proteins are used to characterize canine blood groups, and have been described on mature erythrocytes from dogs. Eight major dog erythrocyte antigen (DEA) types have been recognized, and typing sera have been produced and applied in blood typing in order to minimize transfusion reactions following blood transfusions (Hale 1995). DEA1.1 is the most clinically relevant alloantigen because of its potential to cause acute hemolytic reactions after incompatible blood transfusions (24, 25), and a frequency of approximately 50% in the canine population (Swisher et al. 1961; Hale 1995; Kessler et al. 2010; Lucidi et al. 2011).

Macrophages play a critical role in erythropoiesis and are part of the erythropoietic niche. They form erythroid islands throughout the marrow, in which macrophages are located in the center and are surrounded by erythroid precursors. The roles of the macrophage involve development of erythroid cells, transfer of iron to developing erythroid precursors to synthesize hemoglobin (Chasis et al., 2008), and removal of apoptotic hematopoietic cells or cell nuclei (Testa 2004; Angelucci et al., 2002; Yoshida et al., 2005; Chasis et al., 2008). They also have a role in clearance of senescent RBCs from the circulation.

Erythropoiesis and apoptotic signals are closely related. Phosphatidylserine (PS), a phospholipid present in cell membranes, is normally restricted to the inner leaflet of the plasma membrane (Balasubramanian et al., 2003). This asymmetric assembly is promoted by two enzymes: aminophospholipid translocase, which specifically transports

PS from the outer to the inner leaflet of the plasma membrane in an ATP-dependent manner, and scramblase, which randomly translocates lipids across the lipid bilayer in a Ca^{2+}-dependent but ATP-independent manner. Extruded nuclei from metarubricytes rapidly expose phosphatidylserine on their surface, promoting phagocytosis by adjacent macrophages (Yoshida et al., 2005; Chasis et al., 2008).

Apoptosis is involved in the maintenance of normal homeostasis of the erythropoietic system. In vitro studies suggest that in health, a small number of erythroid precursors die prematurely in the bone marrow before reaching full development (Samson et al., 1981). Death is due to apoptotic changes, and involves ligands and cell death receptors of the tumor necrosis factor (TNF) and TNF receptor (TNF-R) superfamilies, respectively (de Maria et al., 1999; Testa 2004). These molecules are down-regulated in response to EPO, allowing for increased erythropoiesis. On the other hand, these molecules are increased and provide the main mechanism for control of erythropoiesis when EPO production is low. One other proposed mechanism for regulation of erythropoiesis is that more mature precursors apply a Fas-mediated cytotoxic effect on more immature precursors within erythroid islands, in a negative-feedback fashion (de Maria et al., 1999). Apoptotic erythroid precursors are rapidly engulfed by adjacent macrophages (Angelucci et al., 2002).

The average life span for canine erythrocytes is approximately 100 days (Garon et al., 2010), and through erythropoiesis, millions of erythrocytes are produced daily in order to maintain enough mature erythrocytes to provide adequate oxygenation of body tissues. Processes leading to anemia may involve increased erythrocyte loss, increased erythrocyte destruction, or decreased production in the bone marrow. These studies will

focus on two types of anemia in dogs, involving primarily increased erythrocyte destruction and decreased bone marrow production.

Canine IMHA

In immune-mediated hemolytic anemia (IMHA), a common and well characterized hematologic disorder in many breeds of dogs, immunoglobulins or complement become associated with the surface of RBCs and mediate erythrocyte destruction through a type II hypersensitivity immune reaction. The most common types of immunoglobulins involved are IgG and IgM, but IgA can also be involved in a minority of cases (Barker et al., 1992; Slappendel 1979; McCullough 2003). IgG molecules are monomeric (i.e., they possess only two antigen binding sites) and mediate RBC destruction mostly through phagocytosis (extravascular hemolysis) resulting from Fc receptors on macrophages binding to the Fc portion of the RBC-associated IgG molecules. Some immunoglobulins that bind to RBCs promote fixation of C1, with subsequent generation of C3 on the cell membrane. This can lead to activation of other complement components (C5, C6, C7, C8, C9) and generation of a membrane attack complex that forms transmembrane channels in the RBC membrane, allowing entry of water and electrolytes, and ultimately promoting intravascular hemolysis. Complement can also mediate RBC phagocytosis through complement receptors on macrophages, which bind C3. Because of their pentameric structure, IgM molecules are more efficient than IgG in activating and fixing complement, as well as in promoting RBC agglutination.

Although rarely documented, if antibodies are directed against RBC autoantigens and there is no underlying cause for a secondary immune response, the condition is considered idiopathic and known as autoimmune hemolytic anemia (AIHA). The

mechanisms that cause primary IMHA (i.e., AIHA) are unknown, but are likely to involve a failure in natural regulation of autoreactive lymphocytes (Day 2010). When IMHA occurs in association with neoplasia, infections, or drug administration, it is generally regarded as secondary. In these cases, antibodies may attach to RBC membranes not through normal RBC epitopes but via surface organisms, altered RBC membrane proteins, or foreign proteins. Immunoglobulins may also bind to RBCs by recognition of alloantigens, such as in blood transfusion reactions or neonatal isoerythrolysis. And in some species, C3-bearing immune complexes can bind to complement receptor on the RBC membrane and cause removal of the RBC (and therefore of the immune-complexes) by a bystander effect. This is not documented in the dog.

Canine IMHA most frequently occurs as a single hematologic disease; however, it may also occur concurrently with other immune-mediated disorders such as immune-mediated thrombocytopenia (IMT), or be part of a systemic immune-mediated condition such as systemic lupus erythematosus (Grindem et al., 1983; Jackson et al., 1985; Klag et al., 1993).

In IMHA, a regenerative bone marrow response and reticulocytosis are expected within 3-5 days of the onset of anemia, restoring the hematocrit by increased erythropoiesis if hemolysis is controlled by treatment. A regenerative anemia is typically considered to be IMHA when present with one or more of the following: 1) positivity for RBC surface-associated immunoglobulin or C3, 2) RBC agglutination, and 3) spherocytosis (Klag et al., 1993; Kellerman et al., 1995; Wilkerson et al., 2000; Quigley et al., 2001; McCullough 2003; Kucinskiene et al., 2005; Wardrop 2005). In addition, plasma may be hemolyzed and hemoglobinuria may be present with overwhelming intravascular hemolysis, and hyperbilirubinemia and ghost cells may occur.

Several immunoassays have been used for detection of canine RBC-associated immunoglobulins and C3, including Coombs' (Overman et al., 2007; Piek et al., 2012; Warman et al., 2008), gel column (Caviezel et al., 2014; Piek et al., 2012), and flow cytometry tests (Wilkerson et al., 2000; Quigley et al., 2001; Kucinskiene et al., 2005). Of these, flow cytometry has been shown to be the most sensitive method for detection of RBC bound antibodies in both canine (Wilkerson et al., 2000; Quigley et al., 2001; Kucinskiene et al., 2005) and human samples (Wang et al., 2001; Chaudhary et al., 2006; Lin et al., 2009).

Despite progress in understanding the disease (McCullough 2003; Day 1999; Tan et al., 2012), relapses (Piek et al., 2008), complications, and mortality are still problematic (Swan et al., 2013), and often associated with steroid therapy or thromboembolic events (Carr et al., 2002; Klein et al., 1989; Reimer et al., 1999; Swan et al., 2013; Piek et al., 2008). Although there is a clear association between IMHA and a prothrombotic state (Fenty et al., 2011), the cause of thromboembolism in dogs with IMHA has not yet been clearly determined (Kidd et al., 2013).

Canine PIMA

In contrast to the classical regenerative presentation of IMHA, as many as 33-58% of dogs reported to have evidence of immune-mediated RBC destruction lack an appropriate regenerative bone marrow response at the time of presentation, in a condition frequently called nonregenerative IMHA (NRIMHA) (Klag et al., 1993; Kellerman et al., 1995; Burgess et al., 1997; Jonas et al., 1987). Although many of these dogs may have been assessed before stimulated erythropoiesis led to reticulocytosis, i.e., in a preregenerative state (Klag et al., 1993; Kellerman et al., 1995; Burgess et al.,

1997), few have had persistent anemia accompanied by impaired erythropoiesis (Klag et al., 1993; Jonas et al., 1987).

Bone marrow assessment of these dogs has usually revealed erythroid hypercellularity, indicating ineffective erythropoiesis, or less commonly erythroid hypocellularity, interpreted as pure red cell aplasia (PRCA) (Jonas et al., 1987; Stockham et al., 1980; Weiss et al 1982; Holloway et al., 1990; Scott-Moncrieff et al., 1995; McManus 2000; Stokol et al., 2000; Weiss 2002; Weiss 2008). An additional group of dogs with persistent hyporegenerative anemia and similar bone marrow findings have no evidence of hemolysis but have a similar clinical course, and have also been considered to have NRIMHA or nonregenerative immune-mediated anemia (NIMA) (Weiss et al 1982; Scott-Moncrieff et al., 1995; McManus 2000; Stokol et al., 2000; Weiss 2002). Because these dogs are suspected to have immune-mediated targeting of their erythroid precursors, the condition will be referred to henceforth as precursor-targeted immune-mediated anemia (PIMA).

Phagocytosis of erythroid precursors, which we refer to as rubriphagocytosis, has been reported sporadically in dogs with suspected PIMA (Jonas et al., 1987; Stockham et al., 1980; Holloway et al., 1990; McManus 2000), but one investigator (McManus 2000) found that many of 44 dogs with NRIMHA had increased marrow macrophages, rubriphagocytosis, and macrophages containing basophilic granules consistent with degraded cellular material. These findings were rare in marrow from dogs with traditional IMHA or hemorrhagic anemia. There is no study to date showing how rubriphagocytosis and the stage at which it occurs during erythroid development may be associated with bone marrow abnormalities and expected clinical outcome in PIMA.

An additional bone marrow abnormality seen in some dogs with suspected PIMA is myelofibrosis. Reticulin myelofibrosis was reported in 16 of 16 tested dogs with PIMA (Stokol et al., 2000), and has been associated with autoimmune disorders in humans in a condition recently characterized as autoimmune myelofibrosis (Vergara-Lluri et al., 2014). A relationship between PIMA and myelofibrosis could also be related to an immune-mediated mechanism, and supports the concept of autoimmune-associated myelofibrosis that has been suggested in dogs (Villiers et al., 1999; Reagan et al., 1992). Alternatively, myelofibrosis could be secondary to a state of chronic inflammation in the bone marrow of PIMA dogs, possibly accompanied by an increase in fibrotic signaling and tumor growth factor β (TGF-β) (Ueha et al., 2012). Other mechanisms for myelofibrosis in anemia that have been considered include: 1) persistent increases in EPO from high pharmacologic doses of recombinant human erythropoietin (Bader et al., 1992) or in dogs with inherited pyruvate kinase deficiency (Prasse et al. 1975; Searcy et al., 1979), 2) hemosiderin accumulation (Hoff et al., 1991), and 3) increased numbers of dysplastic bone marrow megakaryocytes as in people with primary myelofibrosis (Thiele et al., 2009), but there is no strong evidence to indicate these mechanisms could be the cause of myelofibrosis in PIMA. Current lack of systematic assessment of core biopsies of dogs with PIMA limits understanding of how myelofibrosis may relate to this condition.

Over 200 dogs with some form of suspected PIMA have been described (Klag et al., 1993; Kellerman et al., 1995; Burgess et al., 1997; Jonas et al., 1987; Stockham et al., 1980; Weiss et al., 1982; Holloway et al., 1990; Scott-Moncrieff et al., 1995; McManus 2000; Stokol et al., 2000; Weiss 2002; Weiss 2008; Weiss 1986). Suspicion of an immune-mediated pathogenesis (Klag et al., 1993; Kellerman et al., 1995; Burgess et al., 1997; Jonas et al., 1987; Stockham et al., 1980; Holloway et al., 1990; Stokol et al.,

2000; Weiss 2008) is reasonable but unproven, and is based on concurrent evidence of IMHA in many dogs, some favorable responses to immunosuppressive therapy, apparent relapses after withdrawal of steroids, rubriphagocytosis, which may reflect immune-targeting of erythroid precursors, and evidence that some dogs with PRCA have a circulating inhibitor to erythropoiesis that is in the IgG-containing fraction of plasma (Weiss 1986).

In the most complete clinical study published to date, 43 dogs with PRCA or NRIMA were described, including a wide range of breeds and ages with a median age of 6.5 years and over-representation by spayed females and Labrador retrievers (Stokol et al., 2000). Evidence of concurrent IMHA was frequent: 20 of 35 tested animals had Coombs' test positivity, 23 of 43 had many spherocytes, and 2 of 43 dogs had RBC agglutination. Treatment with a variety of immunosuppressive protocols was associated with complete, partial, and poor response in 55%, 18%, and 27% of the dogs, respectively. Time to initial response was 1-10 weeks, and six dogs (21%) relapsed after tapering or stopping treatment. The mortality rate was 28%. Others have reported variable and slow treatment responses with relapses (Jonas et al., 1987; Stockham et al., 1980; Scott-Moncrieff et al., 1995) and mortality rates up to 44% (Weiss 2008). Therapeutic protocols commonly used for regenerative IMHA seem to be less successful in dogs with PIMA. Despite suspicion and evidence for erythroid lineage immune targeting in PIMA, lack of response in many dogs, slow responses for animals that improve with treatment, and absence of evidence of concurrent IMHA in many PIMA dogs suggest that there is more to learn about the pathogenesis and treatment of this condition.

Human PIMA counterpart

Some human cases of PRCA and reticulocytopenic AIHA resemble cases of canine PIMA, with or without evidence of mature RBC targeting (Meyer et al., 1978; Hedge et al., 1977; Conley et al., 1982; van de Loosdrecht et al., 2000; Jacobs et al., 1985; Crosby et al., 1956; Wranne et al., 1970; Liesveld et al., 1987; Cazzola et al., 1983; Keefer et al., 1988). Although rarely reported, plasma and RBC eluates of some patients with reticulocytopenic AIHA contain IgG reactive with erythrocytes and erythroid precursors (Mangan et al., 1984; Meyer et al., 1978), sometimes via distinctly different antibodies (Mangan et al., 1984). Such patient antibodies have been associated with inhibition of in vitro erythropoiesis (Meyer et al., 1978; Mangan et al., 1984), and either bone marrow erythroid hyperplasia (ineffective erythropoiesis) (Conley et al., 1982; Cazzola et al., 1983) or erythroid hypoplasia in vivo (Mangan et al., 1984). Similarly, many patients with acquired idiopathic PRCA have plasma with demonstrable stage-selective inhibition of erythropoiesis prior to the BFU-E, between the BFU- E and CFU-E, or after the CFU-E (Charles et al., 1996). In one isolated case of reticulocytopenic AIHA associated with thymoma, anemia was attributed not only to an antibody-mediated mechanism, but also to T-cell-mediated in vitro suppression of erythropoiesis (Taniguchi et al., 1988). Direct detection of IgG on the surface of erythroid precursors has been shown in one person with reticulocytopenic AIHA (van de Loosdrecht et al., 2000), and phagocytosis of erythroid precursors has been rarely reported in people with reticulocytopenic AIHA (Keefer et al., 1988). Although infrequently reported, human reticulocytopenic AIHA seems to resemble PIMA clinically, since multiple drug protocols are often required, splenectomy is sometimes necessary, responses are typically slow, relapses are not uncommon, and there is substantial morbidity associated with

prolonged therapy (Charles et al., 1996; Dessypris et al., 2003; Kwong et al., 1996; Beard et al., 1978).

Phosphatidylserine and apoptosis in immune-mediated anemias

Apoptosis of nucleated cells is typically characterized by cell shrinkage, chromatin condensation, nuclear fragmentation, mitochondrial membrane permeabilization, cell membrane blebbing, and breakdown of PS asymmetry of the plasma membrane (Ziegler et al., 2004). Cells exposing PS at the cell surface are recognized by macrophages, which are equipped with receptors specific for PS (Fadok et al., 2000) and rapidly engulf and degrade the affected cells. It has been shown that, despite not having a nucleus, erythrocytes can undergo a similar process, eryptosis, which is characterized by cell shrinkage, cell membrane scrambling, exposure of PS, and consequently triggering of phagocytosis by macrophages (Lang et al., 2015). This process is stimulated by increased Ca^{2+} entry into the cell, and seems to be one of the mechanisms involved in programmed RBC death, therefore participating in erythrocyte turnover (Lang et al., 2005; Lang et al., 2012; Kuypers et al., 2004; Bosman et al., 2005; Garratty 2008). Examples of conditions that may promote eryptosis are hyperosmolarity, oxidative stress, energy depletion, and hyperthermia (Lang et al., 2005; Lang et al., 2012; Kuypers et al., 2004).

Oxidative stress of a severity much less than that required to cause morphologic changes indicative of oxidative damage to RBCs (eccentrocytes, Heinz bodies) has been associated with decreased RBC survival, with or without involvement of antibodies. In people with thalassemia, oxidative damage was shown to cause increased PS exposure on RBCs, contributing to increased erythrophagocytosis and development of

anemia (Kuypers et al., 1998; Amer et al., 2008). In vitro treatment of thalassemic RBCs with an antioxidant compound reduced their sensitivity to hemolysis and phagocytosis by macrophages (Amer et al., 2008). In mice, drug-induced oxidative stress has triggered erythrophagocytosis and anemia. Interestingly, in this study, anemia was accompanied by spherocytosis (Noh et al., 2010), suggesting that increased oxidative stress may contribute to spherocyte formation. Also in mice, decreased antioxidant capacity has been related to the appearance of RBCs with increased membrane IgG and increased sensitivity to lysis (Lee et al., 2004). The investigators proposed that a chronic increase in oxidative stress sensitized RBCs with IgG and promoted IMHA (Lee et al., 2004). In contrast, the binding of anti-A IgG to human A antigen (of the ABO group) on human RBCs in vitro induced Ca^{2+} entry and subsequent PS exposure, suggesting that eryptosis may contribute to RBC removal following an immune reaction against the A antigen (Attanasio et al., 2007), and creating controversy as to which event (immunoglobulin binding or increased oxidative stress/PS exposure) happens first. To date, the relationship between increased oxidative stress, PS exposure, and IgG binding to RBCs is not altogether clear.

Oxidative stress and apoptotic-like mechanisms have been suggested to play a role in canine IMHA because of the demonstration of plasma antibodies against two distinct RBC proteins in IMHA dogs: Prx2, which is involved with the antioxidative metabolism, and calpain, which plays a role in apoptotic pathways (Tan et al., 2012). It has been proposed that oxidative stress or binding of autoreactive antibodies could lead to eryptosis in IMHA (Tan et al., 2012). In support of this idea is the finding of increased plasma malondialdehyde (MDA) concentrations, a marker of oxidative stress, in IMHA dogs when compared to healthy dogs (Pesillo et al., 2002). Currently, there is no clearly

established relationship between oxidative stress, PS exposure, IgG binding, and increased RBC destruction in immune-mediated anemias in humans or dogs.

Phosphatidylserine exposure may be involved not only with accelerated destruction of RBCs, but also with destruction of erythroid precursors in hyporegenerative immune-mediated anemias. This has been evidenced by a person with reticulocytopenic AIHA and positivity for IgG and PS on bone marrow erythroid nucleated cells (van de Loosdrecht et al., 2000). Despite PS positivity, erythroid cells with morphologic abnormalities characteristic of apoptosis were not observed in bone marrow cytologic evaluations of this patient. Apoptosis ultimately results in prominent cellular morphologic changes, but early apoptotic events can trigger PS exposure and may mediate macrophage phagocytosis before the cell appearance is affected (Martin et al., 1995). Therefore, apoptosis cannot be excluded as a potential contributor to ineffective erythropoiesis in dogs with PIMA.

Increased PS exposure in eryptotic RBCs has been reported to cause excessive erythrocyte adhesion to the vascular endothelium, potentially promoting microvascular occlusion and contributing to thrombosis (Borst et al., 2012). The presence of PS in the outer leaflet of the plasma membrane of RBCs from beta-thalassemia patients has been suggested to account for the chronic hypercoagulable state, frequent thrombotic events, and platelet activation that are seen in these patients (Borenstain-Ben Yashar et al., 1993; Musallam et al. 2012). A prothrombotic state and development of thromboembolic disease are frequent complications in dogs with IMHA, and are associated with poor prognosis and increased mortality (Carr et al., 2002; Klein et al., 1989; Fenty et al., 2011; Swan et al., 2013; Piek et al., 2008). The pathogenesis of this prothrombotic state in dogs with IMHA has not yet been determined. Thrombosis has been reported in at

least two PIMA dogs (Jonas et al., 1987; Stokol et al., 2000) and deserves further evaluation.

There are no studies to date showing involvement of increased PS exposure on canine RBCs or erythroid precursors in IMHA or PIMA. However, there is enough information to suggest that cell membrane scrambling and eryptotic mechanisms may contribute to canine immune-mediated anemias in an immunologic (secondary to immunoglobulin binding) or non-immunologic (independent of immunoglobulin binding) fashion. Phosphatidylserine exposure may contribute to anemia and/or ineffective erythropoiesis and rubriphagocytosis in dogs, and it may also play a role in thrombotic events in IMHA, and perhaps in PIMA. Therefore, assessment of PS exposure in dogs with immune-mediated anemias is warranted.

SUMMARY

Immune-mediated anemia is one of the most common causes of anemia in dogs, with IMHA being the most well studied type. IMHA is characterized by evidence of immune-mediated targeting and destruction of mature RBCs, most specifically spherocytosis, RBC agglutination, ghost cells, and/or Coombs' test positivity, and it is usually accompanied by erythroid regeneration. The underlying cause of IMHA remains unknown, and mortality remains a problem, particularly for dogs that develop thromboembolic disease.

In a different condition, dogs with nonregenerative anemia have ineffective erythropoiesis that has occasionally been associated with phagocytosis of erythroid precursors (rubriphagocytosis) or myelofibrosis. Based on some responses to immunosuppressive therapy, relapses after therapy withdrawal, and, in some cases, evidence of concurrent IMHA, these dogs have been considered to have immune-mediated nonregenerative anemia, or PIMA. The pathogenesis of PIMA has not yet been determined, but roles for immunoglobulin and/or complement have been suspected. Evidence of involvement of apoptosis-like mechanisms with phosphatidylserine (PS) exposure in immune-mediated anemias of people and dogs also warrants further studies about the role of PS in PIMA and IMHA. Our experience indicates that the diagnosis of PIMA has historically been missed, or only weakly suggested, because patterns of PIMA have not been recognized, rubriphagocytosis has gone undetected, and awareness of PIMA without hemolysis has not been widespread.

We hypothesize that PIMA is part of a spectrum of immune-mediated anemias, including IMHA, in which IgG, with or without the contribution of PS, targets and promotes phagocytosis of different stages of erythroid cells, simultaneously or

independently. Targeting of epitopes expressed only on erythroid precursors would result in PIMA with ineffective erythropoiesis and without evidence of IMHA, and targeting of epitopes present only on mature RBCs would yield classical IMHA with a typical regenerative presentation. Targeting of epitopes shared by erythroid precursors and mature RBCs would yield concurrent hemolysis (IMHA) and ineffective erythropoiesis (PIMA). In support of this hypothesis is knowledge that 1) erythroid precursors and their mature counterparts express shared and independent cell surface markers (Okumura et al., 1992; Chen et al., 2009; Spivak 2005), 2) antiplatelet antibodies often impair thrombopoiesis and mediate platelet destruction in IMT (Olsson et al., 2005), 3) there is evidence of antibody-mediated reticulocytopenic AIHA associated with erythroid maturation arrest in human patients (Conley et al., 1982; Cazzola et al., 1983; Keefer et al., 1988; Crosby et al., 1956; Wranne et al., 1970; Hedge et al., 1977; Hauke et al., 1983; Jacobs et al., 1985; Liesveld et al., 1987), and 4) increased susceptibility of RBCs and erythroid precursors to phagocytosis has been documented in people with AIHA and thalassemia (Conley et al., 1982; Gallagher et al., 1983; Herron et al., 1986).

The research described in this dissertation represents a step toward better characterizing the pathogenesis of PIMA and its association with IMHA, with the ultimate goals of establishing diagnostic criteria, identifying appropriate therapeutic strategies based on knowledge of the pathogenesis of the diseases, and raising veterinary awareness of PIMA.

HYPOTHESIS AND SPECIFIC AIMS

These studies represent the first steps towards exploring the mechanism behind PIMA. The central hypothesis is that PIMA is part of a spectrum of immune-mediated anemias including IMHA, in which IgG, with or without the contribution of phosphatidylserine (PS), target and promote phagocytosis of different stages of erythroid cells, simultaneously or independently. The rationale is that clarification of the mechanism of PIMA will lead to development of appropriate therapeutic strategies and appropriate diagnostic assays. The specific aims are as follows:

Aim 1. Retrospectively characterize clinical and laboratory findings in dogs with rubriphagocytosis and PIMA, with emphasis on bone marrow cytologic and histologic findings in order to better characterize the condition and facilitate its diagnosis (Chapter 2).

Aim 2. Determine if erythroid populations in dogs with PIMA have increased surface IgG, which could promote phagocytosis (Chapter 3 and Chapter 4).

Hypothesis: Erythroid precursors from dogs with PIMA have increased membrane IgG compared to those from healthy and unhealthy non-PIMA dogs (sick dogs without evidence of immune-mediated anemia).

Aim 2 initially included additional assessment of C3 on erythroid precursors of PIMA dogs, but that was not possible. Details about this topic are discussed in Appendix A.

Aim 3. Determine if phosphatidylserine (PS) plays a role in dogs with immune-mediated anemias (Chapter 4).

3.1. Determine if erythroid populations in dogs with PIMA have increased PS exposure.

Hypothesis: Erythroid precursors from dogs with PIMA have increased PS exposure compared to those from healthy and unhealthy non-PIMA dogs.

3.2. Determine if circulating erythrocytes of dogs with IMHA have increased PS exposure.

Hypothesis: PS exposure is increased on erythrocytes of dogs with IMHA compared to those from healthy and unhealthy non-IMHA dogs (sick dogs without evidence of immune-mediated anemia).

Aim 4. Determine if erythroid precursors in dogs with PIMA have increased susceptibility to phagocytosis (Appendix B).

Hypothesis: Erythroid precursors from dogs with PIMA undergo increased phagocytosis compared to those from healthy and unhealthy non-PIMA dogs.

OUTLINE OF STUDIES

My studies are presented in three chapters followed by a conclusion that discusses a summary of findings, limitations and future directions, and two appendices. The goals and brief descriptions of chapters 2 through 4, and appendices A and B, follow:

Chapter 2

Chapter 2 describes a retrospective study of dogs with PIMA that were assessed at the MSU VMC. The main goal of this chapter was to help characterize canine PIMA, with particular focus on bone marrow findings, in order to better understand the condition, facilitate its recognition and diagnosis, and ultimately lead to better therapeutic management and clinical outcomes.

Summary of study: All dogs with paired bone marrow aspirate and core biopsy samples collected at the MSU VMC from January 2006 through December 2009 were reviewed, and PIMA dogs were identified. Laboratory and clinical data were collected, and response to treatment was assessed when available. Three bone marrow patterns were identified in PIMA dogs, depending on the stage of erythroid precursor being phagocytized, and a fourth pattern consisted of dogs with severe myelofibrosis. Response to immunosuppressive therapy took a median time of one month, and approximately half the dogs did not respond to treatment.

Chapter 3

Chapter 3 describes the development of flow cytometric assays for RBC and erythroid precursor IgG, including a Percoll gradient separation for erythroid populations.

The main goal of this study was to develop methods that would be used for isolation of erythroid precursors and their assessment for IgG and PS.

Summary of study: An assay for RBC IgG was developed, characterized, and applied to IMHA, non-IMHA, and healthy dogs. This assay was adapted for assessment of erythroid precursors. A Percoll gradient separation method was developed for separation of different stages of erythroid precursors from other bone marrow cells, and a 3-color flow assay was developed and characterized to be used for IgG detection on erythroid precursors. Bone marrow cells from DEA1.1 negative and positive dogs were incubated with anti-DEA1.1 typing serum in order to prove our capacity to detect IgG on the surface of erythroid precursors. An additional finding of this study was the expression of DEA1.1 on canine erythroid precursors from rubriblasts through metarubricytes.

Chapter 4

Chapter 4 involves testing of IMHA and PIMA dogs for IgG and PS on erythroid cells. The main goal of this study was to test our hypotheses that IgG and PS play a role in immune-mediated anemias in dogs.

Summary of study: IMHA, non-IMHA, and healthy dogs were tested for IgG and PS on RBCs, and results showed significantly higher IgG and PS in IMHA dogs when compared to other groups. A subset of PIMA dogs had increased IgG-positive erythroid precursors when compared to healthy and non-IMHA dogs. Erythroid precursors from most tested PIMA dogs had more PS-positive erythroid precursors than healthy dogs, however no sick dogs without PIMA were tested for comparison. A limitation of this study, especially in regard to PIMA dogs, is the low number of animals that were tested.

Appendix A

This appendix discusses interesting problems found while trying to develop an assay to assess erythroid cells for canine complement (C3) and IgM. Limitations regarding commercially available reagents and development of a positive control hindered addressing involvement of these mediators as part of Aim 2.

Appendix B

This appendix describes the phagocytic assay developed with the goal of assessing Aim 4, and discusses limitations found with the method and why it ultimately was not used. Additionally, it discusses experiments done while developing and characterizing the assay, and describes important results regarding the speed of phagocytosis and degradation of IgG-coated canine erythroid precursors, which may help explain the paucity of rubriphagocytosis in the bone marrows of PIMA dogs.

REFERENCES

REFERENCES

Amer J, Atlas D, Fibach E. N-acetylcysteine amide (AD4) attenuates oxidative stress in beta-thalassemia blood cells. Biochim Biophys Acta. 2008;1780:249-255.

Angelucci E, Bai H, Centis F, et al. Enhanced macrophagic attack on beta-thalassemia major erythroid precursors. Haematologica. 2002;87:578-583.

Attanasio P, Shumilina E, Hermle T, et al. Stimulation of eryptosis by anti-A IgG antibodies. Cell Physiol Biochem. 2007;20:591-600.

Bader R, Bode G, Rebel W, et al. Stimulation of bone marrow by administration of excessive doses of recombinant human erythropoietin. Pathol Res Pract. 1992;188:676-679.

Balasubramanian K, Schroit AJ. Aminophospholipid asymmetry: A matter of life and death. Annu Rev Physiol. 2003;65:701-734.

Barker RN, Gruffydd-Jones TJ, Stokes CR, et al. Autoimmune haemolysis in the dog: relationship between anaemia and the levels of red blood cell bound immunoglobulins and complement measured by an enzyme-linked antiglobulin test. Vet Immunol Immunopathol. 1992;34:1-20.

Beard ME, Krantz SB, Johnson SA, et al. Pure red cell aplasia. Q J Med. 1978;47:339-348.

Borenstain-Ben Yashar V, Barenholz Y, Hy-Am E, et al. Phosphatidylserine in the outer leaflet of red blood cells from beta-thalassemia patients may explain the chronic hypercoagulable state and thrombotic episodes. Am J Hematol. 1993;44:63-65.

Borst O, Abed M, Alesutan I, et al. Dynamic adhesion of eryptotic erythrocytes to endothelial cells via CXCL16/SR-PSOX. Am J Physiol Cell Physiol. 2012;302:C644-651.

Bosman GJ, Willekens FL, Were JM. Erythrocyte aging: a more than superficial resemblance to apoptosis? Cell Physiol Biochem. 2005;16:1-8.

Burgess KE, Rand W, Moore A. Immune mediated hemolytic anemia in dogs: a retrospective study of 60 cases treated with cyclophosphamide [abstract]. J Vet Intern Med. 1997;11:143.

Carr AP, Panciera DL, Kidd L. Prognostic factors for mortality and thromboembolism in canine immune-mediated hemolytic anemia: a retrospective study of 72 dogs. J Vet Intern Med. 2002;16:504-509.

Caviezel LL, Raj K, Giger U. Comparison of 4 direct Coombs' test methods with

polyclonal antiglobulins in anemic and nonanemic dogs for in-clinic or laboratory use. J Vet Intern Med. 2014;28:583-591.

Cazzola M, Barosi G, Ascari E. Cold haemagglutinin disease with severe anaemia, reticulocytopenia and erythroid bone marrow. Scand J Haematol. 1983;30:25-29.

Charles RJ, Sabo KM, Kidd PG, et al. The pathophysiology of pure red cell aplasia: implications for therapy. Blood. 1996;87:4831-4838.

Chasis JA, Mohandas N. Erythroblastic islands: niches for erythropoiesis. Blood. 2008;112:470-478.

Chaudhary R, Das SS, Gupta R, et al. Application of flow cytometry in detection of red cell bound IgG in Coombs negative AIHA. Hematology. 2006;11:295-300.

Chen K, Liu J, Heck S, et al. Resolving the distinct stages in erythroid differentiation based on dynamic changes in membrane protein expression during erythropoiesis. Proc Natl Acad Sci USA. 2009;106:17413-17418.

Conley CL, Lippman SM, Ness PM, et al. Autoimmune hemolytic anemia with reticulocytopenia and erythroid marrow. N Engl J Med. 1982;306:281-286.

Crosby WH, Rappaport H. Reticulocytopenia in autoimmune hemolytic anemia. Blood. 1956;11:929-936.

Day MJ. Antigen specificity in canine autoimmune haemolytic anaemia. Vet Immunol Immunopathol. 1999;69:215-224.

Day MJ. IgG subclasses of canine anti-erythrocyte, antinuclear and anti-thyroglobulin autoantibodies. Res Vet Sci. 1996;61:129-135.

Day MJ. Immune-mediated anemias in the dog. In: Weiss DJ, Wardrop KJ, eds. Schalm’s Veterinary Hematology. 6th ed. Ames, IA: Wiley-Blackwell; 2010:216-225.

de Maria R, Testa U, Luchetti L, et al. Apoptotic role of Fas/Fas ligand system in the regulation of erythropoiesis. Blood. 1999;93:796-803.

Dessypris EN, Lipton JM. Red cell aplasia. In: Greer JP, Foerster J, Lukens JN, Rodgers GM, Paraskevas F, Glader BE, eds. Wintrobe's Clinical Hematology. 11th ed. Philadelphia, PA: Lippincott Williams & Wilkins; 2003:1421-1437.

Fadok VA, Bratton DL, Rose DM, et al. A receptor for phosphatidylserine-specific clearance of apoptotic cells. Nature. 2000;405:85-90.

Fenty RK, Delaforcade AM, Shaw SE, et al. Identification of hypercoagulability in dogs with primary immune-mediated hemolytic anemia by means of thromboelastography. J

Am Vet Med Assoc. 2011;238:463-467.

Gallagher MT, Branch DR, Mison A, et al. Evaluation of reticuloendothelial function in autoimmune hemolytic anemia using an in vitro assay of monocyte-macrophage interaction with erythrocytes. Exp Hematol. 1983;11:82-89.

Garon CL, Cohn LA, Scott MA. Erythrocyte survival time in Greyhounds as assessed by use of in vivo biotinylation. Am J Vet Res. 2010;71:1033-1038.

Garratty G. The James Blundell Award Lecture 2007: do we really understand immune red cell destruction? Transfus Med. 2008;18:321-334.

Grindem CB, Johnson KH. Systemic lupus erythematosus: literature review and report of 42 new canine cases. J Am Anim Hosp Assoc. 1983;19:489-503.

Hale AS. Canine blood groups and their importance in veterinary transfusion medicine. Vet Clin North Am Small Anim Pract. 1995;25:1323-1332.

Hauke G, Fauser AA, Weber S, et al. Reticulocytopenia in severe autoimmune hemolytic anemia (AIHA) of the warm antibody type. Blut. 1983;46:321-327.

Hedge UM, Gordon-Smith EC, Worlledge SM. Reticulocytopenia and “absence” of red cell autoantibodies in immune haemolytic anaemia. Br Med J. 1977;2:1444-1447.

Herron R, Clark M, Young D, et al. Correlation of mononuclear phagocyte assay results and in vivo haemolytic rate in subjects with a positive direct antiglobulin test. Clin Lab Haematol. 1986;8:199-207.

Hoff B, Lumsden JH, Valli VEO, et al. Myelofibrosis: Review of clinical and pathological features in fourteen dogs. Can Vet J. 1991;32:357-361.

Holloway SA, Meyer DJ, Mannella C. Prednisolone and danazol for treatment of immune-mediated anemia, thrombocytopenia, and ineffective erythropoiesis in a dog. J Am Vet Med Assoc. 1990;197:1045-1048.

Jackson ML, Kruth SA. Immune-mediated hemolytic anaemia and thrombocytopenia in the dog: a retrospective study of 55 cases diagnosed from 1969 through 1983 at the Western College of Veterinary Medicine. Can Vet J. 1985;26:245-250.

Jacobs AD, Champlin RE, Golde DW. Pure red cell aplasia characterized by erythropoietic maturation arrest. Am J Med. 1985;78:515-517.

Jonas LD, Thrall MA, Weiser MG. Nonregenerative form of immune-mediated hemolytic anemia in dogs. J Am Anim Hosp Assoc. 1987;23:201-204.

Keefer MJ, Solanki DL. Dyserythropoiesis and erythroblast-phagocytosis preceding pure

red cell aplasia. Am J Hematol. 1988;27:132-135.

Kellerman DL, Lewis DC, Bruyette DS. Canine immune-mediated hemolytic anemia: a retrospective analysis of 37 cases [abstract]. J Vet Intern Med. 1995;9:189.

Kessler RJ, Reese J, Chang D, et al. Dog erythrocyte antigens 1.1, 1.2, 3, 4, 7, and Dal blood typing and cross-matching by gel column technique. Vet Clin Pathol. 2010;39:306-316.

Kidd L, Mackman N. Prothrombotic mechanisms and anticoagulant therapy in dogs with immune-mediated hemolytic anemia. J Vet Emerg Crit Care (San Antonio). 2013;23:3-13.

Klag AR, Giger U, Shofer FS. Idiopathic immune-mediated hemolytic anemia in dogs: 42 cases (1986-1990). J Am Vet Med Assoc. 1993;202:783-788.

Klein MK, Dow SW, Rosychuk RA. Pulmonary thromboembolism associated with immune-mediated hemolytic anemia in dogs: ten cases (1982-1987). J Am Vet Med Assoc. 1989;195:246-250.

Kucinskiene G, Schuberth H, Leibold W, et al. Flow cytometric evaluation of bound IgG on erythrocytes of anaemic dogs. Vet J. 2005;169:303-307.

Kuypers FA, de Jong K. The role of phosphatidylserine in recognition and removal of erythrocytes. Cell Mol Biol (Noisy-le-grand). 2004;50:147-158.

Kuypers FA, Yuan J, Lewis RA, et al. Membrane phospholipid asymmetry in human thalassemia. Blood. 1998;91:3044-3051.

Kwong YL, Wong KF, Liang RH, et al. Pure red cell aplasia: clinical features and treatment results in 16 cases. Ann Hematol. 1996;72:137-140.

Lang E, Lang F. Mechanisms and pathophysiological significance of eryptosis, the suicidal erythrocyte death. Semin Cell Dev Biol. 2015;39:35-42.

Lang E, Qadri SM, Lang F. Killing me softly - Suicidal erythrocyte death. Int J Biochem Cell Biol. 2012;44:1236-1243.

Lang KS, Lang PA, Bauer C, et al. Mechanisms of suicidal erythrocyte death. Cell Physiol Biochem. 2005;15:195-202.

Lee JM, Chan K, Kan YW, et al. Targeted disruption of Nrf2 causes regenerative immune-mediated hemolytic anemia. Proc Natl Acad Sci USA. 2004;101:9751-9756.

Liesveld JL, Rowe JM, Lichtman MA. Variability of the erythropoietic response in autoimmune hemolytic anemia: analysis of 109 cases. Blood. 1987;69:820-826.

Lin J, Hao T, Lyou J, et al. Clinical application of a flow cytometric direct antiglobulin test. Transfusion. 2009;49:1335-1346.

Lucidi CA, de Resende CLE, Jutkowitz LA, et al. Cytologic and histologic bone marrow findings in dogs with suspected marrow-directed immune-mediated anemia [abstract]. Vet Clin Pathol. 2010;39:560.

Lucidi CA, Takahira RK, Gerlach JA, et al. Flow cytometric assessment of canine erythrocytes and platelets for dog erythrocyte antigen 1.1. Vet Clin Pathol. 2011;40:435-443.

Mangan KF, Besa EC, Shadduck RK, et al. Demonstration of two distinct antibodies in autoimmune hemolytic anemia with reticulocytopenia and red cell aplasia. Exp Hematol. 1984;12:788-793.

Martin SJ, Reutelingsperger CP, McGahon AJ, et al. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. J Exp Med. 1995;182:1545-1556.

Mathias LA, Fisher TC, Zeng L, et al. Ineffective erythropoiesis in beta-thalassemia major is due to apoptosis at the polychromatophilic normoblast stage. Exp Hematol. 2000;28:1343-1353.

McCullough S. Immune-mediated hemolytic anemia: understanding the nemesis. Vet Clin North Am Small Anim Pract. 2003;33:1295-1315.

McManus P. Marrow and erythrocyte changes associated with canine non-regenerative immune-mediated anemia: 44 cases (1996-2000) [abstract]. Vet Pathol. 2000;37:526.

Meyer RJ, Hoffman R, Zanjani ED. Autoimmune hemolytic anemia and periodic pure red cell aplasia in systemic lupus erythematosus. Am J Med. 1978;65:342-345.

Musallam KM, Taher AT, Karimi M, et al. Cerebral infarction in β-thalassemia intermedia: breaking the silence. Thromb Res. 2012;130:695-702.

Noh JY, Park JS, Lim KM, et al. A naphthoquinone derivative can induce anemia through phosphatidylserine exposure-mediated erythrophagocytosis. J Pharmacol Exp Ther. 2010;333:414-420.

Okumura N, Kohichiro T, Nakahata T. Changes in cell surface antigen expressions during proliferation and differentiation of human erythroid progenitors. Blood. 1992;80:642-650.

Olsson B, Andersson PO, Jacobsson S, et al. Disturbed apoptosis of T-cells in patients

with active idiopathic thrombocytopenic purpura. Thromb Haemost. 2005;93:139-144.

Olver CS. Erythropoiesis. In: Weiss DJ, Wardrop KJ, eds. Schalm's Veterinary Hematology. 6th ed. Ames, IA: Wiley-Blackwell; 2010:36-42.

Overmann JA, Sharkey LC, Weiss DJ, et al. Performance of 2 microtiter canine Coombs' tests. Vet Clin Pathol. 2007;36:179-183.

Pesillo SA, Freeman LM, Stone MS, et al. Assessment of oxidative stress in dogs with immune mediated hemolytic anemia [abstract]. Proc 8th Int Vet Emerg Crit Care Symp. (IVECCS). 2002;781.

Piek CJ, Junius G, Dekker A, et al. Idiopathic immune-mediated hemolytic anemia: treatment outcome and prognostic factors in 149 dogs. J Vet Intern Med. 2008;22:366-373.

Piek CJ, Teske E, van Leeuwen MW, et al. Good agreement of conventional and gel-based direct agglutination test in immune-mediated haemolytic anaemia. Acta Vet Scand. 2012;54:10.

Prasse KW, Crouser D, Beutler E, et al. Pyruvate kinase deficiency anemia with terminal myelofibrosis and osteosclerosis in a beagle. J Am Vet Med Assoc. 1975;166:1170-1175.

Quigley KA, Chelack BJ, Haines DM, et al. Application of a flow cytometric erythrocyte immunofluorescence assay in dogs with immune-mediated hemolytic anemia and comparison to the direct antiglobulin test. J Vet Diagn Invest. 2001;13:297-300.

Reagan W, Scott-Moncrieff C, DeNicola D, et al. Immune mediated non-regenerative anemia and myelofibrosis in female beagle dogs [abstract]. Vet Clin Pathol. 1992;21:127.

Reimer ME, Troy GC, Warnick LD. Immune-mediated hemolytic anemia: 70 cases (1988-1996). J Am Anim Hosp Assoc. 1999;35:384-391.

Samson D, Tikerpae J, Crowne H. A simple in vitro method for the assessment of ineffective erythropoiesis. Blood. 1981;58:782-787.

Schrier SL, Centis F, Verneris M, et al. The role of oxidant injury in the pathophysiology of human thalassemias. Redox Rep. 2003;8:241-245.

Scott-Moncrieff JCR, Reagan WJ, Glickman LT, et al. Treatment of nonregenerative anemia with human γ-globulin in dogs. J Am Vet Med Assoc. 1995;206:1895-1900.

Searcy GP, Tasker JB, Miller DR. Animal model of human disease: pyruvate kinase deficiency. Am J Pathol. 1979;94:689-692.

Slappendel RJ. The diagnostic significance of the direct antiglobulin test (DAT) in anemic dogs. Vet Immunol Immunopathol. 1979;1:49-59.

Spivak JL. The anaemia of cancer: death by a thousand cuts. Nat Rev Cancer. 2005;5:543-555.

Stockham SL, Ford RB, Weiss DJ. Canine autoimmune hemolytic disease with a delayed erythroid regeneration. J Am Anim Hosp Assoc. 1980;16:927-931.

Stokol T, Blue JT, French TW. Idiopathic pure red cell aplasia and nonregenerative immune-mediated anemia in dogs: 43 cases (1988-1999). J Am Vet Med Assoc. 2000;216:1429-1436.

Swann JW, Skelly BJ. Systematic review of evidence relating to the treatment of immune-mediated hemolytic anemia in dogs. J Vet Intern Med. 2013;27:1-9.

Tan E, Bienzle D, Shewen P, et al. Potentially antigenic RBC membrane proteins in dogs with primary immune-mediated hemolytic anemia. Vet Clin Pathol. 2012;41:45-55.

Taniguchi S, Shibuya T, Morioka E, et al. Demonstration of three distinct immunological disorders on erythropoiesis in a patient with pure red cell aplasia and autoimmune haemolytic anaemia associated with thymoma. Br J Haematol. 1988;68:473-477.

Testa U. Apoptotic mechanisms in the control of erythropoiesis. Leukemia. 2004;18:1176-1199.

Thiele J, Kvasnicka HM. The 2008 WHO diagnostic criteria for polycythemia vera, essential thrombocythemia, and primary myelofibrosis. Curr Hematol Malig Rep. 2009;4:33-40.

Ueha S, Shand FH, Matsushima K. Cellular and molecular mechanisms of chronic inflammation-associated organ fibrosis. Front Immunol. 2012;3:71.

van de Loosdrecht AA, Hendriks DW, Blom NR, et al. Excessive apoptosis of bone marrow erythroblasts in a patient with autoimmune haemolytic anaemia with reticulocytopenia. Br J Haematol. 2000;108:313-315.

Vergara-Lluri ME, Piatek CI, Pullarkat V, et al. Autoimmune myelofibrosis: an update on morphologic features in 29 cases and review of the literature. Hum Pathol. 2014;45:2183-2191.

Villiers EJ, Dunn JK. Clinicopathological features of seven cases of canine myelofibrosis and the possible relationship between the histological findings and prognosis. Vet Rec. 1999;145:222-228.

Wang Z, Shi J, Zhou Y, et al. Detection of red blood cell-bound immunoglobulin G by flow cytometry and its application in the diagnosis of autoimmune hemolytic anemia. Int J Hematol. 2001;73:188-193.

Wardrop KJ. The Coombs' test in veterinary medicine: past, present, future. Vet Clin Pathol. 2005;34:325-334.

Warman SM, Murray JK, Ridyard A, et al. Pattern of Coombs' test reactivity has diagnostic significance in dogs with immune-mediated haemolytic anaemia. J Small Anim Pract. 2008;49:525-530.

Weiss DJ, Stockham SL, Willard MD, et al. Transient erythroid hypoplasia in the dog: report of five cases. J Am Anim Hosp Assoc. 1982;18:353-359.

Weiss DJ. Antibody-mediated suppression of erythropoiesis in dogs with red blood cell aplasia. Am J Vet Res. 1986;47:2646-2648.

Weiss DJ. Bone marrow pathology in dogs and cats with non-regenerative immune-mediated haemolytic anaemia and pure red cell aplasia. J Comp Pathol. 2008;138:46-53.

Weiss DJ. Primary pure red cell aplasia in dogs: 13 cases (1996-2000). J Am Vet Med Assoc. 2002;221:93-95.

Wilkerson MJ, Davis E, Shuman W, et al. Isotype-specific antibodies in horses and dogs with immune-mediated hemolytic anemia. J Vet Intern Med. 2000;14:190-196.

Wranne L, Bonnevier JO, Killander A, et al. Pure red-cell anaemia with pro-erythroblast maturation arrest. Scand J Haematol. 1970;7:73-75.

Yoshida H, Kawane K, Koike M, et al. Phosphatidylserine-dependent engulfment by macrophages of nuclei from erythroid precursor cells. Nature. 2005;437:754-758.

Ziegler U, Groscurth P. Morphological features of cell death. News Physiol Sci. 2004;19:124-128.

CHAPTER 2

Histologic and Cytologic Bone Marrow Findings in Dogs with Ineffective Erythropoiesis and Phagocytosis of Erythroid Precursors – Suspected Precursor-Targeted Immune-Mediated Anemia

ABSTRACT

Background: Precursor-targeted immune-mediated anemia (PIMA) has been suspected in dogs with nonregenerative anemia and bone marrow findings varying from erythroid hyperplasia to pure red cell aplasia. Phagocytosis of erythroid precursors (rubriphagocytosis) reported in some affected dogs suggests a destructive component to the pathogenesis of PIMA.

Objectives: To characterize laboratory and clinical findings in dogs with suspected PIMA and rubriphagocytosis, with emphasis on bone marrow cytologic and histologic findings.

Methods: Dogs with PIMA and rubriphagocytosis were identified by review of paired bone marrow aspirate and core biopsy slides collected over a 4-year period. Samples were systematically assessed and characterized along with other pertinent laboratory data and clinical findings.

Results: Twenty-five dogs met criteria for PIMA and had rubriphagocytosis that was relatively stage-selective. Erythropoiesis was expanded to the stage of erythroid precursor undergoing most prominent phagocytosis, yielding patterns characterized by a hypo-, normo-, or hypercellular erythroid lineage. A fourth pattern involved severe collagen myelofibrosis, and there was a spectrum of mild to severe collagen myelofibrosis overall. Evidence of immune-mediated hemolysis was rare. Immunosuppressive therapy was associated with remission in 77% of dogs known to be treated for at least the median response time of 2 months.

Conclusions: Bone marrow patterns in dogs fulfilling criteria for PIMA were aligned with stage-selective phagocytosis of erythroid precursors and the development of collagen myelofibrosis, a common feature in dogs with PIMA. Recognition of these patterns and

detection of rubriphagocytosis facilitates diagnosis of PIMA, and slow response to immunosuppressive therapy warrants further investigation into its pathogenesis.

INTRODUCTION

Since 1980, nearly 270 dogs have been described with nonregenerative anemia suspected to be caused by an immune-mediated mechanism (Weiss et al., 1982; Scott-Moncrieff et al., 1995; McManus 2000; Stokol et al., 2000; Weiss 2002; Jonas et al., 1987: Klag et al., 1993; Kellerman et al., 1995; Burgess et al., 1997; Weiss 2008; Holloway et al., 1990; Weiss 1986; Stockham et al., 1980; Reagan et al., 1992; Villiers et al., 1999), and commonly suggested to involve targeting of erythroid precursors (Klag et al., 1993; Kellerman et al., 1995; Burgess et al., 1997; Jonas et al., 1987; Stockham et al., 1980; Holloway et al., 1990; McManus 2000; Stokol et al., 2000; Weiss 2008; Villiers et al. 1999). Approximately 170 of these dogs had evidence of concurrent erythrocyte attack, some with a positive Coombs' test, suggesting a relationship to immune-mediated hemolytic anemia (IMHA) and leading to use of the term nonregenerative IMHA (NRIMHA) whether hemolysis was detected or not (Klag et al., 1993; Kellerman et al., 1995; Burgess et al., 1987; Jonas et al., 1987; Stokol et al., 2000; Weiss 2008; Scott-Moncrieff et al., 1995; Weiss 1986; Reagan et al., 1992; Villiers et al., 1999). Others have considered dogs with similar findings to have nonregenerative immune-mediated anemia (NRIMA, NIMA), a more general term consistent with the lack of hemolysis in some cases (Weiss et al. 1982; Scott-Moncrieff et al., 1995; McManus 2000; Stokol et al., 2000; Weiss 2002; Jonas et al., 1987; Weiss 1986). Although some of these dogs may have been presented within a few days of becoming acutely anemic, while in a preregenerative stage of IMHA (Kellerman et al., 1995; Burgess et al., 1987; Weiss 2008), dogs in these reports have generally had persistent nonregenerative anemias indicative of impaired erythropoiesis.

Comparisons among these cases are complicated by inconsistent inclusion criteria, terminology, and degrees to which bone marrow aspirate and core biopsy findings were reported. Bone marrow findings in dogs with suspected NIMA or NRIMHA have varied markedly from what was considered to be pure red cell aplasia (PRCA) to erythroid hyperplasia often accompanied by erythroid maturation arrest or left shift (Stokol et al., 2000; Weiss 2008; Weiss 1986; Jonas et al., 1987; Holloway et al., 1990; Stockham et al., 1980; Weiss et al. 1982; Scott-Moncrieff et al., 1995; McManus 2000; Weiss 2002). A few reports describe the presence of erythroid precursor phagocytosis (Stockham et al., 1980; Jonas et al., 1987; Holloway et al., 1990; McManus 2000), which we refer to as rubriphagocytosis (RP) to differentiate it from phagocytosis of erythrocytes (erythrophagocytosis) and to reflect the “rubri” component in all stages of erythroid precursors from rubriblasts through metarubricytes. In one of these studies, many of 44 dogs diagnosed with NRIMA, but not dogs with IMHA or hemorrhage-induced anemias, had increased marrow macrophages, RP, and macrophages containing basophilic granules consistent with degraded cellular material (McManus 2000).

We, too, have detected RP in the vast majority of dogs with nonregenerative anemia, ineffective erythropoiesis, and an ultimate working diagnosis of what we refer to as precursor-targeted immune-mediated anemia (PIMA). This term avoids “hemolytic” because of the inconsistency and common absence of a hemolysis in these dogs, and reflects the suspected immune-mediated targeting of erythroid precursors based on nonregenerative anemia with 1) concurrent evidence of immune-mediated hemolysis in some dogs (Holloway et al., 1990; Klag et al., 1993; Kellerman et al., 1995; Burgess et al., 1997; Jonas et al., 1987; Stokol et al., 2000; Weiss 2008; Scott-Moncrieff et al., 1995; Weiss 2002), 2) favorable responses to immunosuppressive therapy in some

dogs (Klag et al., 1993; Jonas et al., 1987; Stockham et al., 1980; Weiss et al 1982; Holloway et al., 1990; Scott-Moncrieff et al., 1995; Stokol et al., 2000; Weiss 2002), 3) apparent relapses after withdrawal of immunosuppressive therapy (Stockham et al., 1980; Weiss et al 1982; Scott-Moncrieff et al., 1995; Stokol et al., 2000; Weiss 2002), 4) RP, which may reflect immune-targeting of erythroid precursors (Stockham et al., 1980; Jonas et al., 1987; Holloway et al., 1990; McManus 2000), and 5) evidence that some dogs with PRCA have a circulating inhibitor to erythropoiesis that is in the IgG-containing fraction of plasma (Weiss 1986). However, the pathogenesis of disease in these dogs is unknown, and bone marrow findings have not been comprehensively reviewed, particularly with regard to RP and its relationship to other findings.

The major goal of this study was to better characterize clinical and laboratory findings in PIMA dogs, with emphasis on bone marrow cytologic and histologic abnormalities. It is hoped that this will facilitate recognition and diagnosis of this condition, and eventually lead to a better understanding of the disease and its optimal management.

MATERIAL AND METHODS

Samples

PIMA cases were identified through the authors' review of archived slides for all dogs with paired bone marrow aspirate and core biopsies collected within 24 h of a complete blood count (CBC) at the Michigan State University Veterinary Medical Center (MSU VMC) from January 2006 through June 2009. Wright-stained aspirate smears were reviewed without knowledge of previous descriptions, but with knowledge of CBC results and indications for bone marrow assessment. Additional cases were acquired from July through December 2009 through assessment of marrow reports and review of archived slides for dogs with a possible PIMA component; reportedly inadequate samples were reviewed for adequacy and evidence of PIMA. Thin (3 μm target) histologic sections of B5-fixed tissue stained with hematoxylin and eosin were subsequently reviewed without knowledge of previous core biopsy findings, but with knowledge of aspirate reassessment findings. This process paralleled routine clinical diagnostic service and provided consistent and complete assessments of all cases without the variability inherent in using original reports from multiple pathologists.

Each aspirate and core bone marrow sample was assessed for routine features (Table 1) and RP, and findings were systematically recorded. Left shifts were considered maturation arrest if there was a prominent diminution of a lineage at a certain nucleated precursor stage and beyond. Cases were excluded if either sample was considered inadequate and lacking in any useful information regarding the indication for marrow assessment.

Table 1: *Features and categories for cytologic and histologic assessment of bone marrow aspirate and core specimens.*

ASPIRATE AND CORE	
Quality	Inadequate Marginal Adequate
Hematopoietic Cellularity	Low (< 25%) Moderate (25 – 50%) High (> 75%)
Megakaryocyte Number	Decreased Within limits of health Increased
Megakaryocyte Maturation	Orderly Left shifted
Myeloid to Erythroid Ratio	Decreased Within limits of health Increased Unclear
Maturation of Cell Lineages (Myeloid and Erythroid)	Orderly and complete Left shifted Maturation arrest
Numbers of Other Cell Types*	Within limits of health Increased
Cell Abnormalities	Atypical cell populations Dysplastic changes Cytophagia†
Hemosiderin	Absent Small amounts Moderate amounts Large amounts

Table 1 (cont'd)

CORE	
Erythroid Islands (number and size)	Decreased Within limits of health Increased
Necrosis	Absent Present
Bone Abnormalities	Increased osteoclasts Bone lysis Bone formation
Vascular Lesions	Sinusoidal dilation Hemorrhage Vasculitis Thrombosis
Collagen Fibrosis	Mild Moderate Marked

* Lymphocytes, plasma cells, macrophages, mast cells, osteoclasts, osteoblasts
† Specific cell types and stages noted; most apparent cytologically

Dogs were classified as having PIMA when the following criteria were met: 1) persistent nonregenerative anemia of enough significance to direct the attending clinicians to collect bone marrow (on the day of marrow collection, all had evidence of anemia for at least 5 days, unexplained Hct ≤ 25%, and no reticulocytosis (≤76,000/μL)), 2) ineffective erythropoiesis as evidenced by persistent anemia with concurrent expanded or arrested erythroid islands (cores) or increased particle hematopoietic cellularity with decreased myeloid to erythroid ratios (aspirates), 3) lack of significant dysplasia, and 4) clear selective phagocytosis of intact erythroid precursors by intact normal-appearing macrophages (Figure 1). Although PIMA was diagnosed in several other dogs over this time period when RP was suspected by cytoplasmic fragments of macrophages containing intact erythroid precursors or by increased degraded cellular material within macrophage cytoplasm (Figure 2), these cases were excluded from this

study to avoid any concerns about whether RP was actually present. Dogs with suspected nonselective cytophagic disorders and concurrent phagocytosis of neutrophils or other nucleated cell types, e.g., hemophagocytic syndrome or histiocytic sarcoma, were excluded.

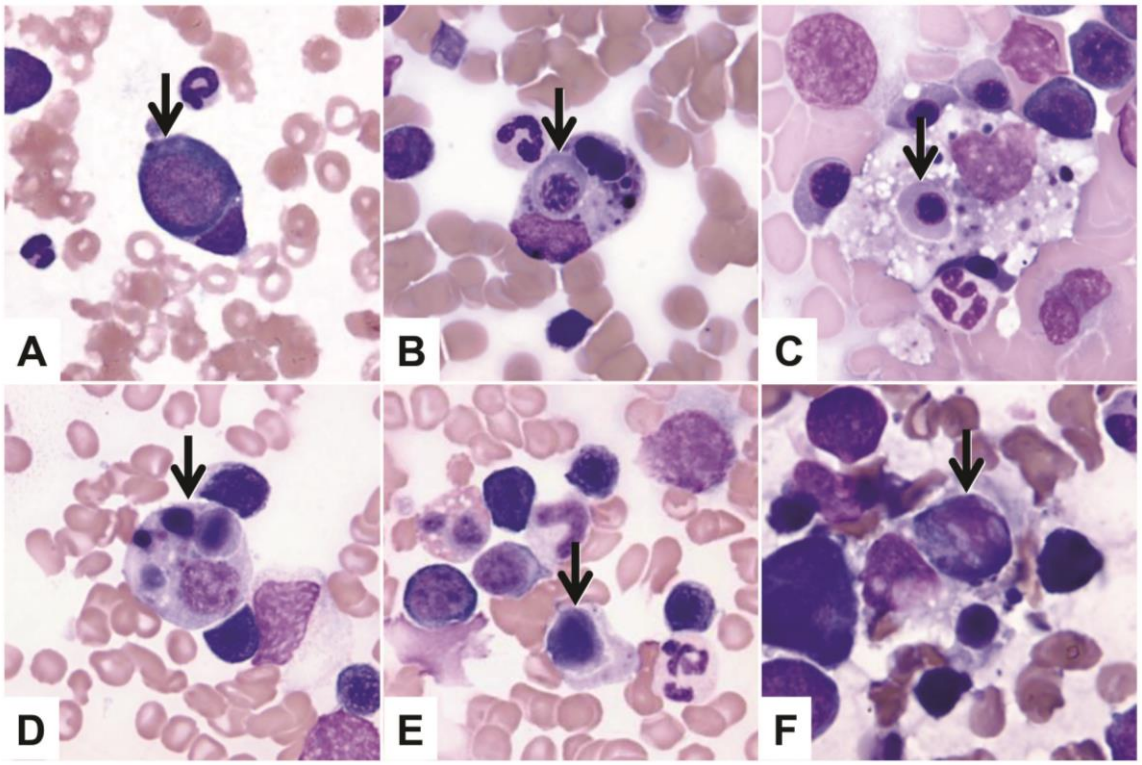

Figure 1: *Cytophagia in aspirates of canine bone marrow.* Rubriphagocytosis (RP) of intact early- (rubriblast, A), mid- (rubricyte, B), and late-stage (C) erythroid precursors by normal-appearing macrophages in dogs with PIMA. Although frequently present with intact RP, dogs whose macrophages (D) or macrophage cytoplasmic fragments (E) contained only degraded cells with indistinct limiting membranes and chromatin patterns were excluded, even when the nuclear material formed too large an inclusion to have been an extruded metarubricyte nucleus (D, E). Dogs with phagocytosis only of cells with apoptotic features were also excluded, though this was rarely seen and was not seen in dogs considered to have PIMA (F). Wright-Giemsa stain, 100× magnification.

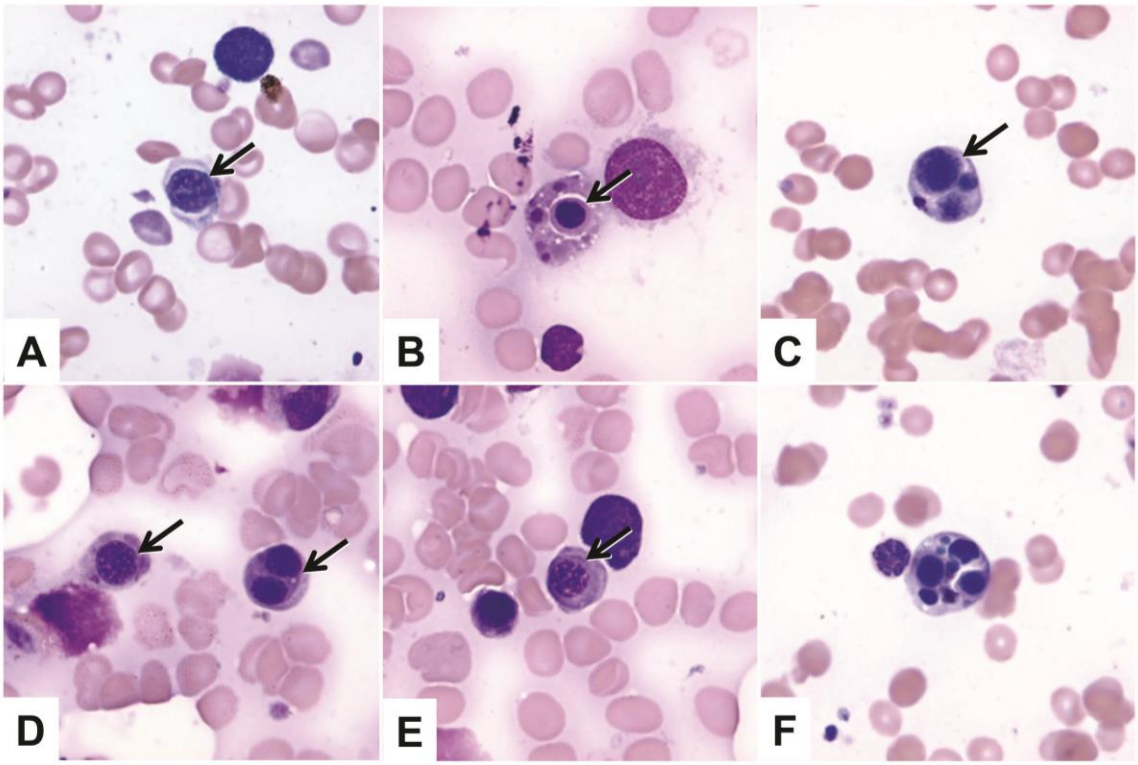

Figure 2: *Macrophage cytoplasmic fragments containing erythroid precursors.* Although treated clinically as PIMA cases, cases were excluded if the evidence of RP was restricted to cytoplasmic fragments of macrophages containing intact erythroid precursors (A-E), in case there was any question as to the nature of these fragments. The arrows point to the outer edge of the erythroid precursor within the macrophage fragments, several of which contain other inclusions typical of macrophages. These fragments are contrasted by fragments containing only degraded cellular material or extruded metarubricyte nuclei (F). Wright-Giemsa stain, 100× magnification.

Clinical and laboratory data

For each case, clinical and laboratory data were retrospectively collected from patient files. Results for CBC at admission and serum color were recorded for all dogs, and results for the following initial tests were recorded when generated: Coombs' test,

serum protein and bilirubin concentrations, hemostasis profile, and serologic and molecular tests for Babesia canis, Borrelia burgdorferi, Anaplasma phagocytophilum, Ehrlichia canis, and Rickettsia rickettsii. These results were generated by the MSU Diagnostic Center for Population and Animal Health. Radiographic, ultrasonographic, cytologic, and histologic results were also recorded when available.

Other recorded clinical information for each dog included signalment, transfusion and disease history, recent or current medications, presenting complaint, days after beginning immunosuppressive therapy at MSU until response and until remission, achievement of second remission, and survival days based on the last known information obtained from the owner or referring veterinarian. Response to therapy was considered an increase in the reticulocyte concentration to exceed the upper reference limit or an increase in Hct to within reference interval (WRI). Remission was defined as a return of Hct to WRI. When there was a relapse of anemia after remission, a second remission was defined by either resolution of anemia or by owner-reported resolution of all clinical signs.

Data analysis

Dogs with PIMA were grouped by the stage of erythroid precursor undergoing RP combined with cellularity of the erythroid lineage and the presence of severe myelofibrosis. Groups were tested for normality using D'Agostino-Pearson test. Comparisons between two groups were done using the Mann Whitney test, and comparisons among three groups or more were done using the Kruskal-Wallis test; $p<0.05$ was considered significant. Prism 6 (GraphPad Software Inc, La Jolla, CA, USA) was used for all statistics.

RESULTS

Paired bone marrow aspirate and core biopsy samples were examined for 121 patients, and 16 dogs were excluded because of at least one inadequate sample. Inclusion criteria for PIMA were fulfilled for 25 of the remaining 105 dogs. Indications for marrow assessment in these 25 patients were either isolated nonregenerative anemia (17, 68%) or nonregenerative anemia with one other cytopenia (8, 32%). PIMA dogs accounted for 17 of the 32 dogs (53%) assessed for nonregenerative anemia alone, and 8 of the 24 dogs (33%) assessed for nonregenerative anemia and neutropenia (8) or nonregenerative anemia and thrombocytopenia (16).

Of the 25 PIMA dogs, 11 were male (2 intact) and 14 were female (1 intact), ranging in age from 1 to 15 years (median = 7 years). Four were of mixed breeding, two were Labrador retrievers, two were Pembroke Welsh corgis, and the other 17 were from 17 other distinct breeds. At the time of admission, six dogs were receiving immunosuppressive therapy, four for their current anemias and two for IMHA diagnosed 5 months or 1 year prior. One other dog had a history of relapsing immune-mediated thrombocytopenia (IMT).

Presenting clinical complaints were lethargy (23/25), anorexia/hyporexia (13/25), weight loss (9/25), collapse (7/25), exercise intolerance (6/25), vomiting (3/25), seizures (1/25, on phenobarbital and potassium bromide), and pica (1/25, also vomiting). One patient had received a blood transfusion eight days prior to admission, and two had transfusions less than 24 hours before a CBC was obtained, raising their Hcts to 23% and 31%. The latter dog had undergone an exploratory laparotomy for vomiting the previous day, and a surgical biopsy yielded a diagnosis of moderate chronic-active lymphoplasmacytic hepatitis with fibrosis.

CBC findings

Other than nonregenerative anemia, hematologic abnormalities were variable (Table 2). Anemia was moderate to severe in the majority of dogs, and clearly nonregenerative in all dogs at the time of bone marrow evaluation. Ten dogs had decreased reticulocyte concentrations; these dogs had Hcts of 8 to 23%. Most anemias were normocytic normochromic (6 of 25), macrocytic normochromic (5 of 25), or macrocytic hypochromic (7 of 25). Nucleated RBCs (nRBCs) were reported in six dogs, with a median for those six dogs of 0.4×10^3/μL (low-high: 0.1-1.0×10^3/μL).

Features common in IMHA were rare in these PIMA dogs. No erythrocyte agglutination was observed in blood tubes, in blood films, or on the ADVIA 120 analyzer scatterplot of any PIMA patient. Mild spherocytosis was reported for one dog, but it was not confirmed on later review. A polyvalent direct Coombs' test was positive in two of the nine dogs that were tested.

Leukocyte findings were variable, with leukocytosis in 40% (10/25) of the dogs, mild leukopenia with neutropenia in three dogs, and mild neutropenia in two others (Table 2). Thrombocytosis (low-high: 500,000-882,000/μL; median = 610,000/μL) was present in five dogs, and thrombocytopenia (11,000/μL; RI: 155,000-393,000) was present in one dog suspected of having concurrent IMT.

Table 2: *Hematologic findings in the 25 dogs fulfilling diagnostic criteria for PIMA.*

	Unit	Median	Low - High	< LRL	WRI	> URL	RI
Hct	%	18†	8 - 31†, +	n = 25	n = 0	n = 0	40 - 55
Reticulocytes	/µL	17,000	<5,000 - 102,000+	n = 10	n = 14	n = 1+	12,000 - 76,000
MCV	fL	71	61 - 100	n = 2	n = 11	n = 12	62 - 71
CHCM	g/dL	34.2	27.4 - 38.1	n =11	n = 12	n = 2	33.5 - 36.4
WBCs	/µL	10,260	5,240 - 55,950	n = 3	n = 12	n = 10	5,900 - 11,600
Neutrophils	/µL	7,090	2,600 - 38,430	n = 5	n = 8	n = 12	4,000 - 8,200*

† Includes two dogs with transfusions less than 24 h before obtaining Hcts of 23% and 31%.
+ The dog with highest Hct and reticulocyte concentration at admission had decreased values one day prior to bone marrow collection (3 days after admission) – Hct = 21%, reticulocyte concentration = 42,900, and on the day of bone marrow collection (4 days after admission) – Hct = 19%, reticulocyte concentration = 29,200.
* RI for microscopic differentials (18 of 25 dogs); RI for automated differentials is 3,800-7,800/µL (7 of 25 dogs)
LRL = lower reference limit; URL = upper reference limit; WRI = within reference interval.

Bone marrow findings

Selective RP was detected in bone marrow aspirates from 23 dogs, in only cytologic smears of the spleen for one dog, in only histologic sections of marrow for one dog, and in histologic sections and cytologic smears for 8 dogs. RP was typically less prominent than indirect evidence of RP, including cytoplasmic fragments containing morphologically intact precursors and increased numbers of macrophages containing basophilic inclusions consistent with degraded cells (Figure 2). These findings were present in the marrow and spleen of the dog with clear RP only in the spleen. RP was prominent in some cases, but in other cases it was detected only after careful examination of one or several smears. It was often best found along or near the side edges of aspirate smears, where nucleated cells were somewhat concentrated, relatively well spread, and intact. In tissue sections, RP was less clear because of high cell density and diminished cellular detail, but it was detectable when the plane of sectioning included the macrophage nucleus along with the phagocytized cell surrounded by macrophage cytoplasm containing hemosiderin or other inclusions.

Although all PIMA dogs had ineffective erythropoiesis and RP, four patterns emerged from their combined cytologic and histologic bone marrow samples. These patterns were defined by differences in erythroid cellularity, maturation stage of cells undergoing phagocytosis, and degree of collagen myelofibrosis (Table 3; Figures 3 and 4). With all patterns, expanded erythroid islands on histologic sections provided evidence of stimulated erythropoiesis up to the stage of most prominent RP, which corresponded with the stage of maturation arrest, when present.

Table 3: *Features of the four bone marrow patterns apparent in the 25 dogs with PIMA.*

PIMA Pattern	Predominant Cells Undergoing RP	Erythroid Cellularity	M:E Ratio	Collagen MF	Number
Early-stage	Rubriblasts, prorubricytes	Low*	High	Absent	3
Mid-stage	Prorubricytes, early rubricytes	Moderate	Moderate	Absent	3
Late-stage	Late rubricytes, metarubricytes	High	Low	Absent to moderate	14
Severe MF	Rubricytes to metarubricytes	Low	Low	Severe	5

MF = myelofibrosis; M:E = myeloid to erythroid ratio
* with expanded erythroid islands

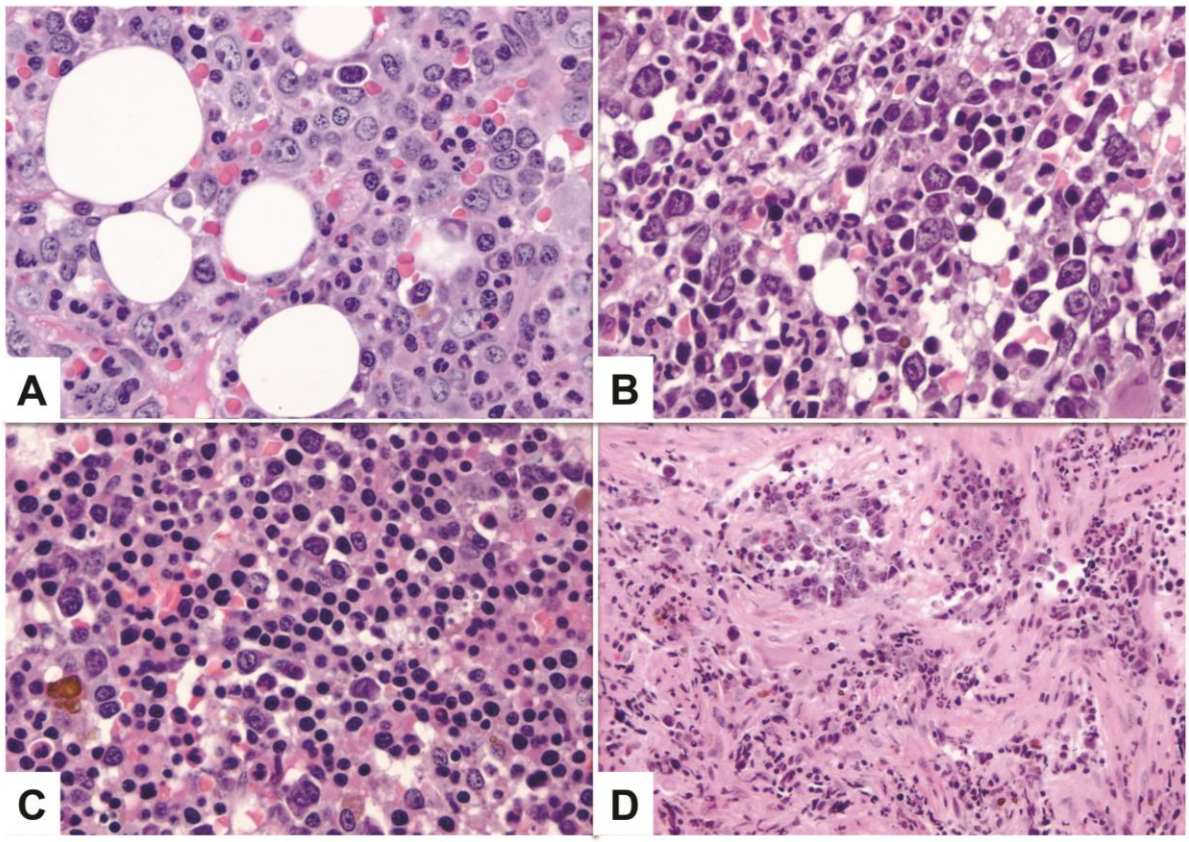

Figure 3: *Four histologic patterns identified in the 25 dogs with PIMA.* Low erythroid cellularity with a marked erythroid left shift (A), moderate erythroid cellularity with a moderate erythroid left shift or maturation arrest (B), high erythroid cellularity with many mid- and late-stage erythroid precursors (C), and severe collagen fibrosis combined with erythroid hypocellularity but expanded and typically left-shifted erythroid islands (D). In all cases, erythropoiesis was ineffective and characterized by expanded erythropoiesis in active areas. B5 fixation, H&E stain, 50× magnification (A-C), 20× magnification for D to better show the extent of fibrosis.

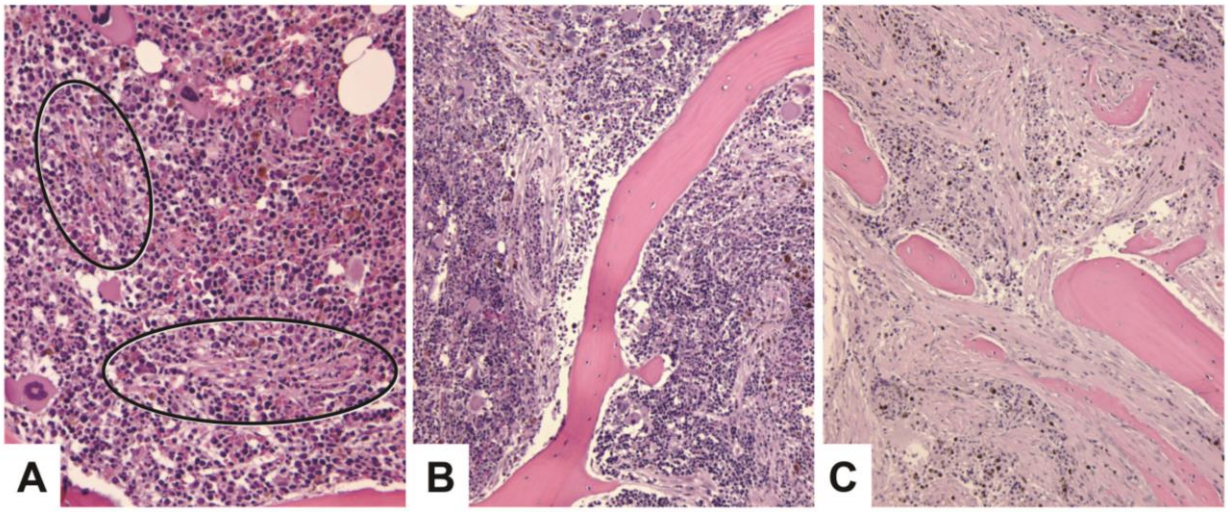

Figure 4: *Mild (A), moderate (B), and severe (C) collagen myelofibrosis in dogs with late-stage PIMA.* Fibrosis progressed from small, indistinct linear eosinophilic regions (A) to narrow and moderately wide intertrabecular and/or paratrabecular bands of fibroblasts and collagen (B), to effacing fibrosis with new bone formation (C). B5 fixation, H&E stain, 20× (A) and 10× (B, C) magnification.

Early-PIMA dogs (3/25) had erythroid hypocellularity associated with early-stage RP and early maturation arrest at the rubriblast (2) or prorubricyte (1) stage (Figure 3A). Rubriblasts varied from rare to increased. Overall hematopoietic cellularity was moderate or high because of active myelopoiesis. Concurrent ineffective neutropoiesis was suspected in the one dog with high hematopoietic cellularity. All three dogs in this group had mild increases in lymphocytes and/or plasma cells.

Mid-PIMA dogs (3/25) had no clear increase or decrease in erythroid cellularity, but they had mostly mid-stage RP (Figure 3B) associated with erythroid maturation arrest or left shift and decreased metarubricytes. Although RP of prorubricytes and rubricytes

predominated, there was additional phagocytosis of metarubricytes in one dog. One dog had a mild increase in plasma cells.

Late-PIMA dogs (14/25) had increased hematopoietic cellularity with erythroid hypercellularity and mostly late-stage RP (Figure 3C), but phagocytosis of basophilic rubricytes was additionally seen in two dogs. Myeloid to erythroid ratios were consistently decreased because of erythroid hyperplasia. Maturation arrest or left shift in the nucleated erythroid series was detected in 9 of 14 dogs (64.3 %). Seven of these 14 dogs had mild (3) to moderate (4) collagen myelofibrosis characterized by narrow to moderately wide intertrabecular or paratrabecular bands of fibroblasts and collagen in histologic sections (Figure 4).

SMF-PIMA dogs (5/25) had severe myelofibrosis effacing most medullary tissue (Figure 3D), with bone remodeling and new bone formation within some fibrous bands. Remaining foci of hematopoietic tissue contained expanded islands of erythropoiesis, and had decreased myeloid to erythroid ratios with late-stage RP in three dogs, mid- and late-stage RP in one dog, and only mid-stage RP in one dog.

Altogether, 12 of the 25 PIMA dogs had mild to severe collagen myelofibrosis (Table 4), all with mostly mid- to late-stage RP. When collectively compared to late-PIMA dogs without collagen myelofibrosis, late-PIMA dogs with any degree of myelofibrosis were older ($p=0.03$). SMF-PIMA dogs had greater MCV than dogs with no myelofibrosis ($p=0.04$).

Table 4: *Selected findings in late-stage PIMA dogs grouped by degree of collagen myelofibrosis.*

	No MF	Any MF	Mild MF	Moderate MF	Severe MF	RI
Number	7	12	3	4	5	-
Age (yr)	5.0	**8.0***	8.0	9.5	7.0	-
Hct (%)	20.0	17.0	18.0	16.0	16.0	40-55
MCV (fL)	71	72	69	65	**76***	62-71
Number < or > RI	3H, 1L	7H	1H	1H	5H	
CHCM (g/dL)	33.1	34.0	33.2	35.6	33.7	33.5-36.4
Number < or > RI	5L	2H, 5L	2L	2H, 1L	2L	
Reticulocytes (×10^4/μL)	5.7	**1.8***	0.5	1.8	1.8	1.2-7.6
Number < or > RI	2L	4L	2L	1L	1L	

All values are medians. RI = reference interval. H = high and L = low with respect to RI. *Significantly different from the No MF group.

Hemosiderin was present in the bone marrow of all 25 PIMA dogs, with moderate to large amounts in all but three dogs (two with late-PIMA and one with early-PIMA). Large amounts were noted in 6 of 14 late-PIMA dogs, in 1 of 3 early-PIMA dogs (multifocal dense accumulations), and in 2 of 5 SMF-PIMA dogs (one with multifocal dense accumulations). Macrophages subjectively appeared increased in six dogs, four late-PIMA dogs, one early-PIMA dog, and one SMF-PIMA dog. Eight PIMA dogs had megakaryocytic hyperplasia; given the association between megakaryocytes and fibrosis in people, it is noteworthy that seven of these eight dogs lacked collagen myelofibrosis, and no megakaryocyte dysplasia was detected. No necrosis, vascular disease, inflammation, or atypical cell populations were detected in any of the 25 dogs.

Other findings

One of the Coombs'-positive dogs had mild hyperbilirubinemia, with a total bilirubin concentration of 1.4 mg/dL (RI = 0.1-0.4), most of which was indirect (1.1 mg/dL; RI = 0.1-0.4). Four other dogs had slight hyperbilirubinemia (0.5-0.6 mg/dL) of questionable significance.

Of the 19 dogs that underwent abdominal imaging, five had splenomegaly, and splenic tissue was cytologically evaluated for two of them and for three other dogs with suspected splenic abnormalities. Increased splenic hematopoiesis was reported in four of the five dogs, one of which also had erythroid maturation arrest and destruction of erythroid precursors in the spleen. Similar findings were not reported in the other dogs, but their slides were not available for review. Of the 14 dogs assessed for coagulation abnormalities, four had decreased antithrombin III, increased D-dimers, and increased

fibrin degradation products. None of the 11 dogs assessed for tick-borne disease had results indicative of disease.

Clinical outcomes

Follow-up information was available for all but one of the 25 dogs, a dog with SMF-PIMA that was lost to follow-up upon discharge. All other dogs were treated with immunosuppressive therapy and, when indicated, packed erythrocyte transfusions. Dogs were initially treated with prednisone alone (7) or in combination with cyclosporine (4) or azathioprine (13); azathioprine or cyclosporine were added later in five dogs. There was no apparent relationship between initial treatment regimen and outcome.

Of the 24 dogs for which follow-up information was available, 12 had evidence of response, although two were lost to follow-up before a response might be expected (lost at 6 and 20 days after admission). The median time from the beginning of immunosuppressive therapy at MSU until response was 23 (1-48) days for the 10 dogs with follow-up reticulocyte concentrations, and 27 (1-48) days when excluding the three dogs on immunosuppressive therapy at admission. After further exclusion of two dogs with early (days 1 and 7) transient reticulocytosis that may not have been an appropriate indicator of response, the remaining five dogs had a similar median response time of 32 (21-48) days.

Ten of the twelve known responders reached clinical remission, and immunotherapy was stopped in at least eight of them. The median time from beginning immunosuppressive therapy at MSU until documented remission for the six dogs with Hct follow-up data was 67 (31-123) days. Eight of the ten remission-achieving dogs lived at least 1 year after initial presentation; the two dying sooner were a dog that underwent

euthanasia for aggression and a dog whose owners declined recommended therapy at the time of relapse. Five of the eight dogs lived at least 3 years and two more lived at least 2 years after initial presentation. The eighth died of pneumonia 14 months after presentation. The two responders not reaching remission had increases in reticulocyte concentrations from < 20,000/μL to greater than 200,000/μL within 5-7 weeks after therapy was initiated, but died within two months, one with a portal vein thrombosis and acute renal failure, and the other of unknown cause.

Five of the ten known remission-achieving dogs had relapses of PIMA, three did not, and relapse information was not available for two. Three of the five dogs that relapsed had a second remission; one died from PIMA when the relapse occurred and recommended therapy was not accepted, and one was still on treatment at last follow-up. An additional two dogs were treated for IMT after remission of PIMA.

Of the 12 dogs with some follow-up information and no evidence of response to therapy, one died on day 17 and six underwent euthanasia within 45 days because of no response (3), economic reasons (1), septic peritonitis (1), or for unknown reasons (1). Another underwent euthanasia 120-150 days after presentation. The other four dogs were lost to follow-up after not responding to initial or altered therapeutic protocols for 6, 20, 90, or 270 days. Overall, at least four of these dogs lived 45 days and at least three lived three months without response. Eight of the twelve dogs had collagen myelofibrosis, including three with SMF-PIMA; five of the eight were known to have undergone euthanasia 11 days to 5 months after admission.

Group differences

Dogs with early-PIMA were younger than dogs in other groups (p=0.04), and all three were female and had reticulocytopenia. Moreover, in contrast to all but one of the other PIMA dogs, they all had increased lymphoid cells in the bone marrow (increases that did not appear to be just relative). When comparing early-PIMA dogs to all other PIMA dogs combined, early-PIMA dogs had lower reticulocyte concentrations (p=0.01) and tended to have lower Hcts (p=0.05).

The two Coombs'-positive dogs had late-stage RP, one with SMF-PIMA and one with late-PIMA. Two late-PIMA dogs were known to have had previous episodes of spherocytic, regenerative IMHA (5 months or 1 year prior to presentation for PIMA). Both dogs that developed IMT after PIMA also had late-PIMA, as did four of the five dogs that relapsed. Four of the seven dogs that survived the disease to last follow-up had late-PIMA; the other three belonged one each to the other three groups. One dog each with mild, moderate, and severe collagen myelofibrosis was in the group of responders, and one achieved remission and lived for at least 33 months after admission; the other two died within 2 months, one with PIMA, and one with acute renal failure and portal vein thrombosis. There was no difference in days until response among groups.

DISCUSSION

PIMA was prevalent in this MSU VMC population, being the single most common diagnosis in dogs evaluated for nonregenerative anemia alone, and common in dogs assessed for nonregenerative anemia accompanied by either neutropenia or thrombocytopenia. There was no apparent breed or sex predisposition, and clinical signs were nonspecific or related to anemia. Immunosuppressive therapy was associated with apparent responses in 50% of the dogs, though the response rate might have been higher with greater therapeutic efforts and complete follow-ups. Responders had median times to response and remission of approximately 1 and 2 months, respectively. Some dogs lived years after remission and discontinuation of therapy, while at least 40% relapsed one or more times after steroid withdrawal or tapering, and other dogs did not respond despite months of immunosuppressive therapy.

Bone marrow findings formed a spectrum of four patterns, none of which predicted outcome. Each pattern included evidence of stimulated erythropoiesis up to the stage of erythroid precursor undergoing phagocytosis. When rubriblasts and prorubricyte phagocytosis was primarily detected, the pattern mimicked severe erythroid hypoplasia or pure red cell aplasia. However, the presence or expansion of early erythroid stages together with their phagocytosis supports an erythroid stimulus and response accompanied by arrested maturation, not hypoplasia in the sense of a decreased erythropoietin stimulus or decreased marrow response. If that were the case, one would expect a more proportionate decrease in all erythroid stages without early expansion or RP.

In contrast, most PIMA dogs had hypercellular marrows with erythroid hyperplasia and usually a maturation arrest or more subtle left-shift associated with mostly late-stage

RP. In dogs without obvious left shifts, the presence of RP and a markedly expanded and coalescing erythroid islands in the face of persistent nonregenerative anemia allowed differentiation from early pre-reticulocytosis responses to anemia. Five dogs with evidence of mid- or late-stage PIMA were classified separately because they had severe collagen myelofibrosis and an overall decrease in the erythroid lineage associated with marrow effacement; however, stimulated erythropoiesis was evidenced by decreased myeloid to erythroid ratios and expanded foci of erythropoiesis in core sections. In the fourth pattern characterized by mostly mid-stage RP and mid-stage maturation arrest, erythroid islands were not clearly expanded or reduced, hence there was erythroid normocellularity, but early-stage precursors were increased, again reflecting an erythropoietic stimulus.

In all cases, whether a cause or an effect of ineffective erythropoiesis, RP appeared to be relatively stage-restricted, and it was typically aligned with the most mature stage that was well represented. This may have occurred because of relatively stage-specific targeting or because the most abundant stage would be phagocytized most often with RP that was not stage restricted. In either case, the cells undergoing phagocytosis appeared intact and lacked morphologic features that would identify them as apoptotic cells. If antibodies or complement mediate RP in PIMA, as they mediate IMHA, restricted phagocytosis may relate to restricted expression of target epitopes on different stages of development, and concurrent evidence of hemolysis in some cases may relate to shared epitopes on developing and mature cells (Okumura et al., 1992; Spivak 2005) or to multiple distinct antibodies (Mangan et al., 1984). RP associated with maturation arrest in the spleen of one study dog, as we have seen in others (Lucidi et al., 2011), supports that PIMA is a systemic disorder not restricted to the marrow.

If RP mediates PIMA, the question arises as to why it has been so uncommonly reported. This might relate to varied and sometimes more limited degrees of attack, a rapid rate of phagolysosomal degradation (Oh et al., 1996), and difficulties in detecting RP when present. Cytologic detection requires a sensitized eye, evaluation in the most optimal regions of smears, and a directed search. Because the cells undergoing phagocytosis are intact, they blend into their surroundings until being too degraded to be recognized, though the presence of large nuclear masses suggests something other than physiologic clearance of extruded metarubricyte nuclei. Additionally, macrophages containing phagocytized cells commonly break into cytoplasmic fragments, which are more difficult to recognize (Figure 2). A more intense search for RP is warranted when there is an increase in the number of macrophages containing basophilic material consistent with degraded cells (McManus 2000). In our experience, RP is seen in most PIMA dogs, but is rarely present in dogs with no evidence of PIMA; its detection adds confidence to a clinical diagnosis of PIMA. Of note, RP was detected even in the hemodiluted and poorly cellular smears from the five SMF-PIMA dogs, and together with decreased myeloid to erythroid ratios and erythroid left shifts, supports PIMA as an underlying disorder in dogs that might otherwise be diagnosed as having idiopathic myelofibrosis.

The presence of mild or moderate collagen myelofibrosis in seven other study dogs, with 48% of the dogs affected overall, is evidence that fibrosis may develop secondarily over time in dogs with PIMA. This aligns with previous reports of reticulin (Stokol et al., 2000) or collagen (Scott-Moncrieff et al., 1995; Stokol et al., 2000; Reagan 1993; Villiers et al., 1999) myelofibrosis in dogs with suspected immune-mediated anemia, and with reports of some Coombs'-positive or corticosteroid-responsive anemia

in dogs with severe myelofibrosis (Hoff et al., 1991). The mechanism of fibrosis in these dogs is unknown, but, as has been suggested by others (Villiers et al., 1999), it may be similar to that of autoimmune myelofibrosis (AIMF) reported in human patients with such conditions as systemic lupus erythematosus and autoimmune hemolytic anemia (Vergara-Lluri et al., 2014; Pullarkat et al., 2003). Alternatively, myelofibrosis in PIMA dogs may be related to a state of chronic inflammation in the bone marrow tissue, which can increase fibrotic signaling and lead to organ fibrosis (Ueha et al., 2012).

Whatever the mechanism, myelofibrosis may have further impaired erythropoiesis and hematopoiesis in general, thus affecting treatment responses, but it may also have simply limited treatment because of concerns about prognosis in dogs with myelofibrosis. Of the 12 nonresponders, 67% had some degree of collagen myelofibrosis in contrast to only 25% of the responders, and four underwent euthanasia within 45 days of diagnosis. It is noteworthy that one of the dogs with collagen myelofibrosis lived at least 33 months after diagnosis. However, it is not known if fibrosis resolved in any dog, because bone marrows were not reassessed. Recovery from presumed PIMA with collagen myelofibrosis has been described, though again without follow-up marrow assessment (Villiers et al., 1999; Hoff et al., 1991; Reagan et al., 1992), and resolution of reticulin fibrosis has been reported in PIMA dogs (Stokol et al., 2000). In people, including those with AIMF, reticulin myelofibrosis is often reversible (Kuter et al., 2007; Vergara-Lluri et al., 2014; Pullarkat et al., 2003), while collagen myelofibrosis is considered less likely reversible and perhaps irreversible (Kuter et al., 2007). However, findings indicate that dogs with some degree of collagen myelofibrosis may respond and achieve remission from PIMA, so clinical pessimism related to treating dogs with collagen myelofibrosis may be excessive.

While severe collagen myelofibrosis may have contributed to neutropenia in two of the five dogs with neutropenia, ineffective neutropoiesis was suspected in two of the other three dogs because of ample to increased neutropoiesis and no evidence of overwhelming inflammatory lesions. Two other dogs were excluded from the PIMA group because of neutrophil phagocytosis accompanied by evidence of ineffective neutropoiesis in addition to findings typical of early-PIMA. Although excluded because of the potential for nonselective phagocytosis, we suspect that phagocytosis in these and similar dogs (Lucidi et al., 2011) is selective, with selective targeting of more than one cell type. This is not surprising, as multiple (Michel et al., 2009; Martino et al., 1995) or shifting (Day 2010; Taniguchi et al., 1988; Meyer et al., 1978; Mangan et al., 1984; Tohda et al., 1992; Crosby et al., 1956) targets in immune-mediated hematologic disorders are not uncommon and may be further evidenced in this study population by two dogs that had recovered from IMHA prior to developing PIMA, by one dog with a history of IMT, by two dogs that developed IMT after remission from PIMA, similar to a dog in a previous study (Stokol et al., 2000), and by one study dog with a clinical diagnosis of concurrent IMT.

Although PIMA appears to be part of a spectrum of cytopenic disorders, pathogeneses may vary. The early-PIMA group differed from mid- and late-stage PIMA by an over-representation of females and younger dogs, lower reticulocyte concentrations, the absence of collagen myelofibrosis, and by mild increases in bone marrow lymphocytes and/or plasma cells, which were identified in only one other dog. Group numbers were low, but these findings mirror those reported for dogs with PRCA (Weiss 2002; Weiss 2008), a similar condition, and suggest that the pathogenesis of early-PIMA and PRCA may differ from that of mid- and late-PIMA. We currently classify

the pattern of early maturation arrest with a predominance of rubriblasts accompanied by early-stage RP as early-PIMA, whereas we apply the PRCA label to cases with an absence or marked paucity of erythroid precursors without a left shift or RP. This may or may not be an artificial division.

Several factors may explain why evidence of hemolysis in dogs with PIMA has varied from common (Stokol et al., 2000) to uncommon (Scott-Moncrieff et al., 1995), the latter being the case in this study. In our hospital, dogs with nonregenerative and possibly preregenerative anemia but obvious signs of hemolytic disease are typically treated for IMHA without marrow examination, and therefore would not have entered our study group unless they failed to respond to treatment. In contrast, such dogs may have been included in other studies of nonregenerative immune-mediated anemia, some of which actually required evidence of hemolysis and erythrocyte targeting for inclusion (Jonas et al., 1987; Klag et al., 1993; Kellerman et al., 1995; Burgess et al., 1997; Weiss 2008). Interestingly, we have detected selective late-stage RP in dogs with IMHA when bone marrow was sampled despite classic presentations and regenerative anemias, but because such samples from classic IMHA patients are rare in our hospital, it is unknown how frequently this occurs and therefore the degree to which impaired erythropoiesis may potentially contribute to anemia in dogs with acute, regenerative IMHA. Other reasons for less hemolysis in our PIMA population may relate to varied diagnostic interpretations. Dogs with no hemolytic signs but with persistent nonregenerative anemia and evidence of ineffective erythropoiesis may be interpreted as having erythroid hypoplasia (early- and mid-PIMA), myelodysplasia, or, in the case of late-PIMA, erythroid hyperplasia without a provided cause. Additionally, our population differed from others by the requirement for RP, which may have selected a different set

of dogs, including those with severe collagen myelofibrosis that might otherwise have been considered to have idiopathic myelofibrosis.

Dogs with IMHA are prothrombotic and prone to thromboembolic disease that is associated with increased mortality (Carr et al., 2002; Klein et al., 1989; Fenty et al., 2011), and thrombotic disease has also been reported in a few dogs with nonregenerative immune-mediated anemia (Jonas et al., 1987; Stokol et al., 2000). However, these dogs may have had a hemolytic component to their anemias, whereas, in our study, one dog lacking evidence of hemolysis developed portal vein thrombosis and had increased FDPs and D-dimers, while four other dogs, including one the day after exploratory surgery, had decreased antithrombin III, increased D-dimers, and increased FDPs without detected thrombosis. Further studies are warranted to determine if thromboembolic events are a significant complication in dogs with PIMA as they are in dogs with IMHA.

In conclusion, we evaluated 25 dogs with PIMA, defined by persistent nonregenerative anemia and evidence of ineffective erythropoiesis with selective RP. Three bone marrow patterns were identified based on early-, mid-, or late-stage RP and maturation arrest, and a fourth pattern was based on the presence of concurrent severe collagen myelofibrosis. Response to immunosuppressive therapy, and in some cases relapse upon withdrawal of therapy, supports an immunologic condition, as does detection of RP in bone marrow and spleen. However, remission required weeks to months and evidence of hemolysis was rare, supporting that PIMA should be distinguished from IMHA. Mild to severe myelofibrosis is common in dogs with PIMA, and does not prevent clinical remission. If severe collagen myelofibrosis is detected in canine bone marrow cores, PIMA should be a differential diagnosis and further evidence

of PIMA should be sought (i.e., expanded erythroid islands, erythroid left shift, selective RP). Further investigations to determine the pathogenesis of PIMA, the cause of RP, and the best therapeutic approach for managing affected patients are warranted.

REFERENCES

REFERENCES

Burgess KE, Rand W, Moore A. Immune mediated hemolytic anemia in dogs: a retrospective study of 60 cases treated with cyclophosphamide [abstract]. J Vet Intern Med. 1997;11:143.

Carr AP, Panciera DL, Kidd L. Prognostic factors for mortality and thromboembolism in canine immune-mediated hemolytic anemia: a retrospective study of 72 dogs. J Vet Intern Med. 2002;16:504-509.

Crosby WH, Rappaport H. Reticulocytopenia in autoimmune hemolytic anemia. Blood. 1956;11:929-936.

Day MJ. Immune-mediated anemias in the dog. In: Weiss DJ, Wardrop KJ, eds. Schalm's Veterinary Hematology. 6th ed. Ames, IA: Wiley-Blackwell; 2010:216-225.

Fenty RK, Delaforcade AM, Shaw SE, et al. Identification of hypercoagulability in dogs with primary immune-mediated hemolytic anemia by means of thromboelastography. J Am Vet Med Assoc. 2011;238:463-467.

Hoff B, Lumsden JH, Valli VEO, et al. Myelofibrosis: Review of clinical and pathological features in fourteen dogs. Can Vet J. 1991;32:357-361.

Holloway SA, Meyer DJ, Mannella C. Prednisolone and danazol for treatment of immune-mediated anemia, thrombocytopenia, and ineffective erythropoiesis in a dog. J Am Vet Med Assoc. 1990;197:1045-1048.

Jonas LD, Thrall MA, Weiser MG. Nonregenerative form of immune-mediated hemolytic anemia in dogs. J Am Anim Hosp Assoc. 1987;23:201-204.

Kellerman DL, Lewis DC, Bruyette DS. Canine immune-mediated hemolytic anemia: a retrospective analysis of 37 cases [abstract]. J Vet Intern Med. 1995;9:189.

Klag AR, Giger U, Shofer FS. Idiopathic immune-mediated hemolytic anemia in dogs: 42 cases (1986-1990). J Am Vet Med Assoc. 1993;202:783-788.

Klein MK, Dow SW, Rosychuk RA. Pulmonary thromboembolism associated with immune-mediated hemolytic anemia in dogs: ten cases (1982-1987). J Am Vet Med Assoc. 1989;195:246-250.

Kuter DJ, Bain B, Mufti G, et al. Bone marrow fibrosis: pathophysiology and clinical significance of increased bone marrow stromal fibres. Br J Haematol. 2007;139:351-362.

Lucidi CA, Steficek B, Jutkowitz LA, et al. Case review – Histologic assessment of a dog's spleen: pitfalls and possibilities (Ineffective erythropoiesis associated with

precursor-directed immune-mediated anemia, ineffective neutropoiesis, and thrombocytosis). Proc 46th Annual Meeting Am Soc Vet Clin Pathol. Nashville, TN, December 3-7, 2011.

Mangan KF, Besa EC, Shadduck RK, et al. Demonstration of two distinct antibodies in autoimmune hemolytic anemia with reticulocytopenia and red cell aplasia. Exp Hematol. 1984;12:788-793.

Martino R, Muñiz-Díaz E, Arilla M, et al. Combined autoimmune cytopenias. Haematologica. 1995;80:305-310.

McManus P. Marrow and erythrocyte changes associated with canine non-regenerative immune-mediated anemia: 44 cases (1996-2000) [abstract]. Vet Pathol. 2000;37:526.

Meyer RJ, Hoffman R, Zanjani ED. Autoimmune hemolytic anemia and periodic pure red cell aplasia in systemic lupus erythematosus. Am J Med. 1978;65:342-345.

Michel M, Chanet V, Dechartres A, et al. The spectrum of Evans syndrome in adults: new insight into the disease based on the analysis of 68 cases. Blood. 2009;114:3167-3172.

Oh YK, Swanson JA. Different fates of phagocytosed particles after delivery into macrophage lysosomes. J Cell Biol. 1996;132:585-593.

Okumura N, Tsuji K, Nakahata T. Changes in cell surface antigen expressions during proliferation and differentiation of human erythroid progenitors. Blood. 1992;80:642-650.

Pullarkat V, Bass RD, Gong JZ, et al. Primary autoimmune myelofibrosis: definition of a distinct clinicopathologic syndrome. Am J Hematol. 2003;72:8-12.

Reagan W, Scott-Moncrieff C, DeNicola D, et al. Immune mediated non-regenerative anemia and myelofibrosis in female beagle dogs [abstract]. Vet Clin Pathol. 1992;21:127.

Reagan WJ. A review of myelofibrosis in dogs. Toxicol Pathol. 1993;21:164-169.

Scott-Moncrieff JCR, Reagan WJ, Glickman LT, et al. Treatment of nonregenerative anemia with human γ-globulin in dogs. J Am Vet Med Assoc. 1995;206:1895-1900.

Spivak JL. The anaemia of cancer: death by a thousand cuts. Nat Rev Cancer. 2005;5:543-555.

Stockham SL, Ford RB, Weiss DJ. Canine autoimmune hemolytic disease with a delayed erythroid regeneration. J Am Anim Hosp Assoc. 1980;16:927-931.

Stokol T, Blue JT, French TW. Idiopathic pure red cell aplasia and nonregenerative immune-mediated anemia in dogs: 43 cases (1988-1999). J Am Vet Med Assoc. 2000;216:1429-1436.

Taniguchi S, Shibuya T, Morioka E, et al. Demonstration of three distinct immunological disorders on erythropoiesis in a patient with pure red cell aplasia and autoimmune haemolytic anaemia associated with thymoma. Br J Haematol. 1988;68:473-477.

Tohda S, Nara N, Tanikawa S, et al. Pure red cell aplasia following autoimmune haemolytic anaemia. Cell-mediated suppression of erythropoiesis as a possible pathogenesis of pure red cell aplasia. Acta Haematol. 1992;87:98-102.

Ueha S, Shand FH, Matsushima K. Cellular and molecular mechanisms of chronic inflammation-associated organ fibrosis. Front Immunol. 2012;3:71.

Vergara-Lluri ME, Piatek CI, Pullarkat V, et al. Autoimmune myelofibrosis: an update on morphologic features in 29 cases and review of the literature. Hum Pathol. 2014; 45:2183-2191.

Villiers EJ, Dunn JK. Clinicopathological features of seven cases of canine myelofibrosis and the possible relationship between the histological findings and prognosis. Vet Rec. 1999;145:222-228.

Weiss DJ, Stockham SL, Willard MD, et al. Transient erythroid hypoplasia in the dog: report of five cases. J Am Anim Hosp Assoc. 1982;18:353-359.

Weiss DJ. Antibody-mediated suppression of erythropoiesis in dogs with red blood cell aplasia. Am J Vet Res. 1986;47:2646-2648.

Weiss DJ. Bone marrow pathology in dogs and cats with non-regenerative immune-mediated haemolytic anaemia and pure red cell aplasia. J Comp Pathol. 2008;138:46-53.

Weiss DJ. Primary pure red cell aplasia in dogs: 13 cases (1996-2000). J Am Vet Med Assoc. 2002;221:93-95.

CHAPTER 3

Flow Cytometric Assays for Canine Immune-mediated Anemias; Detection of DEA1.1 on Canine Erythroid Precursors

ABSTRACT

Objective: To develop and characterize flow cytometric assays for IgG on erythroid populations from the blood and bone marrow of dogs, ultimately to explore the pathogenesis of suspected precursor-targeted immune-mediated anemia.

Samples: Blood from 20 healthy and 61 sick dogs with (33) or without (28) immune-mediated hemolytic anemia, and bone marrow from 39 healthy or sick dogs.

Procedures: A flow cytometric assay for RBC IgG and a positive control were developed, and the assay's analytical and diagnostic performance were characterized. The assay was combined with Percoll fractionation of marrow cells and a 2-color erythroid precursor (nRBC) assay to detect nRBC IgG. Sorting and cytology were used to confirm target populations, and anti-DEA1.1 blood typing serum was used to make IgG-positive nRBCs.

Results: The RBC IgG assay had within-run and between-run coefficients of variation of 0.1-13.9%, and >90% of spiked positive cells were detected. Clinical assessment yielded diagnostic sensitivity, specificity, and accuracy of 87.9%, 92.9%, and 90.2%, respectively. Cytologic assessment of cell-sorted early-, mid-, and late-stage nRBC regions from 3 healthy dogs showed 89-98% nRBC purity. IgG was detected on nRBCs from DEA1.1-positive dogs, but not DEA1.1-negative dogs, after IgG coating with anti-DEA1.1 typing serum.

Conclusions and Clinical Relevance: The RBC IgG assay had favorable analytical and diagnostic performance and can be used as a diagnostic or research tool for immune-mediated erythrocyte destruction. The assay was successfully applied to nRBCs isolated from canine bone marrow, and IgG-coated nRBCs were detected. In addition, the findings support the presence of DEA1.1 on nRBC.

INTRODUCTION

Precursor-targeted immune-mediated anemia (PIMA) has been reported in several studies (Weiss et at., 1982; McManus 2000; Scott-Moncrieff et al., 1995; Stockham et al., 1980; Stokol et al., 2000; Jonas et al., 1987; Weiss 2008; Holloway et al., 1990), and accounts for approximately 25% of the canine bone marrow diagnoses at the Michigan State University Veterinary Medical Center (MSU VMC). However, its pathogenesis is unclear. The condition is characterized by nonregenerative anemia, ineffective erythropoiesis, and, in most MSU VMC cases, by rubriphagocytosis – phagocytosis of intact erythroid precursors (nucleated red blood cells, nRBCs). We have found that rubriphagocytosis in PIMA dogs is quite stage-selective, and that the stage of erythroid precursor being phagocytized (i.e., early-, mid-, or late-stage nRBCs) aligns with a spectrum of cytologic and histologic bone marrow patterns that commonly include mild to severe myelofibrosis (Lucidi et al., 2010). An immune-mediated mechanism has been proposed, but not proven, based on phagocytosis of erythroid precursors (Stockham et al., 1980; Jonas et al., 1987; Holloway et al., 1990; McManus 2000), responses to immunosuppressive therapy (Jonas et al., 1987; Stockham et al., 1980; Weiss et al., 1982; Holloway et al., 1990; Scott-Moncrieff et al., 1995; Stokol et al., 2000), apparent relapses after withdrawal of immunosuppressive therapy (Stockham et al., 1980; Weiss et al., 1982; Scott-Moncrieff et al., 1995; Stokol et al., 2000), and evidence of concurrent immune-mediated hemolytic anemia (IMHA) in some cases (Holloway et al., 1990; Jonas et al., 1987; Stokol et al., 2000; Weiss 2008; Scott-Moncrieff et al., 1995). However, responses to immunosuppressive drugs are slow, relapses are common, and many of the affected dogs die or undergo euthanasia due to clinical pessimism or financial constraints.

Flow cytometry has been used to diagnose and characterize immune-mediated hemolytic anemias in dogs (Wilkerson et al., 2000; Quigley et al., 2001; Kucinskiene et al., 2005; Morley et al., 2008) and people (Chaudhary et al., 2006; Lin et al., 2009; Wang et al., 2001), and the method has been shown to be more sensitive than standard agglutination tests for IgG detection (Wilkerson et al., 2000; Quigley et al., 2001; Kucinskiene et al., 2005; Chaudhary et al., 2006; Lin et al., 2009; Wang et al., 2001). A potential PIMA counterpart which may be mediated by IgG directed against nRBCs has been identified in human patients (Mangan et al., 1984; Meyer et al., 1978; van de Loosdrecht et al., 2000), and flow cytometry has been used to identify IgG on bone marrow-derived nRBCs in one of these cases (van de Loosdrecht et al., 2000). These studies indicate that flow cytometry could also be used to assess PIMA dogs for increased nRBC IgG.

The goal of this work was to develop a method that would allow isolation and flow cytometric assessment of IgG on different stages of nRBCs from the bone marrow of dogs. To this end, an assay for red blood cells (RBC) IgG was first developed and validated, and this method was applied to marrow-derived nRBCs. These assays will facilitate a variety of blood and stage-selective marrow assessments in dogs with PIMA, as well as other disorders, and help determine if there is a role for IgG in the pathogenesis of canine PIMA. Better understanding of the mechanism of PIMA may lead to improved management strategies and therapeutic responses in affected dogs.

MATERIAL AND METHODS

Dogs

For assessment of IgG on RBCs, blood was collected by routine venipuncture into K_2EDTA from 20 dogs enrolled in the MSU VMC Blood Donor Program and deemed clinically and hematologically healthy based on annual physical exam, CBC, serum chemistry, and screening for infectious diseases (heartworm disease, babesiosis, ehrlichiosis, leishmaniasis, and hemotropic mycoplasmosis). Blood was similarly collected from 61 MSU VMC patients as part of their routine diagnostic evaluation. These patients included 33 dogs with IMHA and 28 dogs with non-IMHA conditions. Dogs were classified as having IMHA if they had a clinical diagnosis of IMHA associated with regenerative anemia (all had hematocrit below 33%) and at least one of the following with no other explanation: 1) spherocytosis, 2) erythrocyte agglutination, or 3) Coombs' test positivity. The unhealthy non-IMHA group comprised sick dogs with no clinical suspicion or hematologic evidence of immune-mediated anemia.

For development and evaluation of a flow cytometric assay for IgG on nRBCs, bone marrow was collected from 39 dogs: 21 clinically healthy colony dogs and 18 MSU VMC patients. Some colony dogs received 1-2 physical examinations per month, while the others received physical examinations twice per year. All dog and sample use was in compliance with the Michigan State University and MPI Research Institutional Animal Care and Use Committees.

Bone marrow collection

Approximately 2-5 mL of bone marrow was aspirated from the proximal humerus or wing of the ilium of anesthetized dogs using a 15 or 18 gauge Illinois bone marrow

needle. Marrow was collected into a syringe containing 0.5 mL of 3% K_2EDTA in saline, and then dispensed into a Petri dish for particle harvesting and preparation of cytologic smears; the remaining sample was used for flow cytometric analysis.

RBC sample preparation

Blood samples collected routinely into EDTA-anticoagulated vacuum tubes were used on the day of collection or stored at 4°C and used the next day, and always processed at room temperature (20 to 22°C). Whole blood was centrifuged at 1200 g for 5 minutes, and after aspiration of the plasma and buffy coat, 10 µL of packed RBCs were withdrawn and washed twice by resuspension in 1.5 mL PBS-0.3%BSA (PBS: 137 mM NaCl, 2.7 mM KCl, 9.6 mM NaH_2PO_4, 1.5 mM KH_2PO_4, containing 0.3% bovine serum albumin and 0.025% sodium azide, pH 7.3) followed by centrifugation at 1200 g for 1 minute. The washed pellet was resuspended in 800 µL PBS-0.3%BSA.

Flow cytometric assay for RBC IgG

The following procedure was applied after optimization studies that included assessment of signal to noise ratios and number of wash steps. For each dog, 50 µL of washed RBC suspension were incubated with 15 µL of fluorescein isothiocyanate (FITC)-conjugated caprine anti-[dog IgGγ] IgG (IgG test antibody, KPL, Gaithersburg, MD) in the dark for 30 minutes. RBCs were washed once as described above, resuspended in 1.5 mL PBS-0.3%BSA, and analyzed on a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA) by acquiring 10,000 events and using CellQuest Pro software (Becton-Dickinson, San Jose, CA). The electronic settings for RBC analysis were: forward scatter (FSC) voltage = E00, AmpGain = 2.25; side scatter (SSC) voltage

= 304, AmpGain = 1.32, FL1 voltage = 703; and FSC threshold = 100. RBCs were recognized and gated based on FSC and SSC, as previously described (Lucidi et al., 2011).

Positive control samples were developed and negative and positive control samples were assessed with each test sample. Negative controls were made by incubating washed RBCs from healthy dogs with IgG test antibody (negative control for IgG), and by incubating test RBCs from each tested dog with FITC-conjugated caprine anti-[chicken IgG-heavy and light chain] IgG (nonsense antibody, KPL, Gaithersburg, MD) as a control for nonspecific binding. Positive control samples consisted of dog erythrocyte antigen (DEA)1.1-positive RBCs treated with a polyclonal anti-DEA1.1 typing serum (Animal Blood Resources International, Stockbridge, MI) to yield IgG-coated RBCs. More specifically, positive control cells were made by a 30 minute room temperature incubation of 800 µL washed RBC suspension of DEA1.1-positive RBCs with 800 µL of anti-DEA1.1 typing serum diluted with PBS-0.3%BSA. Cells were washed two times by resuspension in 1.5 mL PBS-0.3%BSA followed by centrifugation at 1200 g for 1 minute, and the washed pellet was resuspended in 800 µL PBS-0.3%BSA. This positive control was assessed for stability, and new batches were made approximately once a month, as needed. IgG positivity of control cells varied from moderate to strong, depending on the cells and serum dilution used.

Various methods of determining IgG positivity were assessed, and it was concluded that results would be reported as % positive events, with the cutoff for positive fluorescence of each test sample being at the level defining the top 2% of events for the nonsense antibody (nonspecific binding). When RBC agglutinates were identified on flow plots based on RBC FSC and SSC (Figure 5), they were gated out and

excluded from the analysis in order to prevent falsely decreased IgG positivity caused by setting a higher 2% fluorescence cutoff to accommodate the increased fluorescence of agglutinates with the nonspecific control (Arndt et al., 2010).

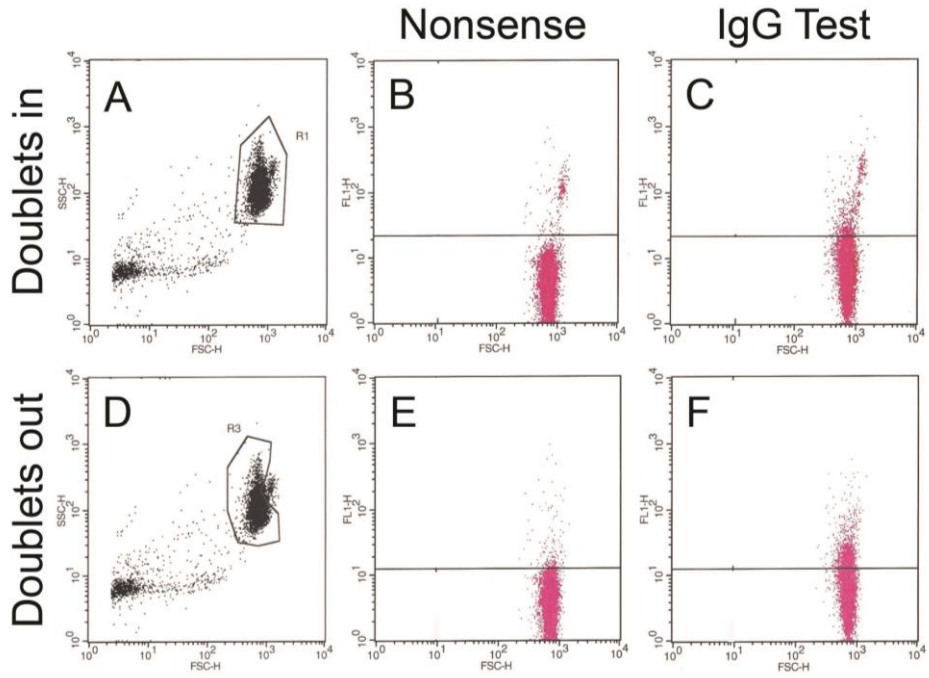

Figure 5: *Effect of RBC agglutinates on IgG detection.* When included in the gate of interest (A), RBC agglutinates result in a higher 2% fluorescence cutoff based on the nonsense antibody (B), and falsely lower IgG positivity based on the test antibody (C, 5.7% positive events), compared to when agglutinates are excluded from the gate of interest (D), thereby lowering the cutoff (E) and allowing more accurate detection of IgG positivity (F, 18.7% positive events).

Analytical precision of negative and positive samples was assessed, and the final coefficients of variation (CVs) were calculated based on % positive events. For within-run precision, 20 different RBC suspensions were prepared from a single negative blood sample, and 40 µL of each was spiked with 10 µL of positive control cells to yield 20%

positive cells. Negative control cells were similarly tested in parallel. For assessment of between-run precision and stability over time, IgG negative and positive control RBCs were stored in Alsever's solution (Sigma-Aldrich, Saint Louis, MO) with 0.5% BSA (Sigma-Aldrich, Saint Louis, MO) at 4°C, and RBCs were tested for 10 consecutive weekdays, followed by weekly testing for 3 weeks. Prior to each assessment, 50 µL of RBC suspension were washed with 1.5 mL PBS-0.3%BSA, resuspended in 50 µL of the same buffer, and tested for IgG positivity with test and nonsense antibodies as described above.

Analytical accuracy was assessed in triplicate by a mixing experiment in which IgG negative control RBCs were spiked with IgG positive control RBCs to target 0, 20, 40, 60, 80, and 100% positive events. The percentage of targeted positivity that was detected was determined. To assess changes in % positive events and IgG signal (median fluorescence intensity of test minus nonsense antibody) with decreasing amount of IgG per RBC, DEA1.1-positive RBCs incubated with doubling dilutions of anti-DEA1.1 typing serum from 1/16 to 1/512 in PBS-0.3%BSA were assessed.

Clinical assessment of the RBC IgG assay

RBCs from healthy dogs, dogs with IMHA, and unhealthy dogs without IMHA were tested, and diagnostic sensitivity, specificity, and accuracy were calculated. The mean % positive events + 3 standard deviations of samples from 20 healthy dogs was used as a cutoff to classify IMHA and unhealthy non-IMHA dogs into IgG-negative (below cutoff) and IgG-positive (above cutoff) groups. To assess the effects of testing day-old samples, and to generate comparison values if they differed, the values for healthy dogs

were determined for fresh and day-old samples. Pertinent clinical and laboratory information was gathered for each of these dogs.

Fractionation of bone marrow cells

Bone marrow samples were transported to the laboratory in a cooled container and processed at room temperature within 30 minutes of collection in order to minimize changes in cell membrane or density, which might promote nonspecific binding or affect Percoll gradient separation, respectively. However, we did not evaluate if such changes would in fact occur with storage. Samples were mixed with 40 mL PBS-1%BSA (PBS containing 1% bovine serum albumin and 0.025% sodium azide, pH 7.3) and centrifuged at 500 g for 10 minutes before supernatant was removed and cell pellets were resuspended in 5 mL PBS-1%BSA. Cell suspensions were applied to a 3-layer Percoll (Sigma-Aldrich, Saint Louis, MO) gradient (3 mL each of 52%, 64%, 68% Percoll in PBS-1%BSA) modified from previous studies (Weiss et al., 2000). After centrifugation at 500 g for 30 minutes, the three cell fractions that will be referred to as top, middle, and bottom fractions were harvested from top to bottom using plastic Pasteur pipettes. Each was dispensed into a 5 mL tube, washed twice with alternating PBS-1%BSA (10 mL and then 3 mL) and centrifugation at 500 g. Washed pellets were resuspended in 0.5-3 mL PBS-1%BSA depending on pellet size, and nucleated cell concentrations were determined by hemocytometer. Desired cell separation was confirmed by microscopic examination of stained (Wright-stain), air-dried, stop-flow preparations of cells from each layer.

Preparation of bone marrow fractions for flow cytometry

A 2-color assay was developed using the nucleic acid stain LDS751 (Life Technologies, Grand Island, NY) in combination with labeling for CD18 on non-erythroid cells. The following processing procedure was adopted after trial assessments and optimization steps. For each sample, 10^6 bone marrow cells in 100 µL PBS-1%BSA were incubated with 2 µL of murine anti-[dog CD18] monoclonal antibody (clone CA1.4E9, isotype IgG1, AbD Serotec, Raleigh, NC) under mechanical rotation for 30 minutes, washed twice with 1.5 mL PBS-0.3%BSA, and resuspended in 100 µL of the same buffer. Cells were then incubated with 5 µL of polyclonal phycoerythrin (PE)-conjugated goat anti-[mouse IgG] (AbD Serotec, Raleigh, NC) antibody under mechanical rotation for 30 minutes in the dark, washed twice with 1.5 mL PBS-0.3%BSA, resuspended in 400 µL of the same buffer, and transferred to a flow cytometer analysis tube. These cells were then incubated for at least 20 minutes (progressively longer for samples analyzed later in a batch) in the dark with 160 µL of LDS751 working solution prepared by diluting 2 µL stock into 1.5 mL PBS-0.3%BSA (Saad et al., 2000).

Flow cytometric identification of bone marrow nRBCs

nRBCs from each of the top, middle, and bottom Percoll fractions were expected to stain strongly with LDS751 and not label for CD18. This was assessed by processing bone marrow samples from eight dogs as described above, and then sorting them with a BD Influx cell sorter (Becton-Dickinson, San Jose, CA) using BD FACS Sortware sorter software (Becton-Dickinson, San Jose, CA). Sorted fractions of interest were collected into buffered saline solution to which was immediately added bovine fetal serum (Sigma-Aldrich, Saint Louis, MO) at a ratio of 50 µL serum per 1 mL of saline. The cell content of

each erythroid fraction of three dogs was assessed by differential cell counts on modified Wright-stained cytocentrifuge preparations. Certain other sorted fractions from these and other dogs were also assessed cytologically to determine the location of potentially confounding cell populations.

IgG detection on nRBCs

Top, middle, and bottom Percoll fractions of bone marrow were assessed for IgG-positive nRBCs by essentially combining the assay for RBC IgG with the 2-color bone marrow assay described above to yield a 3-color assay for nRBC IgG. For each fraction, 10^6 nucleated cells were processed as described for the 2-color assay with the addition of test anti-IgG and nonsense antibodies at the time of incubation with goat anti-[mouse IgG]-PE. Assay optimization studies showed no false positive IgG signal caused by binding of IgG test antibody to anti-CD18 antibodies. Cells were analyzed on a FACSCalibur flow cytometer by acquiring 10,000 events, using CellQuest software. The electronic settings for analysis of erythroid populations were: FSC voltage = E00, AmpGain = 2.0; SSC voltage = 400, AmpGain = 1.0, FL1 voltage = 673, FL2 voltage = 610, FL3 voltage = 540; FSC threshold = 100, and compensation settings of FL1 - 15% FL2; FL2 - 21%FL1; FL2 - 6.7%FL3; FL3 - 6.7%FL2. As for the RBC assay, nRBC IgG results were reported as % positive events in the gates of interest.

To assess the ability of the assay to detect IgG on nRBCs, we used an approach parallel to the RBC IgG assay positive control and explored the generation of IgG-positive DEA1.1-positive nRBCs by incubation with anti-DEA1.1 typing serum. For 3 DEA1.1-positive dogs, 100 µL containing 10^6 cells from each bone marrow fraction were incubated with 100 µL of anti-DEA1.1 typing serum at low (1/16) or high (1/128) dilutions

(v/v) in PBS-0.3%BSA for 30 minutes. Low and high dilutions were used to assess detection of stronger and weaker signals. Cells were washed twice with 1.5 mL PBS-0.3%BSA and resuspended in 100 µL of the same buffer prior to 3-color testing as described above. Bone marrow fractions of 3 DEA1.1-negative dogs were processed similarly, and samples from 10 healthy dogs were also assessed for nRBC IgG (without the DEA antiserum steps). Hematologic health of these colony dogs was based on bone marrow aspirate evaluation, semi-annual CBCs in some dogs, and CBCs at the time of marrow collection in the others. DEA1.1-positive and negative dogs were identified by flow cytometric blood typing (Lucidi et al., 2011). The same controls used for the RBC IgG assay were used for each bone marrow sample.

Statistical analysis

Significant differences ($p<0.05$) in RBC and nRBC IgG signal among groups were assessed by Kruskal Wallis and Dunn's multiple comparison post hoc tests using a statistical software (GraphPad Prism, GraphPad Software Inc, San Diego, CA).

RESULTS

RBC IgG assay characterization

The CV for within-run precision was 6.8% for a modestly positive sample with a mean of 20.9% positive events, and 13.4% for negative control cells with a mean of only 6.8% positive events. Between-run precision of negative (mean of 4.4% positive events) and strongly positive (mean of 99.9% positive events) control cells yielded 10-day CVs of 13.9% and 0.1%, respectively. A 10-day assessment is reported because mild shifts in positivity and progressive hemolysis were apparent beyond that time, but control cells were clearly negative and positive for at least one month, with the negative control IgG positivity varying from 4.9% (day 1) to 2.8% (day 34), and that of the positive control varying from 100% (day 1) to 99.8% (day 34). IgG-uncoated RBCs spiked with positive cells targeting 0, 20, 40, 60, 80, and 100% positive events yielded 0, 18, 37, 56, 78, and 100% positive cells, with the percentage of detected to targeted cells varying from 90% to 100%. When using samples with decreasing amounts of canine IgG per RBC, a constant number of 100% positive events was detected, but median fluorescence intensity decreased curvilinearly with IgG signal, ranging from 2,372 to 190 (Figure 6).

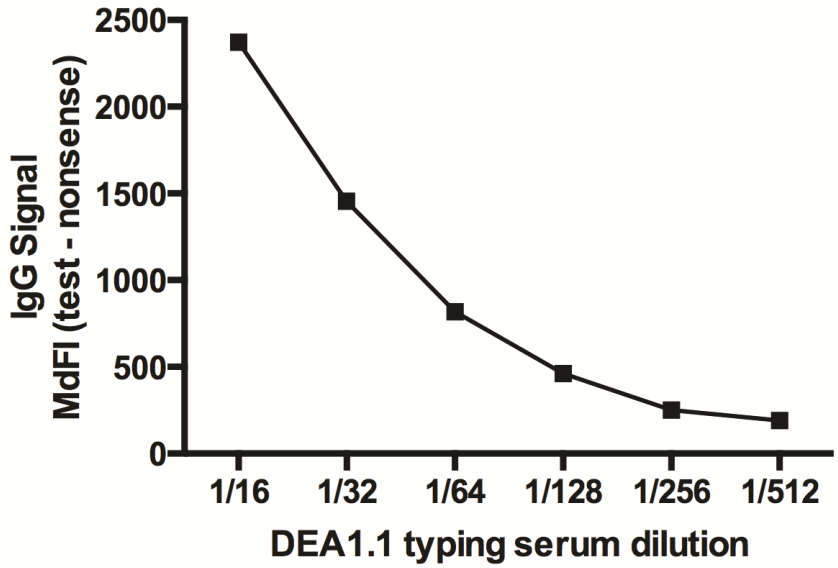

Figure 6: *Dose-response curve for RBC IgG.* DEA1.1-positive RBCs incubated with doubling dilutions of anti-DEA1.1 typing serum yielded a curvilinear reduction in signal with reduction in IgG.

Clinical assessment of the RBC IgG assay

The mean RBC IgG positivity for fresh and day-old samples from 20 healthy dogs, reported as % positive events, was 4.8% and 5.2%, respectively, yielding similar cutoffs for IgG positivity of 9.4% and 8.9% for fresh and day-old samples, respectively. Results were positive in 88% of IMHA dogs and in 7% of unhealthy non-IMHA dogs (Table 5), and results were the same whether matched fresh and day-old cutoffs were used or not. Differences in IgG positivity were significant between the IMHA group and each other group, but not between unhealthy non-IMHA and healthy groups. Based on these results, the diagnostic sensitivity, specificity, and accuracy for detecting IMHA were 87.9%, 92.9%, and 90.2%, respectively.

Table 5: *Number of IMHA and unhealthy non-IMHA dogs with negative and positive RBC IgG results.*

IgG Result	Number of IMHA dogs (median % positive, low-high)	Number of non-IMHA dogs (median % positive, low-high)
Negative	4 (3.2%, 2.6-6.7%)	26 (4.9%, 1.2-8.2%)
Positive	29 (82.5%, 10.4-100%)	2 (17.4%, 10.5-24.2%)

Bone marrow fractions and detection of nRBCs

Percoll separation yielded a top fraction enriched in early-stage erythroid precursors, with fewer mid-stage erythroid precursors, many early to mid-stage myeloid cells, and few megakaryocytes, macrophages, and lymphocytes. The middle fraction was enriched in mid-stage (with few late-stage) erythroid precursors, and also contained a mixture of mid- to late-stage granulocytes, few lymphocytes, and rare plasma cells. The bottom fraction contained mostly late-stage (with few mid-stage) erythroid precursors and segmented neutrophils (Figure 7). All three fractions also contained low to moderate numbers of mature erythrocytes and reticulocytes, with greater numbers in the bottom fraction.

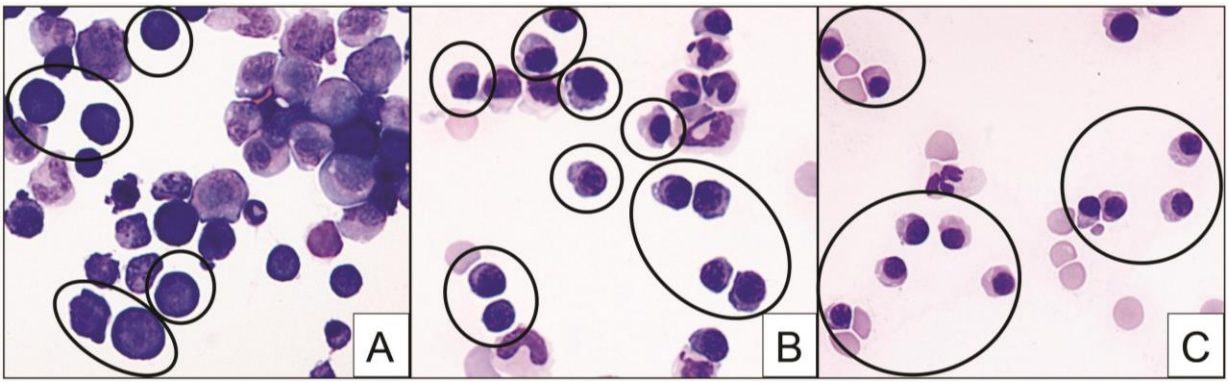

Figure 7: *Canine bone marrow fractions after separation using a Percoll gradient.* Percoll separation yielded top (A), middle (B), and bottom (C) fractions enriched in early, mid-, and late-stage erythroid precursors, respectively. Other cell types such as granulocytes, macrophages, lymphocytes, mature erythrocytes and reticulocytes were also present.

Erythroid populations were identified as CD18-negative (FL2 channel) and LDS751-positive (FL3 channel) events (Figure 8A-C, gate R1), which were shown by cell sorting and cytologic examination to have little contamination with other cell types (Figure 9B-D). Cytologic assessment of cell-sorted nRBC regions from 3 healthy dogs showed ≥ 89% nRBCs in all samples, with 89-99% in the early-stage nRBC region and 98-100% nRBCs in the mid- and late-stage nRBC regions. Additionally, populations that were CD18-negative and LDS751-moderate, and CD18-negative and LDS751-negative, consisted of reticulocytes (Figure 8A-C, gate R2; and 9E) and mature erythrocytes (Figure 8A-C, gate R3; and 9F), respectively, making it possible to assess erythroid precursors, reticulocytes, and erythrocytes simultaneously if enough cells were acquired.

Most of the contaminating cells in the nRBC regions appeared lymphoid, and because of the greater contamination in the nRBC gate of top Percoll fractions, this region was further assessed by sorting and cytologic evaluation after splitting it into top and bottom sections. The top and bottom sections had similar numbers of lymphocytes, and the bottom was found to include many mid-stage nRBCs in mitosis. Consequently, to maximize interrogation of early-stage nRBCs, only the top section of the nRBC region from the top Percoll fraction was used to assess for IgG on nRBC. Additional sorting of non-erythroid regions of selected clinical samples demonstrated where signals of other potentially contaminating populations fell, including plasma cells, macrophages, lymphocytes, and myeloid cells (Figure 8A-C).

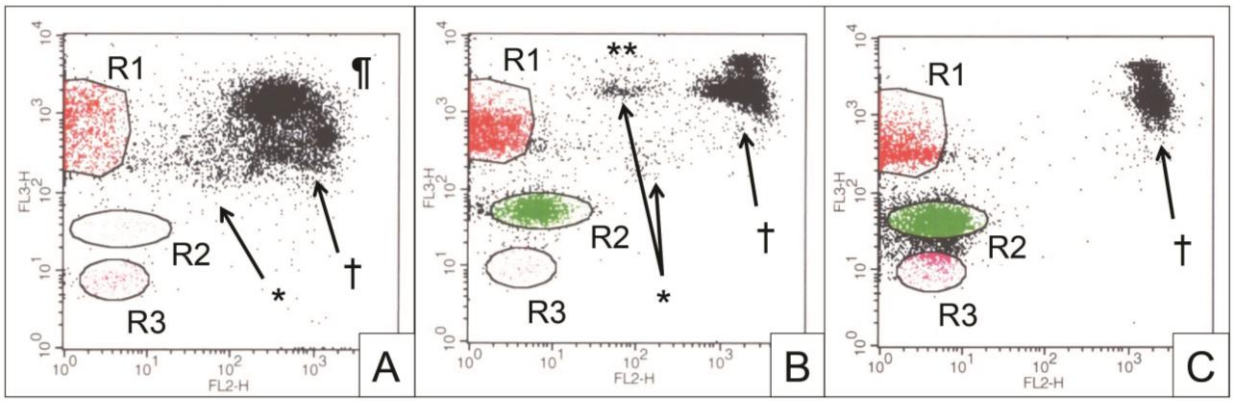

Figure 8: *Flow cytometric identification of potentially contaminating cell populations based on cell sorting of non-erythroid regions.* Representative dot plots of bone marrow cells in the top (A), middle (B), and bottom (C) Percoll fractions show different positivity patterns for CD18 (horizontal axis) and LDS751 (vertical axis). Erythroid precursors (R1), reticulocytes (R2), and mature erythrocytes (R3) were CD18-negative and LDS751-positive, CD18-negative and LDS751-moderate, and CD18-negative and LDS751-negative, respectively. Additionally, the following non-erythroid cell populations

Figure 8 (cont'd): were identified in the labeled regions: * lymphocytes (A-B), † myeloid cells (A-C), ¶ macrophages (A, not present in this sample), and ** plasma cells (B).

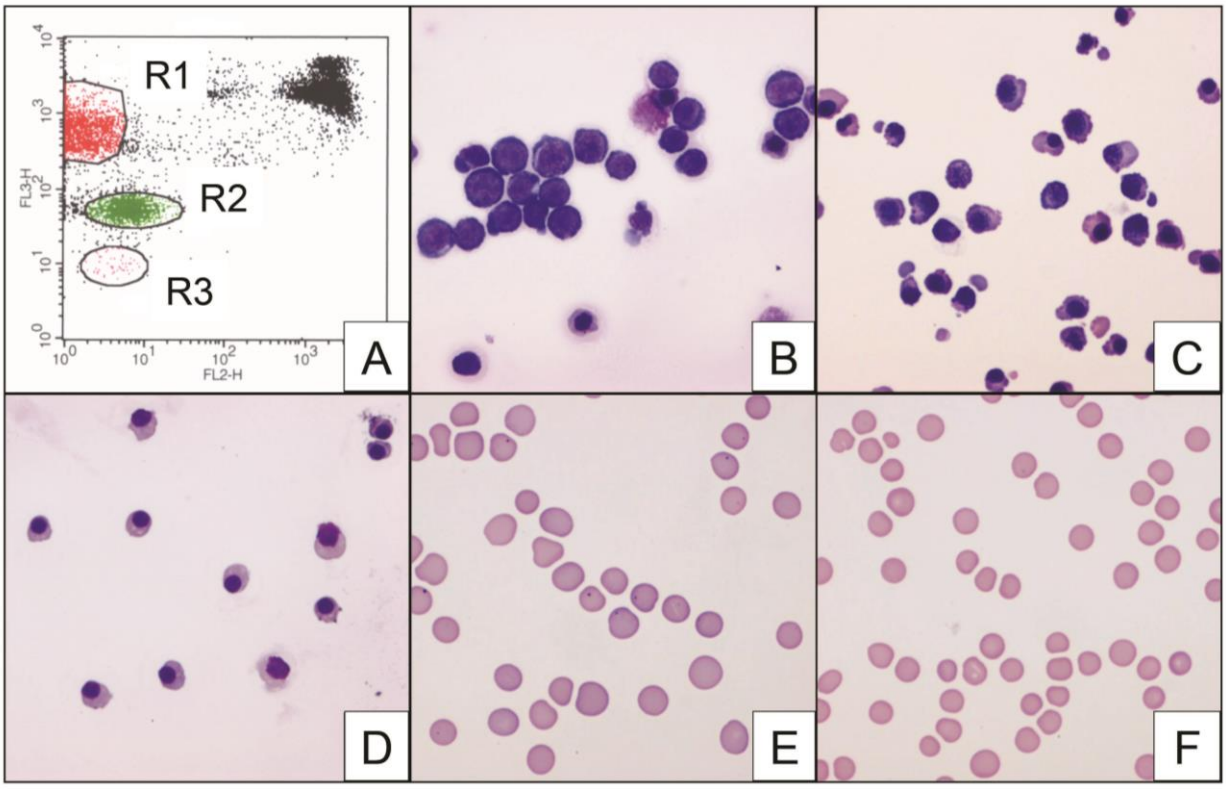

Figure 9: *Cytologic findings in sorted samples from erythroid regions of LDS751 versus CD18 dot plots.* A) Representative dot plot of bone marrow cells in the middle Percoll fraction with CD18 labeling (horizontal axis) and LDS751 staining (vertical axis) showing erythroid precursor (R1), reticulocyte (R2), and mature erythrocyte (R3) gates, respectively. Cell sorting of top, middle, and bottom fractions confirmed R1 gates of each to be enriched in early- (B), mid- (C), and late-stage (D) erythroid precursors, respectively, with little contamination of other cell types, and R2 and R3 gates (A) to consist mostly of reticulocytes (E) and mature erythrocytes (F), respectively.

IgG detection on nRBCs

The median percentages of IgG positive events in the nRBC regions of 10 healthy dogs were 5.7% (range, 1.6-12.3%) for the early nRBCs, 8.6% (range, 2.5-15.2%) for mid-stage nRBCs, and 6.8% (range, 4.2-17.5%) for the late-stage nRBCs. Reticulocytes were abundant enough in the bottom Percoll fractions of each dog to assess, and they had a median of 5% positive IgG events (range, 2.1-15.9%). Mature RBCs were not generally acquired in enough concentration to assess for IgG; they were instead assessed using blood samples.

Median IgG signals for the 3 DEA1.1-negative dogs after incubation with anti-DEA1.1 typing serum were 4.2% (range, 3.6-7.3%), 6.0% (range, 5.3-10.2%), 16.1% (range, 13.1-16.3%), and 12.6% (range, 11.8-25.6%) positive events for the top, middle, and bottom Percoll fractions and the bottom-fraction reticulocytes, respectively. These cell populations from the 3 DEA1.1-positive dogs after sample incubation with anti-DEA1.1 typing serum yielded median IgG results of 71.1% (range, 49.1-90.1%), 98.1% (range, 95.7-99.3%), 99.4% (range, 99.3-99.5%), and 100% positive events, respectively. There were significant differences in % positive events for all nRBC fractions and bottom-fraction reticulocytes when comparing DEA1.1-positive dogs and healthy dogs. Although median fluorescence (not shown) and % positivity results were clearly different for nRBCs from DEA1.1-negative and DEA1.1-positive dogs (Figure 10), the differences were not statistically significant.

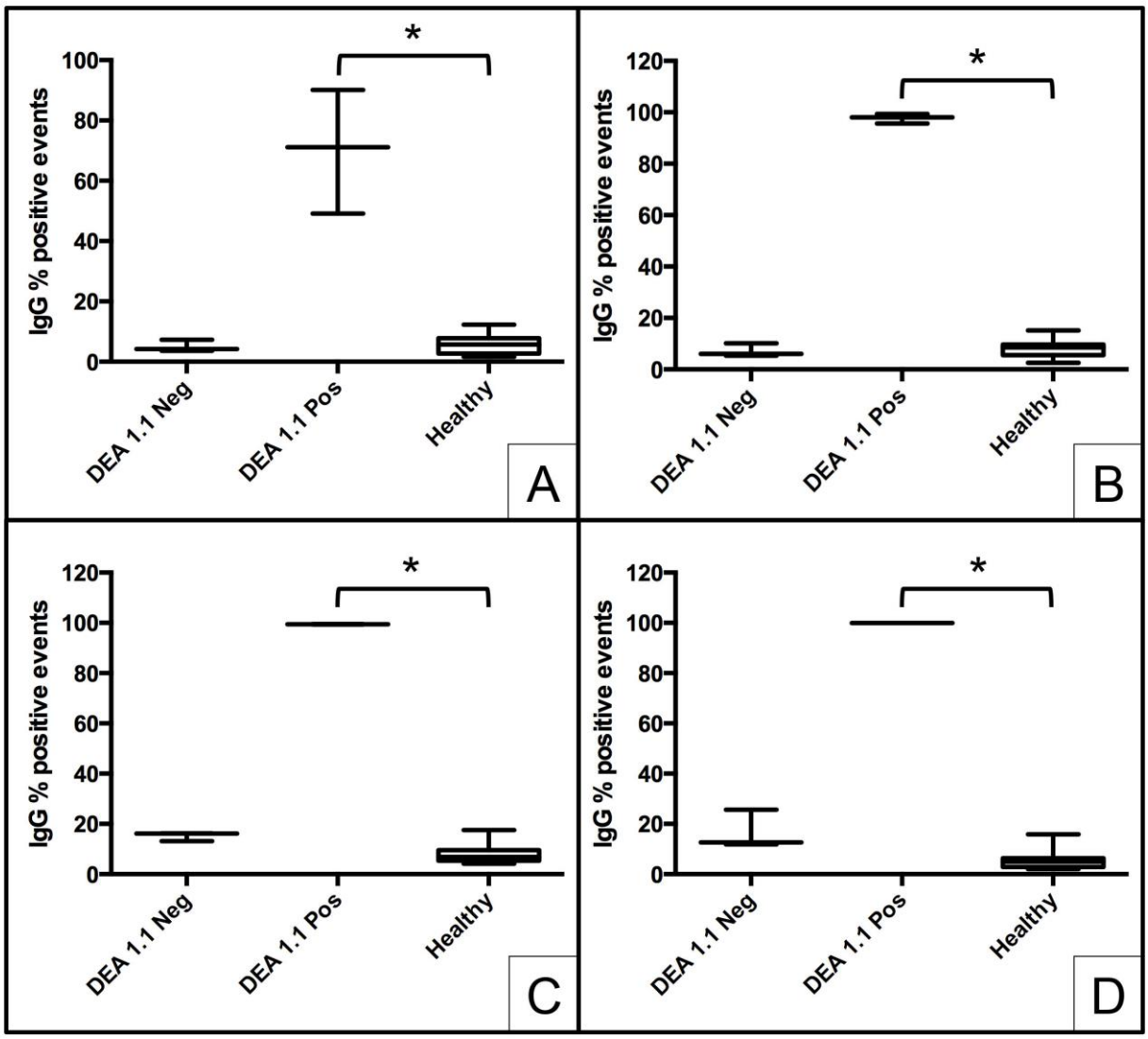

Figure 10: *nRBC IgG detection using DEA1.1-positive cells coated with anti-DEA1.1 IgG.* Erythroid precursors from the top (A), middle (B) and bottom (C) Percoll fractions (i.e., early-, mid-, and late-stage precursors, respectively), and reticulocytes (D) from the bottom Percoll fraction had strong signals for IgG in DEA1.1-positive dogs in contrast to those from healthy (*, $p<0.05$) and DEA1.1-negative dogs after cells from DEA1.1-positive or -negative dogs had been incubated with anti-DEA1.1 typing serum. Median, minimum, and maximum values are shown for each group.

DISCUSSION

In order to investigate the role of IgG in canine immune-mediated anemias, including IMHA and PIMA, we first developed and herein characterized a flow cytometric assay for RBC IgG using a heavy-chain specific antiglobulin reagent and positive and negative control cells. The assay proved to have good analytical precision and accuracy, and its diagnostic accuracy was good when applied to a population of sick dogs with or without IMHA. This RBC IgG assay was then applied to early-, mid-, and late-stage nRBCs after developing a process to harvest them from bone marrow and identify them by a 2-color flow cytometric assay. This nRBC assay was shown to reliably detect IgG on early-, mid- and late-stage nRBCs, and reticulocytes. Additionally, we describe for the first time, based on immunologic detection, the expression of DEA1.1 on early- to late-stage canine nRBCs, and reticulocytes.

During initial stages of assay development, we attempted to detect IgM and C3 on erythroid cells, in addition to IgG, as IgM and C3 may also play roles in mediating cell destruction. However, the IgM assay was hindered by difficulty in developing a reliable positive control solely for IgM, and despite development of a reliable positive control for canine complement, extensive testing of dogs with IMHA yielded only one questionable positive result for canine C3. These findings together with differences in canine and human complement results in vitro (data not shown) and the negative findings of others with similar assays (Wilkerson et al., 2000; Quigley et al., 2001), led us to question the reliability of commercially available anti-[dog C3] antibodies to detect complement-mediated opsonization of canine erythrocytes. Therefore, assay development was limited to detection of IgG, further ensured by use of a heavy-chain specific antiglobulin

reagent, which was selected to minimize cross reactivity with other classes of immunoglobulins.

Although there are different approaches to defining positivity in flow cytometric assays for cell-associated antibody (Kucinskiene et al., 2005; Lucidi et al., 2011; Quigley et al., 2001; Morley et al., 2008; Wilkerson et al., 2000), we used % positive events after accounting for nonspecific binding of a nonsense antibody because this approach appeared to be most sensitive and reliable. It was more sensitive than median or mean fluorescence intensity because many dogs with IMHA had only a subpopulation of detectably positive cells, and the positive events could be clearly increased without significantly shifting the median fluorescence (Figure 11). Regarding nonspecific binding, amounts varied among individuals, and increased amounts of autologous canine IgG on the cell surface was associated with greater nonspecific binding of the nonsense antibody and thus presumably the test antibody (Figure 11). Therefore, use of the nonsense antibody binding to set the cutoff for each sample was important. One particularly noteworthy dog with zinc toxicity had nonspecific binding with the nonsense antibody that was as great as a strong positive reaction, so it was impossible to assess IgG positivity in this sample and the dog was excluded from the study. Similar to other dogs with zinc toxicosis (Gurnee et al., 2007), this dog had a severe regenerative anemia (hematocrit = 13%) associated with marked hemolysis, Heinz bodies, moderate spherocytosis (not pyknocytosis), many ghost cells, and schizocytosis. Although this dog may truly have been positive for RBC IgG, the strong nonspecific signal made analysis impossible, perhaps because of high autofluorescence or membrane changes causing increased adherence of nonsense and test antibodies.

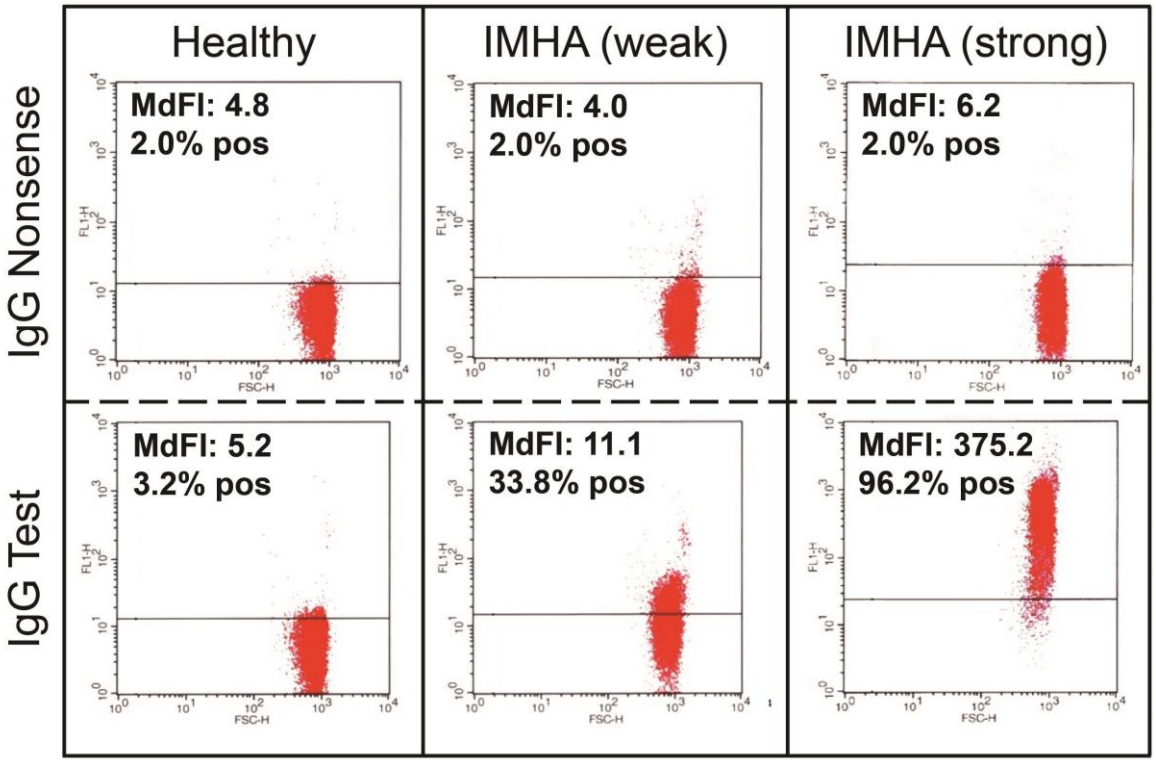

Figure 11: *Median fluorescence intensity (MdFI) versus % positive events for assessing IgG positivity.* Among different ways to assess RBC IgG positivity, % positive events was the most sensitive as represented here by a 2-fold increase in MdFI, but a 10-fold increase in % positive events (% pos) when comparing RBCs incubated with IgG test antibody (bottom row) from a weakly positive IMHA dog to a healthy dog. An IgG nonsense antibody was used to set a cutoff for IgG positivity for every dog in order to account for nonspecific binding (top row), and nonspecific binding tended to be greater for individuals with more IgG – IMHA (strong) – than for those with lower amounts of autologous canine IgG on the cell surface – Healthy and IMHA (weak).

Clinical evaluation of the RBC IgG assay in dogs with and without IMHA were favorable, with few (4 of 33) negative results in dogs with IMHA and few (2 of 28) positive results in unhealthy dogs without a clinical diagnosis of IMHA. The four negative

results in IMHA dogs may have been related to mediation by IgM (C3) or IgA without involvement of IgG (Piek et al., 2012; Wilkerson et al., 2000; Slappendel 1979; Day 1996; Segel et al., 2014), or, in one dog, to 4 days of immunosuppressive therapy prior to testing, as IgG positivity by flow cytometry has been shown to decrease with treatment (Wilkerson et al., 2000). Low amounts of cell-bound IgG (Sachs et al., 2006; Segel et al., 2014) or insufficient assay sensitivity may also have yielded negative results, but flow cytometric assays have been described as having greater diagnostic sensitivity for IMHA than the Coombs' test in both human and canine patients (Wilkerson et al., 2000; Quigley et al., 2001; Kucinskiene et al., 2005; Wang et al., 2001; Chaudhary et al., 2006; Lin et al., 2009). Finally, false-negative results could have been caused by low affinity IgG removed by cell washes not conducted at 4°C or with low ionic strength buffers (Segel et al. 2014). Regarding the two positive results for RBC IgG in the 28 unhealthy non-IMHA dogs, one result was very weak in a nonanemic dog with a pheochromocytoma, and one was mildly increased in an anemic dog (hematocrit = 33%) with hypoadrenocorticism and hypothyroidism. Further evaluation of the anemia in this dog was not done.

Although it has been reported that storage of blood samples for up to 7 days did not affect Coombs' test results when compared to fresh samples (Caviezel et al., 2014), effects on a flow cytometric assay, which may be more sensitive, were not assessed. Our results from 20 healthy dogs showed that testing blood-derived RBCs for IgG on the day of collection versus the day after collection following storage at 4°C did not affect results or clinical interpretation, and we have not seen clinically significant differences with 1-2 day storage in sporadic paired assessments, but we have not systematically

assessed effects of storage over longer periods of time or on samples from unhealthy or positive dogs.

Favorable analytical and diagnostic performance of the RBC IgG assay led to efforts to apply it to nRBCs from the bone marrow. Initial attempts involved lysis of RBCs to remove them as interfering events from bone marrow samples. However, pre- and post-lysis cytologic preparations indicated that tris ammonium chloride, ammonium chloride, and a commercial erythrocyte lysing buffer (Erythrolyse Red Blood Cell Lysing Buffer, AbD Serotec, Raleigh, NC) all resulted in lysis of nRBCs in addition to mature RBCs (data not shown). By combining a Percoll gradient separation method with a 2-color flow cytometric assay, concentrations of mature RBCs and reticulocytes were markedly reduced and easily discriminated from nRBCs, and omission of a lysis step decreased potential cell damage and loss.

Initial studies also explored an anti-canine CD45 (Rat anti dog CD45:RPE monoclonal antibody, AbD Serotec, Raleigh, NC) antibody to separate erythroid cells from other populations (Cobbold et al., 1994; Weiss 2004), but it yielded poorer discrimination (results not shown) of cell populations than did the anti-[dog-CD18] antibody, so its use was discontinued. If anti-[dog nRBC] antibodies (e.g. anti-[dog transferrin receptor], anti-[dog glycophorin]) become available, they should be explored as better labor- and time-saving options that would mirror human methods for nRBC analysis (Loken et al., 1987; Civin et al., 1987; Chen et al., 2009; Bony et al., 1999).

As finally developed, the assay's CD18-negative and LDS751-positive populations contained very few non-erythroid cells, and combined use of Percoll fractionation and 2-color separation allowed independent analysis of largely early-, mid-, and late-stage nRBCs. All three of these nRBC fractions from DEA1.1-positive dogs had strong signals

for IgG after incubation with anti-DEA1.1 typing serum. This was in contrast to the nRBC fractions from DEA1.1-negative dogs submitted to the same treatment, all of which had results similar to those of 10 healthy dogs. Detection of this IgG on the DEA1.1-positive nRBCs demonstrated the ability of the assay to detect nRBC IgG, and supports its application to dogs with PIMA. Additionally, it provided evidence that DEA1.1 is expressed on early through late stages of nRBCs in DEA1.1 positive dogs, something not reported to date, to the authors' knowledge. Further evidence of DEA1.1 expression on canine nRBCs could be sought by nonimmunologic approaches such as mass spectrometry of RBC membranes or detection of DEA1.1 mRNA. The presence of DEA1.1 on precursors could be of interest given that certain blood group antigens expressed on human erythroid precursors (Blacklock et al., 1984; Bony et al., 1999; Wada et al., 1990) and human RBC-incompatible allogeneic bone marrow transplantations can have deleterious consequences such as immediate and delayed hemolytic transfusion reactions, delayed RBC recovery, and pure red cell aplasia (Blacklock et al., 1984; Rowley et al., 2011). Even though current donor bone marrow conditioning and immunosuppressive regimens appear to overcome these deleterious effects, crossmatching is still warranted, and RBC-compatible bone marrow transplants are preferable (Rowley et al., 2011). DEA1.1 expression on canine erythroid precursors may be relevant to allogeneic bone marrow transplantations in dogs.

In conclusion, we have developed and described a new method for isolation and identification of early-, mid-, and late-stage nRBCs from the bone marrow of dogs, and demonstrated that we can detect IgG on all erythroid populations by applying the fundamentals of a flow cytometric assay for IgG detection on RBCs. In the process, we obtained evidence that DEA1.1 is expressed on rubriblasts through erythrocytes of

DEA1.1-positive dogs. These assays allow assessment of IgG on all erythroid populations from bone marrow and blood of PIMA dogs, and they will be used to help clarify the mechanism and appropriate management for this hematologic disease.

REFERENCES

REFERENCES

Arndt PA, Garratty G. A critical review of published methods for analysis of red cell antigen-antibody reactions by flow cytometry, and approaches for resolving problems with red cell agglutination. Transfus Med Rev. 2010;24:172-194.

Blacklock HA, Katz F, Michalevicz R, et al. A and B blood group antigen expression on mixed colony cells and erythroid precursors: relevance for human allogeneic bone marrow transplantation. Br J Haematol. 1984;58:267-276.

Bony V, Gane P, Bailly P, et al. Time-course expression of polypeptides carrying blood group antigens during human erythroid differentiation. Br J Haematol. 1999;107:263-274.

Caviezel LL, Raj K, Giger U. Comparison of 4 direct Coombs' test methods with polyclonal antiglobulins in anemic and nonanemic dogs for in-clinic or laboratory use. J Vet Intern Med. 2014;28:583-591.

Chaudhary R, Das SS, Gupta R, et al. Application of flow cytometry in detection of red-cell-bound IgG in Coombs-negative AIHA. Hematology. 2006;11:295-300.

Chen K, Liu J, Heck S, et al. Resolving the distinct stages in erythroid differentiation based on dynamic changes in membrane protein expression during erythropoiesis. Proc Natl Acad Sci USA. 2009;106:17413-17418.

Civin CI, Loken MR. Cell surface antigens on human marrow cells: dissection of hematopoietic development using monoclonal antibodies and multiparameter flow cytometry. Int J Cell Cloning. 1987;5:267-288.

Cobbold S, Metcalfe S. Monoclonal antibodies that define canine homologues of human CD antigens: summary of the First International Canine Leukocyte Antigen Workshop (CLAW). Tissue Antigens. 1994;43:137-154.

Day MJ. Serial monitoring of clinical, haematological and immunological parameters in canine autoimmune haemolytic anaemia. J Small Anim Pract. 1996;37:523-534.

Gurnee CM, Drobatz KJ. Zinc intoxication in dogs: 19 cases (1991-2003). J Am Vet Med Assoc. 2007;230:1174-1179.

Holloway SA, Meyer DJ, Mannella C. Prednisolone and danazol for treatment of immune-mediated anemia, thrombocytopenia, and ineffective erythropoiesis in a dog. J Am Vet Med Assoc. 1990;197:1045-1048.

Jonas LD, Thrall MA, Weiser MG. Nonregenerative form of immune-mediated hemolytic anemia in dogs. J Am Anim Hosp Assoc. 1987;23:201-204.

Kucinskiene G, Schuberth H, Leibold W, et al. Flow cytometric evaluation of bound IgG on erythrocytes of anaemic dogs. Vet J. 2005;169:303-307.

Lin J, Hao T, Lyou J, et al. Clinical application of a flow cytometric direct antiglobulin test. Transfusion. 2009;49:1335-1346.

Loken MR, Shah VO, Dattilio KL, et al. Flow cytometric analysis of human bone marrow: I. Normal erythroid development. Blood. 1987;69:255-263.

Lucidi CA, Resende CLE, Jutkowitz LA, et al. Cytologic and histologic bone marrow findings in dogs with suspected marrow-directed immune-mediated anemia [abstract]. 61st Annual Meeting Am College Vet Pathol and 45th Annual Meeting Am Soc Vet Clin Pathol., Baltimore, MD, Oct-Nov 2010.

Lucidi CA, Takahira RK, Gerlach JA, et al. Flow cytometric assessment of canine erythrocytes and platelets for dog erythrocyte antigen 1.1. Vet Clin Pathol. 2011;40:435-443.

Mangan KF, Besa EC, Shadduck RK, et al. Demonstration of two distinct antibodies in autoimmune hemolytic anemia with reticulocytopenia and red cell aplasia. Exp Hematol. 1984;12:788-793.

McManus P. Marrow and erythrocyte changes associated with canine non-regenerative immune- mediated anemia: 44 cases (1996-2000) [abstract]. Vet Pathol. 2000;37:526.

Meyer RJ, Hoffman R, Zanjani ED. Autoimmune hemolytic anemia and periodic pure red cell aplasia in systemic lupus erythematosus. Am J Med. 1978;65:342-345.

Morley P, Mathes M, Guth A, et al. Anti-erythrocyte antibodies and disease associations in anemic and nonanemic dogs. J Vet Intern Med. 2008;22:886-892.

Piek CJ, Teske E, van Leeuwen MW, et al. Good agreement of conventional and gel-based direct agglutination test in immune-mediated haemolytic anaemia. Acta Vet Scand. 2012;54:10.

Quigley KA, Chelack BJ, Haines DM, et al. Application of a flow cytometric erythrocyte immunofluorescence assay in dogs with immune-mediated hemolytic anemia and comparison to the direct antiglobulin test. J Vet Diagn Invest. 2001;13:297-300.

Rowley SD, Donato ML, Bhattacharyya P. Red blood cell-incompatible allogeneic hematopoietic progenitor cell transplantation. Bone Marrow Transplant. 2011;46:1167-1185.

Saad A, Palm M, Widell S, et al. Differential analysis of rat bone marrow by flow cytometry. Comp Haematol Int. 2000;10:97-101.

Sachs UJ, Röder L, Santoso S, et al. Does a negative direct antiglobulin test exclude

warm autoimmune haemolytic anaemia? A prospective study of 504 cases. Br J Haematol. 2006;132:655-656.

Scott-Moncrieff JCR, Reagan WJ, Glickman LT, et al. Treatment of nonregenerative anemia with human γ-globulin in dogs. J Am Vet Med Assoc. 1995;206:1895-1900.

Segel GB, Lichtman MA. Direct antiglobulin ("Coombs") test-negative autoimmune hemolytic anemia: a review. Blood Cells Mol Dis. 2014;52:152-160.

Slappendel RJ. The diagnostic significance of the direct antiglobulin test (DAT) in anemic dogs. Vet Immunol Immunopathol. 1979;1:49-59.

Stockham SL, Ford RB, Weiss DJ. Canine autoimmune hemolytic disease with a delayed erythroid regeneration. J Am Anim Hosp Assoc. 1980;16:927-931.

Stokol T, Blue JT, French TW. Idiopathic pure red cell aplasia and nonregenerative immune-mediated anemia in dogs: 43 cases (1988-1999). J Am Vet Med Assoc. 2000;216:1429-1436.

van de Loosdrecht AA, Hendriks DW, Blom NR, et al. Excessive apoptosis of bone marrow erythroblasts in a patient with autoimmune haemolytic anaemia with reticulocytopenia. Br J Haematol. 2000;108:313-315.

Wada H, Suda T, Miura Y, et al. Expression of major blood group antigens on human erythroid cells in a two phase liquid culture system. Blood. 1990;75:505-511.

Wang Z, Shi J, Zhou Y, et al. Detection of red blood cell-bound immunoglobulin G by flow cytometry and its application in the diagnosis of autoimmune hemolytic anemia. Int J Hematol. 2001;73:188-193.

Weiss DJ, Blauvelt M, Sykes J, et al. Flow cytometric evaluation of canine bone marrow differential counts. Vet Clin Pathol. 2000;29:97-104.

Weiss DJ, Stockham SL, Willard MD, et al. Transient erythroid hypoplasia in the dog: report of five cases. J Am Anim Hosp Assoc. 1982;18:353-359.

Weiss DJ. Bone marrow pathology in dogs and cats with non-regenerative immune-mediated haemolytic anaemia and pure red cell aplasia. J Comp Pathol. 2008;138:46-53.

Weiss DJ. Flow cytometric evaluation of canine bone marrow based on intracytoplasmic complexity and CD45 expression. Vet Clin Pathol. 2004;33:96-101.

Wilkerson MJ, Davis E, Shuman W, et al. Isotype-specific antibodies in horses and dogs with immune-mediated hemolytic anemia. J Vet Intern Med. 2000;14:190-196.

CHAPTER 4

Exploring the Roles of IgG and Phosphatidylserine in Canine Regenerative and Nonregenerative Immune-Mediated Anemias

ABSTRACT

Background: Many cases of suspected immune-mediated precursor-targeted anemia (PIMA) have been described and suggested to be caused by immune targeting of erythroid precursors, but the pathogenic mechanism of anemia is unknown.

Objective: To test the hypothesis that IgG and phosphatidylserine (PS) are involved in mediating PIMA, and that PS is involved in mediating IMHA.

Animals: Blood and bone marrow samples from healthy colony dogs, and client-owned dogs with IMHA, PIMA, or other (non-IMHA, non-PIMA) conditions were assessed prospectively.

Methods: A Percoll gradient separation method was used to separate different stages of erythroid precursors from bone marrow samples. Flow cytometric assays were used to assess for IgG and PS on erythrocytes (RBCs) and erythroid precursors.

Results: RBC IgG was increased on 9 of 11 and 0 of 9 IMHA and non-IMHA dogs, respectively; 10 of 11 and 2 of 9 IMHA and non-IMHA dogs were positive for PS on RBCs, respectively. Erythroid precursor IgG was not statistically different between PIMA and non-PIMA dogs, but 5 of 17 and 0 of 7 PIMA and non-PIMA dogs, respectively, were positive based on values from healthy dogs. Erythroid precursor PS was significantly increased in PIMA dogs compared to healthy dogs, with positivity in 5 of 6 PIMA dogs.

Conclusions and Clinical Importance: Our data suggests a role for PS in canine IMHA, and for IgG and PS in canine PIMA. Additional studies are warranted to further assess multiple mechanisms and optimal therapy.

INTRODUCTION

Precursor-targeted immune-mediated anemia (PIMA) has been described in dogs with nonregenerative anemia and evidence of ineffective erythropoiesis, and although it has been suggested that the pathogenesis may relate to immune targeting of erythroid precursors (Jonas et al., 1987; Stockham et al., 1980; Weiss et al., 1982; Holloway et al., 1990; Scott-Moncrieff et al., 1995; McManus 2000; Stokol et al., 2000; Weiss 2008), this has not been demonstrated. Suspicion of an immune-mediated mechanism is based on: 1) apparent responses to steroid treatment (Jonas et al., 1987; Stockham et al., 1980; Weiss et al., 1982; Holloway et al., 1990; Scott-Moncrieff et al., 1995; Stokol et al., 2000) and relapses when tapering it (Stockham et al., 1980; Weiss et al., 1982; Scott-Moncrieff et al., 1995; Stokol et al., 2000), 2) phagocytosis of intact erythroid precursors (Jonas et al., 1987; Stockham et al., 1980; Holloway et al., 1990; McManus 2000), and 3) association with concurrent evidence of immune-mediated hemolytic anemia (IMHA), such as spherocytosis, red blood cell (RBC) agglutination, or Coombs' positivity (Jonas et al., 1987; Stockham et al., 1980; Holloway et al., 1990; Scott-Moncrieff et al., 1995; Stokol et al., 2000; Weiss 2008), or with other immune-mediated disease such as immune-mediated thrombocytopenia (IMT) (Holloway et al., 1990; Scott-Moncrieff et al., 1995; Stokol et al., 2000). However, dogs diagnosed with PIMA typically respond slowly to immunosuppressive therapy (Jonas et al., 1987; Stockham et al., 1980; Weiss et al., 1982; Holloway et al., 1990; Scott-Moncrieff et al., 1995; Stokol et al., 2000), some do not respond at all (Jonas et al., 1987; Stokol et al., 2000), and many have no evidence of concurrent IMHA (Weiss et al., 1982; Scott-Moncrieff et al., 1995; Stokol et al., 2000), leaving unanswered questions about the mechanism(s) of anemia in these dogs.

Dogs with an ultimate diagnosis of PIMA account for approximately 25% of canine bone marrow submissions to the Diagnostic Center for Population and Animal Health (DCPAH) at the Michigan State University Veterinary Medical Center (MSU VMC), and for approximately 50% of those submissions for dogs with the main complaint of nonregenerative anemia (Chapter 2). In our experience, phagocytosis of intact erythroid precursors, rubriphagocytosis (RP), by normal-appearing macrophages is found in bone marrow samples from most dogs with PIMA. This RP is usually selective for the erythroid lineage and for selective stages of erythroid development, with RP of different stages associated with different bone marrow patterns (Lucidi et al., 2010; Chapter 2). This suggests a role for phagocytosis in the ineffective erythropoiesis and nonregenerative anemia in these dogs. The different bone marrow patterns seen in these dogs may reflect a spectrum of disease caused by a single mechanism, or they may result from multiple mechanisms.

Phagocytosis by macrophages may proceed through recognition of several cell membrane mediators, including IgG, complement, and phosphatidylserine (PS). Although the role of antibodies in the pathogenesis of IMHA is well demonstrated, membrane exposure of PS through oxidative stress (Pesillo et al., 2004; Tan et al., 2012) and an apoptotic-like mechanism (Tan et al., 2012) has been suggested to contribute to RBC destruction in dogs and mice (Lee et al., 2004). Eryptosis, which is apoptosis of mature RBCs, is characterized by cell shrinkage, cell membrane scrambling, and PS exposure triggering phagocytosis by macrophages (Lang et al., 2015). Apoptosis of erythroid precursors may occur as in other nucleated cells, and increased PS exposure of bone marrow-derived erythroid precursors has been

demonstrated in association with IgG positivity in one human patient with reticulocytopenic immune-mediated anemia (van de Loosdrecht et al., 2000).

The goal of this study was to explore the potential roles of IgG and PS in mediating canine immune-mediated anemias. We hypothesized that PS exposure on RBCs and erythroid precursors plays a role in canine IMHA and PIMA, respectively, and that IgG on erythroid precursors contributes to PIMA as it does to IMHA.

MATERIAL AND METHODS

Animals

RBCs from suspected IMHA dogs admitted to the MSU VMC from October 2013 through November 2014 were assessed for IgG and PS. RBCs from unhealthy dogs thought to lack immune-mediated anemia and collected within 24 h of samples from suspected IMHA samples were also tested for IgG and PS in order to evaluate the specificity of results for IMHA. Some dogs from each group were excluded after testing if they were later found to not fulfill criteria. Inclusion criteria for IMHA required a clinical diagnosis of IMHA associated with regenerative anemia (all had hematocrit [Hct] below 33%) and at least one of the following with no other explanation: 1) spherocytosis, 2) erythrocyte agglutination, and 3) Coombs' test positivity. Unhealthy non-IMHA dogs were defined as any sick dogs with no clinical suspicion or hematologic evidence of immune-mediated anemia, including the absence of spherocytosis, RBC agglutination, and Coombs' positivity.

Dogs from which a bone marrow aspirate sample was collected for cytologic examination at the MSU VMC from April 2012 through December 2014 were assessed for IgG on erythroid precursors and RBCs. Samples of dogs assessed after October 2013 were also tested for PS. The following data were collected: signalment, clinical history and presenting complaint, bone marrow aspirate (and core, when available) findings, relevant diagnostic test results, clinical diagnosis and therapeutic regimen, and clinical outcome with emphasis on hematologic response. Clinical and diagnostic data were reviewed in order to identify dogs with clear diagnoses of PIMA and dogs that clearly did not have PIMA (non-PIMA dogs). Inclusion criteria for PIMA dogs were as follows: 1) unexplained persistent nonregenerative or inappropriately regenerative

anemia (on the day of bone marrow collection, all had Hct ≤ 29%, inadequate erythroid regeneration for the degree of anemia, and documented anemia or anemia-related signs for at least 5 days), 2) ineffective erythropoiesis, as evidenced by persistent anemia with erythroid hyperplasia or by erythroid maturation arrest or left-shift with RP, 3) lack of erythroid dysplasia, and 4) a clinical diagnosis of PIMA. Inclusion criteria for non-PIMA dogs were the same as described for unhealthy non-IMHA dogs, with the addition of 1) no evidence of erythroid precursor targeting, including absence of ineffective erythropoiesis, and 2) a clear clinical diagnosis of a condition other than PIMA. Dogs were excluded from the study if they had any evidence of neoplastic cells in bone marrow samples, or if the cell yield from bone marrow aspirates was insufficient for flow cytometric assessment.

A control population for IgG and PS on RBCs consisted of 20 clinically and hematologically healthy adult dogs enrolled in the MSU VMC Blood Donor Program (Chapter 3). Similarly, IgG was assessed on erythroid precursors from 10 clinically and hematologically healthy adult colony dogs (Chapter 3), and PS was assessed for 5 of these dogs. Use of leftover blood and bone marrow specimens from clinical patients, and sample collection procedures from all dogs were performed in compliance with the MSU and MPI Research Institutional Animal Care and Use Committees.

RBC samples and isolation

RBCs from clinical patients were isolated from ethylenediaminetetraacetic acid (EDTA)-anticoagulated blood submitted to the DCPAH laboratory for a CBC or Coombs' test. Blood was tested on the day of collection or stored at 4°C and tested on the next day. RBCs were isolated as described in a previous study (Chapter 3). Briefly, 10 µL of

packed RBCs were washed twice and resuspended in 800 µL phosphate buffered saline (PBS) containing 0.3% bovine serum albumin (BSA) to make a RBC suspension.

Bone marrow samples and erythroid precursor isolation

Erythroid precursors of bone marrow-tested dogs were isolated from samples collected into 0.5 mL of 3% EDTA for cytologic assessment. After collection, bone marrow material was transferred to a Petri dish for particle collection and slide preparation, transported to the laboratory in a cooled container, and sample processing was initiated within 60 minutes of collection. Erythroid precursors were isolated as described in a previous study (Chapter 3). Briefly, 2-5 mL of bone marrow aspirate was washed with PBS-1%BSA, cells were applied to a Percoll density gradient with 68, 64, and 52% Percoll layers, and centrifuged for 30 minutes at 500 g. The three layers of cells formed by centrifugation were harvested from top to bottom into individual tubes. Cells were washed, and nucleated cell concentration was determined by hemocytometer. The mixtures of cells in these top, mid, and bottom Percoll fractions were shown by cell sorting (Chapter 3) to be enriched in early (rubriblasts and prorubricytes), mid (early- to mid-rubricytes), and late (mid- to late-stage rubricytes and metarubricytes) erythroid precursors, respectively. This separation allowed for IgG and PS assessment of distinct stages of erythroid development. For every sample, stop-flow cytologic preparations of each fraction were prepared and assessed microscopically in order to ensure proper separation. For 5 healthy dogs, bone marrow samples were transported in a cooled container from MPI Research in Mattawan, MI to MSU after harvesting the Percoll layers and before continuation of the protocol for IgG testing.

RBC testing for IgG

As described previously (Chapter 3), RBC suspensions were incubated with a fluorescein isothiocyanate (FITC) conjugated anti-[dog IgGγ] antibody (IgG test antibody, KPL, Gaithersburg, MD) and analyzed on a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA) with CellQuest Pro software (Becton-Dickinson, San Jose, CA). For each sample, 10,000 events with target FSC and SSC properties (Lucidi et al., 2011) were acquired, and RBC agglutinates were excluded by gating (Arndt et al., 2010). Controls for every patient sample were: 1) RBCs from a healthy dog incubated with IgG test antibody (negative control for IgG), 2) dog erythrocyte antigen (DEA)1.1-positive RBCs treated with a polyclonal anti-DEA1.1 typing serum (Animal Blood Resources International, Stockbridge, MI) and then incubated with IgG test antibody (positive control for IgG), and 3) RBCs from each tested patient incubated with an anti-[chicken IgG-heavy and light chain]-FITC-labeled antibody (nonsense antibody, KPL, Gaithersburg, MD) to control for nonspecific binding. IgG positivity was determined by % positive events as follows: RBCs from each tested dog incubated with nonsense antibody were used to set a marker for 2% positive events, based on a dot plot distribution. RBCs incubated with IgG test antibody that fell above this marker were considered positive events for IgG. The same strategy was applied to results of 20 healthy dogs, and cutoffs for IgG positivity were calculated based on the mean percent positive events plus 3 standard deviations (SDs). Patients with greater % positivity were considered positive for RBC IgG (Figure 12).

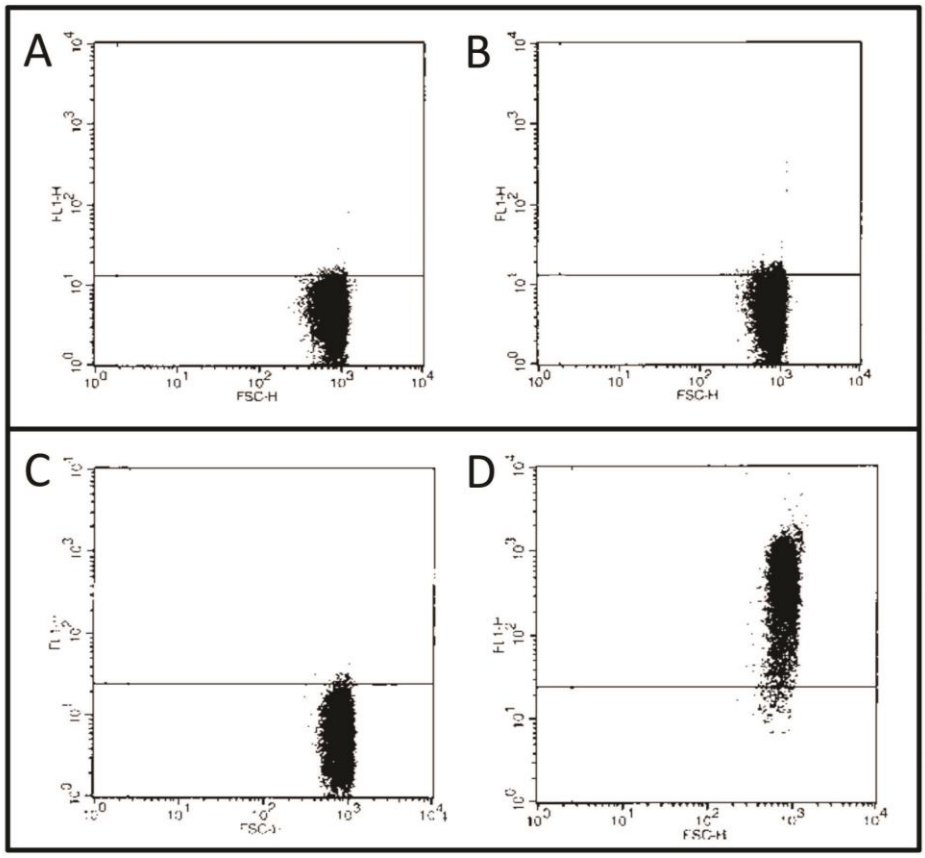

Figure 12: *Representative dot plots of flow cytometric IgG fluorescence (vertical axis) versus forward scatter (horizontal axis) of red blood cells (RBCs).* Markers for positivity were set at 2% positive events for each tested dog after incubation with nonsense antibody (A, C), and RBCs falling above this marker after incubation with IgG test antibody were used to determine positivity (B, D). Examples are of a healthy dog, 3.2% positive events (A, B), and an IgG-positive dog, 96.2% positive events (C, D).

RBC testing for PS

RBCs were prepared for PS analysis by washing 20 µL of RBC suspension with annexin buffer (140 mM NaCl, 5 mM $CaCl_2$, and 10 mM HEPES, pH 7.4; filter sterilized) and then resuspending and incubating the RBCs in the dark for 15 minutes in 100 µL of

incubation buffer mixed with 2 µL of 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester-conjugated annexin V (annexin V-FLUOS, Roche Diagnostics, Indianapolis, IN) in the presence of calcium (AnV-Ca). RBCs were then resuspended into 400 µL of annexin buffer and analyzed by flow cytometry.

Controls consisted of: 1) RBCs from a healthy dog incubated with AnV-Ca (negative control for PS), 2) PS-positive control RBCs incubated with AnV-Ca (positive control for PS), 3) RBCs from each tested patient incubated as described above, but in the absence of annexin V-FLUOS (control for RBC autofluorescence), and 4) RBCs from each tested patient incubated as described above, but with a calcium-free annexin control buffer (annexin buffer without $CaCl_2$) used in every step (AnV-Ca-free) to control for nonspecific binding of annexin, since binding to PS requires calcium (Tait et al., 1992). PS-positive control cells were made by twice washing 10 µL of packed RBCs from a healthy dog with a BSA-NaCl solution (147.5 mM NaCl, 2.5 mM KCl, 5 mM D-glucose, 20 mM HEPES, 0.1% BSA, and 0.025% sodium azide; pH 7.5), resuspending the cell pellet into 800 µL of the same solution without BSA or sodium azide, and incubating 200 µL of this RBC suspension with 2 µL of 10 mM 2′(3′)-O-(4-benzoylbenzoyl)adenosine 5′-triphosphate triethylammonium (BzATP, Sigma-Aldrich, St. Louis, MO) stock solution at 37°C for 4 hours (Sluyter et al., 2007). RBCs were then washed and resuspended in 200 µL of annexin buffer for use.

PS positivity was determined by percent positive events as follows: control RBCs from each tested dog were incubated with AnV-Ca-free and used to set a visual marker to include the main population of RBCs in the fluorescence histogram. This resulted in 0.1-0.7% positive events outside the main peak. Those test RBCs incubated with AnV-Ca that had greater fluorescence were considered positive PS events. This strategy was

applied to results of 20 healthy dogs, and cutoffs for PS positivity were calculated based on the mean % positive events plus 3 standard deviations (SD). Patients with % positive events above the cutoff determined based on healthy dogs were considered positive for PS on RBCs (Figure 13). IgG and PS results from same-day and next-day patient samples were interpreted using same-day and next-day reference cutoffs, respectively.

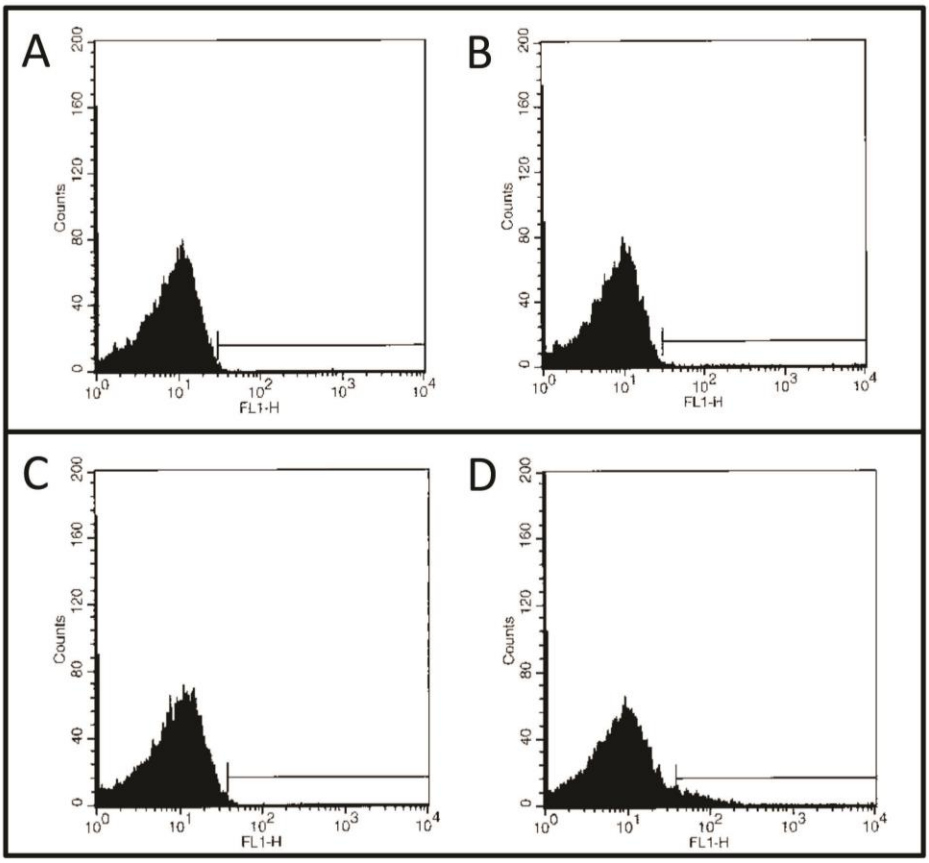

Figure 13: *Representative frequency histograms of flow cytometric phosphatidylserine (PS) fluorescence (horizontal axis) on red blood cells (RBCs).* Markers for positivity were set at the end of the main population for each tested dog after incubation with annexin V-FLUOS in the absence of calcium (A, C), and RBCs with fluorescence greater than this marker after incubation with annexin V-FLUOS in the presence of calcium were

Figure 13 (cont'd): used to determine positivity (B, D). Examples are of a healthy dog, 0.3% positive events (A, B), and a PS-positive dog with IMHA and 5.4% positive events C, D).

Erythroid precursor testing for IgG and PS

Assessment of IgG on erythroid precursors was performed as previously described (Chapter 3). Briefly, nucleated bone marrow cells from each Percoll fraction were incubated with a monoclonal murine anti-[dog CD18] antibody (AbD Serotec, Raleigh, NC) and then with a phycoerythrin (PE) conjugated polyclonal goat anti-[mouse IgG] antibody (AbD Serotec, Raleigh, NC) and an IgG test antibody. Cells were then incubated for 20 minutes to approximately 1 hour (longer times for samples analyzed later in a batch) with the nucleic acid stain LDS751 (Life Technologies, Grand Island, NY) as previously described (Saad et al., 2000) immediately prior to flow cytometric analysis. At least 10,000 total events were acquired, depending on erythroid precursor concentrations, and erythroid precursors were identified as CD18-low, LDS751-high events. Negative and positive IgG controls were the same as used for RBC testing, with the addition of Percoll fractions of test-dog bone marrow cells incubated with nonsense antibody. Percent positive results for IgG were determined based on IgG nonsense and test antibodies in the same manner as for RBCs (Figure 14).

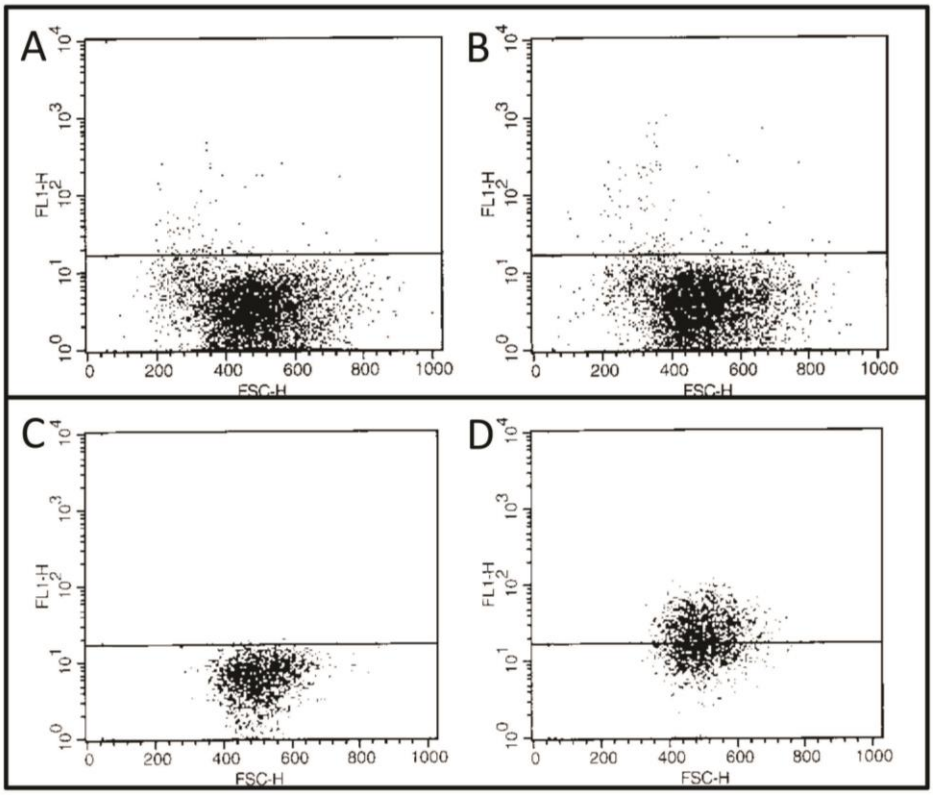

Figure 14: *Representative dot plots of flow cytometric IgG fluorescence (vertical axis) versus forward scatter (horizontal axis) of erythroid precursors (nRBCs).* Markers for positivity were set at 2% positive events for each tested dog after incubation with nonsense antibody (A, C), and events falling above this marker after incubation with IgG test antibody were used to determine positivity (B, D). Examples are of nRBCs from the mid Percoll fractions of a healthy dog, 2.5% positive events (A, B), and an IgG-positive PIMA dog, 57.6% positive events (C, D).

Erythroid precursor testing for PS was done by applying the same method used for RBCs, but assessing 10^6 bone marrow-derived nucleated cells from each Percoll fraction. After resuspending cells in 400 µL of annexin buffer, cells were mixed with 160 µL of LDS751 working solution (Saad et al., 2000) and incubated for 20 minutes to

approximately 1 hour in the dark before flow cytometric analysis. Erythroid precursors were identified based on CD18 labeling and LDS751 staining. Negative and positive PS controls were the same as those used for RBCs, and nucleated cells from Percoll fractions were additionally incubated with LDS751 to test for autofluorescence and nonspecific annexin binding. PS positivity was determined by % positive events as follows: erythroid precursors from each Percoll fraction of each dog were incubated with AnV-Ca-free and used to set a marker with 2% positive events based on dot plot distributions. All test sample events with fluorescence greater than this cutoff were considered positive (Figure 15). Because PS positivity was high on erythroid precursors compared to RBCs, effects of processing and time were individually assessed on unfractionated marrow nucleated cells by testing them immediately and 5.5 hours later, the duration of the assay. No effects of processing (i.e., Percoll fractionation, washing, or time) on PS results were detected, but nucleated erythroid populations could not be assessed alone, as incubations with CD18 and LDS751 reagents would have been required, thus adding additional variables of time and washing.

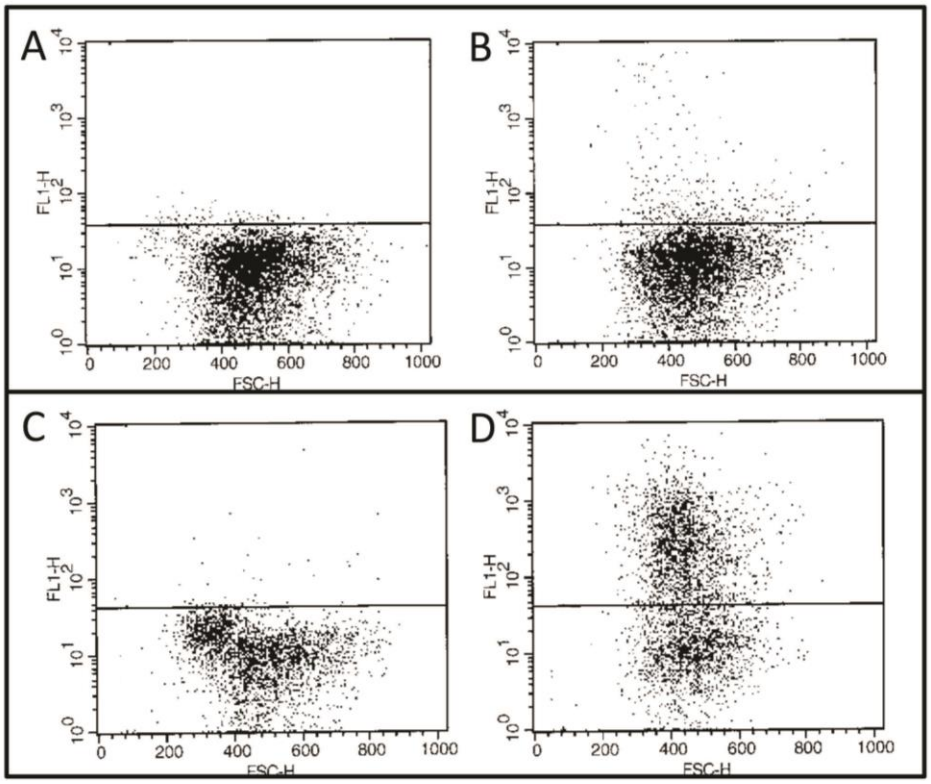

Figure 15: *Representative dot plots of flow cytometric phosphatidylserine (PS) fluorescence (vertical axis) versus forward scatter (horizontal axis) of erythroid precursors (nRBCs).* Markers for positivity were set at 2% positive events for each tested dog after incubation with annexin V-FLUOS in the absence of calcium (A, C), and events falling above this marker after incubation with annexin V-FLUOS in the presence of calcium were used to determine positivity (B, D). Examples are of nRBCs from the mid Percoll fractions of a healthy dog, 7.5% positive events (A, B), and a PS-positive PIMA dog, 53.1% positive events (C, D).

Statistical analysis

Comparisons among 3 or more groups were performed using Kruskall-Wallis and Dunn's multiple comparison tests. Comparisons between healthy dogs tested for IgG

and PS on same-day or next-day were done using the paired t-test, and the two-tailed Mann-Whitney test was used to assess differences in erythroid precursor PS between PIMA and healthy dogs. Statistical analyses were performed using statistical software (GraphPad Prism, GraphPad Software Inc., San Diego, CA), and differences were considered to be significant at $p<0.05$.

RESULTS

RBC IgG and PS in IMHA

Eleven IMHA and 9 non-IMHA dogs were assessed. Of the non-IMHA dogs, 1 was not anemic, and the other 8 had regenerative (4 of 8, Hct range: 30-35%) and nonregenerative (4 of 8, Hct range: 28-39%) anemias. Flow cytometric results revealed 9 of 11 IMHA and 0 of 9 non-IMHA dogs positive for IgG (Figure 16). The median % positive events for IgG in IMHA and non-IMHA dogs were 46.8% (range: 2.6-100%) and 4.4% (range: 2.2-8.2%), respectively. One of the two IgG-negative IMHA dogs had been treated with prednisone for 4 days before blood was collected. There was no significant difference in RBC IgG for healthy dogs tested on the day of collection or the day after collection, so same-day results were used for group comparisons with sick animals. RBC IgG results in dogs with IMHA were significantly different from those of healthy and non-IMHA dogs, but no statistical difference was found between healthy and non-IMHA dogs.

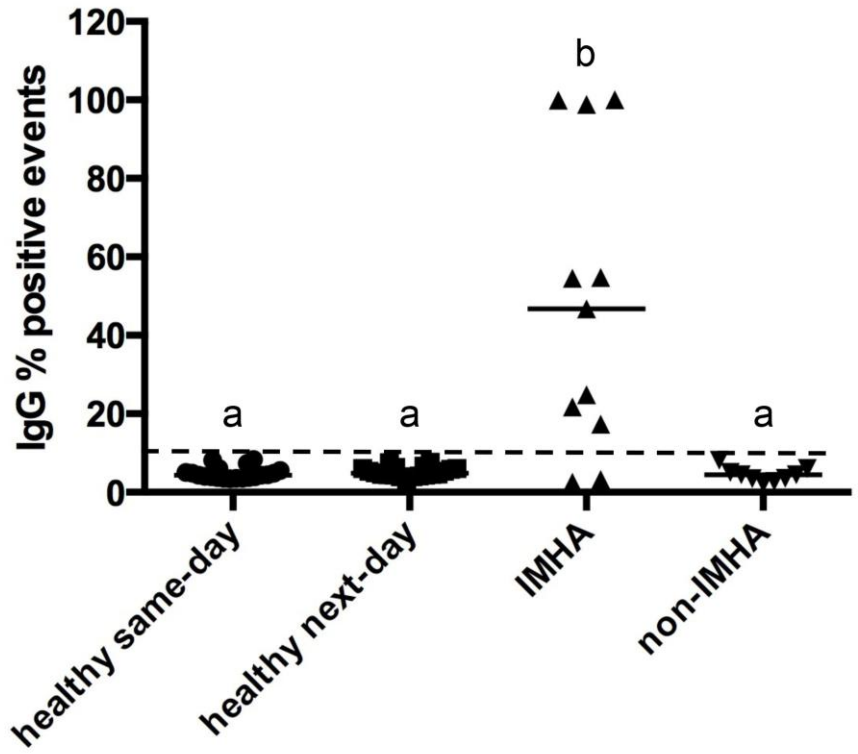

Figure 16: *IgG positivity on RBCs from healthy dogs (n = 20) and dogs with IMHA (n = 11) or without IMHA (non-IMHA, n = 9).* RBCs from healthy dogs were tested on the day of and 1 day following sample collection. Horizontal bars represent group medians and the dashed line represents the cutoff for IgG positivity based on healthy dogs tested on the day of collection. [a] No significant difference, and [b] Significantly different ($p<0.05$).

RBC PS positivity was found on 10 of 11 IMHA dogs and 2 of 9 non-IMHA dogs (Figure 17). The median % positive events for PS in IMHA and non-IMHA dogs were 1.9% (range: 0.8-5.4%) and 0.6% (range: 0.2-1.8%), respectively. One of the two PS-positive non-IMHA dogs had 0.9% positive events, no anemia, and a diagnosis of chronic renal disease, while the other had 1.8% positive events, mild nonregenerative anemia (Hct = 35%), and a diagnosis of immune-mediated neutropenia. There was no

significant difference in RBC PS for healthy dogs tested on the day of collection or the day after, so same-day results were used for group comparisons with sick animals. PS results for dogs with IMHA were significantly different from those of healthy dogs, but PS results for non-IMHA dogs were not significantly different from those of either IMHA dogs or healthy dogs.

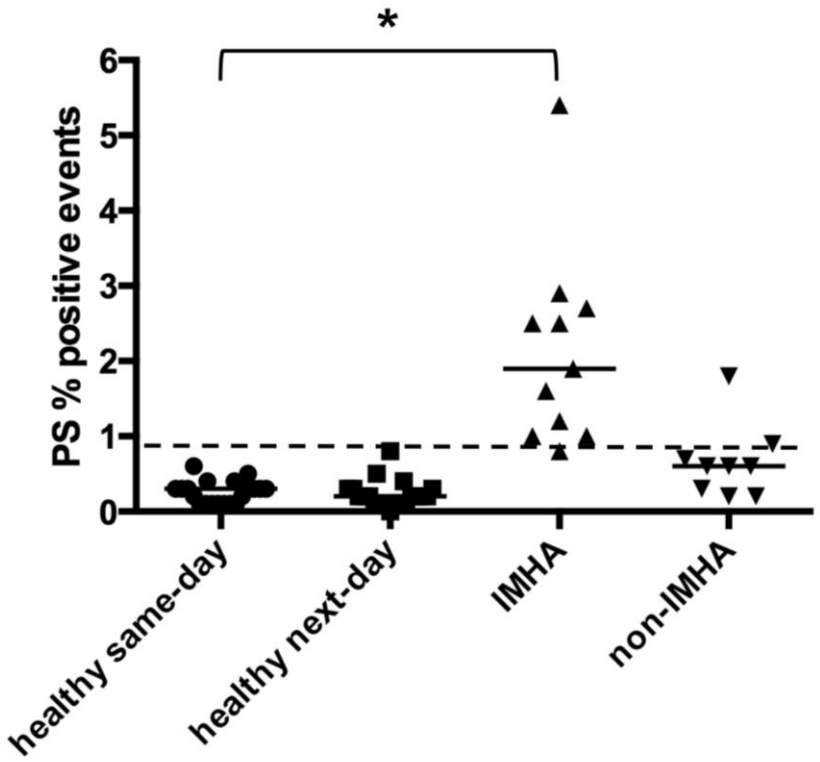

Figure 17: *Phosphatidylserine (PS) positivity on RBCs from healthy dogs (n = 20) and dogs with IMHA (n = 11) or without IMHA (non-IMHA, n = 9).* RBCs from healthy dogs were tested on the day of and 1 day following sample collection, and no significant difference was found in PS positivity. Horizontal bars represent group medians and the dashed line represents the cutoff for PS positivity based on healthy dogs tested on the day of collection. * Significantly different ($p<0.05$).

Erythroid precursor IgG and PS in PIMA

Bone marrow samples from 40 dogs were analyzed, and after review of case materials for each, 17 PIMA and 7 non-PIMA dogs were identified. Of the 16 remaining dogs, one was excluded because of high nonspecific binding of erythroid precursors, one had diagnostically inadequate bone marrow samples, 3 were excluded because of suspected cytopenias secondary to phenobarbital therapy (Plumb 2015), the mechanism of which has not been demonstrated, and 11 were classified as unclear cases because they did not clearly fulfill criteria for either PIMA or non-PIMA groups. Unclear cases consisted of dogs with moderate to severe anemia (Hct < 30%) and erythroid left-shift and/or RP, which suggested a possible immune-mediated component against erythroid precursors, but PIMA criteria were not met for a variety of reasons. For some of these dogs, anemia was too mild (Hct ≥ 30%) or not persistent, or a clinical diagnosis of PIMA was not reached by the attending clinician. For others, there was nonselective phagocytosis involving the erythroid lineage, or unexplained anemia and erythroid hypoplasia, both of which could have involved an immune-mediated component against erythroid precursors. Clinical diagnoses for unclear dogs were immune-mediated neutropenia (IMN) (2 of 11), IMT (1 of 11), hemophagocytic syndrome secondary to ehrlichiosis (1 of 11), chronic large bowel diarrhea (1 of 11), and unknown (6 of 11); the dogs with unknown diagnoses had suspected immune-mediated cytopenias (3), suspected hemophagocytic syndrome or inflammatory/infectious disease (2), or pancytopenia that resolved without therapy (1).

All 35 dogs classified as PIMA, non-PIMA, or unclear were tested for IgG, and 10 of those were also tested for PS on erythroid precursors (6 PIMA, 2 non-PIMA, and 2 unclear). Clean gating of early erythroid precursors in the top Percoll fraction was

problematic in certain clinical samples with a relatively large number of lymphocytes or early myeloid cells, so only the mid and bottom Percoll fractions were used to assess IgG and PS. Because of insufficient cells, not every fraction could be assessed for each dog. Of the 35 IgG-tested dogs, the bottom fraction was assessed in 34, and of the 10 PS tested dogs, the mid and bottom fractions were assessed in 9 and 8 dogs, respectively.

No statistical differences were found among dogs classified as healthy, PIMA, non-PIMA, or unclear regarding IgG positivity on nRBCs. However, compared to fraction-specific cutoffs based on the median percent positive events plus 3 SD for 10 healthy dogs, 5 of 17 PIMA dogs (Figure 18) were positive for IgG on erythroid precursors from at least one of the two Percoll layers (range: 19.0-59.5% positive events), while IgG positivity was not found in any of the 7 non-PIMA dogs. Two of the 12 IgG-negative PIMA dogs had been on immunosuppressive therapy for 8 (prednisone) to 24 (mycophenolate) months, and two had received prednisone in the 5 days prior to bone marrow collection. Additionally, 4 of 11 unclear cases were positive for IgG with one or both fractions (range: 21.2-36.4% positive events). One of these dogs had pancytopenia and RP with potential immune-mediated destruction of all cell lineages. RP was not detected in the other 3 dogs, one with pancytopenia suspected to be secondary to recent bone marrow injury, one with leptospirosis but evidence of secondary hemophagocytic syndrome, and one with an unexplained nonregenerative anemia associated with IMT.

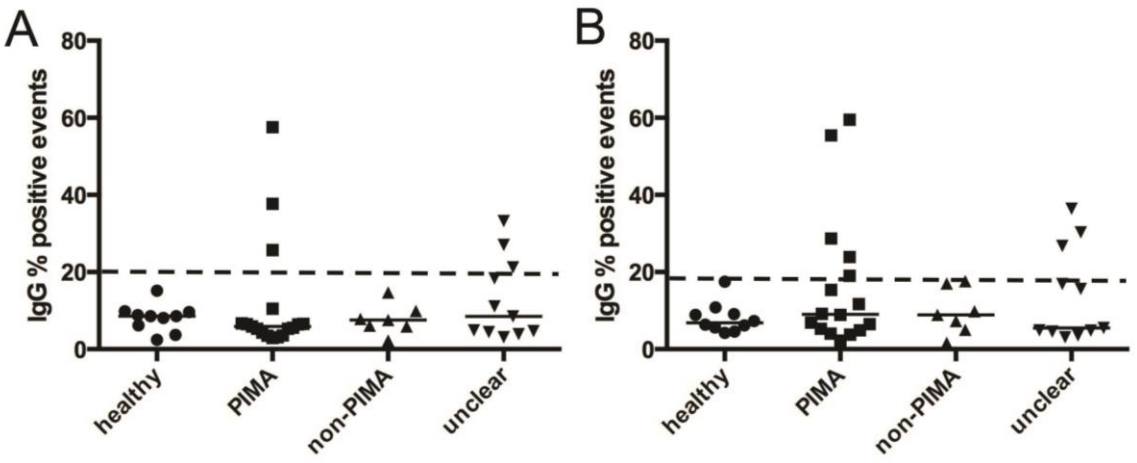

Figure 18: *IgG positivity on erythroid precursors from the mid (A) and bottom (B) Percoll fractions of bone marrow from healthy dogs (n = 10) and dogs with PIMA (n = 17 for mid, n = 16 for bottom), without PIMA (non-PIMA, n = 7), or with unclear diagnoses (n = 11).* Horizontal bars represent group medians and dashed lines represent cutoffs for IgG positivity based on healthy dogs. No statistically significant differences were detected, but increased values were present in some dogs with PIMA or unclear diagnoses that may have had an immunologic component.

When comparing groups for PS on erythroid precursors, PIMA dogs (n = 5) were statistically different from healthy dogs (n = 5) for both the mid and bottom Percoll fractions, but there were too few (n = 2) non-PIMA dogs to compare statistically (Figure 19). When compared to fraction-specific cutoff values based on median % positive events plus 3 SD for 5 healthy dogs, 5 of 6 tested PIMA dogs were positive, four in the mid fraction (range: 36.9-77.8% positive events) and three in the bottom fraction (range: 64.6-70.0% positive events). Neither of the 2 non-PIMA dogs was positive for PS; 1 of the 2 unclear dogs was positive for PS (33.7% positive events) in the mid fraction. This dog had pancytopenia suspected to be secondary to recent bone marrow injury.

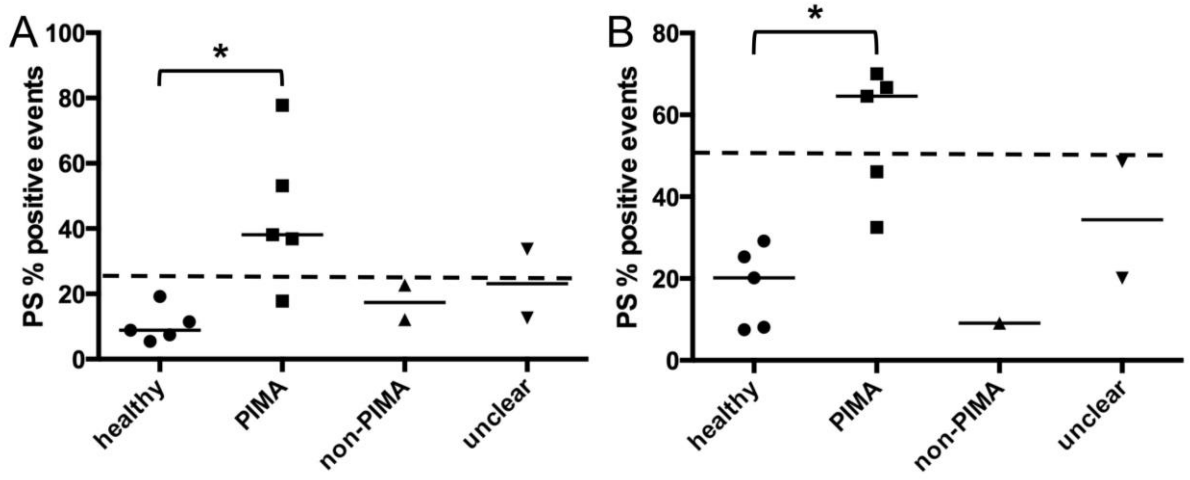

Figure 19: *PS positivity on erythroid precursors from the mid (A) and bottom (B) Percoll fractions of bone marrow from healthy dogs (n = 5) and dogs with PIMA (n = 5 for mid and bottom), without PIMA (non-PIMA, n = 2 for mid, n = 1 for bottom), or with unclear diagnoses (n = 2).* Horizontal bars represent group medians and dashed lines represent cutoffs for PS positivity based on healthy dogs. Dogs with PIMA had greater PS positivity than healthy dogs (*, $p<0.05$), but dogs without PIMA were too few for statistical comparisons.

IgG was assessed on RBCs of 14 of 17 PIMA dogs, 6 of 7 non-PIMA dogs, and in 9 dogs with unclear diagnoses; positivity was found for 5 of 14 PIMA (9.5, 10.2, 16.0, 76.9, and 99.4% positive events), 2 of 6 non-PIMA (9.9 and 23.1% positive events), and 2 of 9 unclear (11.0 and 23.8% positive events) dogs (Figure 20). Three of the 5 PIMA dogs with IgG positivity on RBCs were also IgG positive on erythroid precursors, and one of them was also Coombs' test positive. PS was assessed on the RBCs of 7 PIMA, 2 non-PIMA, and 4 unclear dogs, and all of them yielded negative results (Figure 20),

including one PIMA and one non-PIMA dog that each had IgG-positive RBCs (76.9 and 23.1% positive events, respectively).

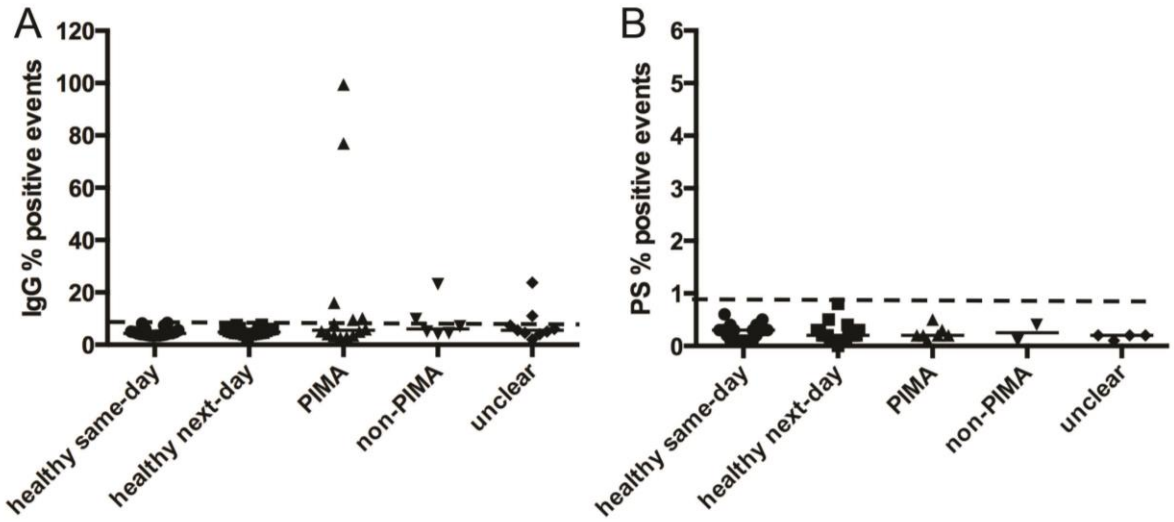

Figure 20: *IgG (A) and PS (B) positivity on RBCs from healthy dogs (n = 20) and dogs with PIMA (n = 14 for IgG, n = 7 for PS), without PIMA (non-PIMA, n = 6 for IgG, n = 2 for PS), or with unclear diagnoses (n = 9 for IgG, n = 4 for PS).* RBCs from healthy dogs were tested on the day of and 1 day following sample collection, and no significant difference was found in IgG or PS positivity. Horizontal bars represent group medians and dashed lines represent cutoffs for IgG or PS positivity based on healthy dogs tested on the day of collection.

RP was seen in all 17 PIMA dogs, which, combined with bone marrow findings as previously described (Chapter 2), allowed characterization of these dogs' conditions as early (n = 2), mid (n = 4), or late PIMA (n = 11). The relationship between the Percoll fractions with IgG and/or PS positivity and stage of RP in PIMA dogs is shown in Table 6.

Table 6: *Relationship between the stage of rubriphagocytosis (RP) reported on bone marrow examination and the Percoll fractions of bone marrow with IgG and/or phosphatidylserine (PS) positivity in PIMA dogs.*

Stage of RP	Fraction with IgG positivity	Fraction with PS positivity
Late	Mid, bottom	ND
Late	Bottom	ND
Late	Mid, bottom	ND
Late	Mid, bottom	Bottom*
Late	Bottom	None
Mid	None	Mid
Late	None	Mid, bottom
Late	None	Mid**
Mid	None	Mid and bottom

*Mid fraction not tested because of insufficient cells.
**Bottom fraction not tested because of insufficient cells.
ND = not done

Clinical and diagnostic findings in PIMA dogs

Nine PIMA dogs were spayed females, 7 were castrated males, and 1 was an intact male. Their ages ranged from 4 to 13 years old, with most dogs (n = 13) being middle-aged (5-10 years old). Dogs were of the following breeds: mixed (n = 5), dachshund (n = 3), miniature dachshund (n = 1), Labrador retriever (n = 2), Chihuahua (n = 1), German shepherd (n = 1), fox terrier (n = 1), Boston terrier (n = 1), Pembroke Welsh corgi (n = 1), and Havanese (n = 1). One dog had evidence of previous immunologic disease, having been diagnosed with PIMA 25 months prior to assessment for this study.

Evidence of concurrent RBC targeting was seen in 2 of 17 PIMA dogs: mild spherocytosis in one mid-PIMA dog, and Coombs' positivity at 20-21°C (but not 37°C) in one dog with late-PIMA. Only 3 dogs were Coombs' tested.

Mild to severe collagen myelofibrosis (MF) was detected in core biopsies of 4 of 17 PIMA dogs. All 4 dogs with MF were negative for IgG on erythroid precursors, and the only MF dog tested for PS was positive in both fractions. None of the 12 non-PIMA or unclear dogs assessed had MF, but 6 could not be assessed for MF because bone marrow core biopsy samples were inadequate (3) or not collected (3).

All PIMA dogs were treated with immunosuppressive doses of prednisone, with or without other immunosuppressive drugs. Fourteen out of 17 PIMA dogs achieved remission (10) or responded (4), with remission based on achieving a Hct > 35%, and response based on either an increase in reticulocyte concentration to above the upper reference limit (76,000/μL) or a Hct increment of at least 5 percentage points without transfusion. Three dogs did not respond to treatment, one dying 90 days after diagnosis and two undergoing euthanasia 35 and 39 days after diagnosis because of clinical deterioration and suspected neoplasia in one of them. Of the 5 IgG-positive PIMA dogs, all responded and at least 4 achieved remission. Of the 5 dogs with increased PS, 4 of 5 responded, with at least one of the four achieving remission. The only PIMA dog that was positive for both IgG and PS achieved remission.

DISCUSSION

These findings suggest that IgG contributes to PIMA by binding to erythroid precursors in at least a subset of dogs, and that PS exposure on erythrocytes and erythroid precursors may contribute to erythroid cell destruction in canine IMHA and PIMA. Further testing in a greater number of dogs is indicated.

Increased RBC PS exposure in IMHA dogs supports a potential role for eryptosis in IMHA. Eryptosis has been associated with a variety of anemia-inducing conditions and can be triggered by cell injury from hyperosmolarity, oxidative stress, energy depletion, and hyperthermia (Lang et al., 2015). Increased oxidative stress has been suggested in some IMHA dogs based on their increased plasma malondialdehyde, decreased serum vitamin E (Pesillo et al., 2004), and increased RBC-associated Prx 2, an enzyme involved in antioxidant defense in RBCs (Tan et al., 2012). Additionally, calpain 1, a protease involved in apoptosis (Lang et al., 2015), was increased in RBC membranes of IMHA dogs, further supporting contributions of an apoptosis-like mechanism in these dogs. Alternatively, increased oxidative injury of RBC membranes and PS exposure may be secondary to free iron due to hemolysis, as has been previously demonstrated in people with thalassemia (Musallam et al. 2012) and considered in dogs with IMHA (Pesillo et al. 2004). Although oxidative stress has not been demonstrated to play an important role in human patients with IMHA (Garratty 2008), there is evidence that increased oxidative stress can exacerbate IMHA in mice with decreased antioxidant capacity (Konno et al., 2013).

IgG binding to RBCs with concurrent increase in oxidative stress or PS exposure has been documented in vitro with murine and human cells (Lee et al., 2004; Konno et al., 2013; Attanasio et al., 2007). It is controversial if increased oxidative stress and PS

exposure trigger immunoglobulin binding to RBCs (Lee et al., 2004) and an autoimmune reaction (Konno et al., 2013), or if, conversely, immunoglobulin binding comes first and promotes PS exposure on RBCs (Attanasio et al., 2007). Increased PS positivity in RBCs of 4 IgG-negative dogs in our study, 2 IMHA and 2 non-IMHA dogs, suggests that PS exposure can occur independently of IgG binding. Although IgG levels may have been too low to detect, or another class of immunoglobulin may have been present in these two IMHA dogs, an additional IMHA dog that was strongly positive for IgG (100% positive events) was PS negative, and two bone marrow-tested dogs had positive IgG but negative PS results on RBCs, suggesting that IgG binding does not necessarily cause PS exposure. To test this hypothesis further, and to exclude the possibility of nonspecific binding of annexin V-FLUOS to RBC-bound IgG molecules resulting in false PS-positive results in IgG-positive dogs, we tested DEA1.1-positive RBCs from a healthy dog for both IgG and PS before and after incubation with anti-DEA1.1 typing serum. PS did not increase and remained below the cutoff based on 20 healthy dogs, while IgG shifted from negative to strongly positive (100% positive events with 50-140× increases in MdFI) after incubation with typing serum. Therefore, IgG binding did not promote PS exposure or nonspecific binding of annexin V-FLUOS.

Increased RBC PS can likely occur for multiple reasons, so it is not surprising that RBC PS was increased in two non-IMHA dogs in our study, one with chronic renal failure and one with immune-mediated neutropenia. It is possible that PS exposure accelerated erythrocyte destruction in these dogs. Chronic renal disease has been associated with increased oxidative stress in dogs (Buranakarl et al., 2009), as well as with increased oxidative stress and PS exposure on human RBCs (Bonomini et al.,

1999; Lang et al., 2015). However, there is no clear association between immune-mediated neutropenia and eryptosis.

Positivity for erythroid precursor IgG in a subset of PIMA dogs and negativity in all non-PIMA dogs suggests that IgG may play a role in the pathogenesis of PIMA in at least some dogs. This is further supported indirectly by the finding that all 5 IgG-positive dogs appeared to respond to immunosuppressive therapy, with four of them achieving remission, and that 3 of the 4 IgG-positive dogs that were tested were also positive for RBC IgG. These findings align with the previous demonstration of a circulating inhibitor of erythropoiesis in the IgG containing plasma fraction of some dogs with nonregenerative anemia associated with pure red cell aplasia (Weiss 1986). Involvement of IgG has also been documented in a few human patients with reticulocytopenic immune-mediated anemia, either by detecting plasma or RBC-eluate IgG that was reactive with RBCs and erythroid precursors (Mangan et al., 1984; Meyer et al., 1978), or by detecting surface-bound IgG on erythroid precursors by flow cytometry (van de Loosdrecht et al., 2000).

Although only 2 early-PIMA dogs were tested and IgG results were not reported for the top Percoll fractions, the stage of erythroid precursor with IgG positivity matched the stage of RP in all IgG-positive dogs (all late PIMA), with additional positivity of the mid Percoll fraction for 3 of 5 dogs. Positivity in both fractions could have been caused by imperfect separation of erythroid stages among Percoll fractions or by targeting of an epitope common to both mid and later erythroid precursors. Additionally, the stage of PS positivity also matched the stage of erythroid precursor attack in PS-positive dogs, although the bottom fraction was not tested (insufficient cells) in one late-PIMA dog that was PS positive in the mid fraction.

Negative results for IgG on erythroid precursors of 12 PIMA dogs may have occurred for several reasons. Four PIMA dogs had received immunosuppressive therapy, which could have been associated with decreased IgG positivity if treatment was effective. As in IMHA, IgM, C3, or IgA rather than IgG may have mediated PIMA in some dogs (Piek et al., 2012; Wilkerson et al., 2000; Slappendel 1979; Day 1996); we attempted to develop RBC and erythroid precursor assays for IgM and C3, but, despite being able to develop a positive control RBCs for canine C3, testing was unsuccessful due to lack of proper IgM control cells and our continued inability to clearly detect C3 in clinical IMHA patients. Negative IgG results may also have occurred in some PIMA dogs because of inadequate assay sensitivity for low amounts of IgG. Additionally, cell-mediated destruction or involvement of unknown mediators cannot be excluded as potential mediators of PIMA in some dogs.

nRBC IgG positivity in the 4 dogs with unclear diagnoses is difficult to interpret, but several of these dogs could certainly have had an immunologic component to their anemias. Phagocytosis of multiple cell lines was present in the bone marrow of two of these dogs, and it may have reflected immune-mediated disease. However, nonselective phagocytosis may also occur through nonimmunologic mechanisms. A third dog had concurrent IMT and may have had additional immunologic disease directed at erythroid precursors.

In agreement with our previous findings (Chapter 2), PIMA dogs in this study had rare evidence of RBC targeting (e.g., spherocytosis, Coombs' test positivity) based on routine testing; however, 5 of 14 tested PIMA dogs were positive for RBC IgG by flow cytometry. This indirectly supports an IgG-mediated targeting of erythroid precursors as well, and raises the question of what erythroid epitopes are being attacked in PIMA.

Although it is tempting to suspect antibodies targeting a single epitope shared by RBCs and precursors, potentially via the same antibodies (Meyer et al., 1978), distinctly different concurrent antibodies to RBCs and erythroid precursors have been documented in a human patient (Mangan et al., 1984).

The increase in erythroid precursor PS, but not RBC PS, in PIMA dogs compared to healthy dogs suggests that PS may contribute to RP in at least some dogs. However, RP in PIMA dogs is characterized by morphologically normal cells within macrophages, and this contrasts with phagocytosis of apoptotic-appearing cells as is seen, for example, in the bone marrow of some dogs with myelodysplastic syndrome. Interestingly, in the only such report found, one person with reticulocytopenic IMHA and increased erythroid precursor IgG and PS had no morphologic abnormalities of erythroid precursors by light microscopic examination (van de Loosdrecht et al., 2000). These findings may in part be explained by reports that PS exposure occurred before morphologic changes on cytologic preparations and before detection of cell death using propidium iodide (Martin et al., 1995; Shiratsuchi et al., 1998). Externalized PS by itself can induce phagocytosis of cells in early apoptosis (Shiratsuchi et al., 1998), supporting the idea that PS exposure could have mediated RP of intact-appearing erythroid precursors in PIMA. However, it is unclear what might cause increased PS exposure in PIMA dogs.

No relationship between IgG and PS positivity was apparent for PIMA dogs, either on erythroid precursors or RBCs. This suggests that IgG and PS could be involved independently of each other, in different subsets of affected dogs. Additionally, no evidence was found to suggest a relationship between IgG or PS positivity and MF, which was present only in PIMA dogs of this study, supporting our previous findings

(Chapter 2) of an association between MF and PIMA, and further suggesting that MF may be secondary to the pathogenic mechanism(s) causing anemia (Villiers et al., 1999; Stokol et al., 2000).

Limited availability of bone marrow samples from healthy and non-PIMA dogs was a limitation of this study, warranting further studies using larger populations. Unfortunately, bone marrow collection, especially of core samples, is not without some risk, so collection is difficult to justify in dogs that do not require bone marrow evaluation for their diagnostic work-up.

In conclusion, data generated with a novel assay for IgG on canine marrow erythroid precursors suggest that IgG may mediate anemia in at least a subset of PIMA dogs, and increased PS exposure and apoptotic mechanisms may play a role in canine IMHA and PIMA. PS and IgG findings did not completely parallel each other, so multiple mechanisms may be involved. Additional studies are needed to further assess the pathogeneses of these anemias so that patient management can be optimized.

REFERENCES

REFERENCES

Arndt PA, Garratty G. A critical review of published methods for analysis of red cell antigen-antibody reactions by flow cytometry, and approaches for resolving problems with red cell agglutination. Transfus Med Rev. 2010;24:172-194.

Attanasio P, Shumilina E, Hermle T, et al. Stimulation of eryptosis by anti-A IgG antibodies. Cell Physiol Biochem. 2007;20:591-600.

Bonomini M, Sirolli V, Settefrati N, et al. Increased erythrocyte phosphatidylserine exposure in chronic renal failure. J Am Soc Nephrol. 1999;10:1982-1990.

Buranakarl C, Trisiriroj M, Pondeenana S, et al. Relationships between oxidative stress markers and red blood cell characteristics in renal azotemic dogs. Res Vet Sci. 2009;86:309-313.

Day MJ. Serial monitoring of clinical, haematological and immunological parameters in canine autoimmune haemolytic anaemia. J Small Anim Pract. 1996;37:523-534.

Garratty G. The James Blundell Award Lecture 2007: do we really understand immune red cell destruction? Transfus Med. 2008;18:321-334.

Holloway SA, Meyer DJ, Mannella C. Prednisolone and danazol for treatment of immune-mediated anemia, thrombocytopenia, and ineffective erythropoiesis in a dog. J Am Vet Med Assoc. 1990;197:1045-1048.

Jonas LD, Thrall MA, Weiser MG. Nonregenerative form of immune-mediated hemolytic anemia in dogs. J Am Anim Hosp Assoc. 1987;23:201-204.

Konno T, Otsuki N, Kurahashi T, et al. Reactive oxygen species exacerbate autoimmune hemolytic anemia in New Zealand Black mice. Free Radic Biol Med. 2013;65:1378-1384.

Lang E, Lang F. Mechanisms and pathophysiological significance of eryptosis, the suicidal erythrocyte death. Semin Cell Dev Biol. 2015;39:35-42.

Lee JM, Chan K, Kan YW, et al. Targeted disruption of Nrf2 causes regenerative immune-mediated hemolytic anemia. Proc Natl Acad Sci USA. 2004;101:9751-9756.

Lucidi CA, de Resende CLE, Jutkowitz LA, et al. Cytologic and histologic bone marrow findings in dogs with suspected marrow-directed immune-mediated anemia [abstract]. Vet Clin Pathol. 2010;39:560.

Lucidi CA, Takahira RK, Gerlach JA, et al. Flow cytometric assessment of canine erythrocytes and platelets for dog erythrocyte antigen 1.1. Vet Clin Pathol. 2011;40:435-443.

Mangan KF, Besa EC, Shadduck RK, et al. Demonstration of two distinct antibodies in autoimmune hemolytic anemia with reticulocytopenia and red cell aplasia. Exp Hematol. 1984;12:788-793.

Martin SJ, Reutelingsperger CP, McGahon AJ, et al. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. J Exp Med. 1995;182:1545-1556.

McManus P. Marrow and erythrocyte changes associated with canine non-regenerative immune-mediated anemia: 44 cases (1996-2000) [abstract]. Vet Pathol. 2000;37:526.

Meyer RJ, Hoffman R, Zanjani ED. Autoimmune hemolytic anemia and periodic pure red cell aplasia in systemic lupus erythematosus. Am J Med. 1978;65:342-345.

Pesillo SA, Freeman LM, Rush JE. Assessment of lipid peroxidation and serum vitamin E concentration in dogs with immune-mediated hemolytic anemia. Am J Vet Res. 2004;65:1621-1624.

Piek CJ, Teske E, van Leeuwen MW, et al. Good agreement of conventional and gel-based direct agglutination test in immune-mediated haemolytic anaemia. Acta Vet Scand. 2012;54:10.

Plumb DC. Phenobarbital sodium. In: Plumb DC, ed. Plumb's Veterinary Drug Handbook, 8th ed. Stockholm, WI: PharmaVet Inc.; 2015:840–843.

Saad A, Palm M, Widell S, et al. Differential analysis of rat bone marrow by flow cytometry. Comp Haematol Int. 2000;10:97-101.

Scott-Moncrieff JCR, Reagan WJ, Glickman LT, et al. Treatment of nonregenerative anemia with human γ-globulin in dogs. J Am Vet Med Assoc. 1995;206:1895-1900.

Shiratsuchi A, Osada S, Kanazawa S, et al. Essential role of phosphatidylserine externalization in apoptosing cell phagocytosis by macrophages. Biochem Biophys Res Commun. 1998;246:549-555.

Slappendel RJ. The diagnostic significance of the direct antiglobulin test (DAT) in anemic dogs. Vet Immunol Immunopathol. 1979;1:49-59.

Sluyter R, Shemon AN, Hughes WE, et al. Canine erythrocytes express the P2X7 receptor: greatly increased function compared with human erythrocytes. Am J Physiol Regul Integr Comp Physiol. 2007;293:R2090-2098.

Stockham SL, Ford RB, Weiss DJ. Canine autoimmune hemolytic disease with a delayed erythroid regeneration. J Am Anim Hosp Assoc. 1980;16:927-931.

Stokol T, Blue JT, French TW. Idiopathic pure red cell aplasia and nonregenerative immune- mediated anemia in dogs: 43 cases (1988-1999). J Am Vet Med Assoc. 2000;216:1429-1436.

Tait JF, Gibson D. Phospholipid binding of annexin V: effects of calcium and membrane phosphatidylserine content. Arch Biochem Biophys. 1992;298:187-191.

Tan E, Bienzle D, Shewen P, et al. Potentially antigenic RBC membrane proteins in dogs with primary immune-mediated hemolytic anemia. Vet Clin Pathol. 2012;41:45-55.

van de Loosdrecht AA, Hendriks DW, Blom NR, et al. Excessive apoptosis of bone marrow erythroblasts in a patient with autoimmune haemolytic anaemia with reticulocytopenia. Br J Haematol. 2000;108:313-315.

Villiers EJ, Dunn JK. Clinicopathological features of seven cases of canine myelofibrosis and the possible relationship between the histological findings and prognosis. Vet Rec. 1999;145:222-228.

Weiss DJ, Stockham SL, Willard MD, et al. Transient erythroid hypoplasia in the dog: report of five cases. J Am Anim Hosp Assoc. 1982;18:353-359.

Weiss DJ. Antibody-mediated suppression of erythropoiesis in dogs with red blood cell aplasia. Am J Vet Res. 1986;47:2646-2648.

Weiss DJ. Bone marrow pathology in dogs and cats with non-regenerative immune-mediated haemolytic anaemia and pure red cell aplasia. J Comp Pathol. 2008;138:46-53.

Wilkerson MJ, Davis E, Shuman W, et al. Isotype-specific antibodies in horses and dogs with immune-mediated hemolytic anemia. J Vet Intern Med. 2000;14:190-196.

CHAPTER 5

Summary and Conclusions

SUMMARY OF FINDINGS

The primary focus of my dissertation research was to characterize and explore the bone marrow findings in dogs with suspected precursor-targeted immune-mediated anemia (PIMA). Methods were developed and studies were done to test the hypotheses that PIMA is mediated by IgG and PS, and that PS, in addition to immunoglobulins, plays a role in IMHA by promoting erythroid cell destruction and anemia.

In characterizing 25 clinical cases of PIMA with rubriphagocytosis (RP), we found three different bone marrow patterns reflecting ineffective erythropoiesis at different stages of erythroid attack. When late-stage precursors are attacked, there is erythroid hyperplasia, typically with a left shift (i.e., an increased ratio of immature to mature erythroid precursors). When mid-stage erythroid precursors are targeted, there is a normocellular erythroid series with mid-stage maturation arrest (i.e., a paucity of erythroid precursors beyond the point of mid-stage of development). And when early-stage erythroid precursors are phagocytized, we see a reduced erythroid lineage with early-stage maturation arrest (i.e., few erythroid precursors beyond the early-stage of development). A fourth pattern was characterized by severe, effacing collagen myelofibrosis with foci of left-shifted erythropoiesis associated with mostly late-stage rubriphagocytosis.

We also documented that PIMA is not a rare condition in our bone marrow submission population, accounting for approximately 25% of all canine cases, and for most of the dogs presenting with a poorly regenerative or nonregenerative anemia. Evidence of concurrent IMHA was rare in these dogs, and mild to severe myelofibrosis was common, being found in up to 48% of the studied canine populations. Many dogs had slow or no apparent response to therapy, and one third of the dogs that appeared to

respond to therapy relapsed after a few weeks or months. Most of the patients with a clinical follow-up (13/20) died or underwent euthanasia within days to weeks after diagnosis, often apparently because of sluggish regenerative responses, relapses, or clinical pessimism about a successful outcome. However, many dogs (7) lived for years.

In order to test if PIMA is mediated by IgG binding to erythroid precursors, a one-color flow cytometric assay was first developed to assess RBCs of IMHA dogs (Chapter 3). The assay had high diagnostic sensitivity (88%), specificity (93%), and accuracy (90%) for IgG detection on RBCs of IMHA dogs when compared to a variety of sick dogs not affected by IMHA. Validation of the RBC assay showed good within- and between-day precision; coefficients of variation for negative control cells with negligible IgG were 13% and 14%, respectively, and they were 7% and 0.1% for positive control cells, respectively, using modest positivity for the within-day testing and markedly positive cells for the between-day positivity. Stability of negative and positive IgG control RBCs was good for up to one month, and accuracy was high based on mixing experiments yielding recoveries of 0, 18, 37, 56, 78, and 100% IgG-positive RBCs after spiking with 0, 20, 40, 60, 80, and 100% IgG-positive cells.

Initial plans to assess the role of C3 as a mediator of erythroid precursor destruction in PIMA were thwarted by unsuccessful concerted attempts to develop an assay for detection of complement (C3) on RBCs of IMHA dogs. In vitro experiments and clinical testing resulted in the conclusion that commercially available anti-[dog C3] antibodies could not reliably detect the appropriate canine C3 fragment on canine RBCs, as discussed on Appendix A. IgM testing was considered an alternative to C3, but the relative importance of IgG over IgM in most immune-mediated cytopenias, and

difficulties in developing a positive control for IgM discouraged continuation of these studies (Appendix A).

To assess erythroid precursors for IgG and PS, a method was developed to isolate different stages of erythroid precursors from canine bone marrow samples, and to separate them from other cell types (Chapter 3). We aimed to separate different stages of erythroid cells in order to assess if potential IgG and/or PS positivity could have any relationship with the stage of erythroid precursor undergoing RP in PIMA dogs. That goal was based on our retrospective study findings of early-, mid-, and late-stage RP associated with distinct bone marrow patterns, suggesting that attacks may occur at different stages of development in different dogs (Chapter 2). Percoll gradient separation allowed fractionation of bone marrow samples into a top fraction enriched in rubriblasts and prorubricytes (early stages), a middle fraction enriched in early- to mid-stage rubricytes (mid stages), and a bottom fraction enriched in late-stage rubricytes and metarubricytes (late stages). Regarding identification of erythroid precursors, sorting experiments showed good separation of erythroid cells from other cell populations in canine bone marrow samples from three different dogs, by using a two-color flow assay. Gating strategy relied on CD18 antibody labeling and LDS751 staining. Gated erythroid precursor populations from the top, mid, and bottom Percoll fractions contained, respectively, ≥ 89%, 98%, and 98% erythroid precursors. Using this flow cytometric assay, we were also able to gate on reticulocytes and RBCs. However, we opted to assess IgG and PS on RBCs from blood because of inconsistent RBC concentrations in Percoll fractions of bone marrow samples.

This flow cytometric bone marrow assay was then applied, with the addition of IgG antibodies used for RBCs, to assess IgG binding to erythroid precursors (Chapter 3).

The first step was to assess the assay's ability to detect IgG on erythroid precursors. To that end, erythroid precursors from DEA1.1 negative and positive dogs were incubated with anti-DEA1.1 typing serum, in the same fashion as was done for IgG control RBCs (Chapter 3). Results from this experiment showed clear differences in median IgG signals for 3 DEA1.1 negative dogs (4.2%, 6.0%, and 16.1% positive events for the top, mid and bottom Percoll fractions, respectively) compared to 3 DEA1.1 positive dogs (71.1%, 98.1%, and 99.4% positive events for the top, mid and bottom Percoll fractions, respectively) and compared to healthy dogs. Expression of certain blood type antigens on erythroid precursors has been described in humans, but to the author's knowledge, this was the first time that DEA1.1 expression has been reported on canine erythroid precursors, from rubriblasts through metarubricytes.

The 3-color flow cytometric assay was then used to prospectively test bone marrow samples of PIMA, sick non-PIMA, and healthy dogs, in order to assess if IgG binding to erythroid precursors is involved in PIMA (Chapter 4). Based on cutoffs generated from 10 healthy dogs, 5 of 17 PIMA dogs were positive for IgG on erythroid precursors from the middle or bottom Percoll layers, while non-PIMA dogs were negative. Additionally, 4 of 11 dogs with unclear diagnoses were positive for IgG, and 3 of these had findings that could have reflected immune targeting of erythroid precursors. These results offer support for the hypothesis that PIMA is mediated by IgG, at least in some dogs, but further assessment is required.

PS positivity was increased on erythroid precursors from 5 of 6 of the tested PIMA dogs compared to 5 healthy animals, and these differences were significantly different (Chapter 4). This supports a potential role for PS in PIMA.

In addition, the stage of erythroid precursor undergoing RP generally matched the Percoll fraction with IgG and/or PS positivity in most positive dogs (Chapter 4), indicating that RP may be mediated by Fc receptor or PS receptor signaling. However, only two early-PIMA dogs were tested, and we were unable to assess erythroid precursors in the top Percoll fraction, so the role of IgG and PS in early-stage RP of early-PIMA dogs was not assessed.

Involvement of an apoptotic-like mechanism has been previously suggested in IMHA, in addition to an immune-mediated attack. Our results support this idea and our hypothesis that PS plays a role in IMHA, by showing that 10 of 11 IMHA dogs but only 2 of 9 non-IMHA dogs were positive for RBC PS (Chapter 4). Statistical analysis confirmed significant differences between PS results of IMHA versus healthy dogs. Our data from client-owned dogs and experiment results suggest that IgG and PS positivity are independent events, and warrant further studies to clarify the mechanism and potential consequences of PS exposure in IMHA dogs.

Despite our repeated attempts to optimize and apply an assay to assess for susceptibility of nRBC to phagocytosis, we found several limitations that hindered these studies (Appendix B). However, our data suggested that in vitro phagocytosis and extensive degradation of IgG-coated nRBCs by macrophages can happen in as little as 15 minutes, suggesting that rapid clearance of abnormal cells by macrophages may limit detection of RP in the bone marrow of PIMA dogs.

LIMITATIONS AND UNANSWERED QUESTIONS

The main limitation of this work was the availability of bone marrow samples from healthy dogs and from dogs with clear diagnoses of PIMA or non-PIMA, particularly after the PS assay was developed. Obtaining samples from healthy dogs was especially problematic, because owners are understandably unlikely to give consent for a procedure with very low, but real risk, of bone fracture, infection, or, in the case of core biopsies, anesthetic complications, and purchase and conditioning of healthy dogs for just bone marrow collection was cost prohibitive. Consequently, sample sizes for early-PIMA, non-PIMA, and healthy dogs were low for IgG and especially PS assessment on erythroid precursors. Despite testing all but two bone marrow samples that qualified for this study and that were collected at the Michigan State University Veterinary Medical Center over 32 months, only 40 usable dogs were identified, and those included 17 PIMA and 7 clear non-PIMA cases. Most (11) of the remaining 16 dogs had unclear diagnoses, and could not be classified as either PIMA or non-PIMA. The retrospective study included 25 PIMA cases over a period of 4 years, which proportionally matches the frequency of 17 PIMA cases identified in a period of 32 months, resulting in approximately 6 to 7 clear PIMA cases identified in our hospital per year. A prospective study with longer duration, with recruitment of clinical cases from other clinics, and increased numbers of healthy dogs would potentially allow for larger sample sizes and stronger statistical results.

Only 2 early-PIMA dogs were tested for erythroid precursor IgG, and both were negative, but neither could be tested for PS at the time. In addition, the top Percoll fraction could not be reliably assessed for some of the dogs in this study because of imperfect flow cytometric separation between erythroid precursors and other cell types.

These two complications left open an important question, proposed in chapter 2, regarding the pathogenesis of early-PIMA. Early-PIMA dogs differed from mid- and late-PIMA dogs in their bone marrow, laboratory, and signalment findings, raising the idea that the pathogenesis of the former may differ from that of mid- and late-PIMA.

Additionally, 4 of 17 PIMA and 1 of 7 non-PIMA dogs could not be assessed for IgG and/or PS in at least one of the Percoll fractions because of insufficient numbers of cells or high nonspecific binding. The protocol used for these studies required $0.5\text{-}1\times10^6$ cells to be tested per tube, ultimately requiring three to six million cells per Percoll fraction to allow for both IgG and PS testing when including all controls.

FUTURE DIRECTIONS

This work provides direct evidence for IgG involvement in at least a subset of PIMA dogs, but 12 of 17 were negative for IgG on erythroid precursors. Of these 12, all that were tested for PS were positive (4 of 4) suggesting PS involvement in a different subset of PIMA dogs. Larger sample sizes are needed to confirm these findings, and future studies are warranted to explore alternative mechanisms for PIMA, including the involvement of C3 and cell-mediated targeting, particularly for early-PIMA, which is typically characterized by a marrow lymphocytosis not seen in mid-and late-PIMA.

Our results support involvement of PS in IMHA as well as in PIMA, but it is unclear what promotes PS exposure in immune-mediated anemias, if PS exposure is associated with apoptotic events, if oxidative stress occurs and if it is a primary or secondary event, if and how increased PS exposure may be involved in thromboembolic events in these dogs, and how this may impact therapeutic approach. The following could be explored to help address these issues: 1) a possible correlation of PS exposure and positivity for canine C3 in erythroid cells, 2) an association between positivity for necrotic markers (such as propidium iodide) and PS positivity in PIMA, 3) assessment of caspase (i.e., caspases 5, 6) activation to explore if PS exposure is a consequence of apoptotic/eryptotic mechanisms or if it is a membrane event independent of apoptosis/eryptosis, 4) the levels of expression of antioxidant proteins by erythroid cells in dogs with immune-mediated anemia versus control dogs, and 5) the potential induction of erythroid oxidative stress in vitro by incubating plasma of dogs with immune-mediated anemia and erythroid cells of control dogs.

Candidate erythroid epitopes have been identified in canine IMHA, but no information is currently available regarding which epitopes may be involved in canine

PIMA. Our results show that targeting of erythroid cells by IgG in PIMA dogs may be directed to precursors, RBCs, or both concurrently, suggesting an immune-mediated attack of epitopes that are sometimes shared by erythroid cells in different stages of development. It is also unknown if attacked epitopes are public (i.e., common to all dogs) or specific to individuals. Further studies to help identify the targeted epitopes in IgG-positive PIMA dogs could involve identification of IgG-bound membrane proteins by methods such as Western blot and immunoprecipitation. Additionally, incubation of eluates from nRBCs or plasma from IgG-positive PIMA dogs with erythroid precursors from healthy dogs or sick dogs not affected with PIMA could be performed to explore if nRBC-reactive antibodies are public.

The data suggest that increased membrane-associated IgG and PS exposure may mediate RP in PIMA. We had hypothesized that erythroid precursors in PIMA undergo RP because of their increased susceptibility to phagocytosis, be it from IgG, PS, or other mechanisms. Attempts to demonstrate increased susceptibility of PIMA erythroid precursors to phagocytosis by using a cell culture-based phagocytic assay were unsuccessful because of the high numbers of cells needed for assessment, and the low assay sensitivity (Appendix B). However, we demonstrated rapid phagocytosis of IgG-coated erythroid precursors, which is detected as early as 15 minutes after incubation, with intact-looking erythroid precursors inside macrophages (Appendix B). This mirrors the appearance of phagocytized cells in clinical samples. If RP is mediated by PS receptors on macrophages in PS-positive PIMA dogs, a question that remains unanswered is why phagocytized erythroid precursors look intact rather than apoptotic inside macrophages prior to their degradation. This may be because PS-positive cells are in early stages of apoptosis, and phagocytosis takes place rapidly, before later

apoptotic morphologic changes occur. Further mechanistic experiments could be directed at assessing other markers of apoptosis and phagocytosis of PS-positive canine nRBCs. Rapid removal of these cells may also help explain the lack of appreciable apoptotic-appearing cells in the bone marrow of PIMA dogs.

Autoimmune MF is a recently characterized condition in humans, but it has not been described, as such, in dogs. In our studies (Chapters 2 and 4), collagen MF was common in PIMA dogs, and the spectrum of MF suggests progression over time in affected dogs. The mechanism that induces MF in these dogs is unknown. There is some evidence that dogs with even severe collagen MF may improve with treatment, but a better understanding of the pathogenesis of MF in these dogs may improve treatment responses. PIMA and control dogs could be assessed for fibrosis-related cytokines and growth factors such as TGF-β in the serum and bone marrow, and for the potential involvement of peripheral monocytes and CD4-positive lymphocytes, as these cells have been proposed to be sources for increased serum TGF-β and substance P in autoimmune MF in people.

Lastly, further studies could be performed to confirm DEA expression on canine erythroid precursors, including mass spectrometry of RBC membrane proteins and detection of DEA1.1 mRNA. Additionally, more studies may be warranted to determine if RBC-incompatible allogeneic bone marrow transplantations in dogs can have the same deleterious consequences as demonstrated in human patients.

APPENDICES

APPENDIX A

Assessment of Canine C3

Complement involvement in immune-mediated anemias

Briefly, in human autoimmune hemolytic anemia (AIHA), the classical complement pathway is activated by IgM or doublets of IgG molecules bound to the erythrocyte, ultimately leading to C3 activation and fixation to the cell membrane as C3b. Cell-bound C3b can lead to cleavage and fixation of C5, C6, C7, C8, and C9 to the erythrocyte membrane promoting the formation of the membrane attack complex (MAC) and lysis of the erythrocyte, or C3b can be cleaved into C3d and C3c. In the latter case, C3c is released into the plasma and C3d remains bound to the circulating erythrocytes (Garratty 1984).

Human macrophages express receptors for C3b (CR1), but not for C3d (CR2), which are expressed only by lymphocytes (Fearon 1985). It has been shown that C3b-coated erythrocytes are recognized by C3b receptor-bearing macrophages and removed from the circulation, while C3d-coated erythrocytes remain in the blood stream and have a normal survival time (Brown et al., 1970). Therefore, the C3 fragment that predominates on erythrocytes in AIHA is C3d, and this is the fragment that should be detected in human Coombs' tests (Garratty 1984; Engelfriet et al., 1970). Polyvalent antiglobulin reagents are commonly used to screen patients for AIHA, and monovalent reagents (i.e., anti-IgG and anti-C3d) are used later for further characterization of the disease in patients with positive results for the polyvalent reagents (Leger 2011). The complement fragment (C3b versus C3d) bound to erythrocyte membrane in canine IMHA has not yet been characterized.

Detection of complement fragment C3 in dogs

In order to address Aim 2 and explore the involvement of complement in canine

immune-mediated anemias, we developed a flow cytometric assay for detection of canine IgG and C3 using antibodies from Bethyl Laboratories. Bethyl antibodies for IgG detection were FITC-labeled nonspecific sheep IgG and sheep FITC-anti-[dog whole IgG] test antibodies. FITC labeled nonspecific goat IgG and goat FITC-anti-[dog C3] test antibodies were used for C3, and goat FITC-anti-[dog IgM] was used for IgM detection. Antibodies used for IgG detection were later purchased from Kirkegaard & Perry Laboratories for studies described in chapters 3 and 4, because of their specificity for the gamma chain of the IgG molecule, minimizing the chances of heavy chain cross reactivity and detection of other immunoglobulin types.

Bethyl antibodies were used to assess erythrocytes from left over EDTA-anticoagulated CBC samples of 29 dogs with suspected immune-mediated hemolytic anemia (IMHA). Fifteen of 29 IMHA suspects had a final clinical diagnosis of IMHA, based on the presence of regenerative anemia with one or more of the following: spherocytosis, erythrocyte agglutination, and Coombs' test positivity. At the time of testing, 13 IMHA dogs were not undergoing any immunosuppressive treatment, and two dogs received one dose of dexamethasone within 24 hours of blood collection; one of the latter two was also receiving cyclosporine. Six healthy blood donor dogs were tested to assess IgG and C3 binding in health. Positivity for IgG and C3 was defined by net signal or net median fluorescence intensity (median fluorescence intensity of test minus nonsense antibody) as compared to the median plus 3 standard deviations of IgG or C3 net signals in 6 healthy dogs: 3.9 and 2.9 for IgG and C3, respectively. Preliminary results showed IgG positivity in none of the healthy dogs and in 14 of 15 IMHA dogs: 7 of 14 had weak positivity (range of IgG net signal = 5.2 – 16.2), and 7 of 14 had strong positivity (range of net IgG signal = 32.3 – 352). Both of the dogs that received

immunosuppressive drugs prior to blood collection had strong IgG positivity.

In contrast, only one of the 15 IMHA dogs had a positive signal for surface-associated C3, while all other 14 IMHA dogs were negative for C3, all with C3 net signals below 1.9. C3 positivity in the one C3-positive patient was weak (net signal = 6.2), and it had the highest IgG (352) net signal of all tested dogs. IgM was also assessed, and the C3-positive dog had the highest IgM net signal (76.1), while the 14 C3-negative dogs had low IgM net signals (< 5.1). High IgM net signal supported the presence of C3 in this patient, but the C3 signal was too weak to be a clear and reliable positive. In every run, a positive control for C3 (sucrose-treated canine erythrocytes) yielded strong flow cytometric positivity for the test antibody (median fluorescence intensity = 176 – 457), while erythrocytes washed with buffer (PBS, 0.3% BSA) yielded negative results (median fluorescence intensity < 18). Despite the low numbers of healthy dogs tested for comparison, it was clear that none of the 15 IMHA dogs had clear and strong C3 positivity. Five of the 15 IMHA dogs had ghost cells reported, and 7 had hemolyzed samples (mild to marked), increasing the likelihood of complement-mediated intravascular hemolysis in these dogs.

Similar to these findings, most studies reported over the last 30 years show that assessment of C3 in IMHA dogs usually yielded negative or only weakly positive results (Jackson et al., 1985; Jones et al., 1987; Barker et al., 1992; Jones et al, 1992; Day 1996; Overmann et al., 2007; Piek et al, 2012) using either Coombs'- or ELISA-based methods and various sources of antibodies: rabbit anti-[dog C3] made in-house (Jones et al, 1987; Jones et al., 1992), goat anti-[dog C3] from Nordic Laboratories (Barker et al., 1992; Day 1996; Piek et al., 2012; Warman et al., 2008) and goat anti-[dog C3] from MP Biomedicals (Overmann et al., 2007). Only in one report are many strong positive

C3 results reported for dogs, and those data were generated with a rabbit anti-[dog C3b] from the Netherlands Red Cross Blood Transfusion Service (Slappendel 1979). In some studies, positivity for canine C3 most often occurred at 4°C rather than at room temperature or 37°C (Warman et al., 2008; Quigley et al., 2001), but the significance of positive results at only 4°C is questionable, since the clinical relevance of C3 bound to cells at nonphysiologic temperatures is not clear. In human patients, the direct antiglobulin test (DAT) is usually performed at room temperature (Roback 2011), and C3 is involved in most cases of autoimmune hemolytic anemia (AIHA) (Leger 2011).

Flow cytometry has been used to detect canine C3 in two studies (Wilkerson et al., 2000; Quigley et al., 2001), both of which used the same goat anti-[dog C3] from Bethyl Laboratories that was initially used for our samples. In one study, none of the 12 IMHA dogs was positive for C3, but all were positive for IgG and/or IgM (Wilkerson et al., 2000). Negativity for C3 could have been due to true negativity, inappropriate reactivity of the anti-C3 antibody, or to analytical error, since C3-bound positive control cells were not used. In a different flow cytometric study (Quigley et al., 2001), 6 of 13 dogs with IMHA tested positive for C3; however, none were strong positives, and the accuracy of C3 detection in this study was questionable because positivity was defined by values greater than the mean plus 2 standard deviations (SD) for 13 clinically normal dogs. Using 3 SD or the greatest healthy value to set a cutoff would have been a more conservative approach that may have resulted in reclassification of weak positive results as negative.

Few and weak positive results for canine C3 in IMHA, based on our data and that of others, has led us to the following questions: 1) Are the commercially available anti-[dog C3] antibodies reliable for detection of the C3 fragment fixed on canine

erythrocytes? 2) What is the fragment of C3 fixed on canine erythrocytes? 3) How is the complement system involved in canine IMHA? 4) Can complement be detected on canine erythrocytes as a mark of opsonization in immune-mediated anemias? Experiments designed to address some of these questions are summarized below.

C3-coated positive control cells

Several methods have been described for preparing C3-coated erythrocytes for use in experimental studies or as positive controls for diagnostic tests. Well accepted protocols are the sucrose and the Fruitstone methods, which fix complement to the erythrocyte membrane through the classical and alternative complement pathways, respectively. Briefly, in the sucrose method, whole blood is incubated with a low-ionic strength sucrose solution at 37°C, allowing immunoglobulins from the plasma to fix C3b and C4b on the erythrocytes (Garratty et al, 1975). In the Fruitstone method, whole blood is incubated with a sucrose solution containing EDTA, for inhibition of the classical pathway, and magnesium chloride is added to accelerate C3 coating through the alternative pathway. This is carried out at low pH (5.1) and temperature (0°C), and results in erythrocytes coated with only C3b (Chaplin et al., 1980; Fruitstone 1978). A few methods are also available for cleavage of C3b into C3d, including trypsinization (Morton et al., 1947; Issitt et al., 1976) and treatment of cells with heat-inactivated serum (Ross et al., 1982). Table 7 shows a summary of in vitro complement fixing methods, and the complement fragment(s) expected to be bound to erythrocytes. Anticipation that complement-fixation methods used for humans would work for dogs was based on evidence that canine and human C3 molecules and their cleavage fragments have similar structure and molecular weight (Johnson et al., 1985; Kay et al., 1985;

Ameratunga et al., 1998).

Table 7: *Methods of complement fixation to erythrocytes, and complement fragments expected to be fixed as a result.*

	Sucrose treatment	Fruitstone treatment	Trypsinization
Complement fragment	C3b/C4b	C3b only	C3d (C4d)

Reactivity of anti-[dog C3] antibodies to canine C3b and/or C3d

In an attempt to investigate if the FITC-labeled anti-[dog C3] antibody from Bethyl Laboratories can detect C3d (presumably the C3 fragment on canine erythrocytes in IMHA), erythrocytes were sucrose-treated and then trypsinized. Human erythrocytes were tested in parallel with canine cells to ensure that the methods of C3 fixation and cleavage were performed correctly. The Fruitstone method was also performed to assure that antibody signal was not a result of C4b detection. A primary polyclonal chicken anti-[human C3d] antibody from Quidel Laboratories, and a secondary caprine anti-[chicken IgG]-FITC labeled antibody from Bethyl Laboratories were used for human C3 detection. Sucrose- and Fruitstone-treated human erythrocytes yielded strong positive results, and remained positive after trypsinization (Table 8), though with some loss of signal, which could have occurred due to damage to the C3 molecule, or because the polyclonal anti-[human C3] antibody detects epitopes on C3b which are not present on the C3d molecule. Communication with the technical support at Quidel Laboratories supported that their anti-[human C3d] antibody may yield stronger reactivity to C3b than to purified C3d. For further characterization, sucrose-, Fruitstone- and trypsin-treated human erythrocytes could be tested with anti-[human C3c] and anti-

[human C3d] monoclonal antibodies in order to prove loss of signal for C3c and stability of signal for C3d after trypsinization (Lachmann et al., 1983), but we did not pursue these studies.

The FITC-anti-[dog C3] antibody from Bethyl Laboratories yielded strong flow cytometric signals on sucrose-treated canine erythrocytes (expected to bear C3b) but weak signals on trypsinized sucrose-treated canine cells (expected to bear C3d), suggesting either lack of reactivity of the antibody with canine C3d, or lack of C3d generation with the trypsinization technique (Table 8). The Fruitstone method did not work for the dog (Table 8), despite performing the test separately with four different concentrations of magnesium chloride (i.e., the recommended concentration for the human protocol and three higher concentrations; data not shown for higher concentrations). Greater concentrations were tested because of species differences in magnesium requirements for alternative pathway activation (Gorman 1984; McConnell et al., 1978; Gorman 1983). C3-coated canine and human erythrocytes incubated with nonsense (for canine) or only secondary (for human) antibodies, and negative control washed canine and washed human erythrocytes incubated with their respective species-specific anti-C3 test antibodies all had low mean fluorescence intensity (≤ 7).

Table 8: *C3 detection on canine and human erythrocytes after various treatments for C3b and C3d fixation.*

RBC Treatment (C' fragment)	Complement Signal (Canine, MFI)*	Complement Signal (Human, MFI)**
Sucrose (C3b+C4b)	487.0	100.9
Sucrose/trypsin (C3d+C4d)	6.3	31.1
Fruitstone (C3b)	8.2	305.1
Fruitstone/Trypsin (C3d)	-	55.2
None	≤ 7	≤ 7

MFI = mean fluorescence intensity
*Bethyl FITC-anti-[dog C3]
**Quidel chicken anti-[human C3d] + FITC-anti-[chicken IgG]
All results with negative control antibodies were ≤ 7.

We also tested the anti-[dog C3]-FITC labeled antibody from Nordic Laboratories, which has been used in other studies of canine IMHA (Barker et al., 1992; Day 1996; Piek et al., 2012; Warman et al., 2008). This antibody yielded strong flow cytometric positivity for sucrose-treated canine erythrocytes, but most of the signal was lost after trypsinization (Table 9). Even if the remaining signal was real, its weakness under experimental conditions to promote a strong positive suggested that the antibody would have little utility in clinical samples. Each of these experiments was repeated on different days and with different samples, some with modifications of trypsin incubation times, and the findings were always similar, though the magnitude of signals differed because of differing degrees of complement fixation.

Table 9: *C3 detection on canine erythrocytes using the Nordic anti-[dog C3c] antibody.*

RBC Treatment	Complement Signal (MFI)
Sucrose (C3b+C4b)	128.6
Sucrose/Trypsin (C3d+C4d)	17.9
None	7.6

MFI = mean fluorescence intensity

Conclusions and future directions

Experiments with human erythrocytes suggested appropriate generation of complement fragments with each technique, and the sucrose method fixed C3 on canine erythrocytes as expected. However, C3 was not detected via the Fruitstone fixation method or after trypsinization. This suggests either that these methods do not work for canine C3 because of differences between the C3 molecule of humans and dogs, or that the antibodies used for canine C3 detection cannot accurately detect canine C3b and C3d molecules fixed on erythrocytes by these methods. Therefore, it is still unclear if the tested and reported anti-[dog C3] antibodies can detect C3d, and if C3d is formed from C3b in dogs.

The canine C3 gene is predicted to produce a pre-pro-C3 protein, which is identical to the pre-pro-C3 protein in people (Ameratunga et al., 1998). Critical structures in the C3 molecule seem to have high homology between dogs and humans, including amino acid sequences involved in: 1) the thioester bond formation between the erythrocyte and the C3 molecule, 2) ligand binding (including binding sites for CR1 and CR2), 3) C3 inactivation (including the two factor I cleavage sites), and 4) terminal cleavage of C3dg into C3d and C3g (such as the trypsin cleavage site). However, the third factor I (I3)

cleavage site in dogs is not conserved with respect to the human sequence, instead being similar to that of guinea pigs and mice (Ameratunga et al., 1998).

Factor I, also known as C3b/C4b inactivator, cleaves C3b (the “active” form) into C3bi (the “inactive” form), and then into C3c (which is released into the plasma), leaving the remaining C3dg fragment bound to the cell (Lachmann 2009). Soluble (e.g., factor H) or cell membrane-bound (e.g., CR1) cofactors are required for these actions of factor I (Minta et al., 1996; Ross et al., 1982). It has been shown that factor I has a divergent segment in mice, human, chicken, and *Xenopus* (Minta et al., 1996). The lack of homology of the I3 cleavage site in dogs with respect to people, the knowledge gap in characterization of CR1 on canine erythrocytes, and the absence of complete homology between the factor I molecule in mice and humans indicate that the cleavage of C3b into C3bi, and further cleavage into C3c and C3dg, mediated by factor I, may be different among these species, and therefore the erythrocyte-bound C3 fragment may not be the same in dogs and people with IMHA. These findings impeded the pursuit of a role for C3 in canine immune-mediated anemias, and led to the idea that currently available monospecific anti-[dog C3] antibodies are not useful for detecting C3 fragments on circulating canine erythrocytes in IMHA.

Identification of the C3 cleavage products in canine IMHA, and assessment of accuracy of currently commercially available anti-[dog C3] antibodies would prevent use of suboptimal or inadequate antibodies in diagnostic tests, and potentially lead to development of more accurate reagents for the diagnosis of canine IMHA. In future studies, our hypothesis may be addressed as described below:

Aim 1. Determine if Bethyl and Nordic anti-[dog C3] antibodies can detect the C3

fragment on canine erythrocytes after cleavage promoted by serum proteins.

Hypothesis: Currently available anti-[dog C3] antibodies cannot detect the canine erythrocyte-bound C3 fragment after cleavage.

An attempt to convert canine C3b into C3d would be done by treating C3b-coated cells with canine heat-inactivated serum (i.e., in the presence of canine factor I and its cofactors). The same would be done using human erythrocytes and heat-inactivated serum as a control. If, after species-specific serum treatment of sucrose-treated canine and human erythrocytes, the positive signal for C3 on canine cells and C3d on human cells remained strong, and the signal for human C3c decreased, by using species-specific anti-[dog C3], anti-[human C3d], and anti-[human C3c] antibodies, respectively, we would be able to suggest that: 1) treatment with heat-inactivated serum cleaves human C3b into C3d, but does not cleave the C3b molecule bound to canine erythrocytes, or 2) canine C3b is cleaved into C3d, and the tested antibodies are able to detect C3d. Negative results for canine cells after treatment with heat-inactivated serum would likely be more useful, and would suggest that the anti-[dog C3] antibodies cannot detect canine C3d (or the natural degradation product of canine C3b). Destruction of the canine C3b molecule after treatment with heat-inactivated serum is unlikely, since this is a more physiologic approach, and it is in theory less damaging to the cells and the C3 molecules than treatment with trypsin. Ideally these cleavage experiments would be done with cells that were C3b-coated by the Fruitstone method, to ensure coating by C3b only. For that, a modified Fruitstone method using EGTA instead of EDTA could be applied if, as expected, it is shown to work on canine erythrocytes in addition to human erythrocytes (Gorman 1983).

Aim 2. Characterize canine C3 degradation. *Hypothesis: Canine C3 fragments differ from human fragments and are not detected by currently available monospecific anti-[dog C3] antibodies.*

We have tried to elute C3 from human sucrose- and sucrose/trypsin-treated erythrocytes by using a high ionic strength solution (0.5 M NaCl), with the goal of characterizing the eluted C3 fragment(s) by SDS-PAGE, and reproducing the method with canine erythrocytes. However, elution was unsuccessful because of covalent binding of C3 to erythrocytes through a thioester bond. Characterization of canine C3 cleavage products may be pursued by purification of C3 from plasma, followed by in vitro cleavage of C3, and characterization of C3 fragments by SDS-PAGE and immunoblots (Johnson et al., 1985; Minta et al., 1977; Eggertsen et al., 1985; Ross et al., 1983). However, development of monoclonal antibodies against canine C3 fragments might be necessary for detection and full characterization of C3 cleavage products in the dog (Lachmann et al., 1980).

REFERENCES

REFERENCES

Ameratunga R, Winkelstein JA, Brody L, et al. Molecular analysis of the third component of canine complement (C3) and identification of the mutation responsible for hereditary canine C3 deficiency. J Immunol. 1998;160:2824-2830.

Barker RN, Gruffydd-Jones TJ, Stokes CR, et al. Autoimmune haemolysis in the dog: relationship between anaemia and the levels of red blood cell bound immunoglobulins and complement measured by an enzyme-linked antiglobulin test. Vet Immunol Immunopathol. 1992;34:1-20.

Brown DL, Lachmann PJ, Dacie JV. The in vivo behavior of complement-coated red cells: studies in C6-deficient, C3-depleted and normal rabbits. Clin Exp Immunol. 1970;7:401-421.

Chaplin H Jr, Freedman J, Massey A, et al. Characterization of red blood cells strongly coated in vitro with C3 via the alternative pathway. Transfusion. 1980;20:256-262.

Day MJ. Serial monitoring of clinical, haematological and immunological parameters in canine autoimmune haemolytic anaemia. J Small Anim Pract. 1996;37:523-534.

Eggertsen G, Hellman U, Lundwall A, et al. Characterization of tryptic fragments of human complement factor C3. Mol Immunol. 1985;22:833-841.

Engelfriet CP, Pondman KW, Wolters G, et al. Autoimmune haemolytic anaemias. 3. Preparation and examination of specific antisera against complement components and products, and their use in serological studies. Clin Exp Immunol. 1970;6:721-732.

Fearon DT. Human complement receptors for C3b (CR1) and C3d (CR2). J Invest Dermatol. 1985;85:53s-57s.

Fruitstone MJ. C3b-sensitized erythrocytes [letter]. Transfusion. 1978;18:125.

Garratty G, Petz LD. Letter: Quality control of antiglobulin serum (A.G.S.). Transfusion. 1975;15:397-398.

Garratty G. The significance of complement in immunohematology. Crit Rev Clin Lab Sci. 1984;20:25-56.

Gorman NT. The interaction of cells persistently infected with canine distemper virus with antiviral antibody and complement. Cell Immunol. 1983;77:242-248.

Gorman NT. Activation of the alternative complement pathway by lymphoblasts isolated from canine thymic lymphomas. Vet Immunol Immunopathol. 1984;7:213-225.

Issitt PD, Smith TR. Evaluation of antiglobulin reagents. In: Myhre BA, editor. A seminar

on performance evaluation. Washington, DC: AABB; 1976. p. 25-73.

Jackson ML, Kruth SA. Immune-mediated hemolytic anemia and thrombocytopenia in the dog: a retrospective study of 55 cases diagnosed from 1979 through 1983 at the Western College of Veterinary Medicine. Can Vet J. 1985;26:245-250.

Johnson JP, Hammer CH, Winkelstein JA. Purification of the third component of canine complement. Vet Immunol Immunopathol. 1985;8:377-389.

Jones DR, Gruffydd-Jones TJ, Stokes CR, et al. Use of a direct enzyme-linked antiglobulin test for laboratory diagnosis of immune-mediated hemolytic anemia in dogs. Am J Vet Res. 1992;53:457-465.

Jones DR, Stokes CR, Gruffydd-Jones TJ, et al. An enzyme-linked antiglobulin test for the detection of erythrocyte-bound antibodies in canine autoimmune haemolytic anaemia. Vet Immunol Immunopathol. 1987;16:11-21.

Kay PH, Dawkins RL, Penhale JW. The molecular structure of different polymorphic forms of canine C3 and C4. Immunogenetics. 1985;21:313-319.

Lachmann PJ, Oldroyd RG, Milstein C, et al. Three rat monoclonal antibodies to human C3. Immunology. 1980;41:503-515.

Lachmann PJ, Voak D, Oldroyd RG, et al. Use of monoclonal anti-C3 antibodies to characterize the fragments of C3 that are found on erythrocytes. Vox Sang. 1983;45:367-372.

Lachmann PJ. The amplification loop of the complement pathways. In: Alt FW, editor. Advances in immunology. Volume 104. San Diego, CA: Elsevier; 2009. p. 115-149.

Leger RM. The positive direct antiglobulin test and immune-mediated hemolysis. In: Roback JD, editor. AABB Technical Manual. 17th ed. Bethesda, MD: AABB; 2011. p. 497-522.

McConnell I, Klein G, Lint TF, et al. Activation of the alternative complement pathway by human B cell lymphoma lines is associated with Epstein-Barr virus transformation of the cells. Eur J Immunol. 1978;8:453-458.

Minta JO, Man D, Movat HZ. Kinetic studies on the fragmentation of the third component of complement (C3) by trypsin. J Immunol. 1977;118:2192-2198.

Minta JO, Wong MJ, Kozak CA, et al. cDNA cloning, sequencing and chromosomal assignment of the gene for mouse complement factor I (C3b/C4b inactivator): identification of a species specific divergent segment in factor I. Mol Immunol. 1996;33:101-112.

Morton JA, Pickles M. Use of trypsin in the detection of incomplete Rh antibodies.

Nature. 1947;159:779.

Overmann JA, Sharkey LC, Weiss DJ, et al. Performance of 2 microtiter canine Coombs' tests. Vet Clin Pathol. 2007;36:179-183.

Piek CJ, Teske E, van Leeuwen MW, et al. Good agreement of conventional and gel-based direct agglutination test in immune-mediated haemolytic anaemia. Acta Vet Scand. 2012;54:10.

Quigley KA, Chelack BJ, Haines DM, et al. Application of a direct flow cytometric erythrocyte immunofluorescence assay in dogs with immune-mediated hemolytic anemia and comparison to the direct antiglobulin test. J Vet Diagn Invest. 2001;13:297-300.

Roback JD, editor. Methods Section 3. Antibody detection, identification, and compatibility testing. In: AABB Technical Manual. 17th ed. Bethesda, MD: AABB; 2011. p. 905-906.

Ross GD, Lambris JD, Cain JA, et al. Generation of three different fragments of bound C3 with purified factor I or serum. I. Requirements for factor H vs. CR1 cofactor activity. J Immunol. 1982;129:2051-2060.

Ross GD, Newman SL, Lambris JD, et al. Generation of three different fragments of bound C3 with purified factor I or serum. II. Location of binding sites in the C3 fragments for factors B and H, complement receptors, and bovine conglutinin. J Exp Med. 1983;158:334-352.

Slappendel RJ. The diagnostic significance of the direct antiglobulin test (DAT) in anemic dogs. Vet Immunol Immunopathol. 1979;1:49-59.

Warman SM, Murray JK, Ridyard A, et al. Pattern of Coombs' test reactivity has diagnostic significance in dogs with immune-mediated haemolytic anaemia. J Small Anim Pract. 2008;49:525-530.

Wilkerson MJ, Davis E, Shuman W, et al. Isotype-specific antibodies in horses and dogs with immune-mediated hemolytic anemia. J Vet Intern Med. 2000;14:190-196.

APPENDIX B

Development and Characterization of a Phagocytic Assay for Canine Erythroid Cells

Introduction

Aim 4 was to determine if erythroid precursors in dogs with suspected precursor-targeted immune-mediated anemia (PIMA) have increased susceptibility to phagocytosis, whether or not they have increased surface IgG or PS. The hypothesis was that erythroid precursors from dogs with PIMA undergo increased phagocytosis compared to those from healthy dogs and from sick non-PIMA dogs. This hypothesis was based on the presence of rubriphagocytosis in most dogs with PIMA, and the idea that rubriphagocytosis may mediate anemia in this condition. The paragraphs to follow include: 1) a review of literature to further support this hypothesis, 2) information on a phagocytic assay we developed to test this hypothesis, and 3) limitations we found with the assay and proposed future studies that could help achieve this aim.

Mediators of phagocytosis

Macrophages use several mechanisms to internalize particles, depending mostly on particle size and signals. Pinocytosis and endocytosis are the mechanisms used for internalization of fluid and small particles, respectively. Phagocytosis is the mechanism used for internalization of larger particles (> 0.5 μm) and targeted cells, and through this process macrophages participate in important homeostatic events involved with development, remodeling, inflammation, and immune responses (Aderem et al., 1999).

The main mediators of phagocytosis on target cells are antibodies, the third component of the complement system (C3), and phosphatidylserine (Aderem et al., 1999), which are recognized by macrophage Fc (Shaw et al., 1981), C3 (Shaw et al., 1981; Carrol 1998), and phosphatidylserine (Fadok et al., 2000) receptors, respectively. Macrophages also have a mannose receptor, which mediates engulfment and removal

of some microorganisms (Stahl et al., 1998). Phagocytosis is a complex event, in which most particles are recognized by more than one receptor (Aderem et al., 1999; Shaw, et al., 1981).

Antibodies can be found on erythroid cells as a consequence of targeting by the immune system because of surface senescence antigens (Bratosin et al., 1998), autoantigens, or secondary molecules bound or expressed on the erythroid membrane secondary to infectious agents, neoplasia, or drug administration (McCullough 2003). Proteins of the complement system can be fixed on the erythroid surface through membrane-bound antibodies, especially IgM, but also by IgG (Ravetch et al., 1998). Phosphatidylserine, a phospholipid found in the inner leaflet of the plasma membrane of erythroid precursors and erythrocytes, can be flipped to the outer leaflet secondary to senescence (Bratosin et al., 1998; Lang et al., 2015) or apoptotic signals (Mathias et al., 2000; Merchant et al., 2001; Matthes et al., 2000; Lang et al., 2015). Through recognition of these mediators of phagocytosis, macrophages play a critical role in the uptake and removal of senescent or damaged erythroid cells. Macrophages also play a critical role in engulfing extruded nuclei from metarubricytes in the bone marrow during erythropoiesis, thus preventing erythroid nuclear cell remnants from being free in the tissue and triggering inflammation (Yoshida et al., 2005).

An alternative mechanism of macrophage recognition of target cells is decreased levels of CD47 on the target cell. CD47, considered a membrane marker of self, is decreased in aging erythrocytes (Oldenborg et al., 2000; Ishikawa-Sekigami et al., 2006), triggering macrophage activation and erythrophagocytosis (Olsson et al., 2006). Even though a role for decreased CD47 has been proposed in human autoimmune

hemolytic anemia (AIHA) (Olsson et al., 2006), several studies refute this idea (Arndt et al., 2004; Ahrens et al., 2006; Barros et al., 2009).

Erythroid precursor phagocytosis in PIMA

As described in chapters 1, 2, and 4, phagocytosis of erythroid precursors (rubriphagocytosis) can be found in the bone marrow of most PIMA dogs at the Michigan State University Veterinary Medical Center. Rubriphagocytosis has also been reported in PIMA by others (Jonas et al., 1987; Stockham et al., 1980; Holloway et al., 1990; McManus 2000). It is important to notice that rubriphagocytosis can also be found in the spleen of affected dogs (Lucidi et al. 2010), supporting a systemic process rather than one localized to the bone marrow. Phagocytosis of erythroid precursors has also been reported in human patients with reticulocytopenic AIHA, a potential counterpart of canine PIMA (Keefer et al., 1988).

Phagocytosis of erythroid precursors in PIMA is selective of the erythroid lineage, and relatively restricted to a specific stage of development (early-, mid-, or late-stage erythroid precursors), suggesting an abnormality of the erythroid cells, rather than the macrophages. This is in contrast to certain other hematologic disorders involving the monocyte-macrophage lineage in the bone marrow. Hemophagocytic syndrome and histiocytic sarcoma (Brown et al., 1994; Moore et al., 2006; Weiss 2007) are associated with nonselective phagocytosis of multiple lineages and multiple stages of development, because the phagocytosis is related to abnormal phagocyte behavior rather than abnormal or tagged target cells. In the case of histiocytic sarcoma, atypical cell features may be present (Brown et al., 1994; Weiss 2001; Moore et al., 2006).

Erythrocyte phagocytosis in IMHA

One of the main mechanisms of anemia in IMHA is the removal of IgG- or C3-coated erythrocytes by macrophages. An in vitro mononuclear phagocyte assay has been applied to demonstrate increased erythrophagocytosis in people with IMHA (Herron et al., 1986; Zupańska et al., 1987; Gallagher et al., 1983; Biondi et al., 2004; Biondi et al., 2001). A correlation was seen between the rate of phagocytosis and the degree of hemolysis in vivo (Gallagher et al., 1983) and the number of erythrocyte-bound IgG molecules (Zupańska et al., 1987). Most importantly, by use of a phagocytic assay, increased erythrophagocytosis was demonstrated in IMHA patients with both positive and negative direct antiglobulin test results (Biondi et al., 2001). These results suggest that a phagocytic assay could be used to assess either IgG/C3-negative or positive erythroid cells in dogs with suspected immune-mediated anemia.

Erythroid cell phagocytosis in apoptotic conditions

Receptors for phosphatidylserine on macrophages mediate phagocytosis of erythrocytes and erythroid precursors undergoing apoptosis. Beta-thalassemia is a condition known to cause oxidative stress, phosphatidylserine exposure, and apoptosis of erythroid cells in people. By applying an in vitro phagocytic assay, using either murine (Knyszynski et al., 1979) or human macrophages (Rachmilewitz et al., 1980), people with thalassemia have been shown to have circulating erythrocytes with increased susceptibility to phagocytosis when compared to healthy individuals. Treatment of erythrocytes of thalassemic patients with an antioxidative compound reduced their susceptibility to in vitro phagocytosis (Amer et al., 2008). In the same study, decrease in susceptibility to phagocytosis was suggested to be associated with decreased

erythrocyte oxidative stress, as demonstrated by reduction in reactive oxygen species (ROS) and increase in erythrocyte reduced glutathione (GSH) levels.

Increased susceptibility to phagocytosis has also been demonstrated in erythroid precursors of human patients with beta-thalassemia major (Angelucci et al., 2002). Enhanced macrophagic attack was attributed to increased apoptosis, but also possibly to concurrent IgG-mediated phagocytosis.

Rationale

Phagocytosis of erythroid cells by macrophages is mediated mainly by IgG, C3, and phosphatidylserine. Selective and stage-selective rubriphagocytosis is common in PIMA, suggesting increased susceptibility of erythroid precursors to phagocytosis. Enhanced phagocytosis of erythrocytes and erythroid precursors has been demonstrated in people with immune- or apoptosis-mediated conditions by use of in vitro phagocytic assays. An in vitro phagocytic assay was developed based on previous studies, and applied to test our hypothesis that erythroid precursors from dogs with PIMA undergo increased phagocytosis compared to those from healthy dogs and from sick control dogs.

Experimental design and methods

Macrophages

J774 murine macrophages (ATCC, Manassas, VA) were used as phagocytic cells. This cell line was chosen based on its expression of receptors for IgG (Sears et al., 1990) and C3 (Ralph et al., 1977), and on its ability to phagocytize IgG-coated cells (Weinshank et al., 1988; Schreiber et al., 1990) and apoptotic cells (McPhillips et al.,

2009). Considering the phagocytic nature of these cells, phagocytosis mediated by alternative mechanisms might also be assessed.

As an alternative, attempts were made to use canine monocyte/macrophage cell lines, anticipating that they would more accurately reflect cell interactions in dogs than a murine cell line. We tested the canine cell line DH82, known to express Fcγ receptors (Sibley et al., 2011; Sibley et al., 2013), but we were not successful in promoting phagocytosis of IgG-coated erythrocytes when using these cells. We also attempted to use the 030-D canine macrophage cell line (Gebhard et al., 1995). However, these cell vials proved to be contaminated with fungus, hindering use of these cells in our studies.

Phagocytic assay

J774 cells were grown in complete medium (DMEM medium with 10% FBS and antimicrobials) and removed from culture dishes by scraping on the day of testing. A total of 200,000 macrophages were cultured in 1 mL of complete medium per well on Millicell EZ slides (4-well glass, sterile), at 37°C, with 5% CO_2, for 3 hours. For each tested dog, 5 x 10^6 nucleated cells in bone marrow-derived Percoll fractions (top, middle, and bottom) were separately layered over J774-coated slides at a 25:1 ratio of nucleated bone marrow cells to phagocytic cells in 0.5 mL of complete medium (Biondi et al., 2004; Biondi et al., 2001; Brajovitch et al., 2009; Angelucci et al., 2002). After 3 hours, slides were washed with PBS, air-dried, stained with a Wright-stain, and examined under oil immersion. Phagocytosis was assessed by the number of nucleated cells phagocytized per 500 macrophages, but percentage of phagocytic macrophages was used in some preliminary experiments. Phagocytized nucleated cells were discriminated as intact erythroid cells, intact myeloid cells, or degraded cells (cell lineage

unrecognizable). Incubations were performed in duplicates when enough bone marrow nucleated cells were available, and average results were calculated.

Initial experiments during assay characterization showed that murine J774 macrophages consistently and robustly phagocytized canine erythrocytes coated with canine IgG, which were made by incubating DEA1.1-positive erythrocytes with anti-DEA1.1 typing serum (Figure 21B,C). Phagocytosis did not occur in negative control washed erythrocytes (not shown), or DEA1.1-negative erythrocytes incubated with anti-DEA1.1 typing serum (Figure 21A). IgG positivity or negativity of control erythrocytes was confirmed by flow cytometry. Phagocytized erythrocytes looked distinct and countable, allowing quantification of the phagocytic activity. J774 macrophages did not phagocytize sucrose-treated canine erythrocytes (positive for canine C3 by flow cytometry, and presumed to be coated with C3b), suggesting failure to assess for canine erythroid cell complement opsonization. However, it is not clear which complement fragment might be present on canine cells in vivo (Appendix A), so ability of these cells to phagocytize naturally complement-opsonized canine erythrocytes could not be excluded.

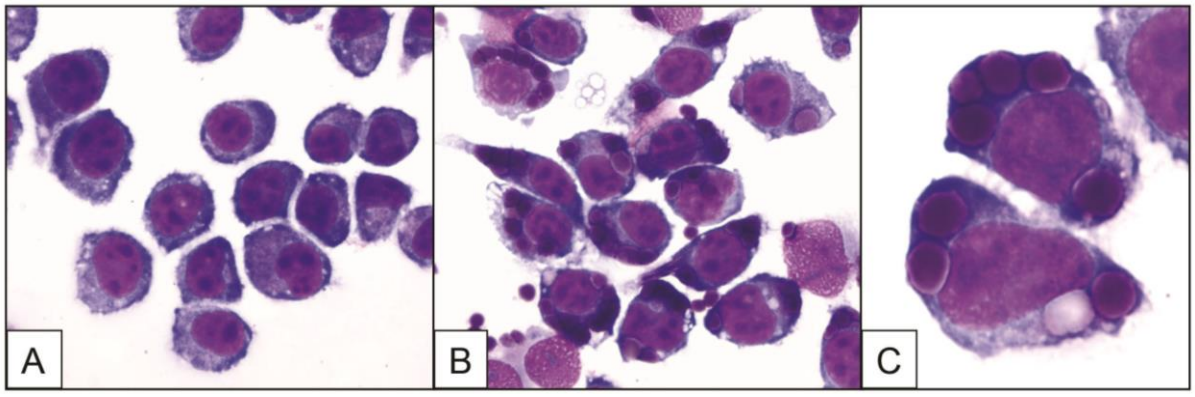

Figure 21: *Representative images of phagocytic assay using murine J774 macrophages adherent to Millicell slides.* Macrophages were washed after incubation with IgG-

Figure 21 (cont'd): negative erythrocytes (DEA 1-negative erythrocytes incubated with anti-DEA 1.1 typing serum) (A) or IgG-positive erythrocytes (DEA 1.1-positive erythrocytes incubated with anti-DEA 1.1 typing serum) (B, C). In B and C, most macrophages contain multiple, but distinct and countable phagocytized erythrocytes. Wright stain, 50× objective (A, B) and 100× objective (C).

Assay sensitivity was assessed by incubating DEA1.1-positive erythrocytes with increasing dilutions of anti-DEA1.1 typing serum in order to coat erythroid cells with decreasing amounts of IgG. Positivity for IgG was determined by flow cytometry, and erythrocytes were then incubated with J774 macrophages. As anticipated, lower numbers of phagocytic macrophages were observed when using erythrocytes coated with lower amounts of IgG (Table 10), characterizing a dose-response of the assay with regard to IgG positivity on the target cells. The lowest amount of IgG, giving a MdFI of 196, equates to a relatively strong positive for RBC IgG in clinical samples. The relatively low amount of phagocytosis at these IgG levels suggested potential problems with assay sensitivity, but testing was begun to assess clinical samples.

Table 10: *Percentage of J774 macrophages engulfing DEA1.1-positive erythrocytes after incubation with different dilutions of anti-DEA1.1 typing serum to yield decreasing IgG positivity documented by flow cytometry.*

DEA1.1 typing serum dilution	MdFI	Phagocytic J774 macrophages (%)
1/32	1,472	96
1/64	828	96
1/128	470	77
1/512	196	16

MdFI = median fluorescence intensity of IgG-coated erythrocytes

Dogs and samples

Bone marrow was collected from dogs with PIMA, healthy dogs, and unhealthy non-PIMA dogs. Mixtures of cells containing early-, mid-, and late-stage erythroid precursors were isolated using a Percoll gradient separation method, as described in Chapter 3.

Expected outcomes

Erythroid precursors of dogs with PIMA were expected to undergo increased phagocytosis compared to those of healthy and unhealthy non-PIMA dogs.

Summary of experiment results and pitfalls encountered

Preliminary results from four unhealthy dogs showed little phagocytosis, with few J774 cells containing degraded nuclei and cellular debris. Impaired identification of phagocytized cells prompted assessment of shorter time periods for cell incubation. IgG positive control erythrocytes were incubated with J774 macrophages for 15 and 30 minutes, in duplicates, and the average number of phagocytized erythrocytes per 200 macrophages was determined. There were averages of 247 and 439 phagocytized erythrocytes per 200 macrophages at 15 and 30 minutes, respectively, indicating that phagocytosis occurs as rapidly as 15 minutes after incubation, and that more phagocytized cells could be found at 30 minutes.

To test if results of this timing experiment were reproducible with bone marrow nucleated cells, precursors from the top Percoll fraction and a mixture of erythroid precursors from the mid and bottom Percoll fractions (in order to have enough cells) from an unhealthy non-PIMA DEA1.1-positive dog were incubated with anti-DEA1.1

typing serum in order to artificially coat erythroid cells with IgG. After washing, cells were incubated with J774 macrophages, and the assay was terminated at 15, 30, 90, and 180 minutes. Additionally, J774 cells were incubated for two hours before adding bone marrow cells, instead of the three hours used previously. IgG coating of erythroid precursors was confirmed by flow cytometry. The number of phagocytized nucleated cells per 500 macrophages was assessed per well. The greatest amount of phagocytosis of any nucleated cell type, intact or degraded, occurred at 30 minutes for the top fraction and at 180 minutes for the mixture of cells from the mid and bottom fractions (Table 11). The greatest number of intact phagocytized erythroid precursors from the top fraction and from the mid and bottom fractions combined was at 30 minutes of incubation (Figure 22). The number of degraded cells within macrophages increased over time for cells from the mid and bottom fractions (Figure 22), but the same was not observed for the top fraction. Based on these findings, the assay was modified for incubation of bone marrow-derived nucleated cells with J774 macrophages for 30 minutes for prospective patient samples.

Table 11: *Phagocytosis of bone marrow-derived nucleated cells after incubation with J774 macrophages for different time periods.*

	Phagocytized nucleated cells* per 500 macrophages			
Percoll Fraction	**15 min**	**30 min**	**90 min**	**180 min**
Top	36	358	152	110
Mixture of mid and bottom	12	73**	99	137

*Intact or degraded, all types
**Average of duplicates

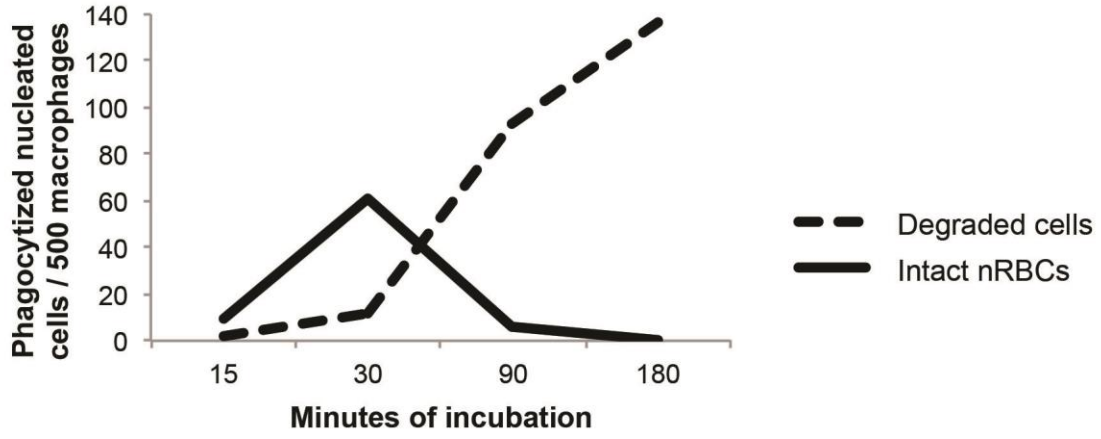

Figure 22: *Number of phagocytized bone marrow-derived nucleated cells from the mid and bottom Percoll fractions of a dog after IgG coating and incubation with J774 macrophages for different time intervals.* The greatest number of intact erythroid precursors within macrophages occurred with 30 minutes of incubation. Degraded cells were present after just 15 minutes of incubation.

Of the 14 dogs tested after optimizing the incubation period to 30 minutes, 2 PIMA, 1 unhealthy non-PIMA, and 5 healthy dogs were identified. The 6 remaining dogs had unclear diagnoses or were excluded because of hematologic neoplasia or high non-specific binding of erythroid precursors on flow cytometric testing. No obvious differences were observed in phagocytic activity in different Percoll fractions among these dogs. The healthy dogs had the following mean (range) number of phagocytic cells per 500 macrophages: 14.1 (3.5-35.5), 10.9 (2.5-19), and 8.1 (4-18.5) for the top, mid, and bottom fractions, respectively, while the three patient samples yielded similar results of 1-26 and 7-18 for the top and mid fractions, respectively. Only one dog had enough cells to assess the bottom fraction, and that fraction had 9 phagocytized cells per 500 macrophages. The greatest degree of phagocytosis was in the top fraction of a

PIMA dog (26 per 500 macrophages), while the greatest degree of phagocytosis in a healthy dog was 35.5 per 500 macrophages, but most cells in the PIMA dog were not identifiable (22 of 26) and may not have been erythroid. At this time, the assay was reassessed.

Unhealthy patients had sometimes markedly varying amounts of erythroid cells in their Percoll fractions, depending on their bone marrow abnormality and the myeloid-to-erythroid ratios (M:E) found in their samples. For instance, late-PIMA dogs tended to have erythroid hyperplasia and low M:E, while dogs with early-PIMA or immune-mediated neutropenia (IMN) had higher M:E, due to erythroid hypoplasia or myeloid hyperplasia, respectively. Based on that, additional steps were taken to evaluate the effect of M:E on phagocytosis of erythroid cells. It was hypothesized that dogs with high M:E could yield falsely low results for phagocytosis of erythroid precursors, because of low concentrations of erythroid cells in their Percoll fractions.

Two experiments were done to address this question. The first one consisted of mixing IgG-coated erythrocytes from packed red cells with white blood cells (WBC) from buffy coat, both from blood, in different proportions in order to reach the following WBC:RBC ratios: 0.2 (mimicking the M:E in late-PIMA), 1.0, and 5.0 (mimicking the M:E in IMN), with an additional cell aliquot containing 100% erythrocytes. These cell aliquots were incubated with J774 cells for 30 minutes, with a fixed number of 5×10^6 nucleated cells (WBC) per well. The percentage of J774 cells containing phagocytized erythrocytes was determined by assessing 200 cells. There was a tendency for decreasing numbers of phagocytic macrophages as the WBC:RBC ratio increased (Table 12), supporting our suspicion that marrows with lower numbers of erythroid cells may yield fewer phagocytized erythroid precursors, even if erythroid cells are targeted for phagocytosis.

Table 12: *Percentage of J774 macrophages engulfing IgG-coated erythrocytes after incubation with mixtures of erythrocytes and white blood cells of varying WBC:RBC ratio but constant total number.*

WBC:RBC ratio	Phagocytic J774 macrophages (%)*
All RBC	78
0.2	82
1.0	69
5.0	43

*average of duplicates based on assessment of 200 J774 cells

The effect of normalizing the number of added erythrocytes with varied WBC:RBC ratios was then assessed by adding 5×10^6 erythrocytes per well with different number of WBCs to give the following WBC:RBC ratios: 0 (100% erythrocytes), 0.2, 1.0, and 5.0. Again, the percentage of J774 macrophages containing phagocytized erythrocytes was determined by assessing 200 macrophages. There was similar phagocytic activity in all wells except the one with the WBC:RBC ratio of 5.0 (Table 13). This suggested that a fixed number of erythroid cells per well would help normalize phagocytic results among patients with different M:E ratios, but high M:E ratios might still be problematic. The lesser phagocytosis with a WBC:RBC ratio of 5.0 might have been caused by an overwhelming total number of nucleated cells per well (30×10^6) leading to 1) physical blockage of macrophages from IgG-coated erythrocytes, 2) insufficient nutrients and oxygen that decreased J774 activity, or 3) removal of IgG-coated erythrocytes by Fc-receptor bearing WBCs present in the cell mixture. So, an Fc-receptor blocker (Fc receptor block, Innovex, Lincoln, RI) was tested, but it did not adequately block phagocytosis. Plans to reduce the total cell number and use only 1×10^6 erythrocytes per well were not completed.

Table 13: *Percentage of J774 macrophages engulfing IgG-coated erythrocytes after incubation with mixtures of erythrocytes and white blood cells (WBC) of varying WBC:RBC ratio and fixed RBC number.*

WBC:RBC ratio	Phagocytic J774 macrophages (%)*
All RBC	97
0.2	96
1.0	96
5.0	73

*average of duplicates based on assessment of 200 J774 cells

Outcome and future directions

After pilot testing and recognition of a potential significant limitation of the assay because of varied numbers of erythroid precursors, this limitation was documented and partially addressed. However, further application was not pursued because of limited time, limited availability of bone marrow samples for testing, and recognition that there would be limited numbers of patients with enough cells to test for IgG, PS, and phagocytosis. Priority was placed on IgG and PS testing.

Remaining concerns about the assay include its sensitivity to detect IgG-coated cells, as phagocytosis was not abundant when test cells were coated with amounts of IgG corresponding to strong clinical positivity for RBC IgG, and such levels were much higher than IgG positivity detected on erythroid precursors of any PIMA dogs. Moreover, phagocytosis of samples from healthy dogs was greater than expected, and recognition and differentiation of degrading nucleated cells proved difficult. Because bone marrow samples were tested for IgG and PS by flow cytometry on the day of collection, and these assays were time consuming, Percoll fractions were stored at 4°C overnight, and the phagocytic assay was performed on the day following collection. Cell storage might

have promoted degradation or expression of cell surface markers, increasing phagocytosis and the number of phagocytized degraded-appearing cells, even in samples from healthy dogs. Alternatively, these problems may have been a consequence of Percoll separation, which could have been damaging to the cell surface. Percoll separation was not assessed to see if it increased susceptibility to phagocytosis.

Continued development and improvement of a phagocytic assay could include assessment of increasing assay sensitivity by stimulating IgG-mediated phagocytic activity of J774 macrophages with IFN-gamma (Weinshank et al., 1988), or by using a canine monocyte/macrophage cell line or fresh cells extracted from canine whole blood (Sampaio et al., 2007) or bone marrow (Tipold et al., 1998), which may have higher affinity for canine IgG. A potentially gentler method of processing bone marrow samples could be assessed, such as removal of nonerythroid cells by antibody coupled to magnetic beads as previously described (Angelucci et al., 2002). One could also assess using macrophages in suspension and assessing phagocytosis by flow cytometry (Bratosin et al., 1997). A novel method of assessing cell internalization by fluorescence and imaging combined is image stream cytometry, which correlates well with results from conventional flow cytometry (Ploppa et al., 2011).

REFERENCES

REFERENCES

Aderem A, Underhill DM. Mechanisms of phagocytosis in macrophages. Annu Rev Immunol. 1999;17:593-623.

Ahrens N, Pagenkopf C, Kiesewetter H, et al. CD47 is expressed at normal levels in patients with autoimmune haemolytic anaemia and/or immune thrombocytopenia. Transfus Med. 2006;16:397-402.

Amer J, Atlas D, Fibach E. N-acetylcysteine amide (AD4) attenuates oxidative stress in beta-thalassemia blood cells. Biochim Biophys Acta. 2008;1780:249-255.

Angelucci E, Bai H, Centis F, et al. Enhanced macrophagic attack on beta-thalassemia major erythroid precursors. Haematologica. 2002;87:578-583.

Arndt PA, Garratty G. Rh(null) red blood cells with reduced CD47 do not show increased interactions with peripheral blood monocytes. Br J Haematol. 2004;125:412-414.

Barros MM, Yamamoto M, Figueiredo MS, et al. Expression levels of CD47, CD35, CD55, and CD59 on red blood cells and signal-regulatory protein-alpha,beta on monocytes from patients with warm autoimmune hemolytic anemia. Transfusion. 2009;49:154-160.

Biondi C, Cotorruelo C, García Borras S, et al. Erythrophagocytosis assay in patients with autoimmune hemolytic anemia. Medicina (B Aires). 2001;61:49-52.

Biondi CS, Cotorruelo CM, Ensinck A, et al. Use of the erythrophagocytosis assay for predicting the clinical consequences of immune blood cell destruction. Clin Lab. 2004;50:265-270.

Brajovich ML, Rucci A, Acosta IL, et al. Effects of aging on antioxidant response and phagocytosis in senescent erythrocytes. Immunol Invest. 2009;38:551-559.

Bratosin D, Mazurier J, Slomianny C, et al. Molecular mechanisms of erythrophagocytosis: flow cytometric quantitation of in vitro erythrocyte phagocytosis by macrophages. Cytometry. 1997;30:269-274.

Bratosin D, Mazurier J, Tissier JP, et al. Cellular and molecular mechanisms of senescent erythrocyte phagocytosis by macrophages. A review. Biochimie. 1998;80:173-195.

Brown DE, Thrall MA, Getzy DM, et al. Cytology of canine malignant histiocytosis. Vet Clin Pathol. 1994;23:118-123.

Carroll MC. The role of complement and complement receptors in induction and

regulation of immunity. Annu Rev Immunol. 1998;16:545-568.

Fadok VA, Bratton DL, Rose DM, et al. A receptor for phosphatidylserine-specific clearance of apoptotic cells. Nature. 2000;405:85-90.

Gallagher MT, Branch DR, Mison A, et al. Evaluation of reticuloendothelial function in autoimmune hemolytic anemia using an in vitro assay of monocyte-macrophage interaction with erythrocytes. Exp Hematol. 1983;11:82-89.

Gebhard DH, Levy MG, Ley D, et al. Isolation and characterization of functionally and phenotypically distinct continuous monocytoid cell lines from a canine malignant histiocytosis [abstract]. Proc Int Union Immunol Soc 4th Int Vet Immunol Symp. 1995:312.

Herron R, Clark M, Young D, et al. Correlation of mononuclear phagocyte assay results and in vivo haemolytic rate in subjects with a positive direct antiglobulin test. Clin Lab Haematol. 1986;8:199-207.

Holloway SA, Meyer DJ, Mannella C. Prednisolone and danazol for treatment of immune-mediated anemia, thrombocytopenia, and ineffective erythropoiesis in a dog. J Am Vet Med Assoc. 1990;197:1045-1048.

Ishikawa-Sekigami T, Kaneko Y, Saito Y, et al. Enhanced phagocytosis of CD47-deficient red blood cells by splenic macrophages requires SHPS-1. Biochem Biophys Res Commun. 2006;343:1197-1200.

Jonas LD, Thrall MA, Weiser MG. Nonregenerative form of immune-mediated hemolytic anemia in dogs. J Am Anim Hosp Assoc. 1987;23:201-204.

Keefer MJ, Solanki DL. Dyserythropoiesis and erythroblast-phagocytosis preceding pure red cell aplasia. Am J Hematol. 1988;27:132-135.

Knyszynski A, Danon D, Kahane I, et al. Phagocytosis of nucleated and mature beta thalassaemic red blood cells by mouse macrophages in vitro. Br J Haematol. 1979;43:251-255.

Lang E, Lang F. Mechanisms and pathophysiological significance of eryptosis, the suicidal erythrocyte death. Semin Cell Dev Biol. 2015;39:35-42.

Lucidi CA, de Resende CLE, Jutkowitz LA, et al. Cytologic and histologic bone marrow findings in dogs with suspected marrow-directed immune-mediated anemia. Vet Clin Pathol. 2010;39:560.

Mathias LA, Fisher TC, Zeng L, et al. Ineffective erythropoiesis in beta-thalassemia major is due to apoptosis at the polychromatophilic normoblast stage. Exp Hematol. 2000;28:1343-1353.

Matthes TW, Meyer G, Samii K, et al. Increased apoptosis in acquired sideroblastic anaemia. Br J Haematol. 2000;111:843-852.

McCullough S. Immune-mediated hemolytic anemia: understanding the nemesis. Vet Clin North Am Small Anim Pract. 2003;33:1295-1315.

McManus P. Marrow and erythrocyte changes associated with canine non-regenerative immune- mediated anemia: 44 cases (1996-2000) [abstract]. Vet Pathol. 2000;37:526.

McPhillips KA, Erwig LP. Assessment of apoptotic cell phagocytosis by macrophages. Methods Mol Biol. 2009;559:247-256.

Merchant SH, Gonchoroff NJ, Hutchison RE. Apoptotic index by Annexin V flow cytometry: adjunct to morphologic and cytogenetic diagnosis of myelodysplastic syndromes. Cytometry. 2001;46:28-32.

Moore PF, Affolter VK, Vernau W. Canine hemophagocytic histiocytic sarcoma: a proliferative disorder of CD11d+ macrophages. Vet Pathol. 2006;43:632-645.

Oldenborg PA, Zheleznyak A, Fang YF, et al. Role of CD47 as a marker of self on red blood cells. Science. 2000;288:2051-2054.

Olsson M, Nilsson A, Oldenborg PA. Target cell CD47 regulates macrophage activation and erythrophagocytosis. Transfus Clin Biol. 2006;13:39-43.

Ploppa A, George TC, Unertl KE, et al. ImageStream cytometry extends the analysis of phagocytosis and oxidative burst. Scand J Clin Lab Invest. 2011;71:362-369.

Rachmilewitz EA, Treves A, Treves AJ. Susceptibility of thalassemic red blood cells to phagocytosis by human macrophages in vitro. Ann N Y Acad Sci. 1980;344:314-322.

Ralph P, Nakoinz I. Antibody-dependent killing of erythrocyte and tumor targets by macrophage-related cell lines: enhancement by PPD and LPS. J Immunol. 1977;119:950-954.

Ravetch JV, Clynes RA. Divergent roles for Fc receptors and complement in vivo. Annu Rev Immunol. 1998;16:421-432.

Sampaio WM, Moura EP, Arruda FC, et al. In vitro binding and survival assays of Leishmania parasites to peripherical blood monocytes and monocyte-derived macrophages isolated from dogs naturally and experimentally infected with Leishmania (Leishmania) chagasi. BMC Vet Res. 2007;3:11.

Schreiber RE, Buku A, Unkeless JC. Expression of murine Fc receptors for IgG. J Immunol. 1990;144:4735-4741.

Sears DW, Osman N, Tate B, et al. Molecular cloning and expression of the mouse high affinity Fc receptor for IgG. J Immunol. 1990;144:371-378.

Shaw DR, Griffin FM Jr. Phagocytosis requires repeated triggering of macrophage phagocytic receptors during particle ingestion. Nature. 1981;289:409-411.

Sibley TA, Fogle JE. Characterization of canine monocyte/macrophages Fcgamma receptors and demonstration of IgG binding [abstract]. Vet Clin Pathol. 2011;40:604.

Sibley TA, Miller MM, Fogle JE. Human intravenous immunoglobulin (hIVIG) inhibits anti-CD32 antibody binding to canine DH82 cells and canine monocytes in vitro. Vet Immunol Immunopathol. 2013;151:229-234.

Stahl PD, Ezekowitz RA. The mannose receptor is a pattern recognition receptor involved in host defense. Curr Opin Immunol. 1998;10:50-55.

Stockham SL, Ford RB, Weiss DJ. Canine autoimmune hemolytic disease with a delayed erythroid regeneration. J Am Anim Hosp Assoc. 1980;16:927-931.

Tipold A, Zurbriggen A, Moore P, et al. Generation and functional characterization of canine bone marrow-derived macrophages. Res Vet Sci. 1998;64:125-132.

Weinshank RL, Luster AD, Ravetch JV. Function and regulation of a murine macrophage-specific IgG Fc receptor, Fc gamma R-alpha. J Exp Med. 1988;167:1909-1925.

Weiss DJ. Cytologic evaluation of benign and malignant hemophagocytic disorders in canine bone marrow. Vet Clin Pathol. 2001;30:28-34.

Weiss DJ. Hemophagocytic syndrome in dogs: 24 cases (1996-2005). J Am Vet Med Assoc. 2007;230:697-701.

Yoshida H, Kawane K, Koike M, et al. Phosphatidylserine-dependent engulfment by macrophages of nuclei from erythroid precursor cells. Nature. 2005;437:754-758.

Zupańska B, Brojer E, Thomson EE, et al. Monocyte-erythrocyte interaction in autoimmune haemolytic anaemia in relation to the number of erythrocyte-bound IgG molecules and subclass specificity of autoantibodies. Vox Sang. 1987;52:212-218.