THE PATHOLOGY OF THE SPLEEN IN CANINE BABESIOSIS.

By

ALISCHA HENNING

Submitted in partial fulfilment of the requirements for the degree

of

Master of Veterinary Medicine (Pathology)

In the Department of Paraclinical Sciences, Faculty of Veterinary Science,

University of Pretoria

Date submitted: February 2019

AKNOWLEDGEMENTS

First of all, I would like to thank both my supervisor, Prof Andrew Leisewitz, and co-supervisor, Dr Sarah Clift for your input, time and patience, without which the completion of this thesis would not have been possible. Thank you to all my colleagues in the histopathology laboratory for the timeous processing, staining etc. of the tissue samples.

Without my husband, Tiaan, life would be an unimaginable reality. From the bottom of my heart, thank you for your endless support, your positive attitude and your undeserved and endless love. Thank you to my two beautiful boys and the constant reminder of the true essence of my life on earth.

Thank you to Nicolize for your support, not only as a colleague, but more importantly as a friend. Thank you for your constant motivation.

Thank you to my dad...your legacy, more than your absence, inspired me more than you will ever know.

Lastly, thank you to LIFE and our GOD that created it. Reality is very rarely what we expect but in the presence of your love and mercy it ends up being far better than what I could have imagined!

DECLARATION

I, **Alischa Henning**, hereby declare that the work on which this thesis is based is my own independent work and that neither the whole work nor part of it has been, is being, or shall be submitted for another degree at this or another university, institution for tertiary education or professional examination body. Declaration of originality attached as appendix 5.

_____

Alischa Henning

_____

Date

February 2019

ETHICS STATEMENT

I, Alischa Henning, have obtained the necessary research ethics approval (V086-15) for the research described in this dissertation; I declare that I have observed the ethical standards required in terms of the University of Pretoria's code of ethics for researchers and the policy guidelines for responsible research.

Contents

LIST OF FIGURES

LIST OF TABLES

Table 1: Antibodies and immunohistochemical methods.....53

Table 2: Mean percentage and comparison across categorical study variables.....70

LIST OF ABBREVIATIONS

ADCC:	Antibody-dependent cell-mediated cytotoxicity
BSA:	Bovine serum albumin
BCG:	Bacillus Calmette-Guerin
CC/s:	Control case/s
CCL19:	Chemokine C-C-motif ligand 19
CCL21:	Chemokine C-C-motif ligand 21
CCR7:	Chemokine C-C-motif receptor 7
CD:	Cluster of differentiation
CXCL12:	Chemokine C-X-C-motif ligand 12
CXCL13:	Chemokine C-X-C-motif ligand 13
CXCR4:	Chemokine C-X-C-motif receptor 4
CXCR5:	Chemokine C-X-C-motif receptor 5
DAB:	Diaminobenzidine
DAFF:	Department of Agriculture, Forestry and Fisheries
DBE:	Disintegrated but evident
DNA:	Deoxyribonucleic acid
DPS:	Department of Paraclinical Sciences
EC/s:	Experimental case/s

EDTA: Ethylenediaminetetraacetic acid

EMH: Extramedullary haematopoiesis

ERTR-9: Anti-SIGN related 1 protein

FVS: Faculty of Veterinary Science

GRi: Gomori's reticulin impregnation

HE: Haematoxylin and eosin

HIER: Heat-induced epitope retrieval

HP: Histopathology

HPF/s: High power field/s

ICAM1: Intercellular adhesion molecule 1

IFN: Interferon

IHC: Immunohistochemistry

IL: Interleukin

M: Molar

MADCAM-1: Mucosal addressin cell adhesion molecule-1

MARCO: Macrophage receptor with collagenous structure

MHC: Major histocompatibility complex

MOMA-1: Monocyte and macrophage-1

No: Number

OVAH: Onderstepoort Veterinary Academic Hospital

PALS: Periarteriolar lymphatic sheaths

PAMP/s: Pathogen-associated molecular pattern/s

PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

PRR/s: Pattern-recognition receptor/s

RLB: Reverse line blot hybridization assay

RNA: Ribonucleic acid

ROI: Region of interest

RP: Red pulp

SIGLEC1: Sialic-acid-binding immunoglobulin-like lectin 1

$SIP_{1/3}$: Sphingosine 1-phosphate receptor 1/3

SOPs: Standard operating procedures

TCR: T lymphocyte receptor

TE: Thromboemboli

TLR/s: Toll-like receptor/s

UP: University of Pretoria

VCAM1: Vascular cell-adhesion molecule 1

WP: White pulp

SUMMARY

THE PATHOLOGY OF THE SPLEEN IN CANINE BABESIOSIS.

by

Alischa Henning

Supervisor: **Prof A L Leisewitz**

Co-supervisor: **Dr S J Clift**

Department: **Paraclinical Sciences**

Degree: **M.Med.Vet (Pathology)**

The spleen is the largest secondary lymphoid organ in the body and therefore not only does it perform many hematopoietic functions but it also mounts an immunological response to blood-borne antigens. Malaria and babesiosis, both haemoparasitic diseases, reveal many similarities in clinical disease, pathogenesis and post mortem findings and are often compared to one another. Both are protozoan diseases transmitted by mosquitoes and ticks, respectively. Malaria has been more extensively studied than babesiosis. Information regarding the effect of babesiosis on various hosts is limited, particularly with respect to the spleen.

The purpose of this study was to investigate the effect of *Babesia rossi* on the spleen of dogs. We aimed to provide a detailed histomorphological analysis of infected spleens, with the addition of immunohistochemical labelling of leukocyte subsets.

One section of splenic tissue from each of 9 *Babesia rossi*-infected dogs and 4 healthy control dogs was examined under the light microscope. Immunohistochemical markers were applied to infected and control spleens in order to characterise different immunocyte populations.

Markers included CD3 (T lymphocytes), CD20 (mature B lymphocytes and normal dog plasma cells), Mum1 (plasma cells), Pax-5 (immature B lymphocytes), Mac387 (monocytes-macrophages of bone marrow origin) and CD204 (resident tissue macrophages). The application of analytic software enabled us to compare leukocyte subpopulations in infected and control spleens in a semi-quantitative manner.

Routine histopathology revealed diffuse intermingling of the white and red pulp in *Babesia rossi*-infected spleens with a clear loss of distinction between these zones. The merging of zones was accentuated by outspoken white pulp dissolution with no discernible germinal centres, mantle or marginal zones. Immunohistochemical labelling revealed a significant increase in the proportion of tissue resident macrophages as well as macrophages of bone marrow origin in the infected spleens. In addition, apart from a few remnant lymphocytes within the peri-arteriolar lymphatic sheaths and follicles, the majority of immunocytes had redistributed to the red pulp, supporting the observation of white and red pulp intermingling. Our study produced novel insights into the pathology of the spleen in canine babesiosis. The majority of our findings are in agreement with histomorphological descriptions of the spleen in a variety of hosts with malaria. The exact causes, consequences and implications for disease pathogenesis require further investigation.

1. CHAPTER ONE: INTRODUCTION

The spleen is the largest secondary lymphoid organ in the body and therefore not only does it perform many hematopoietic functions but it also reacts immunologically in response to blood-borne antigens [11,71]. The spleen does have a remarkable ability to survey the blood for any blood-borne antigens, which makes it indispensable with respect to the immune system's response to disease although the spleen's exact function in relation to these diseases is not yet completely understood. The present review focused on two haemoparasitic diseases – human malaria and canine babesiosis, both of which are vector-borne protozoal diseases and of importance in human and veterinary medicine, respectively [21,34,45]. Both diseases have similar clinical disease, pathogenesis and post mortem findings which all originate as a result of the corresponding effect these diseases have on the host's immune system and more specifically the effect they have on the spleen. Malaria has been extensively studied and although the information regarding its effect on the spleen is limited compared to other organs, a lot more information is available than for canine babesiosis. The literature that is available on equine babesiosis, bovine babesiosis and babesia in mice and its relationship and effect on the spleen specifically can be used to speculate about the effect of canine babesiosis on the spleen [35,69,78]. As malaria and babesia are so similar, they have been compared to one another for many decades.

Although there is still much to learn regarding the role of the spleen in disease, it is becoming clear that the spleen's location, its function and architecture all contribute to its remarkable role especially within the context of multi-systemic diseases [69]. The complex histology related to the multitude of functions performed by the spleen required detailed histological

examination and application of a variety of immunohistochemical stains in order to attempt a better understanding of the spleen and its role in canine babesiosis specifically.

2. CHAPTER TWO: LITERATURE REVIEW

2.1. SPLEEN STRUCTURE AND FUNCTION

2.1.1. Gross anatomy

The dog spleen is an elongated, dark red organ located vertically against the left abdominal wall, in the left cranial abdomen beneath the diaphragm and with ligamentous attachments to the stomach [11,58]. Variable macroscopic appearances, specifically related to shape, are the result of the spleen's ability to contract which in turn depends on the animal's physiological state [11]. The splenic consistency resembles that of normal liver and it appears dorso-ventrally flattened on cross section [11]. The splenic parenchyma is divided into two basic compartments namely the white pulp and the red pulp, so named due to the fresh spleen's macroscopic appearance on cut surface [11,58,60,71].

2.1.2. Basic function

The spleen is the largest secondary lymphoid organ in the body and it performs a variety of different functions. Primary lymphoid organs include the bone marrow and thymus and are responsible for lymphocyte production, whereas secondary lymphoid organs including the spleen, lymph nodes, mucosa-associated lymphoid tissue and tonsils are responsible for lymphocyte activation. Essentially, the red pulp acts as a blood filter removing senescent, damaged or infected erythrocytes and foreign material and it also acts as a store of surplus erythrocytes and platelets [11]. The white pulp houses a significant proportion of the body's lymphocytes and is therefore mainly responsible for the generation of appropriate immune responses against blood-borne antigens [11].

Despite multiple attempts to classify the spleen of different animal species based on differences in its morphology and physiology, some dating as far back as 1723, many aspects are unclear and no simple classification system exists [93]. Dog, human and mouse spleens can store surplus blood, but the dog spleen has the greatest blood storage capacity of all these species, hence its greater contractility [93]. Mouse and human spleens have significantly greater proportions of white pulp but the histomorphological architecture of the white pulp in dog and mouse spleens is similar [93]. The implication is that human spleens are more inclined toward immunological defence (defence spleen) and dog spleens toward storage (storage spleen) [93]. The histology of the spleen in camels revealed many similarities with the splenic structure of, amongst others, the dog [108]. Both the dog and camel spleens are classified primarily as storage spleens with open as well as closed blood flow systems depending on the animals' physiological state. The spleen is significantly involved in both innate and adaptive immunity, the broad subdivisions of the body's immune system.

2.1.2.1. Innate immunity

Innate immunity entails the first-line, nonspecific defence against potentially harmful environmental substances and includes physical barriers (skin epithelium), microenvironments (gastric secretions), molecular products (antimicrobial peptides) and effector molecules (inflammatory mediators) [107]. Microbes have highly conserved ligands called pathogen-associated molecular patterns (PAMPs) which upon contact with the host, encounter cells that express membrane pattern-recognition receptors (PRRs) which include toll-like receptors (TLRs) expressed on the cell surface or cytoplasm [107]. Pathogen-associated molecular patterns (PAMPs) may include lipopolysaccharide in Gram-negative bacterial cell walls, lipoteichoic acids in Gram-positive bacterial cell walls, double-stranded ribonucleic acid

(RNA) in viruses and glycans in fungal cell walls [107]. Upon pattern recognition by receptors such as TLRs on macrophages, dendritic cells and leukocytes (especially neutrophils), these cells release chemokines and cytokines which participate in the innate immune response, acute inflammatory response and therefore the removal of the inciting cause [107].

2.1.2.2. Adaptive immunity

Adaptive immunity consists of an antigen-specific response characterized by the production of antigen-specific antibodies and effector leukocytes which attempt elimination of the inciting cause with the generation of memory cells that make subsequent adaptive immune responses quicker and more successful [107]. Adaptive immunity has two arms namely cell-mediated immunity, mediated by T lymphocytes against intracellular pathogens and humoral immunity, mediated by B lymphocytes against extracellular pathogens and toxins [107]. B lymphocytes are activated by a specific membrane antigen-binding molecule binding to the antigen receptor immunoglobulin and they recognise only antigens. On the other hand, T lymphocytes express T lymphocyte receptor (TCR), a membrane antigen-binding molecule that can only recognise antigens which are associated with cell membrane proteins called major histocompatibility complex (MHC) molecules [107].

2.1.3. Histology

2.1.3.1. Capsule and supportive tissue

The spleen is enclosed by a capsule characterised by an outside layer or visceral peritoneal surface of mesothelial cells overlying dense, irregular connective tissue followed by smooth muscle fibres on the innermost aspect [11,58,60,71]. The mesothelial layer may be difficult to observe under the light microscope due to its fragility and ease of stripping during organ

collection and processing or due to its usual quiescence, whereby mesothelial cells take on the appearance of a single layer of flattened simple squamous epithelium. Multiple trabeculae invest the splenic parenchyma from the capsule and not only lend structural support to the organ but also house the larger blood vessels, lymphatic ducts/vessels and nerves [11,44,60,71]. Trabeculae are composed of elastic fibres, collagen and smooth muscle within an organised, longitudinal arrangement [22]. The smooth muscle component within the capsule and trabeculae is responsible for the spleen's ability to contract in response to decreased circulating erythrocytes and/or platelets, which releases stored erythrocytes and platelets from the red pulp into systemic circulation [60].

2.1.3.2. Vasculature and innervation

The spleen is a highly vascular organ with a complex vascular system (see Figures 1, 2 and 3). The organ receives between 1 and 10% of the total cardiac output [28]. A branch of the celiac artery, namely the splenic artery, is responsible for the organ's afferent blood supply and it diverges into an estimated 25 smaller splenic arteries in the dog before entering the spleen at the hilus [11]. Splenic arteries give rise to trabecular arteries which travel along the trabeculae and enter the white pulp as central arterioles or arteries of the white pulp [11,22,58,60,71]. Central arterioles are surrounded by a cuff of lymphocytes which constitute the periarteriolar lymphatic sheaths (PALS) which, together with lymphoid follicles and marginal zones, represent the white pulp. On cross-section, PALS (diffuse lymphoid tissue) may appear circular and resemble lymphatic nodules (nodular lymphoid tissue), although the presence of a central artery remains the distinguishing feature compared to lymphatic nodules/follicles in other lymphoid tissues [71]. Some branches of the arteries of the white pulp continue to supply capillary beds within the nodular lymphoid tissue (nodular arteries) [22].

Central arterioles send branches to the marginal sinuses, situated within marginal zones, and continue into the red pulp as penicillar arterioles [11,58,60,71]. Penicillar arterioles continue as arterial capillaries/pulp arterioles, some of which are enclosed by macrophages and a meshwork of reticular cells and fibres and are therefore called sheathed capillaries or ellipsoids [11,22,58,60,71]. Apart from a decreasing luminal diameter towards the sheathed capillary or ellipsoid side, the vascular endothelium is also cuboidal and the basal lamina discontinuous within this segment of the vasculature [22]. The specific functions of the ellipsoids are largely unknown although the fact that erythrocytes and lymphocytes can move within these vessels is suggestive of ellipsoid macrophage involvement in the trapping and phagocytosis of blood-borne antigens [7]. On the other hand, marginal zone macrophages are still prerequisite for the trapping of blood-borne antigens especially in animals that do not have ellipsoids, including mice, rats, guinea pigs, rabbits and marsupials (see Figure 2) [7]. In animals with sheathed capillaries or ellipsoids, they continue as unsheathed terminal capillaries [22].

The junction of the terminal capillaries with the venous system within the spleen has caused some controversy which resulted in the closed - versus open theory debate. The closed system is characterised by terminal capillaries which open directly into the splenic sinuses or venules whereas in the case of the open system, the capillaries purportedly open into spaces interspersed between the reticular cells of the red pulp, with blood further entering the venous sinuses through the slits in their walls [22]. Open circulatory flow exists in the human, cat and mouse spleens and is classified as nonsinusal. Sinusal spleens, as in the dog and rat, seem to have a combination of closed and open systems depending on the animal's physiological state. The spaces between endothelial cells lining the sinuses and/or venules within a distended spleen are pulled apart with blood escaping from the open meshwork from

the terminal capillaries into the sinuses and/or venules, whereas the endothelial cells lining sinuses/venules within a contracted spleen are pushed together to form a continuous connection with the terminal capillaries [22]. Therefore it would seem that in the dog, most of the arterial circulation passes directly to the red pulp splenic sinuses; however a portion also arrives within the meshwork of reticular cords in the red pulp [76,77,87]. Efferent blood within the sinuses enters the trabecular veins which then converge at the hilus as the single splenic vein [11,71].

Lymphatic ducts are mainly found within the white pulp near the trabeculae and allow lymphocytes to exit the spleen on demand [71]. The spleen therefore has efferent but no afferent lymphatic supply. Non-myelinated fibres from the celiac plexus and major splanchnic nerves are responsible for splenic innervation [60].

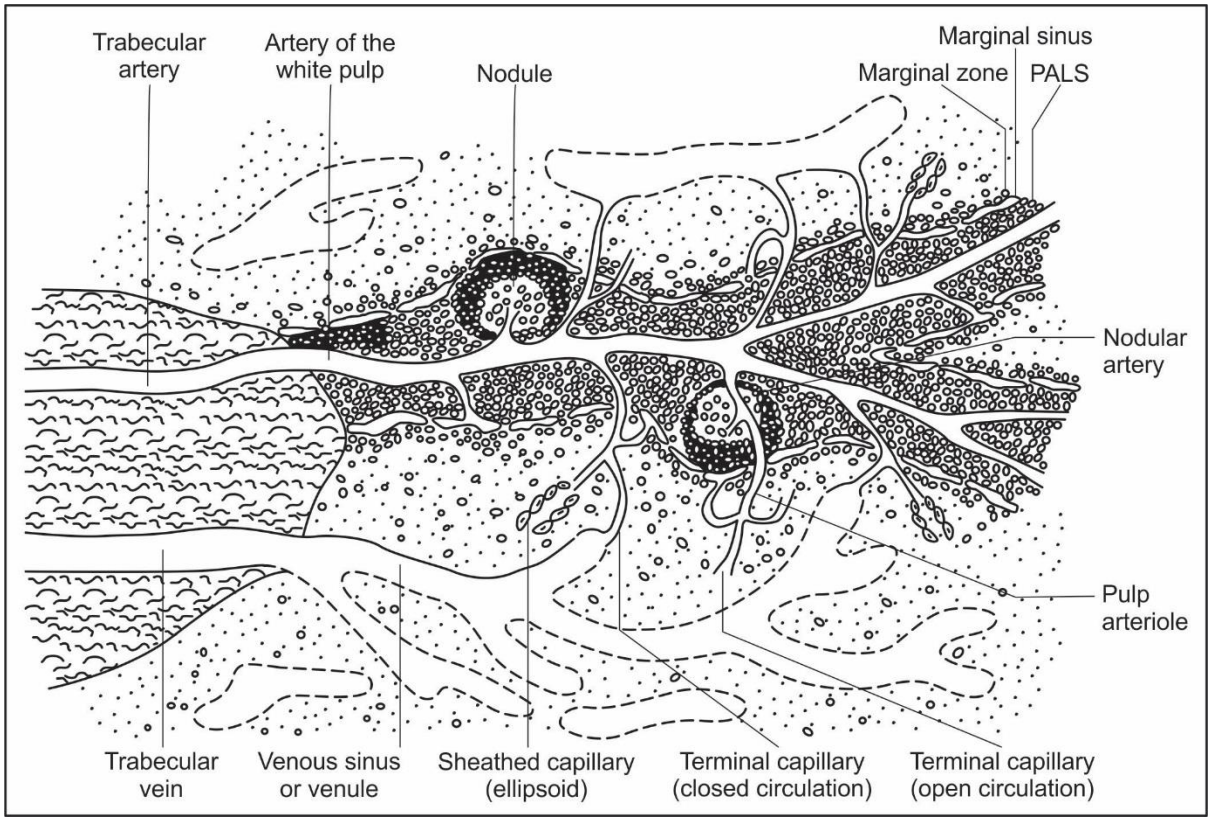

Figure 1: A generic schematic diagram illustrating splenic vasculature (adapted from Eurell JA, Frappier BL: Dellman's Textbook of Veterinary Histology. In: Eurell JA, Frappier BL, eds. *Dellman's Textbook of Veterinary Histology*. Sixth ed. Oxford, UK: Blackwell Publishing Ltd; 2006: 147-151 [22]).

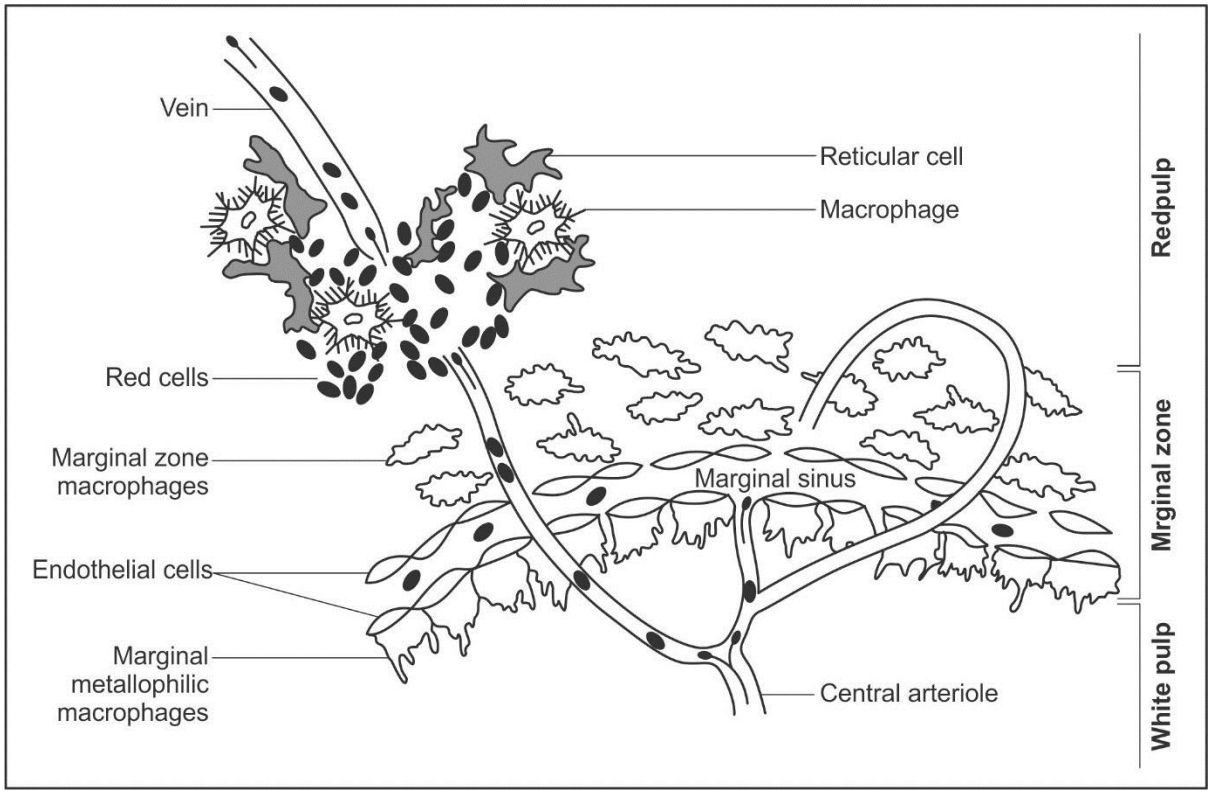

Figure 2: Schematic presentation of the mouse spleen indicating the white pulp, marginal zone and red pulp areas. Blood flow occurs from the central arteriole to the marginal sinus, marginal zone and red pulp. Here blood comes into contact with macrophages and other immune cells whereafter it exits via the vein. Note the absence of ellipsoids compared to figure 3 (adapted from Eurell JA, Frappier BL: Dellman's Textbook of Veterinary Histology. In: Eurell JA, Frappier BL, eds. *Dellman's Textbook of Veterinary Histology*. Sixth ed. Oxford, UK: Blackwell Publishing Ltd; 2006: 147-151 [22]).

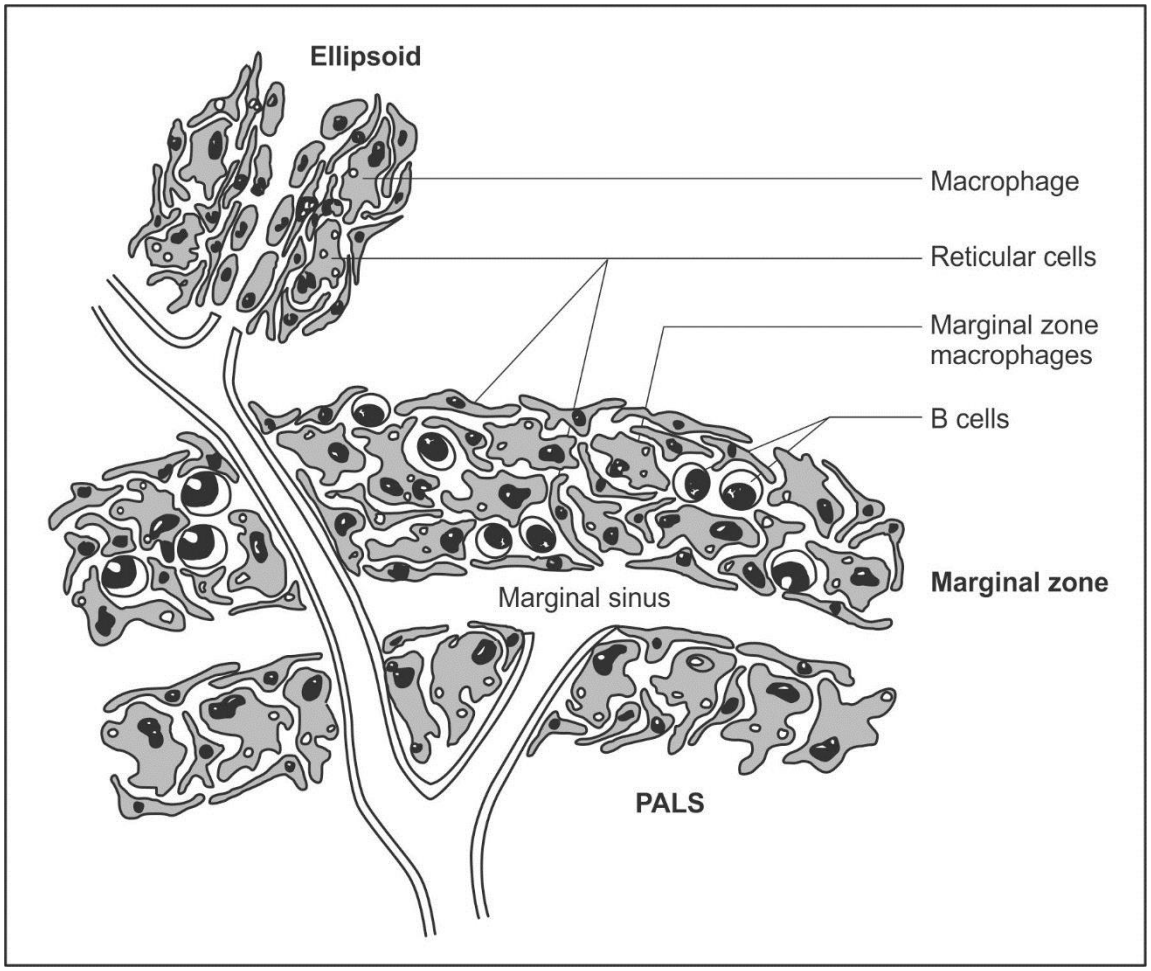

Figure 3: Schematic presentation of the dog spleen. Blood leaves the PALS, passes through a rim of marginal zone metallophilic macrophages and empties into the marginal sinus. Some capillaries pass through the marginal zone and terminate in red pulp ellipsoids (adapted from Eurell JA, Frappier BL: Dellman's Textbook of Veterinary Histology. In: Eurell JA, Frappier BL, eds. *Dellman's Textbook of Veterinary Histology*. Sixth ed. Oxford, UK: Blackwell Publishing Ltd; 2006: 147-151 [22]).

2.1.3.3. White pulp

The white pulp represent roughly 21-25% of the total splenic volume and consists of lymphoid tissue (B and T lymphocytes) within three basic compartments: The PALS, the follicles (germinal/follicular centre and mantle zone or corona) and the marginal zones [11]. The white

pulp constitutes a larger proportion of the total splenic volume in spleens more inclined toward defence i.e. human and mouse spleens. The white pulp also contains macrophages, dendritic cells and plasma cells within a reticular framework [11].

2.1.3.3.1. Periarteriolar lymphatic sheaths

The PALS surrounding the central arterioles consist of inner layers of small T lymphocytes (mainly CD3+ CD4+ T lymphocytes with some CD3+ CD8+ T lymphocytes) and outer layers populated by T and fewer B lymphocytes as well as a few macrophages within a meshwork of reticular cells and fibres [11]. T lymphocyte selection and maturation takes place within the thymus, which gives rise to single positive T lymphocytes that are either CD4+ or CD8+ depending on their expression of cell surface glycoprotein markers. CD4+ T lymphocytes activate cytotoxic T lymphocytes and macrophages, assist in the maturation of B lymphocytes into plasma cells and memory cells and assist other leukocytes in immunologic processes. The CD8+ T lymphocytes are cytotoxic and mainly responsible for the destruction of virus-infected or neoplastic cells. With antigenic stimulation plasma cells also populate the PALS [11].

2.1.3.3.2. Follicles

Primary lymphoid follicles have a uniform internal structure characterised by the absence of appreciable zones compared to secondary follicles which have light and dark zones within the follicular germinal centre [82-84]. The presence of secondary lymphoid follicles depends on the host's immunological status and they develop in secondary lymphoid organs after antigenic stimulations as occurs with infection or immunization [5,22]. Secondary lymphoid follicles consist of a light staining germinal centre with small and large B lymphocytes surrounded by a ring of dark staining small and occasionally medium-sized B lymphocytes called the mantle or corona. This area is surrounded in turn by a marginal zone of medium-sized B lymphocytes

characterised by prominent centrally located nucleoli and more cytoplasm compared to the mantle B lymphocytes [11,60]. T lymphocytes (CD4+ T lymphocytes only) are also found within the follicular centres, mostly within the light zones, although they are far fewer in number than B lymphocytes [11,60]. Upon antigen stimulation, plasmablasts within the white pulp in the follicles and PALS migrate into the red pulp just outside the marginal zone which ensures the rapid entry of antibodies into the bloodstream [58]. The B lymphocyte zones within the follicles are the site for B lymphocyte activation and clonal expansion [58].

2.1.3.3.2.1. Germinal centre

Germinal centres of secondary follicles are polarised into two zones, namely the light and dark zones. The dark zone is situated towards the periphery of the follicle closest to the PALS and contains darker-staining, often larger B lymphocytes that are undergoing differentiation, proliferation and mutation (usually referred to as centroblasts). The B lymphocytes on the opposite aspect of the germinal centre are lighter-staining and are available for antigen dependent selection (usually referred to as centrocytes) [82-84]. Centrocytes are small, cleaved lymphocytes with densely stained chromocenters whereas centroblasts tend to be larger cells with round or deeply clefted nuclei and peripheralised chromatin.

After infection or immunization the centroblasts within the dark zone undergo clonal expansion and hypermutation of B lymphocyte receptors [5,53]. After a few cycles the B lymphocytes with their mutated receptors move to the light zone for exposure to antigens. Apart from the centrocytes, the light zone also consist of a rich network of follicular dendritic cells able to take up antigens which they hold on their surface for extended periods of time until antigen presentation is required [5,53]. The follicular dendritic cell-associated antigen is displayed as an immune complex [5,53]. After the antigen/immune complex is taken up and

processed by B lymphocytes, it can be presented to CD4+ T lymphocytes [5,53]. Centrocyte selection is based on a centrocyte's ability to interact with its cognate antigen displayed by follicular dendritic cells [5,53].

2.1.3.3.3. Marginal zone

The marginal zone is situated at the junction of the red and white pulp and it is an important area with respect to antigen surveillance and processing (see Figures 3 and 4) [11]. Most of the white pulp is involved in adaptive immunity whereas the marginal zone is responsible for innate as well as adaptive immunity due to its macrophage, dendritic cell and B lymphocyte populations, amongst others [58]. The marginal zone blends in with the red pulp cords and receives white pulp capillaries as well as some terminal red pulp capillaries which terminate in the marginal sinus [22]. Thereafter these capillaries drain towards the venous sinuses as discussed earlier [22]. Apart from being a transit area, the marginal zone contains a large population of resident cells which, due to their interdependence, maintain the marginal zone integrity [58]. Marginal zone cells (mainly macrophages and B lymphocytes) and the marginal sinuses separate the white pulp from the red pulp [11,58].

2.1.3.3.3.1. Arrangement and macrophage populations

The inner layer of the marginal zone, immediately adjacent to the white pulp and peripherally delineated by the marginal zone sinus, consists of metallophilic macrophages, a unique subset of macrophages mainly responsible for antigen trapping. The metallophilic macrophages can be visualised with silver-staining or with the monoclonal antibody monocyte-and-macrophage-1 (MOMA-1) [11,58]. Just outside this layer of macrophages are the mucosal addressin cell adhesion molecule-1 (MADCAM1)-positive endothelial cells lining the marginal sinus. Peripheral to the sinus are the marginal zone macrophages, B lymphocytes, dendritic

cells and reticular fibroblasts [11,58,60]. The marginal zone macrophages that stain with monoclonal antibody anti-SIGN related 1 protein (ERTR-9) form the outer ring of cells contained within the marginal sinus [11,58]. Monocyte-and-marophage-1 (MOMA-1), MADCAM1+ and the ERTR-9 antibodies have been applied most extensively in studies on human and mouse spleens and to date, there have been no reports demonstrating their application to the dog spleen. Tissue-resident macrophages of which the metallophilic as well as marginal zone macrophages form part, express high levels of C-type lectin CD169 and F4/80 [30]. The exact function of the metallophilic macrophages is not known, yet the marginal zone macrophages are undoubtedly fundamental with regards to blood-borne pathogen clearance [11]. The marginal zone macrophages express PRRs such as the TLRs and macrophage receptor with collagenous structure (MARCO) which play a role in the uptake of bacteria [11]. Metallophilic macrophages have been known to express the adhesion molecule sialic-acid-binding immunoglobulin-like lectin 1 or sialoadhesin (SIGLEC1) which can bind to sialic-acid containing molecules on the surface of blood cells (for example glycophorin on the erythrocyte cell membrane) or sialic-acid residues on pathogen cell surfaces, which results in their concentration and subsequent removal from the blood [58].

2.1.3.3.3.2. Marginal zone B lymphocytes

These cells have unique properties when compared to follicular B lymphocytes and can be distinguished from one another by their expression of various molecules. Marginal zone B lymphocytes express high levels of IgM, CD1d, CD9, CD21 and CD22 and low levels of IgD, CD23 and B220 [56]. Follicular B cells express high levels of IgD, lower levels of IgM, CD21, CD22 and do not express CD1d or CD9 at all [56].

2.1.3.3.3.3. Human perifollicular zone

Apart from lacking a marginal zone sinus, the human spleen contains an additional compartment outside the marginal zone called the perifollicular zone due to its juxtafollicular location. Blood within this compartment belongs to the open splenic circulation and represents the entry compartment for recirculating lymphocytes into the white pulp, a feature unique to human spleens, the significance of which has not yet been clearly established [83].

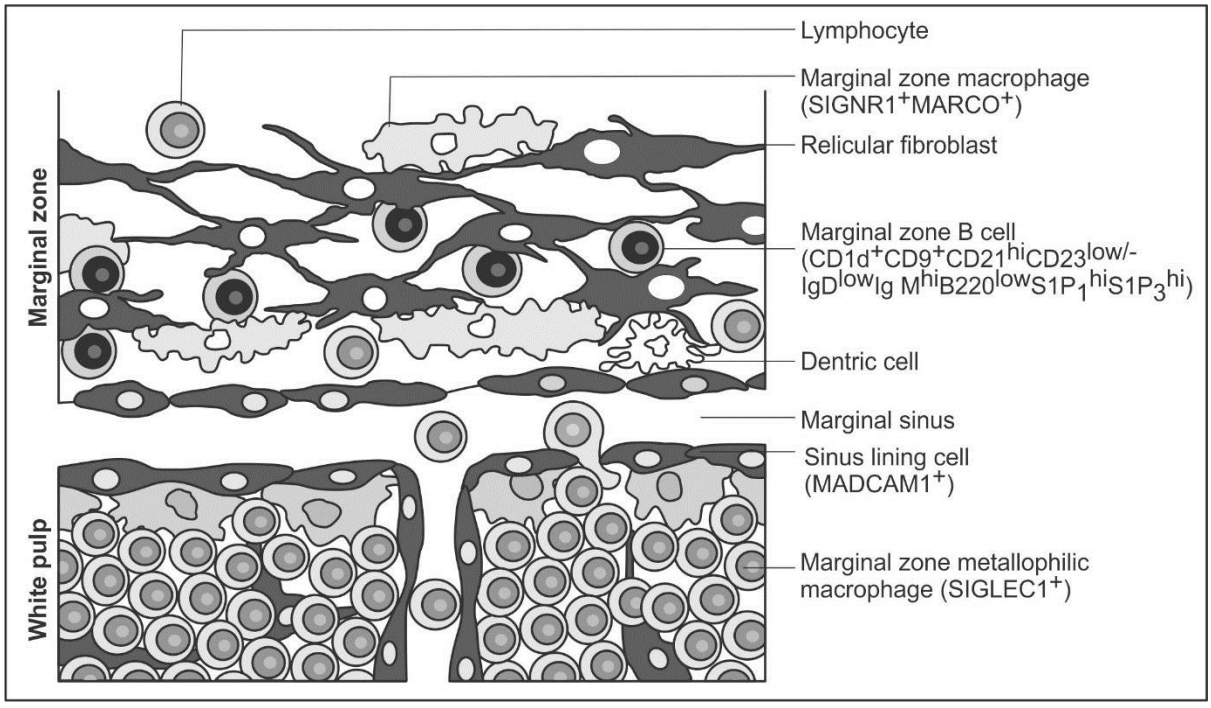

Figure 4: A diagram illustrating the cellular components within the marginal zone (adapted from Mebius RE, Kraal G: Structure and function of the spleen. *Nat Rev Immunol* 2005:5(8):606-616 [58]).

2.1.3.4. Reticular framework

The reticular framework within the spleen is characterised by a meshwork of reticular cells and reticular fibres. Not only does this framework lend structural support to the spleen but more importantly, it forms a distinct microenvironment specifically within the white pulp

(PALS, follicle and marginal zone) [73,88]. The most important contribution emanating from the existence of this microenvironment is related to its ability to assist with chemokine and cytokine homing of diverse white pulp cellular populations [73,88]. This allows for the correct organisation and maintenance of the different areas/zones within the white pulp as well as the interdependence between areas/zones.

According to Tanaka et al (1996) and Satoh et al (1997), the reticular framework in mice and humans have similar characteristics [73,88]. Within the PALS of both humans and mice, several layers of reticular fibres are distributed parallel to the central arterioles [73,88]. Similarly, in the follicles the reticular meshwork is sparse, whilst that of the marginal zone is fine with an intricate and anastomosing arrangement [73,88]. Not much information is available on the arrangement of the reticular framework in the dog spleen. Das et al (2012) looked at the histology of the spleen in the domestic dog and they documented varying densities and reticular fibre arrangements depending on the location within the spleen [16]. Reticular fibres around the central arterioles in the dog are described as coarse, numerous and closely arranged [16]. As previously mentioned, dog and camel spleens are primarily classified as storage spleens with both open and closed blood flow systems depending on the physiological state of the host. The reticular framework of the camel's spleen concurs with that of humans and mice which therefore suggest the possibility of a similar arrangement in dogs [73,88,108].

2.1.3.5. Homing cytokines and chemokines

Specific cytokines with an attractant effect on T and B lymphocytes are responsible for the correct organization and maintenance of the white pulp architecture and therefore function [44]. Chemokine C-X-C-motif ligand 13 (CXCL13), produced by CD35+ follicular dendritic cells and their surrounding stromal cells, ensures the migration of B lymphocytes to the follicles.

On the other hand chemokine C-C-motif ligand 19 (CCL19) and chemokine C-C-motif ligand 21 (CCL21), produced mainly by T lymphocyte zone stromal cells and some dendritic cells, attract T lymphocytes and dendritic cells to the T lymphocyte zone [44,58].

Plasmablasts have their own unique set of chemokine receptors and interactions which include chemokine C-X-C- motif receptor 4 (CXCR4), chemokine C-X-C-motif ligand 12 (CXCL12), chemokine C-X-C-motif receptor 5 (CXCR5) and chemokine C-C-motif receptor 7 (CCR7) which assist the regulation of plasmablast migration [58].

As in other regions of the white pulp, chemokines and other molecules (e.g. adhesion molecules) also play a very important role with regards to the localization and maintenance of the marginal zone. Marginal zone macrophages depend on CCL19 and CCL21 for their localization, whereas without CXCL13, a subset of dendritic cells within the marginal zone will be absent [106]. Although there must be chemokines that induce homing of the metallophilic macrophages, no specific ones have been identified as yet [58].

Studies have confirmed that without marginal zone specific B lymphocytes, metallophilic and marginal zone macrophages would disappear [58]. Macrophage receptor with collagenous structure (MARCO), expressed by marginal zone macrophages, in turn is responsible for the retention of marginal zone B lymphocytes [58]. Marginal zone B lymphocytes express both sphingosine 1-phospahte receptor 1 (SIP_1) and sphingosine 1-phosphate receptor 3 (SIP_3) and their expression is much higher in these lymphocytes compared to follicular B lymphocytes [58]. Sphingosine 1-phosphate receptor 1 (SIP_1) is prerequisite for the correct localization of B lymphocytes to the marginal zone [58]. In the absence of SIP_1, B lymphocytes will migrate to B-cell follicles [58]. Upon an encounter with an antigen, marginal zone B lymphocytes down-regulate SIP_1 and SIP_3 expression and thereafter relocate to the B lymphocyte follicles [58].

Adhesion molecules, called integrins are also involved in the retention of marginal zone B lymphocytes and these integrins interact with intercellular adhesion molecule 1 (ICAM1) and vascular cell-adhesion molecule 1 (VCAM1) [58]. It is now clear that down-regulation of not only the SIP receptors but also the integrins is necessary for marginal zone B lymphocyte relocation [58]. The localization and maintenance of these B lymphocytes requires extensive and complex molecular interactions, a principle applicable to the remaining splenic population as well [58].

2.1.3.6. Red pulp

In storage spleens, the red pulp represents roughly 75-79% of the total splenic volume and consists of a three-dimensional meshwork of splenic cords separated by venous sinuses [11,58,60,71]. These structures enable the spleen to filter blood and remove senescent erythrocytes [58]. Cords consist of fibroblasts, reticular fibres, reticular cells, dendritic cells and macrophages responsible for the structural support of the venous sinuses [11,58,60,71]. Lymphocytes, hematopoietic cells, plasma cells, erythrocytes, granulocytes and platelets may also be associated with the cords [11,58,60,71]. The red pulp macrophages are mainly responsible for the removal and metabolism of senescent and damaged erythrocytes as well as blood-borne antigens [11,71]. The venous sinuses are lined by loosely arranged endothelial cells (with no direct cell-to-cell contact other than through junctional structures) situated upon a basement membrane characterised by apertures or perforations [11,60,71]. The basement membrane is situated between the endothelium and red pulp reticular fibres, with the latter being intermingled with reticular cells, also within the red pulp [11]. The discontinuous endothelial and basement membrane layer results in the formation of slits or fenestrations which allow cells to pass in and out of the sinuses, so embodying the spleen's filtration

function [60,71]. Macrophage and dendritic cell processes also pass through these gaps to survey for the presence of foreign antigens [71].

2.1.3.6.1. Macrophages

Some of the earliest studies suggested that not only were the majority of tissue macrophage populations of blood monocyte origin but that this was also their main source of replenishment [95,96]. This statement was subsequently questioned because of observations which included evidence of local tissue macrophage proliferation as well as the presence of macrophages within the yolk sac before haematopoiesis [61,74]. A study by Sawyer et al in 1982 revealed evidence of self-replenishment of resident macrophages in mice subjected to radiation [74]. Amongst others, these observations led to our current understanding of phagocytic cell lineage divergence. The macrophages within the splenic red pulp are a subset of macrophages seeded before birth, which are maintained by local replenishment without any significant contribution from blood monocytes [32,105]. Apart from the resident tissue macrophages, the spleen also contains a large proportion of extravascular monocytes within the cords of the red pulp [86].

2.1.3.6.2. Dendritic cells

Dendritic cells play a crucial role in the priming of T lymphocyte responses. Dendritic cells express many PRRs (for example TLR) that recognise a variety of PAMPs and this in turn activates the innate immune system. Post TLR activation, inflammatory as well as anti-inflammatory cytokine production is stimulated which triggers dendritic cell maturation [21]. Dendritic cells are then able to migrate to the PALS and present T lymphocytes with antigen [21]. Four dendritic cell populations have been identified in the mouse and at least three populations in humans [21]. In the mouse there are two classical populations of dendritic cells

as well as a plasmacytoid population which appears to play a minimal role in parasite clearance [17]. These populations were classified based on their expression of cell surface molecules. CD8+ and CD8- with CD8- further classified into CD4+ and CD4- [21]. Humans have an interstitial population CD11c+ dendritic cells, Langerhans dendritic cells and plasmacytoid dendritic cells [17,21].

2.1.3.6.3. Erythrocyte metabolism

Another important function of the red pulp which parallels the filtering of the blood includes the removal and processing of old and damaged erythrocytes. Senescent or damaged erythrocytes find passage through the slits/fenestrations much more difficult due to their increased membrane rigidity and subsequently altered pliability [58]. These erythrocytes are phagocytosed by macrophages and hydrolysed by phagolysosomes within the macrophages [58].

In macrophages haemoglobin undergoes proteolytic degradation with the release of haem which is further catabolized into biliverdin, carbon monoxide and ferrous iron [58]. Small amounts of unused iron are stored as the cytosolic protein ferritin and larger amounts of unused iron as hemosiderin, an insoluble partially degraded ferritin complex [44]. Iron can also be used or released by cells if not stored and iron released from macrophages as ferritin can bind plasma transferrin which acts as a transporter protein [44]. Intravascular haemolysis causes haemoglobin to be released into the bloodstream [44]. Free haemoglobin is bound to haptoglobin which is in turn scavenged from the circulation by the spleen [44]. Pigments, especially iron pigments (e.g. hemosiderin) are therefore commonly found within macrophages in the red pulp, the result of erythrocyte breakdown [11]. Other pigments for example ceroid and lipofuscin from lipid oxidation can also be observed in the spleen [11].

2.1.3.6.4. Extramedullary haematopoiesis

Extramedullary haematopoiesis (EMH) is the formation of blood cells outside the designated niches in the medullary spaces of the bone marrow. Any stem cell niche needs to fulfil certain requirements in order for EMH to take place in that location. These requirements include the presence of an extracellular matrix to anchor stem cells and to provide a scaffold, blood vessels to supply nutritional support and neural input to integrate signals from various organs [38]. The spleen and liver are some of the most common extramedullary niches that satisfy these requirements and make them favourable sites for the reactivation of EMH [38]. The specific pathophysiology resulting in the occurrence of EMH in any niche encompasses one or a combination of the following; bone marrow failure (e.g. myelofibrosis) or stimulation (e.g. hypoxia), injury, inflammation or repair of tissues (e.g. splenitis, haematomas and thrombosis) and abnormal cytokine production, whether local or systemic (e.g. experimental overexpression of interleukin (IL)-5 in mice) [38]. Extramedullary haematopoiesis (EMH) in dog spleens occur in association with inflammatory conditions, neoplasia (e.g. histiocytic sarcoma, haemangiosarcoma) and anaemia (e.g. immune-mediated haemolytic anemia) [11,38]. Within the spleen, EMH most often occurs in the red pulp, intrasinusoidally as well as within the cords [38]. Species differences exist regarding the predilection for EMH, although this has not been extensively studied. It is well known that mice are the exception, because EMH occurs throughout their adult life [11,38].

2.2. THE SPLEEN IN HAEMOPARASITIC DISEASE

The spleen's location, its function and architecture all contribute to its remarkable role in disease [69]. Innumerable studies in humans and mice and some in dogs have confirmed the importance of the spleen by examining the outcome of a variety of diseases in splenectomised

patients [4,36,92]. The spleen is the major organ responsible for host defence against haemoparasites for example *Babesia spp., Ehrlichia canis* and *Anaplasma platys* infection in dogs and *Plasmodium falciparum, Babesia spp.* and *Anaplasma phagocytophilum* infection in humans [4,36,92]. There are similarities and differences in the macro- and microscopic pathology in a variety of organs, as well as in the mechanisms underpinning babesiosis in non-human and human malaria [15,45,54,68]. Therefore, it has often been suggested that babesiosis could serve as an animal model for some aspects of malaria research and vice versa [15,45,54,68]. The macro- and microscopic lesions seen in a number of organs in these two diseases are similar to those seen in a multitude of other diseases and are therefore not exclusively babesia – and/or malaria-specific. The main reason for this is that a wide variety of disease processes, whether they be induced by babesia, malaria, sepsis, trauma or burns are dominated by the host's response to the inciting cause, rather that the inciting cause itself [37]. It would appear that T lymphocytes and their associated pro-inflammatory cytokines are especially responsible for the hyperactivation of the host immune response in all of these conditions [13]. The blood flow within the sinuses/sinusoids induces splenic immunosurveillance with resultant antigen detection and an appropriate/inappropriate response [58,69].

2.2.1. The effect of malaria on the spleen

Malaria, a mosquito-borne infectious protozoan disease, is an important cause of death worldwide, but its most devastating effects are experienced in many of the poorest regions and countries, especially in sub-Saharan Africa [21]. Most human malaria is the result of infection with *Plasmodium falciparum* and continues to cause mortality and morbidity rates that are unacceptably high. Poor understanding of the pathophysiological mechanisms

underlying malaria, as well as what actually constitutes a protective immune response has delayed the development of vaccines and more effective treatments.

The spleen of a malaria-infected host is engaged in a variety of different tasks, including the removal of parasitized and damaged erythrocytes, stimulation of immune responses specifically within the white pulp, erythropoiesis and non-erythroid haematopoiesis [21].

2.2.1.1. Removal of parasitized and damaged erythrocytes

Following on the realization that the spleen was of critical importance in the clearance of malaria parasites, Engwerda et al (2005) suggested that the white pulp marginal zone as well as the red pulp, each with their respective macrophage populations were probably the two most important sites for this to occur [21]. Infected erythrocytes contain parasites which decrease erythrocyte deformability and increase their antigenicity [14]. The latter is the result of the insertion of neoantigens into the erythrocyte membranes [14]. An ultrastructural study on human spleens performed by Fujita et al (1985) showed that the spleen is able to remove solid material (Howell-Jolly bodies, Heinz bodies, intraerythrocytic parasites) during the passage of an erythrocyte through endothelial slits [26]. These solid bodies are then phagocytosed by red pulp macrophages in close proximity to the infected erythrocytes [26].

2.2.1.1. White pulp immune responses

Dendritic cells within the marginal zone of the white pulp are situated optimally to acquire antigen for immune stimulation [21,44]. The exact mechanism of antigen uptake remains unclear; however the antigen-presenting dendritic cells undergo regulated maturation and migrate into the T lymphocyte areas (PALS) to stimulate the immune system by activating antigen-specific CD3+ CD4+ T lymphocytes [21,44,51,65]. Mebius et al (2005) also suggested the

involvement of marginal zone B lymphocytes in the process of antigen uptake, presentation and activation of T lymphocytes [58]. Once adaptive immune responses against *Plasmodium spp.* have been initiated, primed B and T lymphocytes need to position themselves in order to function most effectively [21,44]. This explains why plasma cells can be found almost exclusively within the red pulp, allowing released antibodies quick access to the circulation [21]. Activated CD4+ T lymphocytes are responsible for the promotion of antibody-dependent cell-mediated cytotoxicity (ADCC) through inflammatory cytokine production (e.g. interferon (IFN)-γ) and therefore need to be located within the vicinity of phagocytosis in the red pulp, although specific migration to the red pulp is uncertain [21].

2.2.1.2. Extramedullary haematopoiesis

Although not exclusive to malaria, splenomegaly has been viewed a reliable macroscopic indicator of the presence of the disease [27]. Extramedullary hematopoiesis (EMH) is a major contributor to the observed splenomegaly in murine malaria [1,10]. Although it has not been shown in human malaria, murine malaria models have indicated an increased level of haematopoiesis in the spleen of infected mice [21]. The level of haematopoietic activity did however depend on the *Plasmodium* species involved and the associated level of virulence [21].

2.2.1.3. Architectural changes

Many architectural changes ensue in the spleen as a result of malaria infection. The majority of the malaria pathology studies concurred with regard to the observed microscopic changes. However, variations exist and is the result of a multitude of factors including host species (human versus mice versus rat) differences, *Plasmodium spp*. differences and the employed research methodology.

2.2.2. The spleen in canine babesiosis

Canine babesiosis is a tick-borne, infectious haemoprotozoal disease caused by various *Babesia spp.* in dogs [34]. Specific information regarding the effect of canine babesiosis on the macro- and microscopic appearance of the spleen is very limited. The results obtained from the limited number of babesiosis studies performed in cattle, mice and horses serve as the only reference point in relation to the splenic pathology caused by *Babesia spp*. without elaborating on histomorphological specifics [35,69,78].

Babesiosis, like malaria, is a multisystemic disease associated with specific pathology in a wide variety of organs. As with *Plasmodium spp.*, the infection in babesiosis is controlled by the destruction of parasitized erythrocytes or by parasite removal from erythrocytes by splenic macrophages, as well by the production of neutralising antibody directed against the extra- and/or intracellular parasite [9]. Most of the pathology caused by the babesia parasite, as is the case in many other diseases, can be explained by the host's inflammatory response to the parasite and not by direct parasite damage [37]. The innate immune response, for which cells in the red pulp are largely responsible, is cardinal for parasite clearance. As has been mentioned for malaria infection, dendritic cells act as professional antigen presenting cells because they are rich in PRRs which detect PAMPs and all of this takes place for the first time in the spleen [48,51,65]. As for the marginal zone macrophages, dendritic cells also express TLRs which enable the successful detection of parasites [79].

2.2.2.1. Architectural changes

Maegraith et al (1957) noted prominent splenomegaly in a dog with babesiosis and although they found this to be in accordance with changes observed in the spleen of humans infected with *P. falciparum*, the authors suggested that a significant degree of congestion contributed

to the splenomegaly [54]. *Babesia divergence*-infected Mongolian gerbils also exhibited splenomegaly due to increased erythropoiesis as well as increased macrophage densities within the red pulp [20]. Histologically, infected erythrocytes as well as free parasites can be seen within the red pulp and in blood vessels [54]

2.3. IMMUNOHISTOCHEMISTRY

Immunohistochemistry (IHC) entails the demonstration of a wide variety of cell, tissue or microbial antigens within tissue sections by means of specific antibody binding [66]. A coloured histochemical reaction visible by light or fluorescent microscopy demonstrates the specific sought-after antibody-labelled antigen [66]. Therefore the visualisation of specific cell types dependent upon labelling of target cell antigens is possible and IHC facilitates localization of specific antigens [66]. This contributes significantly to the understanding of disease pathogenesis [66].

The characterization and identification of cell markers present on different splenic cell subtypes has enhanced the understanding of overall splenic function within the immune system. Immunohistochemical investigations of the spleen have enabled the study of immune cell populations under normal circumstances as well as during infection [23]. Human and mouse splenic cells that have been studied immunohistochemically include: red pulp macrophages, T lymphocytes, B lymphocytes, metallophilic – and marginal zone macrophages, marginal and mantle zone B lymphocytes, dendritic cells and neutrophils [23]. Fewer cells have been studied immunohistochemically with success in animals (and more specifically in dogs) compared to humans and mice. This study made use of specifically selected cell marker antibodies which have been successfully optimized in dog tissues, including CD3, CD20, Pax-5, Mum1, Mac387, CD204 [23,26,31,33,97,101].

In light of the aforementioned review of the literature, the current study investigated the macroscopic, microscopic and immunohistochemical changes within the spleen of *Babesia rossi*-infected dogs. Additionally, speculation was supplied for the observed changes if and where possible based on the findings. Due to the myriad of similarities between malaria and babesiosis, the results from this study were compared with human and murine malaria study results.

2.4. AIM OF THE STUDY

The aim of this study was to describe the macropathology and histopathology as well as to characterise the proportion and distribution of different immune system cells in the spleen of dogs with *Babesia rossi* infection compared to spleens of healthy dogs without babesiosis.

3. CHAPTER THREE: MATERIALS AND METHODS

3.1. EXPERIMENTAL DESIGN

This was a prospective cohort case control study of dogs that died naturally due to babesiosis (specifically *Babesia rossi* infection) and/or dogs that were humanely euthanized at the owner's request due to financial and/or other constraints whilst suffering from babesiosis. The cohort of control dogs (4 in number) was humanely euthanized at the Onderstepoort Veterinary Academic hospital (OVAH), Faculty of Veterinary Science (FVS), University of Pretoria (UP). Ethical approval for this project was granted by the UP Ethics committee (V086-15).

3.1.1. Experimental animals: Inclusion criteria

Nine dogs of any breed, age, sex and body weight naturally infected with *Babesia rossi* that either died from the disease or were humanely euthanized at the owner's request, were included in the study. Infected animals were those positively diagnosed with the haemoprotozoal parasite on a thin peripheral blood smear. All cases were then proven retrospectively to be positive for a mono-infection of *B. rossi* and negative for infection with other *Babesia* species, *Ehrlichia* species, *Anaplasma* species and *Theileria* species by a polymerase chain reaction (PCR) and reverse line blot hybridization assay (RLB) [57]. All animals had complete post mortems performed and tissues were collected within an hour of death.

3.1.2. Experimental animals: Exclusion criteria

Dogs in the babesia group were excluded if:

- Any other blood parasites were identified on examination of the blood smear or PCR-RLB.

- A significant comorbid disease or condition was diagnosed at post mortem for example ancylostomiasis or poor body condition (body condition score of less than 3).
- The interim (period between death and post mortem) was greater than 12 hours or if the carcass was not immediately refrigerated prior to the post mortem examination.
- They had any haematological abnormality diagnosed on complete blood count (Appendix 1).

3.1.3. Control animals: Exclusion criteria

Dogs were excluded from the control group if:

- They had any blood parasite diagnosed at a molecular level.
- They had any haematological abnormality diagnosed on complete blood count (Appendix 1).
- Babesia parasites were observed on peripheral blood smear.
- They had any significant co-morbid disease.

3.1.4. Experimental animals: Source

Dogs were sourced from the OVAH and included 9 dogs naturally infected with babesiosis. We obtained informed owner consent for complete post mortem examination and for tissue utilisation for the study (Appendix 2 – Informed consent form).

3.1.5. Control animals: Source

The cohort of 4 control dogs was sourced from the OVAH and were healthy dogs admitted for elective euthanasia. The control dogs included in the study had informed owner consent to perform ante-mortem tests and all the dogs had owner consent for the post mortem examination. The 4 control cases were PCR-RLB negative for *Babesia, Ehrlichia, Anaplasma*

and *Theileria* species and routine haematology and biochemistry analysis results were available for these cases (in accordance with protocol V034-14).

3.1.6. Post mortem examination and sample collection

Post mortem examinations and sample collections were performed at the Section of Pathology, Department of Paraclinical Sciences (DPS), FVS, UP by Drs A Henning and CA Martin, with assistance from Prof A Leisewitz. Emphasis was placed on the precise and timeous collection of random but representative splenic samples. A single cubic (1cmx1cmx1cm), randomly selected splenic sample was collected per dog and placed in ample (at least 1 part tissue to 9 parts formalin) 10% buffered formalin for histopathology. The splenic macropathology was described and photographed for each experimental and control case.

3.1.7. Sample processing

The formalin-fixed samples were routinely sectioned into tissue cassettes within 4-5 days of collection to ensure the feasibility of immunohistochemical staining techniques. Each $1cm^3$ splenic sample was further sliced into 2-3mm thick, 0.5cm by 0.5cm wide sections and placed into tissue cassettes supplied by the histopathology laboratory, Section of Pathology, DPS, FVS, UP. Five such splenic sections from 5 different cases/controls were placed in 1 cassette which resulted in a total of 3 cassettes/wax blocks (9 test cases in 2 cassettes and 4 control cases in 1 cassette). Thereafter samples were processed, embedded, sectioned and stained according to Department of Agriculture, Forestry and Fisheries (DAFF)-accredited standard operating procedures (SOPs) in the histopathology laboratory, Section of Pathology, DPS, FVS, UP. The final product was 3 haematoxylin and eosin (HE)-stained tissue sections. In summary:

- 1 x (1cmx1cmx1cm) Piece of spleen sampled per dog
- 9 Experimental cases, 4 control cases
- 5 x (0.5cmx0.5cmx0.3cm thick) Pieces of spleen placed in 1 tissue cassette/wax block
- 2 x Tissue cassettes/wax blocks for the babesia-infected cases
- 1 x Tissue cassettes/wax blocks for the control cases
- Total of 3 tissue cassettes/wax blocks

3.1.8. Tissue sections

From each of the 3 wax blocks the following sections were produced for examination:

- 1 x Haematoxylin and eosin (HE)-stained tissue section per block = total of 3 HE-stained sections
- 1 x Gomori's reticulin impregnation (GRi)-stained tissue section per block = total of 3 GRi-stained sections
- 6 x Immunohistochemistry (IHC)-labelled tissue sections per block = total of 18 IHC-labelled tissue sections

3.1.9. Histopathological examination

All of the experimental as well as control cases were of optimal microscopic quality to allow examination and interpretation. The HE-stained splenic sections were examined using a light microscope at the Section of Pathology, DPS, FVS, UP. Histopathological evaluation was supported by check-lists (Appendix 3 – Histopathology check-list), compiled with the aid of the co-supervisor, which limited reader error due to inexperience. All sections were perused and checked by the co-supervisor in conjunction with the primary investigator. The combination of macro- and histopathology as well as the use of special stains (GRi for the

splenic reticulin framework) and IHC resulted in complete description of the splenic pathology in babesiosis compared to normal healthy control dogs. Splenic extramedullary haematopoiesis (EMH) was graded on a scale of 0 to 3+ based on counting the number of normoblasts over an average of 10 high power fields (HPFs) (400x magnification) per spleen sample as described by O'Keane et al [62]. 1+ Indicated an average number of 1-5 normoblasts per HPF and 2+ indicated an average of 6-10 normoblasts per HPF whereas 3+ indicated more than 10 such cells per HPF [62] .

3.1.10. Immunohistochemical examination

The wax-embedded tissue sections adhered to positively charged superfrost glass slides were placed in a 40°C oven overnight. Thereafter slides were placed in xylene for 10 minutes and then rehydrated in 100%, 96% and 70% ethanol, respectively for 3 minutes each time. Slides were rinsed in distilled water 3 times and placed in 3% hydrogen peroxide in methanol to quench endogenous peroxidase activity for 15 minutes. Slides were microwaved in citrate buffer (pH6) at 96°C and thereafter allowed to cool down on the bench at room temperature. Slides were rinsed in distilled water 3 times followed by 0.1 molar (M) phosphate buffered saline (PBS), pH 7.6, containing 0.1% bovine serum albumin (BSA) buffer solution, rinsing for 10 minutes followed by incubation with the primary antibody (Table 1). Slides were again rinsed 3 times in distilled water followed by 10 minutes in PBS –BSA. The slides were further treated according to BioGenex kit instructions. Slides were again rinsed 3 times in distilled water, repeated 3 times and then rinsed in PBS-BSA buffer for 10 minutes. Using the diaminobenzidine (DAB) kit, slides were checked under the microscope then counterstained with Mayer's haematoxylin for 20 seconds. Lastly, the slides were rinsed under running tap water for 10 minutes, dehydrated through increasing ethanol concentrations, cleared in

xylene, mounted in Entallen and coverslipped. In all cases, positive labelling was brown in colour.

- The following antibodies were used

Table 1: Antibodies and immunohistochemical methods.

Antibody	**Expression/function**	**Animal source**	**Manufacturer**	**Dilution**	**Antigen retrieval HIER***	**Incubation time (minutes)**	**Detection system****
CD3	T lymphocyte co-receptor, restricted in its expression to T lymphocytes [23].	Rabbit polyclonal	Dako Catalogue number: A0452	1:600	Citrate, pH6	60	BioGenex
CD20 (Clone L26)	A transmembrane phosphoprotein expressed predominantly in more mature, circulating B lymphocytes [39]. Also expressed in normal dog and bovine plasma cells (personal observation - S.J Clift 2015).	Mouse monoclonal	Dako Catalogue number: M0755	1:700	EDTA, pH9	60	BioGenex

Antibody	**Expression/function**	**Animal source**	**Manufacturer**	**Dilution**	**Antigen retrieval HIER***	**Incubation time (minutes)**	**Detection system****
Pax-5	B lymphocyte specific activator protein expressed in the early stages of B lymphocyte development, continues to be expressed in the late stages of B lymphocyte differentiation, but not in plasma cells [33].	Mouse monoclonal	BD Bioscience Catalogue number: 610863	1:50	Citrate, pH6	60	BioGenex
Mum1	Interferon regulatory factor family (factor 4) expressed in the final stages of B lymphocyte maturation towards and including plasma cells [33].	Mouse monoclonal	Dako Catalogue number: M7259	1:50	EDTA, pH9	120	BioGenex

Antibody	**Expression/function**	**Animal source**	**Manufacturer**	**Dilution**	**Antigen retrieval HIER***	**Incubation time (minutes)**	**Detection system****
Mac387 (Clone MAC387)	Detects myelomonocytic antigen including a subset of monocyte-macrophages of bone marrow origin and circulating mature myeloid cells [97].	Mouse monoclonal	Source: Dako Catalogue number: M0747	1:800	EDTA, pH9	60	BioGenex
CD204 (Clone SRA-E5)	Scavenger receptor class A expressed on resident/tissue macrophages [40].	Mouse monoclonal	Transgenic Inc.	1:400	Citrate, pH6	60	BioGenex

*HIER = heat-induced epitope retrieval (microwave heat in citrate or ethylenediaminetetraacetic acid (EDTA) buffer solutions, pH of 6 and 9, respectively at 96°C).

**BioGenex Super Sensitive™ Polymer –HRP IHC Detection System (Code number QD420-Yike, BioGenex, Fremont, CA 94538, USA).

3.2. DATA ANALYSIS

The study was primarily descriptive at the microscopic and IHC level. The tissue sections/slides were scanned on an Olympus scanner at the University of Witwatersrand Medical School (VS120-S6-W slide loader system) for the generation of virtual slide images that allowed the application of analytic software (see Figure 5).

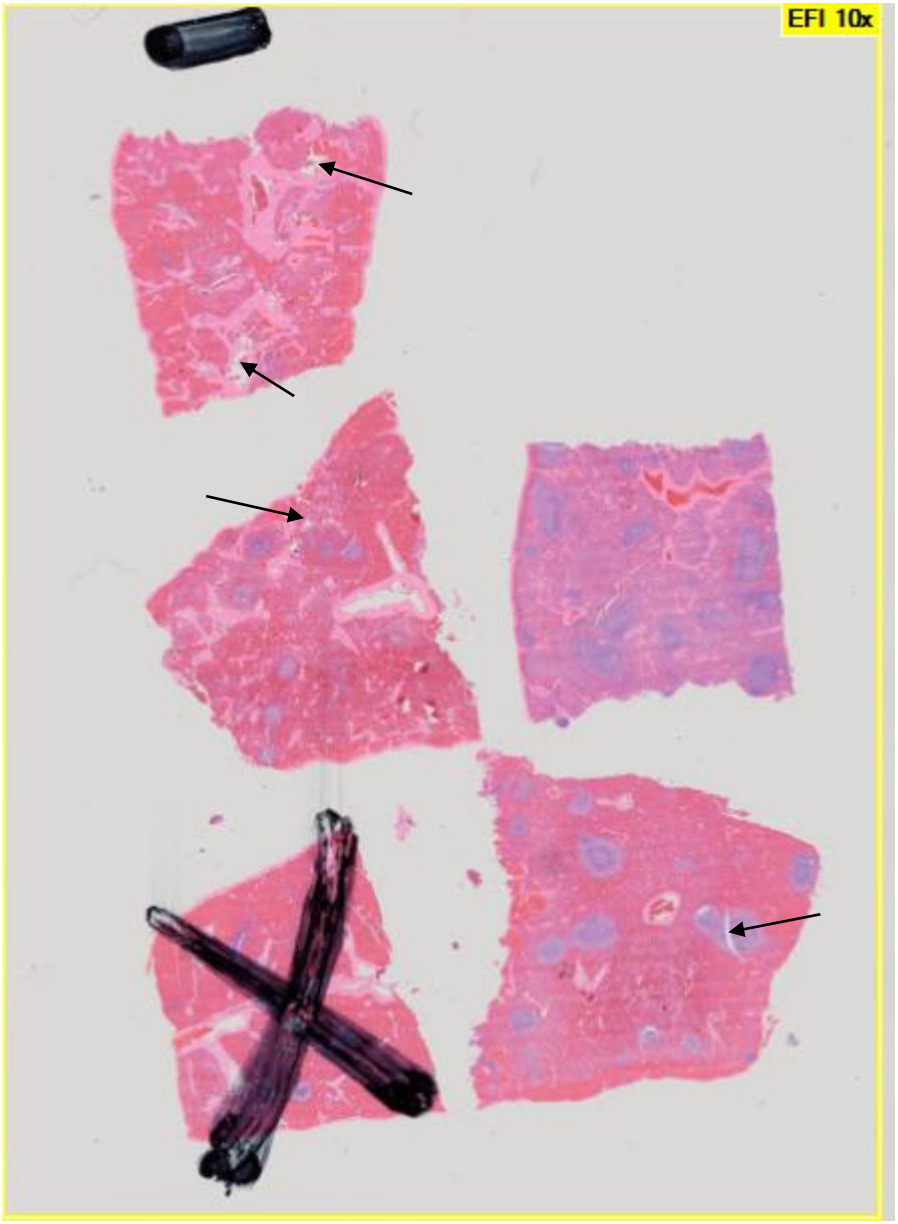

Figure 5: Example of a virtual slide image. The image represents 4 HE-stained sections of spleen (approximately 0.5cm x 0.5cm) each from the 4 control cases (control case number 1

indicated in koki pen, top left). Notice the artifactual spaces and cracks as indicated by line arrows; these areas were specifically excluded during delineation of the respective regions of interest (ROI) per case. The crossed-out tissue section represented one of the cases initially excluded due to poor tissue processing and/or staining.

Figure 6: Example of a virtual slide image. The image represents 3 HE-stained sections of spleen (approximately 0.5cm x 0.5cm) each from 3 experimental cases. This image solely serves as an example of more obvious (compared to Figure 5) artifactual spaces and cracks as

indicated by line arrows which were specifically excluded during delineation of the respective ROI per case. The crossed-out tissue sections represented cases initially excluded due to poor tissue processing and/or staining.

The application of the Count and Measure function from the corresponding Olympus cellSens Dimension software (Olympus CellSens V, Olympus, Japan) enabled analysis of the virtual slide images. Each slide/image was subjected to the channel separation function which allowed maximum contrast and increased visibility for improved detection and analysis of IHC staining. A single ROI was delineated per section of tissue representative of either a test or a control case (see Figure 8). The ROI was set to include the largest possible tissue area whilst attempting to exclude avoidable artefacts such as slits, spaces and areas of uneven staining as well as tissue folds (see Figures 5, 6 and 8). The tissue sections per slide were numbered in a top-to-bottom and left-to-right manner which promoted uniformity with regards to execution of the analyses.

Figure 7: This is the same slide as in Figure 5 but CD20 IHC has been performed on the section. The process of tissue numbering is illustrated (left-to-right and top-to-bottom) in both the slide as well as in a neighbouring schematic diagram.

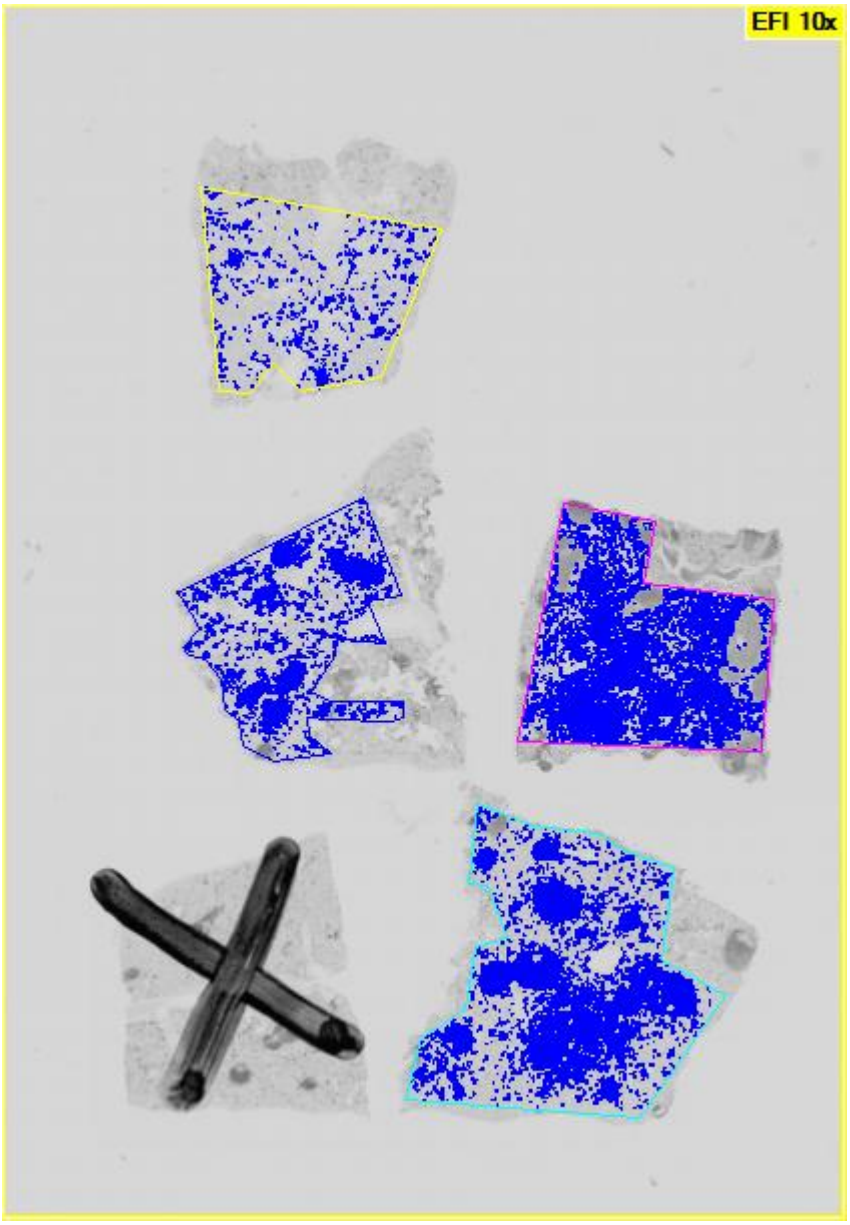

Figure 8: Same slide as seen in Figures 5 and 7 but the ROIs are delineated per section/case as demonstrated by the polygonal areas containing blue variably-sized spherical foci. The largest possible tissue area was included minus any significant slit and fold artefacts (compare to Figure 5). The blue variably-sized spherical foci represent positive IHC labelling as detected by the computer software in each section.

On completion of ROI selection per slide, the Manual Threshold and Count and Measure ROI functions were used to determine the percentage of positive labelling per section per cell marker/antibody per slide. Results for each ROI were correlated with the case number (control and experimental) and tabulated in an Excel spreadsheet (Appendix 4 – Excel spreadsheet).

3.2.1. Statistics

A statistical software package was used for the statistical analysis (SPSS, version 24, IBM). A non-parametric statistical test (Mann-Whitney U) was performed to compare the medians between groups as the samples sizes were small and the Shapiro-Wilk test criterion for normality was not satisfied.

3.3. RECORD KEEPING

The clearly marked formalin-fixed tissues and tissue wax blocks are stored by Prof A Leisewitz in a designated storage facility at the OVAH, FVS, UP. The clearly labelled HE-stained slides are routinely archived in the histopathology laboratory, Section of Pathology, DPS, FVS, UP. All samples will be stored for at least 10 years. All documentation is stored electronically on computers belonging to the primary investigator and supervisors at the FVS, UP and on at least 2 additional back-up storage devices.

4. CHAPTER FOUR: RESULTS

4.1. GROSS PATHOLOGY

4.1.1. Control cases

The macroscopic appearance of the spleens from the healthy dogs conformed to the norm; dark red, elongated, located vertically against the left abdominal wall, with a consistency that resembled normal liver and all were flattened dorso-ventrally on cross section with sharp edges all round (see Figure 9).

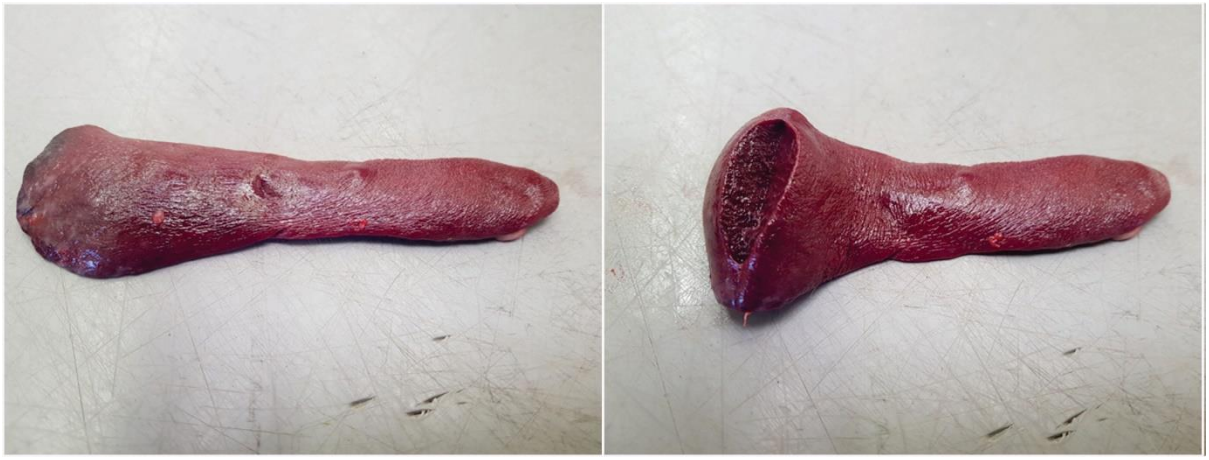

Figure 9: Control case number (no) 3. The normal spleens were dark red, elongated, flattened dorso-ventrally on cross section with sharp edges and a consistency that resembled normal liver.

4.1.2. Experimental cases

The macroscopic appearance of the spleens from the babesia-infected dogs appeared similar to one another; characterised by mild enlargement whilst maintaining an elongated shape (see Figure 10). Splenic enlargement was denoted by an increase in dorso-ventral thickness, an increase in length and edges that were rounded. External palpation of the intact spleens revealed a diffuse soft consistency. On cut surface, all the spleens revealed moderate red pulp

(RP) hyperplasia as evidenced by protrusion of soft, pulpy tissue with no clearly discernible white pulp (WP) areas/tissue and no significant ooze of blood from the cut surface (see Figure 10).

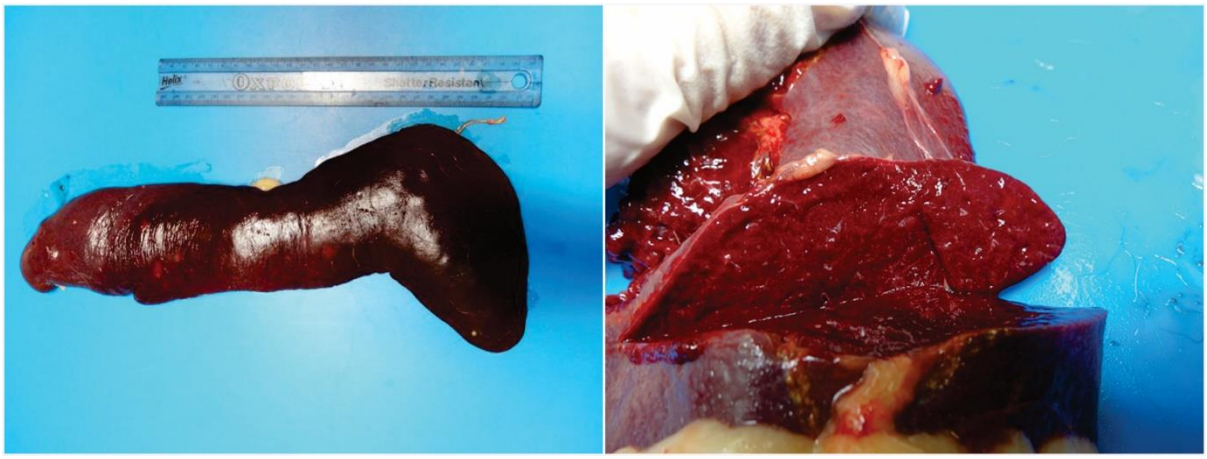

Figure 10: Experimental case no 1. Babesiosis spleens were elongated with mild enlargement characterised by increased dorso-ventral thickness and rounded edges. On cut surface, there was a moderate red pulp hyperplasia.

4.2. HISTOPATHOLOGY

4.2.1. Control cases

4.2.1.1. Demarcation of white pulp and red pulp

The majority of the control cases (CCs) displayed clear demarcation of the splenic tissue into its respective compartments; WP (periarteriolar lymphatic sheaths (PALS), follicles (germinal centres and mantle zones) and marginal zones) versus RP (cords and venous sinuses) (see Plate 1: Figures 11, 12 and 13). Despite the obvious lymphoid follicles, clear mantle zones could only be observed in two of the cases (numbers 3 and 4) (see Plate 1: Figure 12) whereas the line of delineation between the WP and the RP, was in all cases, represented by cells specific to the marginal zone (lymphocytes and macrophages) (see Plate 1: Figure 12). One of

the cases (number 1) contained no discernible follicles and small PALS (see Plate 1: Figure 13). However the WP was still clearly demarcated from the adjacent RP. Arteries of the white pulp (central arterioles) were easily identified, intact and they accommodated only a few erythrocytes (see Plate 1: Figures 11, 12 and 13).

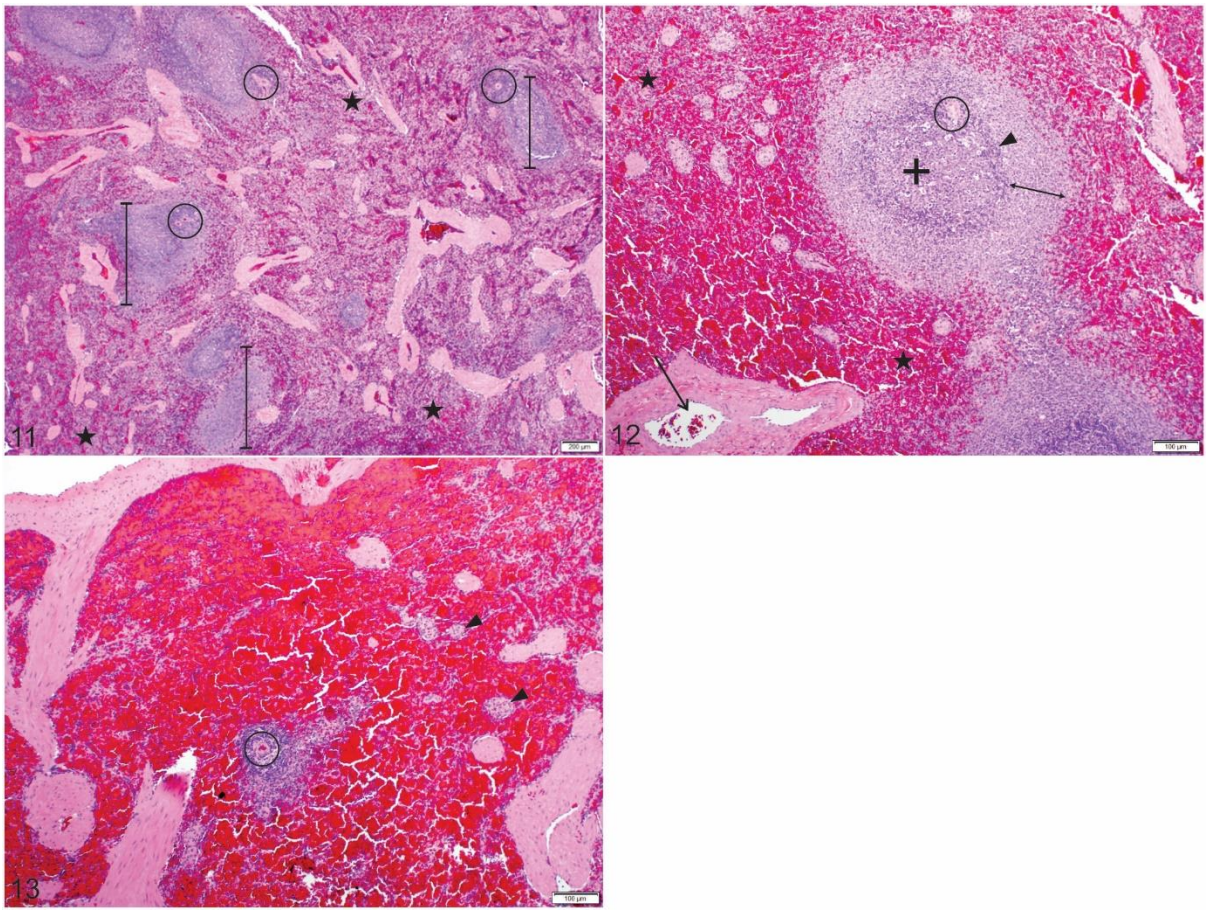

Plate 1: Control cases: Demarcation of white pulp and red pulp.

Figure 11: Control case no 3, haematoxylin and eosin (HE). There is a clear demarcation of white pulp (brackets) and red pulp (stars). Arteries of the white pulp (central arterioles) are easily observed (encircled). **Figure 12:** Control case no 4, HE. Well-circumscribed white pulp areas surrounded by red pulp (stars). Also visible is a large trabecular artery (arrow) containing some erythrocytes. Mantle cell cuffs (arrow head), marginal zone (double arrow), central arteriole (encircled) and germinal centre (cross). **Figure 13:** Control case no 1, HE. The

white pulp is small consisting mainly of the periarteriolar lymphatic sheath present around the central arteriole (encircled) with no obvious follicular development. White pulp and red pulp demarcation is still obvious. Ellipsoids (arrowheads).

4.2.1.2. Plasma cell distribution

The majority of the normal spleens displayed variable numbers of plasma cells within the following locations: RP and WP (PALS, follicular (germinal centre and mantle zone) and marginal zone). Within the RP, the plasma cells were more often observed surrounding the trabeculae (see Plate 2: Figures 14 and 15).

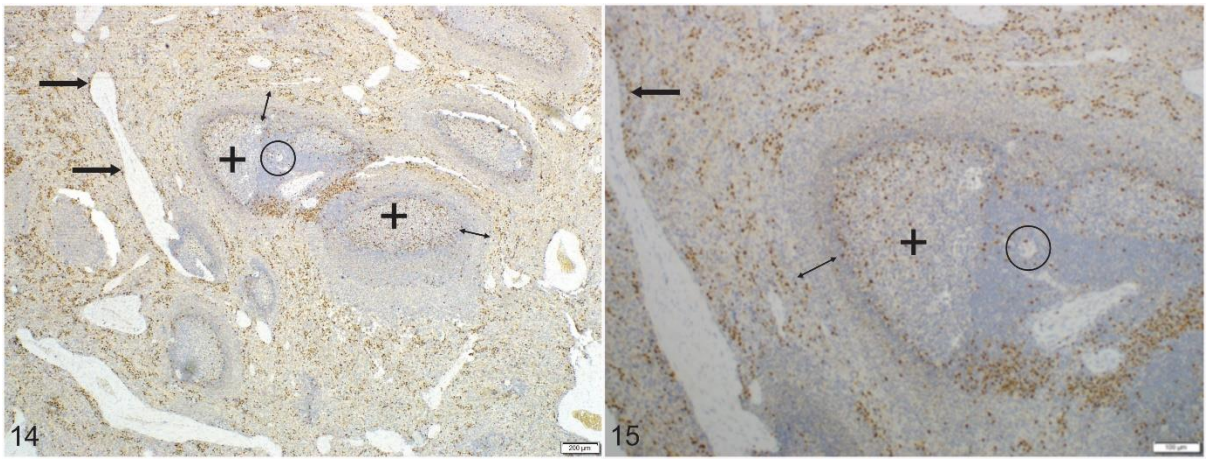

Plate 2: Control cases: Plasma cell distribution.

Figure 14: Control case no 3, immunohistochemistry for Mum1. Immunoreactive cells are observed within the red pulp, germinal centres (crosses), peri-arteriolar lymphatic sheaths (around the encircled central arteriole) and marginal zone (double arrows). Plasma cells are often observed neighbouring trabeculae (block arrows). Central arteriole (encircled). **Figure 15:** Control case no 3, immunohistochemistry for Mum1. Immunoreactive cells are seen in peritrabecular location (block arrow), in peri-arteriolar lymphatic sheaths (scattered positive

cells around the encircled central arteriole), germinal centre (cross) and marginal zone (double arrow).

4.2.1.3. Red pulp

The majority of the CCs revealed severe, diffuse congestion with only one of the cases (number 3) exhibiting mild congestion (see Plate1: Figures 11, 12 and 13). Two control spleens (numbers 1 and 2) exhibited trabeculae in close apposition (see Plate 1: Figure 13). In all control spleens the trabecular arteries were clearly visible and appeared variably congested (see Plate 1: Figure 12 and Plate 3: Figure 16). Sheathed capillaries or ellipsoids were prominent in three of the cases (numbers 1, 2 and 4) but not in the remaining control spleen (number 3) (see Plate 1: Figure 13). Grade 1+ extramedullary haematopoiesis (EMH) was observed in all the CCs.

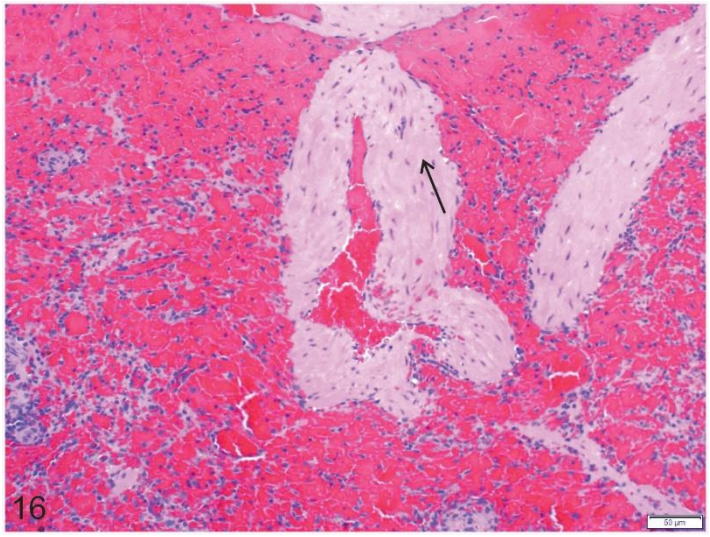

Plate 3: Control cases: Red pulp.

Figure 16: Control case no 1, HE. Large congested trabecular artery with no intraluminal leukocytes visible.

4.2.1.4. Gomori's reticulin impregnation

In all of the CCs, the Gomori's reticulin impregnation (GRi) stain was largely confined to the trabeculae, the periphery of the intervening blood vessels as well as minimal staining, without any specific pattern within some of the white pulp areas.

4.2.2. Experimental cases

4.2.2.1. Demarcation of white pulp and red pulp

The most significant histomorphological observation was characterised by the diffuse intermingling of WP and RP zones with a striking loss of distinction between the two compartments (see Plate 4: Figures 17 and 18). Although the majority of the experimental cases (ECs) revealed some remnant PALS and follicular structures, the latter did not contain any clearly discernible germinal centres, mantle – and/or marginal zones (see Plate 4: Figures 17 and 18). Even with the presence of remnant lymphoid tissue distinction of RP versus WP was difficult suggesting integration of WP and RP zones. Except for two cases (numbers 2 and 3), the WP arteries were still easily identified and accommodated only erythrocytes (see Plate 4: Figures 17 and 18).

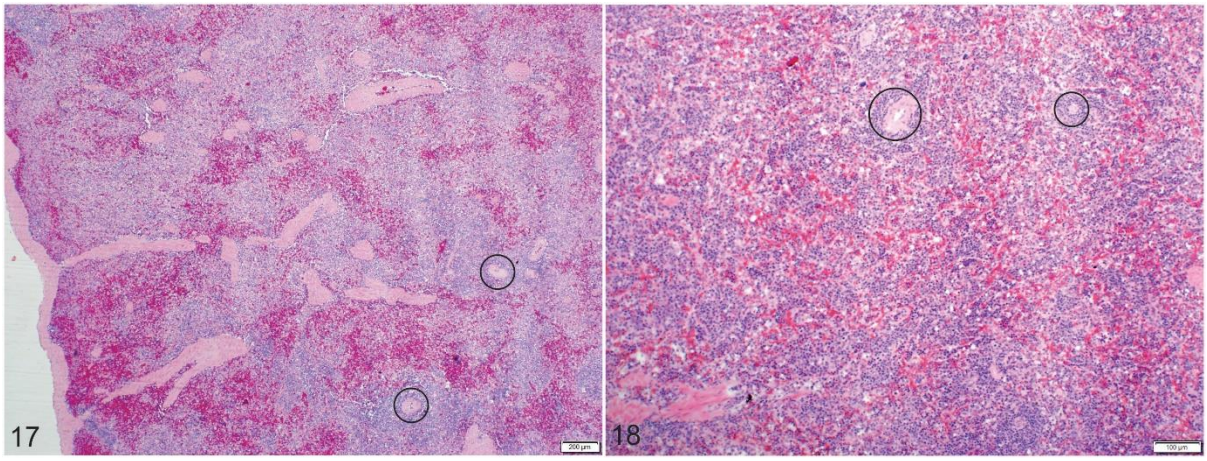

Plate 4: Experimental cases: Demarcation of white pulp and red pulp.

Figure 17: Experimental case no 6, HE. No clear demarcation between white and red pulp. Poorly delineated areas of white pulp are indicated by the presence of central arterioles (encircled). **Figure 18:** Experimental case no 7, HE. No distinction between white and red pulp. The presence of central arterioles (encircled) enables localisation of the remnant white pulp areas.

4.2.2.1. Plasma cell distribution

The vast majority of the plasma cells occurred throughout the splenic RP in the experimental cases and could no longer be readily identified within clearly distinguishable WP areas (see Plate 5: Figures 19 and 20).

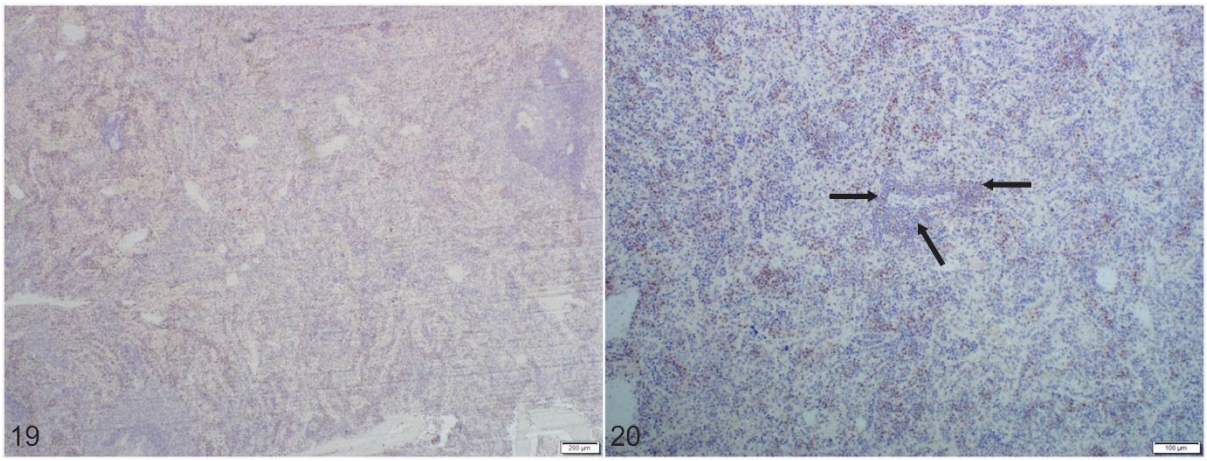

Plate 5: Experimental cases: Plasma cell distribution.

Figure 19: Experimental case no 8, immunohistochemistry for Mum1. Immunoreactive cells are scattered throughout the red pulp with few Mum1+ plasma cells seen in the residual white pulp areas. **Figure 20:** Experimental case no 7, immunohistochemistry for Mum1. Immunoreactive plasma cells are clustered throughout the red pulp with no Mum1+ cells seen in the residual white pulp areas (block arrows).

4.2.2.1. Red pulp

The majority of the ECs exhibited mild congestion. The evenly spaced trabeculae had inconspicuous trabecular arteries in three of the cases (numbers 1, 4 and 6) whereas in the remaining six cases the trabecular arteries were easily identifiable. The majority of the aforementioned revealed leukostasis characterised by lymphocytes, monocytes and plasma cells (highlighted by immunohistochemistry – Mum1) admixed with erythrocytes within arterial lumina (see Plate 6: Figure 21). All of the cases ellipsoids were inconspicuous (could not be detected). Two cases (numbers 1 and 8) displayed necrotic vasculature localised to central arterioles, characterised by outspoken fibrinoid vascular necrosis with associated nuclear pyknosis and karyolysis (see Plate 6: Figure 22). All of the ECs revealed a grade 3+ EMH.

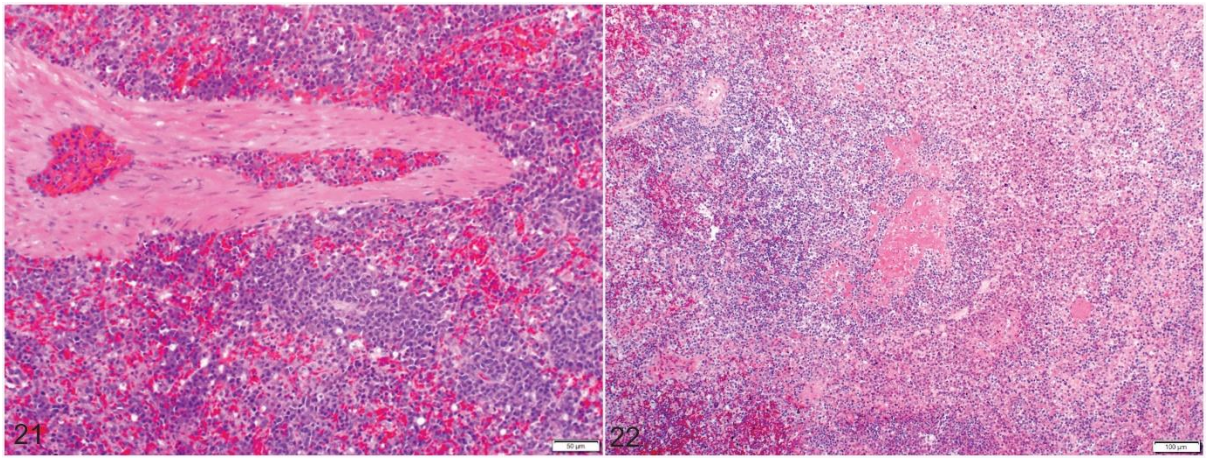

Plate 6: Experimental cases: Red pulp.

Figure 21: Experimental case no 7, HE. Large trabecular artery characterised by mononuclear leukostasis. **Figure 22:** Experimental case no 8, HE. Fibrinoid necrosis of the central arteriole characterised by hypereosinophilia and disintegration of the muscular tunic cytoplasm as well as nuclear pyknosis and karyolysis.

4.2.2.2. Gomori's reticulin impregnation

In all of the ECs, the GRi stain was largely confined to the trabeculae, the periphery of the intervening blood vessels as well as minimal staining, without any specific pattern within some of the white pulp areas.

4.3. IMMUNOHISTOCHEMISTRY

The application of the Olympus cellSens Dimension software determined the percentage of positive labelling per section per cell marker/antibody per slide and the results are listed in Table 2. The Mann-Whitney U test was performed to determine significance between the medians of the groups and significance was set at $P<0.05$ (see Table 2).

Table 2: Mean percentage and comparison across categorical study variables.

Case number	CC(1)*/EC**(2)	Mac387***	CD204***	CD3***	CD20***	Mum1***	Pax5***
1	1	6.69	16.87	6.45	10.28	13.71	7.95
2	1	7.95	14.46	14.67	19.96	21.87	11.87
3	1	5.73	7.17	23.9	28.56	34.8	22.16
4	1	7.27	28.9	22.93	25.84	26.56	15.35
5	2	33.76	37.55	27.59	12.47	21.03	7.15
6	2	18.11	35.96	29.84	12.18	21.66	6.08
7	2	15.05	17.09	22.19	12.19	10.41	14.1
8	2	11.76	38.33	15.54	14.73	3.82	2.65
9	2	17.97	19.34	25.99	16.52	3.71	6.42
10	2	17.64	25.14	18.37	16.93	14.47	7.49
11	2	9.6	37.04	30.81	29.69	23.12	12.76

12	2	14.75	28.4	33.48	19.06	15.78	7.92
13	2	24.45	21.09	34.23	34.2	35.45	13
P-values		**0.005**	**0.045**	0.064	0.643	0.217	0.064

*Control cases are denoted by the number 1 in the table and include case numbers 1 to 4.

**Experimental cases are denoted by the number 2 in the table and include case numbers 5 to 13.

***The values in each column represent the percentage of positive labelling per cell marker per control/experimental case.

4.3.1. Mac 387

The Mac387 antibody detects monocyte-macrophages of bone marrow origin as well as mature circulating myeloid cells (predominantly monocytes and neutrophils). Within the normal spleens the Mac387+ cells were mainly confined to the red pulp and fewer occurred in the marginal zone (see Plate 7: Figure 23). Mac387 labelling assumed a more diffuse pattern due to WP dissolution in the ECs (see Plate 7: Figure 24). Compared to the CCs, ECs also showed a significant increase in the proportion of Mac387 positive cells ($p = 0.005$). Of note was the lack of significant numbers of neutrophils on examination of haematoxylin and eosin (HE)-stained sections in both CCs and ECs.

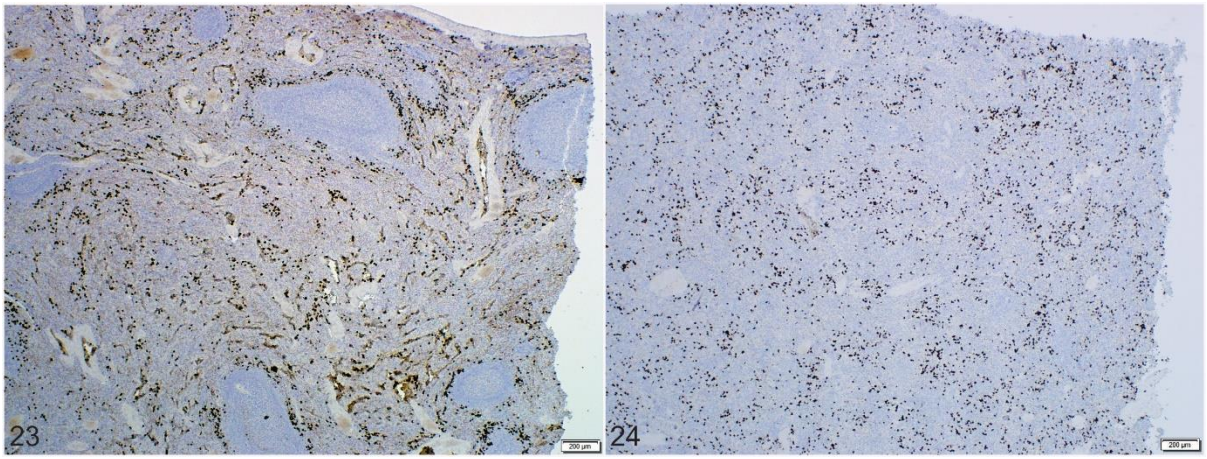

Plate 7: Immunohistochemistry: Mac 387.

Figure 23: Control case no 3, immunohistochemistry for Mac387. Immunoreactive cells are largely confined to the red pulp and marginal zones. **Figure 24:** Experimental case no 7, immunohistochemistry for Mac387. Immunoreactive cells are diffusely scattered throughout the spleen section.

4.3.2. CD204

The CD204 antibody is mainly expressed on tissue/resident macrophages. CD204+ cells were exclusively confined to the RP in both the CCs as well as the ECs (see Plate 8: Figures 25, 26, 27 and 28). The location of the CD204+ macrophages within the RP, of especially the ECs, highlighted the WP dissolution by accentuating the decrease in WP size. A significant increase in the proportion of CD204+ cells was observed in ECs compared to CCs ($p = 0.045$).

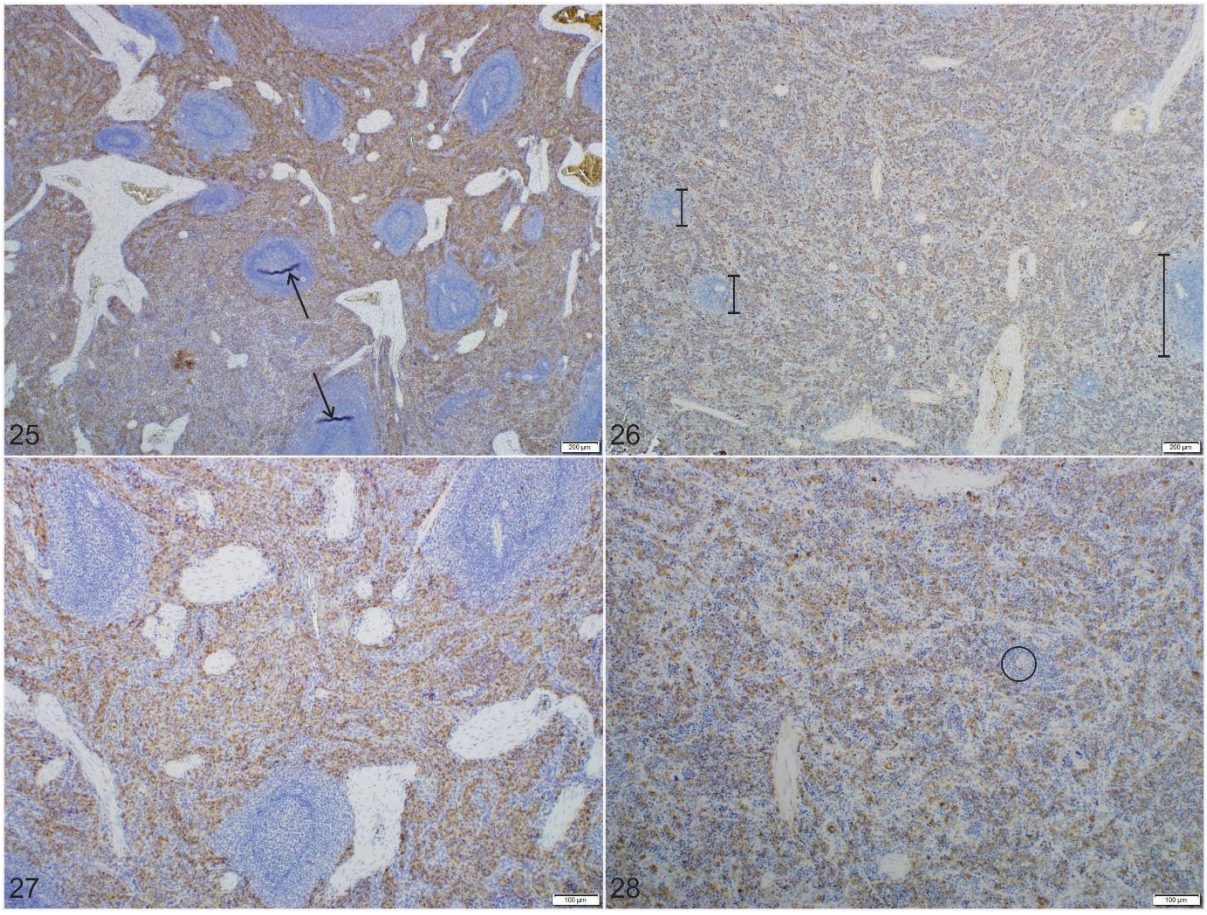

Plate 8: Immunohistochemistry CD204.

Figure 25: Control case no 3, immunohistochemistry for CD204. Immunoreactive cells are confined to the red pulp. This magnification precludes visualisation of individually labelled CD204+ cells. Note artifactual folds in two white pulp areas (arrows). **Figure 26:** Experimental case no 7, immunohistochemistry for CD204. Again, immunoreactive cells are confined to the red pulp. Residual white pulp zones are indicated by the brackets. **Figure 27:** Control case no 3, immunohistochemistry for CD204. Immunoreactive cells are largely confined to the red pulp. Individually labelled cells are more obvious at this magnification. **Figure 28:** Experimental case no 7, immunohistochemistry for CD204. Immunoreactive cells are largely confined to the red pulp interspersed by disintegrated white pulp zones. Central arteriole (encircled) enables localisation of remnant white pulp zone

4.3.3. CD3

The CD3 antibody is expressed in T lymphocytes found mainly within the PALS surrounding central arterioles. The CCs revealed a substantial proportion of T lymphocytes within the PALS, RP and fewer scattered within the follicular germinal centres (see Plate 9: Figure 29). Multifocal small clusters of purely PALS-associated T lymphocytes remained in-between what had become a T lymphocyte-rich red pulp in the ECs (see Plate 9: Figure 30).

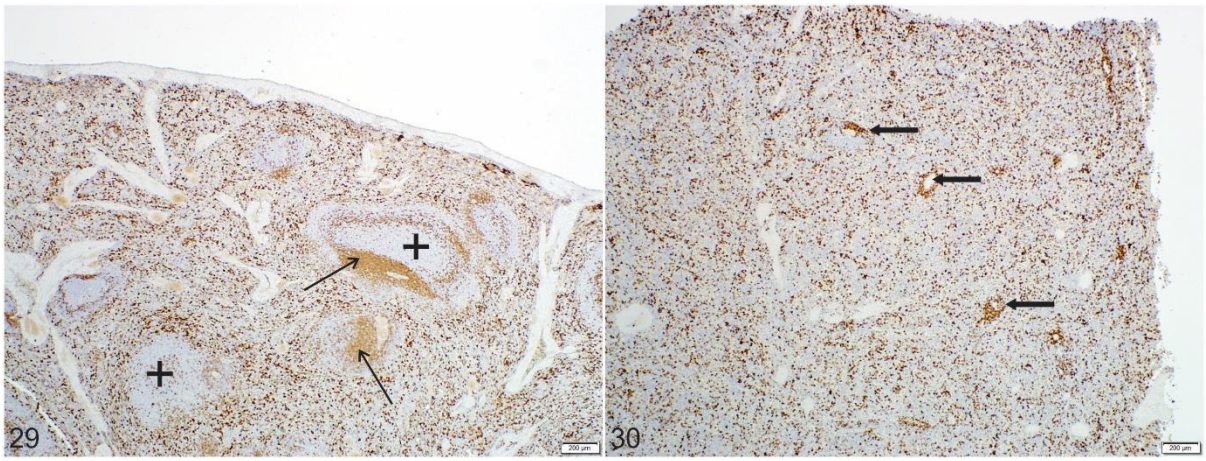

Plate 9: Immunohistochemistry: CD3.

Figure 29: Control case no 3, immunohistochemistry for CD3. Immunoreactive T lymphocytes are seen in the PALS (arrows) and scattered throughout the red pulp with rare T cells in germinal centre (crosses). **Figure 30:** Experimental case no 7, immunohistochemistry for CD3. Immunoreactive T lymphocytes are seen within multifocal small clusters of PALS-associated CD3+ T lymphocytes (block arrows) amongst a T lymphocyte-rich red pulp.

4.3.4. CD20

Mature, circulating B lymphocytes as well as plasma cells in dogs express CD20. The majority of the CD20+ B lymphocytes in the CCs were confined to the lymphoid follicles and scattered within the marginal zone (see Plate 10: Figure 31). A substantial proportion of CD20+ labelling

was also observed within the RP. Multifocal, small, remnant nodular lymphoid tissue consisting of CD20+ B lymphocytes were retained in all the ECs with the majority of the remainder CD20 positivity located to the RP (see Plate 10: Figure 32).

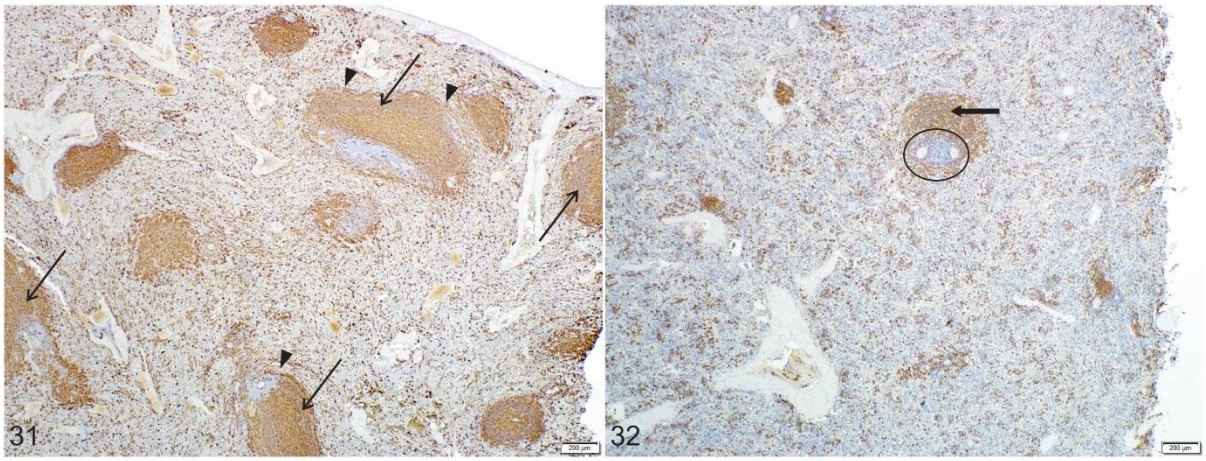

Plate 10: Immunohistochemistry: CD20.

Figure 31: Control case no 3, immunohistochemistry for CD20. CD20+ B lymphocytes confined to lymphoid follicles (arrows) and the marginal zones (arrowheads), with some CD20+ canine plasma cells and fewer B lymphocytes scattered throughout the red pulp. **Figure 32:** Experimental case no 7, immunohistochemistry for CD20. Remnant CD20+ follicular lymphoid tissue (block arrow). Note accentuation of central arterioles due to neighbouring negatively staining T lymphocytes (encircled). Scattered CD20+ B lymphocytes and plasma cells in the red pulp.

4.3.5. Pax-5

Pax-5 expression is limited to early and maturing B lymphocytes. Pax-5 labelling in the CCs corresponded well with that of CD20 expression within the WP; the majority of the positive labelling was confined to the lymphoid follicles and marginal zones (see Plate 11: Figure 33).

In the ECs, the B lymphocyte areas dissipated with greater confluence of WP and RP zones and the Pax-5+ B cells seemed to have re-located to the RP (see Plate 11: Figure 34).

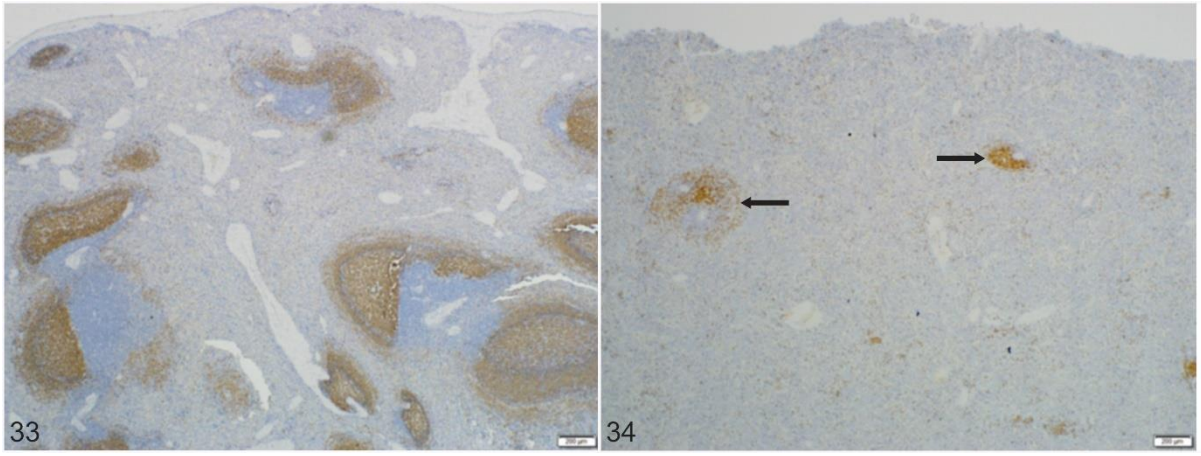

Plate 11: Immunohistochemistry: Pax-5.

Figure 33: Control case no 3, immunohistochemistry for Pax-5. Pax-5 immunoreactivity corresponds with the CD20 immunoreactivity in the white pulp, but far fewer Pax-5+ B lymphocytes (compared to CD20+ cells) are visualised in the red pulp. **Figure 34:** Experimental case no 7, immunohistochemistry for Pax-5. Compared to the control case, there is decreased Pax-5 reactivity within the residual white pulp areas (block arrows) with proportionately more Pax-5 B lymphocytes scattered in the red pulp.

4.3.6. Mum1

Mum1 is expressed in plasma cells. Although in varying proportions, plasma cells were largely confined to the following areas in CCs: PALS, germinal centres, marginal zones and the RP (see Plate 2: Figures 14 and 15). Within the RP, plasma cells were often observed in a juxtatrabecular location. Despite scattered Mum1+ plasma cells within the atrophic lymphoid areas of the ECs, the majority of plasma cells appeared to have re-located to the RP which coincided with the CD20+ plasmacytoid cells in the RP (see Plate 5: Figures 19 and 20).

CHAPTER FIVE: DISCUSSION

The current study provided novel insights into the pathology of the spleen in canine babesiosis.

4.4. Gross pathology

4.4.1. Splenomegaly

Macroscopically the spleens of *Babesia rossi*-infected dogs were enlarged with a soft consistency. The increase in size was the result of an increase in volume i.e. an increase in cell content mainly characterised by erythrocytes but also nucleated cells i.e. macrophages and extramedullary haematopoietic tissue. The degree of extramedullary haematopoiesis (EMH) in ECs superseded that of the CCs. The majority, if not all, haemoprotozoal infections result in splenomegaly albeit to varying degrees. The splenomegaly in the dogs in the current study was attributed to the increased macrophage densities as well as a significant degree of extramedullary haematopoiesis. It should be noted that objectively proving splenomegaly (through body weight : spleen weight ratio or through cell enumeration) is very difficult. Obtaining a normal healthy dog spleen that has not been altered in weight and size is virtually impossible. Drugs used for euthanasia (normally pentobarbitone) causes severe splenomegaly. Spleens removed surgically are usually pathological and also affected by anaesthetic drugs that result in splenomegaly. Spleens collected from dogs that die from non-infectious disease (such as trauma) have almost always been altered through splenic contraction. It may well be worthwhile using ultrasound determined spleen volume as an objective measure of spleen size. This was however not part of this study but should be considered for a future study. Non-lethal infection with *P. chabaudi adami* resulted in significantly increased splenic haematopoiesis whereas fatal *P. berghei* infection resulted in

much lower levels of extramedullary haematopoiesis [11]. Although Schneider et al (2011) also considered the retention of erythrocytes to be a contributing factor in the splenomegaly of *Babesia bovis*-infected calves; they did view acute hyperplasia of nucleated cells to be the most important contributing factor [78]. The acute hyperplasia was characterised by a significantly greater increase in the number of large versus small splenic leucocytes [78]. A study on *P. falciparum* infection in humans by Urban et al (2005) suggested that a combined increase in the weight of the white pulp (WP) and red pulp (RP) caused the observed splenomegaly [94]. In contrast, Carvalho et al (2007) showed B lymphocyte expansion to be the main initiator of the splenomegaly in *P. berghei*-infected mice [10]. The latter authors relied on the examination of Giemsa stained sections to enable visualisation of deoxyribonucleic acid (DNA)/ ribonucleic acid (RNA) material as means of distinguishing cellular populations of interest [10]. The divergent findings are likely caused by different experimental approaches and highlight to difficulty is explaining the reasons for the obviously enlarged spleen.

4.5. Histopathology

4.5.1. Collapse of splenic architecture

The most significant observation in the spleen of *Babesia rossi*-infected dogs was characterised by the loss of distinction between the WP and red RP zones with the diffuse intermingling of these two compartments. The intermingling of the WP and RP zones was not only observed within the spleens of *Babesia divergence*-infected Mongolian gerbils but also within the spleens of *Plasmodium falciparum*-infected humans, *Plasmodium chabaudi chabaudi* and *Plasmodium berghei*-infected mice [1,10,20,94]. To the authors' knowledge, this is the first evidence to support a similar observation within the spleen of *Babesia rossi*-infected dogs. Achtman et al. (2003) infected mice with *P. chabaudi chabaudi* and evaluated the spleen

pathology sequentially over the time course of the disease [1]. They noted the diffuse blending of the splenic compartments during the peak of infection (day 9-10) with complete architectural normality restored by day 60 post infection [1]. Carvalho and co-workers (2007) examined *P. berghei*-infected mice on numerous days post infection and also noted WP and RP intermingling on days 6 to 8 [10]. This was a terminal study; mice were anaesthetised and bled on day 10 and not allowed to recover from the disease and thus later time points were not described [10]. *Babesia divergenc*e in Mongolian gerbils induced a lethal outcome with none of the animals surviving post day 5 [20]. All of the animals displayed signs of illness on day 5 and were anaesthetised and killed to allow for splenic examination [20]. Microscopic examination revealed the loss of distinction between the WP and the RP on day 5 of disease [20]. Similar to the study on humans with fatal natural *P. falciparum* infection, the dogs in the current study were examined once they died or once they were humanely euthanized whilst suffering from severe and advanced forms of the disease [94]. This means that this specific architectural change within the spleen may only be a feature of the peak of the disease when most animals die or are more likely to die (in those that were euthanized on humane grounds). Research models, as with the current study, which do not allow for sequential investigation of the spleen during the course of a disease, deliver a single snapshot in time of the pathology. Research models that do not allow infected animals to recover, whether they are experimentally or naturally infected, are unable to elaborate on the extent and probability of the spleen returning to a normal, pre-infection condition/state.

The immune system's effective response to pathogens is thought to be reliant on the highly organised microarchitecture of the secondary lymphoid organs [2]. This is supported by evidence that reconstitution of splenectomised mice with whole spleen suspensions, did not provide sufficient protection against malaria [29,103]. The cellular organisation within these

organs is responsible for the effective trapping, transport, processing and presentation of antigens [2]. This allows for parasite constraint and induction of a specific immune response [2]. The exact reasons, mechanisms and consequences of complete architectural collapse remain unclear. The fact that normal splenic architecture can be regained once an infection has run its course, as has been extrapolated mainly from malarial studies, is testimony to the organ's phenomenal plasticity. The possibility of the spleen regaining architectural normality in recovered cases of canine babesiosis requires further investigation although it seems likely. In the case of malaria, some workers are of the opinion that the splenic changes, specifically the disorganisation, may prevent the successful development of malarial immunity [10,94,100]. A contrasting opinion argues that the changes promote the interaction between parasitized erythrocytes and T lymphocytes and macrophages and forms a blood-spleen barrier thus protecting developing erythroblasts from the parasite [100,102]. Schneider et al (2011) suggested that the splenic leukocyte redistribution is central to the acute immune response in naïve animals but whether previously exposed animals will reveal a similar/dissimilar finding remained uncertain [78]. Pertaining to the recovery phase in canine babesiosis, it is not possible to determine to what extent the disorganisation of the splenic compartments serves a specific function in canine babesiosis.

The outcome of experimental malarial infections can be manipulated depending on the mouse strain and *Plasmodium* species used in the specific experiment. *Plasmodium chabaudi chabaudi* in BL/6 mice results in a self-resolving infection whereas *Plasmodium chabaudi chabaudi* in A/J mice is lethal [49]. It has long been known that the use of microbial organisms/products can provide non-specific immunity against subsequent malaria infection. Bacillus Calmette-Guerin (BCG) administered to mice provides long-term protection against subsequent malaria and/or *Babesia spp*. infection [49]. Although Leisewitz et al (2008) showed

that BCG-malaria co-infection has contradictory effects in BL/6 versus A/J mice, interestingly both mouse strains revealed the same degree of disorganisation as seen on haematoxylin and eosin (HE) examination [49]. Bacillus Calmette-Guerin (BCG) preinfection resulted in protection of A/J mice but paradoxically resulted in BL/6 mice mortality [49]. The microscopic disorganisation was characterised by significant RP hyperplasia as well as WP hyperplasia, the latter to a lesser degree [49]. Due to the significant RP hyperplasia the WP appeared intact albeit depleted although the opposite was found as determined by flow cytometry analysis [49]. Despite this apparent disorganisation however, the distinction between the RP and WP remained clear [49].

There was no significant difference between the arrangement of the reticular framework within the spleen of the experimental cases compared to the control cases as viewed by Gomori's reticulin impregnation (GRi) in *Babesia rossi*-infected dogs. The immune cell compartmentalisation and localisation in the spleen is the consequence of chemokine gradients and cytokine concentrations together with the presence of the reticular framework [73]. The microanatomical disarray seen on HE-staining may be a consequence of temporary distress in the organ resulting in dramatic alterations in these protein concentrations and gradients with the subsequent loss of ordered structure.

4.5.2. Follicular pathology

In addition to the loss of distinction between the splenic compartments in our study, there was also widespread dissolution of the WP. Remnant follicular structures lacked clearly discernible germinal centres, mantle zones and marginal zones and were interspersed by scattered, remnant periarteriolar lymphatic sheaths (PALS). This dissolution of the WP zones accentuated the intermingling of the WP and RP. *Plasmodium berghei*-infected mice and

humans naturally infected with *Plasmodium falciparum* both revealed an overall loss of germinal centre definition and dissolution on histopathology [10,94]. In contrast to the aforementioned studies, *P. chabaudi chabaudi*-infected mice displayed an increase in follicular size and vigorous germinal centre formation characterised by prominent dark and light zone development [1,50]. Although this observation could be appreciated on histopathology, immunohistochemistry was used to characterise specific immunoblast migratory patterns [1]. Malaria-infected BL/6 mice, specifically involving *P. chabaudi chabaudi* are used as a model for self-resolving non-cerebral malaria infection, whereas *P. berghei*-infected mice are an established model for experimental cerebral malaria. *Plasmodium chabaudi chabaudi*-infected mice are also used to study malaria specific pathogenesis and serve as a model for the study of specific immune mechanisms. Urban et al's (2005) results were derived from natural, acute and fatal malaria infection in humans [94]. These considerations, together with species-related differences in splenic structure and pathogenicity could explain the different but not necessarily contradictory results regarding the presence of significant germinal centre formation and germinal centre dissolution respectively [1,94]. It is also important to note that different methods of study analysing leukocyte quantities and location could contribute to discordant results. The current study included an integrated histological and immunohistochemical analysis of dog spleens. Urban et al's (2005) study used a similar methodology and Carvalho and co-workers (2007) were dependent on bright field microscopy performed on Giemsa-stained sections [10,94]. A blend of not only histopathology but also flow cytometry was employed by Achtman et al (2003) [1]. In the current study, dogs naturally infected with *Babesia rossi* that either died or that were humanely euthanised close to death revealed germinal centre dissolution similar to humans naturally infected with *P. falciparum* and mice infected with *P.berghei* [10,94]. This does however

not mean that follicular expansion with germinal centre formation is not a feature of dogs with babesiosis as splenic examination was only performed soon after death and not at any additional stage during the course of the disease. We also only looked at dogs where the disease was fatal (dogs euthanized were close to death and euthanized on humane grounds) and whether or not a resolving infection would have demonstrated different findings was not investigated. The white pulp dissolution in our current study as well as that of *P. berghei*-infected mice and humans naturally infected with *P. falciparum* suggests a degree of correlation between this observation and the outcome of disease. Due to host reliance on the organised microarchitecture of secondary lymphoid organs in the fight against pathogens, it appears that the WP dissolution in malaria (experimental or natural) and babesiosis may perpetuate a more serious and/or fatal disease outcome. Self-resolving models of malaria (*P. chabaudi chabaudi*) revealed diffuse blending of the splenic compartments albeit vigorous germinal centre formation and subsequent follicular expansion. This model demonstrated that despite these findings at various stages of infection, restoration of normality could be obtained. This suggests that apart from species-specific variation in pathogenicity, the retention of some splenic architecture may be required to promote a positive disease outcome. The current study revealed intermingling of splenic compartments as well as diffuse WP dissolution, which not only speaks to the pathogenicity of *Babesia rossi* but also to the guarded outcome of this disease especially in the absence of or very late application of treatment. Sequential analysis and examination of the spleen during the course of disease will have to be performed to evaluate this.

The spleen is structured in such a manner that the majority of blood passes through the marginal zone. Studies have indicated the essential role of marginal zone macrophages in antigen trapping. An immunohistochemical study by Yadava et al (1996) compared the

functionality of the marginal zone macrophages in murine malaria with that of mice infected with *Salmonella typhimirium* and observed that parasitized erythrocytes avoid a fully functional marginal zone although the underlying mechanisms could not be established [102]. The authors therefore proposed that RP macrophages are the main effector cells involved in malaria parasite clearance [102]. The probability of the marginal zone macrophages' involvement in antigen trapping was significant, but the exact degree of involvement requires further clarification. Aichele et al (2003) evaluated the role of marginal zone macrophages in *Listeria monocytogenes* infection in mice by selectively depleting this population of macrophages and following the subsequent spread of the bacteria within the spleen and systemically [2]. The results from this study found marginal zone macrophages to be crucial to successful antigen trapping [2]. The specific trapping abilities of marginal zone macrophages are enhanced by the reduced blood flow in this area. This, together with their strategic location, optimises antigen trapping. In vitro studies indicate that marginal zone macrophages exhibit specific receptors that promote efficient antigen uptake [2]. These experiments made use of thin spleen slices on slides over which particulate antigen was spread and showed a localisation pattern identical to in vivo studies [12,18,31]. Studies were however not clear as to the exact effect a lack of marginal zone macrophages would have on the host's defence against blood-borne infection [2]. Marginal zone macrophage specific antibodies were not applied in our babesia study. The exact immunohistochemical profile of canine marginal zone macrophages has not yet been fully established. The overall WP dissolution does suggest that the marginal zone forms part of this generalised architectural disorganisation. Red pulp (RP) macrophages are the main effector cell in the post-trapping phase of the host response. The current study revealed a significantly hyperplastic RP macrophage response, which suggests that marginal zone macrophages either trapped antigen optimally preceding disorganisation

or that some remnant functionality remained post disorganisation enabling them to trap antigen to such an extent that a subsequent hyperplastic RP macrophage response was possible. Another possibility may be that RP macrophages either completely or partially take over the function of marginal zone macrophages (i.e. antigen trapping) enabling subsequent RP macrophage hyperplasia. Considering the aforementioned studies, specifically pertaining to the role of the marginal zone macrophages, this hypothesis will need further investigation as it currently seems less likely although not impossible. It may be argued that if antigen trapping occurs within a normal and fully functional marginal zone, other factors must contribute to the poor adaptation of the dog host to the parasite [64]. If the antigen trapping occurs only after splenic disorganisation, the poor tolerance of the dog for this parasite may be in explained one of several ways: the degree of antigen trapping may be insufficient, resulting in a suboptimal host effector phase or the disorganisation interferes with immune cell interaction resulting in a suboptimal host response.

4.6. Immunohistochemistry

4.6.1. Increased macrophage density in the red pulp

The *Babesia rossi*-infected dog spleens revealed a significant increase in the proportion of CD204+ and MAC387+ macrophages specifically within the RP. Apart from the resident tissue macrophage population (amongst others, CD204+), the normal spleen also contains a large proportion of extravascular monocytes [86]. Scavenger receptor A, also known as cluster of differentiation (CD)-204 is a transmembrane protein found primarily on phagocytic cells such macrophages and other antigen presenting cells i.e. splenic dendritic cells [41,63,104]. Scavenger receptors are multi-functional receptors with broad ligand-binding specificity found on a variety of different cell types [41,63,104]. In 1997 Krieger proposed a nomenclature and resultant

classification system for scavenger receptors based on their common structural characteristics (class A, B, C etc.)[46]. The application of CD204 antibodies in dogs in veterinary science has been limited to the diagnosis of histiocytic neoplasia [40]. The MAC387 (S100A/B-Calprotectin) antibody recognizes the intracytoplasmic antigen calprotectin expressed in granulocytes, monocytes, and macrophages as well as the S100 protein family exclusively expressed by cells of myeloid lineage, especially monocytes and neutrophils [8,72,97]. *Plasmodium falciparum*-infected humans and *P. berghei*-infected mice that display germinal centre dissolution also displayed increased macrophage densities throughout the red pulp [10,94]. Carvahlo et al (2007) employed bright field microscopy on Giemsa stained sections to characterise the cellular populations in the spleen of *P. berghei*-infected mice [10]. The RP was expanded due to large numbers of macrophages as well as numerous foci of erythropoiesis and monocytopoiesis [10]. Alterations in macrophage density were investigated mainly by way of histological and quantitative immunohistochemical methods in humans naturally infected with *P. falciparum* [94]. The relative number of macrophages in the RP was significantly increased in malaria [94]. The number of macrophages per examined area was corrected for the observed/documented splenic weight [94]. Achtman et al (2003) found that there was no significant increase in macrophage densities within the RP of *P. chabaudi chabaudi*-infected mice using F4/80 or non-specific esterase staining for macrophages [1]. Another study by Sponaas and co-workers (2009) indicated that a significant proportion of the hyperplastic RP macrophages observed in *P. chabaudi chabaudi*-infected mice were of bone marrow origin as determined by flow cytometry [80]. One of the only babesia-related models was that of *B. divergence*-infected Mongolian gerbils in which flow cytometry attributed the observed splenomegaly amongst others, to increased macrophage densities [20]. The current study is

unique in its attempt to not only determine splenic cell population changes in canine babesiosis, but to also better characterise them immunohistochemically.

Reasons behind increased macrophage densities in human and murine malaria as well as *Babesia*-infected Mongolian gerbils are unclear although there is much speculation. Macrophages contribute substantially to the innate immune response and play a critical role in the fight against protozoan infections. Macrophages not only initiate and perform phagocytosis but they also release cytokines and chemokines all of which mediate pathogen clearance. Selective depletion of macrophages/monocytes in mice chronically infected with *Babesia microti* impairs host protection against subsequent lethal infection with *Babesia rodhani,* showing that these cells are critical for cross-protective immunity against *B. rodhaini*, conferred by *B. microti* in mice [52]. Erythrocyte removal is an important function of the macrophage population of the spleen during haemoprotozoal infections. An older study evaluated the rate of erythrocyte loss in splenectomised *B. rodhaini* and *B. microti*-infected rats [90]. The authors demonstrated that splenectomy does not abolish but only delays the loss of erythrocytes suggesting that macrophages/monocytes of liver and bone marrow origin assume the phagocytic function [90]. The spleen does however play a very important role in the clearance of haemoprotozoal organisms as evidenced by the death of splenectomised rats infected with *Plasmodium berghei* [90]. Another study, similar in methodology, showed the importance of macrophages during the control of both the acute and resolving infections of *Babesia microti* in mice [89]. The authors further demonstrated that the specific absence of marginal zone and red pulp macrophages contributed to the failure to control the parasitaemia [89].

Subversion of macrophage function by protozoa (for example *Plasmodium spp., Trypanosoma spp*., *Toxoplasma spp*. and *Theileria spp*.) is an important indication of the crucial role this immune cell phenotype plays in host defence [43,59,81]. Strategies employed by these organisms include antigenic variation, limited antigen production and altered antigen presentation [43,59,81]. Protozoal infections have also been shown to subvert T cell responses by not only triggering anergy and apoptosis but also by the induction of alternatively activated macrophages, also known as suppression macrophages [70,91]. Alternatively activated macrophages have a distinct phenotype due to different profiles of gene expression[91]. They are characterised by lowered production of pro-inflammatory cytokines, increased levels of scavenger receptors and they promote the killing of parasites [91]. Alternatively activated macrophages can be identified by a characteristic immunohostochemical profile characterised, amongst others, by CD163 positivity which is also the most commonly studied marker of these cells. These macrophages are however also characterised by the expression of CD204, a macrophage scavenger receptor [91].

It should be noted that the examination of the RP on HE-stained sections revealed a stark absence of significant numbers of neutrophils in both the control cases (CCs) as well as experimental cases (ECs). The implication of this is that the increase of especially the MAC387+ cells in the ECs can truly be ascribed to increased macrophage proportion. The increase in the proportion of macrophages (CD204+ and MAC387+), the lack of a significant T lymphocyte response (see later) as well as the immunohistochemical profile of the macrophages (CD204+ specifically) indicate that a significant proportion of macrophages may be alternatively activated macrophages. Considering what other authors have found, the effect of the increase in macrophage densities in the current study is probably multifold. The primary reason for an increase in tissue resident macrophages (of which some or all may be

alternatively activated macrophages) and monocytes/macrophages reflects their role in parasite clearance. Trapping of haemoprotozoal antigens by marginal zone macrophages results in the subsequent signalling to RP macrophages to respond and clear the host of the pathogen. They not only phagocytose the parasitized erythrocytes but they also phagocytose and metabolise non-parasitized erythrocytes that are damaged as a result of the infection [19,30,74]. In addition to what others have shown in natural and experimental malaria infections, the current study also suggests that a macrophage response characterises *Babesia rossi* infection. Future work should focus on better characterising the marginal zone and RP macrophage population and understanding their role in this disease.

4.6.2. T cell response

The current study showed no significant difference between the densities of T lymphocytes in the experimental versus control cases. Alternatively activated macrophages have been shown to suppress T lymphocyte activation in a variety of infectious disease i.e. *Trypanosoma spp., Toxoplasma spp*. and *Theileria spp* [24,25,42,75,85]. Considering the immune-pathogenesis of haemoprotozoal disease, a significant host T lymphocyte response (which should be observed as an increase in splenic T lymphocyte population) would be expected. In the current study mechanisms underlying the lack of a hyperplastic response could be two-fold: a proportion or all of the hyperplastic macrophages may be alternatively activated macrophages which could (as previously described) have a suppressive effect on T lymphocyte activation directly and indirectly on the quantity of T lymphocytes. Another possible explanation for the lack of increase in T lymphocytes could be that various subpopulations of T lymphocytes (i.e. helper T lymphocytes, cytotoxic T lymphocytes) alter their expression of cell surface markers during activation, proliferation and differentiation. A flow cytometric analysis of circulating blood

lymphocyte subpopulations in dogs naturally infected with *Babesia rossi* revealed a significant reduction in peripheral T lymphocyte populations in both complicated and uncomplicated cases of babesiosis [67]. The authors proposed functional immunosuppression as the main reason for this observation [67]. In the current study, there was no significant difference in the density of T lymphocytes in infected versus control dogs. The lowered proportion of positive staining tissue is counter intuitive as one could reasonably expect some degree of hyperplastic response considering the immune stimulation induced by the infecting babesia organism. It could be argued that it may be possible that acute lymphocyte depletion results in immunosuppression in fatal babesia infection of dogs. This may be a unique feature of this particularly pathogenic species of babesia organism infecting dogs. Should this finding indicate an immunosuppressed state, it becomes important to realise that a hypo-responsive immune system can be just as detrimental as an overstimulated response.

4.6.3. B lymphocytes and plasma cells

Neither the CD20+ immunoblasts, Pax-5+ immunoblasts or Mum-1+ plasma cells revealed any significant difference in proportion in the babesiosis cases compared to the control cases. Considering the common splenomegaly caused by the disease, this may not reflect a real drop in cell number, it may in fact be associated with exactly the opposite. Apart from multifocal, small remnant lymphoid follicles, B lymphocytes mostly relocated to the RP. Plasma cell distribution followed that of the B lymphocytes except for the few positive cells observed within the remnant follicular structures. The immunohistochemistry aspect of a study performed on *P. chabaudi chabaudi*-infected mouse spleens found a decrease in the number of marginal zone B lymphocytes perpetuating marginal zone dissolution [1]. However, flow cytometry performed concurrently found no change in the number of marginal zone B

lymphocytes [1]. Immunohistochemistry was unable to determine their exact whereabouts post relocation. In contrast, Urban and co-workers (2005) found a true B lymphocyte depletion, without any evidence of specific migratory patterns in their study of fatal natural *Plasmodium falciparum* infection in the spleens of humans [94]. In contrast, two murine malaria studies showed an increased number of plasma cells throughout the spleens but especially within the RP [1,10]. One of these studies also demonstrated evidence of B lymphocyte proliferation [1].

Various studies have implicated B lymphocytes and their associated antibodies in parasite clearance promoting the survival of the host through enabling parasite clearance [47,55,98]. Having an understanding of the complete B lymphocyte cycle from development through differentiation would assist in understanding the involvement of this phenotype in the evolution of protozoal infections [3]. B lymphocytes originate from the bone marrow. Following a few steps of lineage commitment, B lymphopoeisis yields developmental stages including immature B lymphocytes. Immature B lymphocytes migrate to the spleen to develop into transitional B lymphocytes which in turn develop into conventional B lymphocytes and marginal zone B lymphocytes. Protozoa are able to influence B lymphocyte development and consequently the humoral immune response [1,6,99]. *Plasmodium spp.* for example have been shown to not only have an effect on bone marrow B lymphocyte precursors but also to induce marginal zone B lymphocyte apoptosis and to decrease the size of established memory B lymphocyte populations [1,6,99]. Marginal zone B lymphocytes are known to mediate humoral immune responses against blood-borne antigens, but little is known about their role in protozoal parasite clearance [3]. The results of this current study indicate that the use of immunohistochemistry with a limited number of markers per cell type, may not detect subtle cell population changes. However, without the application of immunohistochemistry it would

not have been possible to observe and appreciate, amongst others, the B lymphocyte relocation to the RP. It is also possible that *Babesia spp*. parasites, like other protozoal species, influence B lymphocyte development and progression. Results from a study performed on *P. chabaudi chabaudi*-infected mice indicated that disturbance of B and T lymphocyte areas do not affect the production of plasma cells within the spleen [1]. The current study did not reveal increased plasma cell proportion although the T and B lymphocyte area disturbance was present as was obvious redistribution from traditional sites of B cell location to the RP. In a flow cytometric study by Rautenbach et al. it was shown that *B. rossi* infection resulted in a depletion of CD21 positive cells in blood [67]. As such, it does appear that *B. rossi* results in a generalized (even if transient) depletion of important cells of acquired immunity.

The pathology studies described here are limited in that they only provide a static snapshot analysis at a specific point during the course of a disease and in the terminal stage of severe disease. An evaluation of the spleen in milder disease may help us understand splenic events in resolving infections which may help discriminate between events associated with death compared to events associated with disease resolution. This would be especially important in the spleen which is an especially plastic organ that is capable of remarkable structural rearrangement in response to disease challenge. These studies are also limited in that they do not quantify cell numbers. Histology provides an architectural description of the organ but not an accurate quantitative assessment of the cells that comprise the organ. An obvious shortcoming of this study was an ultrastructural description of the pathology, which should be addressed in any future study. Detailed immunohistochemical characterisation of lymphocyte, macrophage (specifically marginal zone macrophages) and dendritic cell subpopulations would also be invaluable. Combining this with a flow cytometric assessment of immune cell phenotypes would provide an invaluable additional angle of assessment. The

experimental infection of laboratory dogs with no known previous exposure to disease and with no variability in comorbidities , age, genetic background or environmental influence would also facilitate a more exact study.

CONCLUSION

Our study revealed novel insights, not only into the histopathology but also into the immunohistochemical findings in the spleens of dogs infected with *Babesia rossi* compared to the spleens of uninfected, healthy dogs. The most significant microscopic finding was characterised by the loss of distinction between the red pulp and the white pulp, with the diffuse intermingling of these two compartments. Additionally, there was outspoken white pulp dissolution without clearly discernible white pulp zones compared to the control cases. To the authors' knowledge, these observations are the first of their nature and scope reported in dogs infected with *Babesia rossi*. It is not yet clear what the exact cause or reason for these findings may be or weather the infection is the cause or consequence of these changes. It does however suggest that the architectural disarray may be linked to disease severity and disease outcome in canine babesiosis especially if dogs are left untreated. Immunohistochemistry revealed an increase in the proportion of CD204+ and Mac387+ immunoblasts within the red pulp without any appreciable difference in any of the other markers in the experimental cases versus the control cases. The increase in the red pulp macrophage/monocyte densities as well as a significant increase in the degree of extramedullary haematopoiesis were probably primarily responsible for the observed splenomegaly. It was surprising to find a lack of obvious T and B lymphocyte hyperplasia (as has been described in the blood by flow cytometry) and the consequences and causes of this remain to be investigated. Whether the specific changes help or harm the host need further investigation. Future studies should, amongst others, evaluate the spleen in milder disease to determine whether the findings in our study are indeed linked to disease severity and disease outcome. It would also be important to determine whether the spleen, in a state of complete

architectural disarray, is able to recover and regain normality and how this may relate to disease severity.

REFERENCES

1 Achtman AH, Khan M, MacLennan IC, Langhorne J: Plasmodium chabaudi chabaudi infection in mice induces strong B cell responses and striking but temporary changes in splenic cell distribution. *The Journal of Immunology* 2003:171(1)**:**317-324.

2 Aichele P, Zinke J, Grode L, Schwendener RA, Kaufmann SH, Seiler P: Macrophages of the splenic marginal zone are essential for trapping of blood-borne particulate antigen but dispensable for induction of specific T cell responses. The Journal of Immunology 2003:171(3):1148-1155.

3 Amezcua Vesely MC, Bermejo DA, Montes CL, Acosta-Rodríguez EV, Gruppi A: B-cell response during protozoan parasite infections. *Journal of parasitology research* 2012:2012.

4 Anosa V, Jennings FW, Urquhart GM: The effect of splenectomy on anaemia in Trypanosoma brucei infection of mice. *J Comp Pathol* 1977:87(4)**:**569-579.

5 Bannard O, Horton RM, Allen CD, An J, Nagasawa T, Cyster JG: Germinal center centroblasts transition to a centrocyte phenotype according to a timed program and depend on the dark zone for effective selection. *Immunity* 2013:39(5)**:**912-924.

6 Bockstal V, Geurts N, Magez S: Acute disruption of bone marrow B lymphopoiesis and apoptosis of transitional and marginal zone B cells in the spleen following a blood-stage Plasmodium chabaudi infection in mice. *Journal of parasitology research* 2011:2011.

7 Brendolan A, Rosado MM, Carsetti R, Selleri L, Dear TN: Development and function of the mammalian spleen. *Bioessays* 2007:29(2)**:**166-177.

8 Brodersen R, Bijlsma F, Gori K, Jensen K, Chen W, Dominguez J, et al.: Analysis of the immunological cross reactivities of 213 well characterized monoclonal antibodies with specificities against various leucocyte surface antigens of human and 11 animal species. *Veterinary immunology and immunopathology* 1998:64(1)**:**1-13.

9 Brown WC, Norimine J, Knowles DP, Goff WL: Immune control of Babesia bovis infection. *Vet Parasitol* 2006:138(1-2)**:**75-87.

10 Carvalho LJ, Ferreira-da-Cruz MF, Daniel-Ribeiro CT, Pelajo-Machado M, Lenzi HL: Germinal center architecture disturbance during Plasmodium berghei ANKA infection in CBA mice. *Malaria journal* 2007:6(1)**:**59.

11 Cesta MF: Normal structure, function, and histology of the spleen. *Toxicol Pathol* 2006:34(5)**:**455-465.

12 Cha D, MacPherson GG: Analysis of thymus-independent type 2 antigen uptake by marginal zone macrophages in thin slices of viable lymphoid tissue in vitro. *European journal of immunology* 1990:20(7)**:**1451-1455.

13 Chaves YO, Costa AG, Pereira MLM, Lacerda MVG, Coelho-dos-Reis JG, Martins-Filho OA, et al.: Immune response pattern in recurrent Plasmodium vivax malaria. *Malaria journal* 2016:15(1)**:**445.

14 Chotivanich K, Udomsangpetch R, McGready R, Proux S, Newton P, Pukrittayakamee S, et al.: Central role of the spleen in malaria parasite clearance. *J Infect Dis* 2002:185(10)**:**1538-1541.

15 Clark I, Jacobson L: Do babesiosis and malaria share a common disease process. *Annals of tropical medicine and parasitology* 1998:92(4)**:**483-488.

16 Das S, Alam M, Islam M, Awal M: Histology of the spleen of indigenous dog (Canis familiaris) of Bangladesh. *Bangladesh Journal of Veterinary Medicine* 2012:3(1)**:**59-61.

17 Del Portillo HA, Ferrer M, Brugat T, Martin-Jaular L, Langhorne J, Lacerda MV: The role of the spleen in malaria. *Cell Microbiol* 2012:14(3)**:**343-355.

18 Demoy M, Andreux J-P, Weingarten C, Gouritin B, Guilloux V, Couvreur P: In vitro evaluation of nanoparticles spleen capture. *Life sciences* 1999:64(15)**:**1329-1337.

19 Den Haan JM, Kraal G: Innate immune functions of macrophage subpopulations in the spleen. *Journal of innate immunity* 2012:4(5-6)**:**437-445.

20 Dkhil MA, Al-Quraishy S, Al-Khalifa MS: The effect of Babesia divergens infection on the spleen of Mongolian gerbils. *Biomed Res Int* 2014:2014**:**483854.

21 Engwerda CR, Beattie L, Amante FH: The importance of the spleen in malaria. *Trends in Parasitology* 2005:21(2)**:**75-80.
22 Eurell JA, Frappier BL: Dellman's Textbook of Veterinary Histology. In: Eurell JA, Frappier BL, eds. *Dellman's Textbook of Veterinary Histology*. Sixth ed. Oxford, UK: Blackwell Publishing Ltd; 2006: 147-151.
23 Ferrer L, Fondevila D, Rabanal R, Tarres J, Ramis A: Immunohistochemical detection of CD3 antigen (pan T marker) in canine lymphomas. *J Vet Diagn Invest* 1993:5(4)**:**616-620.
24 Flynn J, Sileghem M: The role of the macrophage in induction of immunosuppression in Trypanosoma congolense-infected cattle. *Immunology* 1991:74(2)**:**310.
25 Fry LM, Schneider DA, Frevert CW, Nelson DD, Morrison WI, Knowles DP: East coast fever caused by Theileria parva is characterized by macrophage activation associated with vasculitis and respiratory failure. *PloS one* 2016:11(5)**:**e0156004.
26 Fujita T, Kashimura M, Adachi K: Scanning electron microscopy and terminal circulation. *Experientia* 1985:41(2)**:**167-179.
27 Good MF, Currier J: The importance of T cell homing and the spleen in reaching a balance between malaria immunity and immunopathology: the moulding of immunity by early exposure to cross-reactive organisms. *Immunology & Cell Biology* 1992:70(6).
28 Groom AC: The Microcirculatory Society Eugene M. Landis award lecture. Microcirculation of the spleen: new concepts, new challenges. *Microvasc Res* 1987:34(3)**:**269-289.
29 Grun J, Long C, Weidanz W: Effects of splenectomy on antibody-independent immunity to Plasmodium chabaudi adami malaria. *Infection and immunity* 1985:48(3)**:**853-858.
30 Gupta P, Lai SM, Sheng J, Tetlak P, Balachander A, Claser C, et al.: Tissue-Resident CD169+ Macrophages Form a Crucial Front Line against Plasmodium Infection. *Cell Reports* 2016:16(6)**:**1749-1761.
31 Han Y, Kelm S, Riesselman MH, Crocker PR, Cutler JE: Mouse sialoadhesin is not responsible for Candida albicans yeast cell binding to splenic marginal zone macrophages. *Infection and immunity* 1994:62(5)**:**2115-2118.
32 Hashimoto D, Chow A, Noizat C, Teo P, Beasley MB, Leboeuf M, et al.: Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes. *Immunity* 2013:38(4)**:**792-804.
33 Herbeck R, Teodorescu Brînzeu D, Giubelan M, Lazăr E, Dema A, Ioniţă H: B-cell transcription factors Pax-5, Oct-2, BOB. 1, Bcl-6, and MUM1 are useful markers for the diagnosis of nodular lymphocyte predominant Hodgkin lymphoma. *Rom J Morphol Embryol* 2011:52(1)**:**69-74.
34 Homer MJ, Aguilar-Delfin I, Telford SR, Krause PJ, Persing DH: Babesiosis. *Clinical microbiology reviews* 2000:13(3)**:**451-469.
35 Hussein HS: Babesia microti and Babesia hylomysci: spleen and phagocytosis in infected mice. *Exp Parasitol* 1979:47(1)**:**1-12.
36 Irvin AD, Omwoyo P, Ledger MA: Comparison of the effects of irradiation and splenectomy on Babesia rodhaini infection in mice. *Int J Parasitol* 1973:3(6)**:**773-781.
37 Jacobson LS, Clark I: The pathophysiology of canine babesiosis: new approaches to an old puzzle. *Journal of the South African Veterinary Association* 1994:65(3)**:**134-145.
38 Johns J, Christopher MM: Extramedullary hematopoiesis: a new look at the underlying stem cell niche, theories of development, and occurrence in animals. *Veterinary pathology* 2012:49(3)**:**508-523.
39 Jubala C, Wojcieszyn J, Valli V, Getzy D, Fosmire S, Coffey D, et al.: CD20 expression in normal canine B cells and in canine non-Hodgkin lymphoma. *Veterinary Pathology Online* 2005:42(4)**:**468-476.
40 Kato Y, Murakami M, Hoshino Y, Mori T, Maruo K, Hirata A, et al.: The class A macrophage scavenger receptor CD204 is a useful immunohistochemical marker of canine histiocytic sarcoma. *Journal of comparative pathology* 2013:148(2)**:**188-196.
41 Kelley JL, Ozment TR, Li C, Schweitzer JB, Williams DL: Scavenger receptor-A (CD204): a two-edged sword in health and disease. *Critical Reviews™ in Immunology* 2014:34(3).

42 Khan IA, Matsuura T, Kasper LH: IL-10 mediates immunosuppression following primary infection with Toxoplasma gondii in mice. *Parasite immunology* 1995:17(4)**:**185-195.
43 Kling JC, Körner H: Different regulatory mechanisms in protozoan parasitic infections. *International journal for parasitology* 2013:43(6)**:**417-425.
44 Kraal G, Mebius R: New insights into the cell biology of the marginal zone of the spleen. *International review of cytology* 2006:250**:**175-215.
45 Krause PJ, Daily J, Telford SR, Vannier E, Lantos P, Spielman A: Shared features in the pathobiology of babesiosis and malaria. *Trends in parasitology* 2007:23(12)**:**605-610.
46 Krieger M: The other side of scavenger receptors: pattern recognition for host defense. *Current opinion in lipidology* 1997:8(5)**:**275-280.
47 Kumar S, Tarleton RL: The relative contribution of antibody production and CD8+ T cell function to immune control of Trypanosoma cruzi. *Parasite immunology* 1998:20(5)**:**207-216.
48 Langhorne J, Albano FR, Hensmann M, Sanni L, Cadman E, Voisine C, et al.: Dendritic cells, pro-inflammatory responses, and antigen presentation in a rodent malaria infection. *Immunol Rev* 2004:201**:**35-47.
49 Leisewitz A, Rockett K, Kwiatkowski D: BCG–malaria co-Infection has paradoxical effects on C57BL/6 and A/J mouse strains. *Parasite immunology* 2008:30(1)**:**1-12.
50 Leisewitz AL, Rockett KA, Gumede B, Jones M, Urban B, Kwiatkowski DP: Response of the splenic dendritic cell population to malaria infection. *Infection and immunity* 2004:72(7)**:**4233-4239.
51 Leisewitz AL, Rockett KA, Gumede B, Jones M, Urban B, Kwiatkowski DP: Response of the splenic dendritic cell population to malaria infection. *Infect Immun* 2004:72(7)**:**4233-4239.
52 Li Y, Terkawi MA, Nishikawa Y, Aboge GO, Luo Y, Ooka H, et al.: Macrophages are critical for cross-protective immunity conferred by Babesia microti against Babesia rodhaini infection in mice. *Infection and immunity* 2012:80(1)**:**311-320.
53 MacLennan IC: Germinal centers. *Annual review of immunology* 1994:12(1)**:**117-139.
54 Maegraith B, Gilles HM, Devakul K: Pathological processes in Babesia canis infections. *Z Tropenmed Parasitol* 1957:8(4)**:**485-514.
55 Magez S, Schwegmann A, Atkinson R, Claes F, Drennan M, De Baetselier P, et al.: The role of B-cells and IgM antibodies in parasitemia, anemia, and VSG switching in trypanosoma brucei–infected mice. *PLoS pathogens* 2008:4(8)**:**e1000122.
56 Martin F, Kearney JF: Marginal-zone B cells. *Nature Reviews Immunology* 2002:2(5)**:**323-335.
57 Matjila PT, Leisewitz AL, Jongejan F, Penzhorn BL: Molecular detection of tick-borne protozoal and ehrlichial infections in domestic dogs in South Africa. *Veterinary Parasitology* 2008:155(1)**:**152-157.
58 Mebius RE, Kraal G: Structure and function of the spleen. *Nat Rev Immunol* 2005:5(8)**:**606-616.
59 Melo MB, Jensen KD, Saeij JP: Toxoplasma gondii effectors are master regulators of the inflammatory response. *Trends in parasitology* 2011:27(11)**:**487-495.
60 Mills SE: Spleen. In: Mills SE, ed. *Histology for pathologists*. Third ed. Philadelphia, USA: Lippincott Williams & Wilkins; 2007: 783-797.
61 Naito M, Umeda S, Yamamoto T, Moriyama H, Umezu H, Hasegawa G, et al.: Development, differentiation, and phenotypic heterogeneity of murine tissue macrophages. *Journal of leukocyte biology* 1996:59(2)**:**133-138.
62 O'Keane JC, Wolf BC, Neiman RS: The pathogenesis of splenic extramedullary hematopoiesis in metastatic carcinoma. *Cancer* 1989:63(8)**:**1539-1543.
63 Ozment TR, Ha T, Breuel KF, Ford TR, Ferguson DA, Kalbfleisch J, et al.: Scavenger receptor class a plays a central role in mediating mortality and the development of the pro-inflammatory phenotype in polymicrobial sepsis. *PLoS Pathog* 2012:8(10)**:**e1002967.
64 Penzhorn BL, Vorster I, Harrison-White RF, Oosthuizen MC: Black-backed jackals (Canis mesomelas) are natural hosts of Babesia rossi, the virulent causative agent of canine babesiosis in sub-Saharan Africa. *Parasites & vectors* 2017:10(1)**:**124.

65 Pouniotis DS, Proudfoot O, Bogdanoska V, Apostolopoulos V, Fifis T, Plebanski M: Dendritic cells induce immunity and long-lasting protection against blood-stage malaria despite an in vitro parasite-induced maturation defect. *Infect Immun* 2004:72(9)**:**5331-5339.
66 Ramos-Vara J, Miller M: When Tissue Antigens and Antibodies Get Along Revisiting the Technical Aspects of Immunohistochemistry—The Red, Brown, and Blue Technique. *Veterinary Pathology Online* 2014:51(1)**:**42-87.
67 Rautenbach Y, Goddard A, Thompson PN, Mellanby RJ, Leisewitz AL: A flow cytometric assessment of the lymphocyte immunophenotypes in dogs naturally infected with Babesia rossi. *Veterinary parasitology* 2017:241**:**26-34.
68 Reyers F, Leisewitz A, Lobetti R, Milner R, Jacobson L: Canine babesiosis in South Africa: more than one disease. Does this serve as a model for falciparum malaria. *Annals of Tropical Medicine and Parasitology* 1998:92(4)**:**503-511.
69 Roberts JA, Kerr JD, Tracey-patte P: Function of the spleen in controlling infections of Babesia rodhaini in mice. *International Journal for Parasitology* 1972:2(2)**:**217-226.
70 Rodrigues V, Cordeiro-da-Silva A, Laforge M, Ouaissi A, Akharid K, Silvestre R, et al.: Impairment of T cell function in parasitic infections. *PLoS neglected tropical diseases* 2014:8(2)**:**e2567.
71 Ross MH, Kaye GI, Pawlina W: Histology a text and atlas *Histology a text and atlas*. Philadephia: Lippincott Williams & Wilkins; 2003: 382-387.
72 Ryckman C, Vandal K, Rouleau P, Talbot M, Tessier PA: Proinflammatory activities of S100: proteins S100A8, S100A9, and S100A8/A9 induce neutrophil chemotaxis and adhesion. *The Journal of Immunology* 2003:170(6)**:**3233-3242.
73 Satoh T, Takeda R, Oikawa H, Satodate R: Immunohistochemical and structural characteristics of the reticular framework of the white pulp and marginal zone in the human spleen. *The Anatomical Record* 1997:249(4)**:**486-494.
74 Sawyer R, Strausbauch P, Volkman A: Resident macrophage proliferation in mice depleted of blood monocytes by strontium-89. *Laboratory investigation; a journal of technical methods and pathology* 1982:46(2)**:**165-170.
75 Schleifer KW, Mansfield JM: Suppressor macrophages in African trypanosomiasis inhibit T cell proliferative responses by nitric oxide and prostaglandins. *The Journal of Immunology* 1993:151(10)**:**5492-5503.
76 Schmidt EE, MacDonald IC, Groom AC: Circulatory pathways in the sinusal spleen of the dog, studied by scanning electron microscopy of microcorrosion casts. *J Morphol* 1983:178(2)**:**111-123.
77 Schmidt EE, MacDonald IC, Groom AC: Direct arteriovenous connections and the intermediate circulation in dog spleen, studied by scanning electron microscopy of microcorrosion casts. *Cell Tissue Res* 1982:225(3)**:**543-555.
78 Schneider DA, Yan H, Bastos RG, Johnson WC, Gavin PR, Allen AJ, et al.: Dynamics of bovine spleen cell populations during the acute response to Babesia bovis infection: an immunohistological study. *Parasite Immunol* 2011:33(1)**:**34-44.
79 Schofield L, Grau GE: Immunological processes in malaria pathogenesis. *Nat Rev Immunol* 2005:5(9)**:**722-735.
80 Sponaas AM, Freitas do Rosario AP, Voisine C, Mastelic B, Thompson J, Koernig S, et al.: Migrating monocytes recruited to the spleen play an important role in control of blood stage malaria. *Blood* 2009:114(27)**:**5522-5531.
81 Stanisic DI, Barry AE, Good MF: Escaping the immune system: how the malaria parasite makes vaccine development a challenge. *Trends in parasitology* 2013:29(12)**:**612-622.
82 Steiniger B, Barth P: Microanatomy and function of the spleen. *Adv Anat Embryol Cell Biol* 2000:151**:**III-IX, 1-101.
83 Steiniger B, Barth P, Hellinger A: The perifollicular and marginal zones of the human splenic white pulp : do fibroblasts guide lymphocyte immigration? *Am J Pathol* 2001:159(2)**:**501-512.
84 Steiniger B, Ruttinger L, Barth PJ: The three-dimensional structure of human splenic white pulp compartments. *J Histochem Cytochem* 2003:51(5)**:**655-664.

85 Sternberg JM: Elevated serum nitrate in Trypanosoma brucei 'rhodesiense'infections: evidence for inducible nitric oxide synthesis in trypanosomiasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 1996:90(4)**:**395.
86 Swirski FK, Nahrendorf M, Etzrodt M, Wildgruber M, Cortez-Retamozo V, Panizzi P, et al.: Identification of splenic reservoir monocytes and their deployment to inflammatory sites. *Science* 2009:325(5940)**:**612-616.
87 Takubo K, Miyamoto H, Imamura M, Tobe T: Morphology of the human and dog spleen with special reference to intrasplenic microcirculation. *Jpn J Surg* 1986:16(1)**:**29-35.
88 Tanaka H, Hataba Y, Saito S, Fukushima O, Miyasaka M: Phenotypic Characteristics and Significance of Reticular Meshwork Surrounding Splenic White Pulp of Mice. *Journal of Electron Microscopy* 1996:45(5)**:**407-416.
89 Terkawi MA, Cao S, Herbas MS, Nishimura M, Li Y, Moumouni PFA, et al.: Macrophages are the determinant of resistance to and outcome of nonlethal Babesia microti infection in mice. *Infection and immunity* 2015:83(1)**:**8-16.
90 Todorovic R, Ferris D, Ristic M: Roles of the spleen in acute plasmodial and babesial infections in rats. *Experimental Parasitology* 1967:21(3)**:**354-372.
91 Tomioka H, Tatano Y, Maw WW, Sano C, Kanehiro Y, Shimizu T: Characteristics of suppressor macrophages induced by mycobacterial and protozoal infections in relation to alternatively activated M2 macrophages. *Clinical and Developmental Immunology* 2012:2012.
92 Tsoukas CM, Bernard NF, Abrahamowicz M, Strawczynski H, Growe G, Card RT, et al.: Effect of splenectomy on slowing human immunodeficiency virus disease progression. *Arch Surg* 1998:133(1)**:**25-31.
93 Udroiu I: Evolution of sinusal and non-sinusal spleens of mammals. *Hystrix, the Italian Journal of Mammalogy* 2007:17(2).
94 Urban BC, Hien TT, Day NP, Phu NH, Roberts R, Pongponratn E, et al.: Fatal Plasmodium falciparum malaria causes specific patterns of splenic architectural disorganization. *Infection and immunity* 2005:73(4)**:**1986-1994.
95 Van Furth R, Cohn Z, Hirsch J, Humphrey J, Spector W, Langevoort H: The mononuclear phagocyte system: a new classification of macrophages, monocytes, and their precursor cells. *Bulletin of the World Health Organization* 1972:46(6)**:**845.
96 van Furth R, Cohn ZA: The origin and kinetics of mononuclear phagocytes. *The Journal of experimental medicine* 1968:128(3)**:**415.
97 Villiers E, Baines S, Law AM, Mallows V: Identification of acute myeloid leukemia in dogs using flow cytometry with myeloperoxidase, MAC387, and a canine neutrophil-specific antibody. *Vet Clin Pathol* 2006:35(1)**:**55-71.
98 von der Weid T, Honarvar N, Langhorne J: Gene-targeted mice lacking B cells are unable to eliminate a blood stage malaria infection. *The Journal of Immunology* 1996:156(7)**:**2510-2516.
99 Weiss GE, Crompton PD, Li S, Walsh LA, Moir S, Traore B, et al.: Atypical memory B cells are greatly expanded in individuals living in a malaria-endemic area. *The Journal of Immunology* 2009:183(3)**:**2176-2182.
100 Weiss L: The spleen in malaria: the role of barrier cells. *Immunology Letters* 1990:25(1–3)**:**165-172.
101 Woo C-H, Lim J-H, Kim J-H: VCAM-1 upregulation via PKCδ-p38 kinase-linked cascade mediates the TNF-α-induced leukocyte adhesion and emigration in the lung airway epithelium. *American Journal of Physiology-Lung Cellular and Molecular Physiology* 2005:288(2)**:**L307-L316.
102 Yadava A, Kumar S, Dvorak JA, Milon G, Miller LH: Trafficking of Plasmodium chabaudi adami-infected erythrocytes within the mouse spleen. *Proceedings of the National Academy of Sciences* 1996:93(10)**:**4595-4599.
103 Yap GS, Stevenson MM: Differential requirements for an intact spleen in induction and expression of B-cell-dependent immunity to Plasmodium chabaudi AS. *Infection and immunity* 1994:62(10)**:**4219-4225.

104 Yi H, Zuo D, Yu X, Hu F, Manjili MH, Chen Z, et al.: Suppression of antigen-specific CD4+ T cell activation by SRA/CD204 through reducing the immunostimulatory capability of antigen-presenting cell. *Journal of molecular medicine* 2012:90(4)**:**413-426.
105 Yona S, Kim K-W, Wolf Y, Mildner A, Varol D, Breker M, et al.: Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. *Immunity* 2013:38(1)**:**79-91.
106 Yu P, Wang Y, Chin RK, Martinez-Pomares L, Gordon S, Kosco-Vibois MH, et al.: B cells control the migration of a subset of dendritic cells into B cell follicles via CXC chemokine ligand 13 in a lymphotoxin-dependent fashion. *The Journal of Immunology* 2002:168(10)**:**5117-5123.
107 Zachary JF, McGavin MD: *Pathologic basis of veterinary disease*. St Louis, Missourie: Elsevier, 2012.
108 Zidan M, Kassem A, Dougbag A, El Ghazzawi E, El A, Abd M, et al.: The spleen of the one humped camel (Camelus dromedarius) has a unique histological structure. *Journal of anatomy* 2000:196(3)**:**425-432.

APPENDICES

Appendix 1: The complete blood count data for the set of 4 control cases (numbers 1-4) and the complete set of 9 babesia-infected cases (numbers 5-13).

Number	CC(1)*/EC(2)**	Haemoglobin (120-180g/L)	Red cell count (5.5-8.5x10^12/L)	Haematocrit (37-55L/L)	MCV[a] (60-77fL)	MCHC[b] (32-36%)	Packed cell volume (%)
1	**1**	176	6.97	0.52	75	33.6	**0**
2	**1**	170	7.28	0.51	69.4	33.7	**0**
3	**1**	178	8.09	0.53	65.8	33.4	**0**
4	**1**	178	7.19	0.51	71.5	34.6	**0**
5	**2**	28	0.97	0.07	76.7	37.3	10
6	**2**	76	3.19	0.22	69.9	34.2	22
7	**2**	78	2.43	0.19	78.7	40.7	20
8	**2**	37	0.75	0.05	72.9	68.1	5
9	**2**	106	5	0.35	69.1	30.8	x

10	**2**	95	3.96	0.28	71.1	33.6	26
11	**2**	35	1.48	0.13	88.8	23.7	26.7
12	**2**	89	3.79	0.25	66.3	23.6	35.5
13	**2**	32	1.48	0.11	76.7	23.8	28.4

* Control cases are represented by the number 1 in the table and include cases number 1 to 4.

** Experimental cases are represented by the number 1 in the table and include case number 5 to 13.

[a] Mean corpuscular volume.

[b] Mean corpuscular haemoglobin concentration.

Appendix 1 continued: Represents continuation of routine haematology results for the set of 4 control cases (numbers 1-4) and the complete set of 9 babesia-infected cases (numbers 5-13).

Number	**CC(1)*/EC(2)****	**White cell count (6-15[a]x 10^9/L)**	**Segmented neutrophils (3-11.5x10^9/L)**	**Band neutrophils (0-0.5x10^9/L)**	**Lymphocytes (1-4.8x10^9/L)**	**Monocytes (0.15-1.35x10^9/L)**	**Eosinophils (0.1-1.25x10^9/L)**	**Platelet count. (200-500x10^9/L)**

1	**1**	10.84	6.83	0.11	2.82	0.22	0.87	271
2	**1**	14.43	7.5	0	4.18	1.44	1.3	142
3	**1**	9.21	5.16	0	3.22	0.18	0.64	236
4	**1**	8.25	5.78	0	1.65	0.33	0.5	180
5	**2**	21.6	15.12	0.86	4.97	0.65	0	25
6	**2**	10.25	6.36	0.51	1.64	1.64	0.1	24
7	**2**	11.31	8.93	0.11	1.81	0.45	0	21
8	**2**	1.1	0.04	0.26	0.66	0.13	0	4
9	**2**	16.74	10.88	2.18	3.18	0.5	0	15
10	**2**	3.35	0.9	1.04	0.77	0.64	0	23
11	**2**	12.5	7.75	1.5	2.75	0.25	0.25	56
12	**2**	2.28	1	0.32	0.73	0.23	0	14
13	**2**	12.17	9.01	0.97	1.22	0.97	0	62

* Control cases are represented by the number 1 in the table and include number 1 – 4.

** Experimental cases are represented by the number 1 in the table and include number 5-13.

[a] Mean corpuscular volume.

[b] Mean corpuscular haemoglobin concentration.

Appendix 2: Informed consent form

(To be completed by the patient's owner / authorized agent)

Encircle Yes or No where necessary

1.	Have you read the information sheet on canine babesiosis?	Yes	No
2.	Have you had the opportunity to ask questions about the research project?	Yes	No
3.	Have you received satisfactory answers to your questions?	Yes	No
4.	Have you received enough information about this study?	Yes	No
5.	Supply the name of the person to whom you have spoken to:		
	.....		
6.	Do you grant consent that blood and urine samples can be drawn from your dog?	Yes	No
7.	Do you grant consent that a post mortem examination can be performed in the case of death?		
		Yes	No

I, ……, hereby give permission that my dog ……., a …….. may participate in this clinical study conducted at the Onderstepoort Veterinary Academic Hospital.

I understand that this study will in no way harm my dog. Furthermore I understand that the costs of the additional tests will be borne by the trial fund, and that I will only be liable for costs pertaining the treatment that would in any event be required by my dog, including any complications that may arise as a result of canine babesiosis.

Signed at Onderstepoort on the ……. day of …… 20.....

Signature Owner/Agent ……..

Home Tel: ……..

Work Tel: ……

Cell No: ……

Appendix 3: Histopathology check-list

Autolysis	Impacts HP*		Not		Hemolysis	Dissolution	
Congested	Severe		Not				
Trabeculae	In close proximity		Not				
<u>Vasculature:</u>							
Trabecular arteries	Identifiable		Not		Leukostasis Yes/No	Neutrophils	Lymphocytes
	Monocytes	Other_____			TE** Yes/No	Eythrocytes	Fibrin
	Neutrophils	Lymphocytes	Monocytes	Other____			
	Necrotic vasculature Yes/No						
Arteries of white pulp	Identifiable		Not		Leukostasis Yes/No	Neutrophils	Lymohocytes
	Monocytes	Other_____			TE** Yes/No	Eythrocytes	Fibrin
	Neutrophils	Lymphocytes	Monocytes	Platelets	Other____		
	Necrotic vasculature Yes/No						
Ellipsoids	Identifiable		Not				
<u>PALS:</u>							
T lymphocyte area	Easily identifiable		Not				
	DBE***						
Plasma cells	Identifiable		Not		Red pulp	T cell area	
	Germinal centre		Mantle/corona		Marginal zone	Peritrabecular	
<u>Follicles: B lymphocytes</u>							
Follicles	Easily identifiable						

	DBE***						
Mantle zone	Easily identifiable		Not				
	DBE***						
Marginal zone:							
Marginal zone macrophages	Easily identifiable						
	DBE***						
Marginal zone lymphocytes	Easily identifiable						
	DBE***						
Cords of Billroth:							
EMH	Grade 0		Grade 1+		Grade 2+	Grade 3+	

*HP - Histopathology

**TE - Thromboemboli

***DBE - Disintegrated but evident

Appendix 4: Excel spreadsheet

Case number	CC(1)*/EC**(2)	Mac387***	CD204***	CD3***	CD20***	Mum1***	Pax5***
1	1	%	%	%	%	%	%
2	1	%	%	%	%	%	%
3	1	%	%	%	%	%	%
4	1	%	%	%	%	%	%
5	2	%	%	%	%	%	%
6	2	%	%	%	%	%	%
7	2	%	%	%	%	%	%
8	2	%	%	%	%	%	%
9	2	%	%	%	%	%	%
10	2	%	%	%	%	%	%
11	2	%	%	%	%	%	%
12	2	%	%	%	%	%	%
13	2	%	%	%	%	%	%
P-values		X	X	X	X	X	X

*Control cases are represented by the number 1 in the table and include case numbers 1 to 4.

**Experimental cases are represented by the number 2 in the table and include case numbers 5 to 13.

***The values in each column represent the percentage of positive labelling per cell marker per control/experimental case.

Appendix 5: Declaration of originality

THE PATHOLOGY OF THE SPLEEN IN CANINE BABESIOSIS.

ORIGINALITY REPORT

22%	19%	20%	13%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	www.ncbi.nlm.nih.gov Internet Source	1%
2	www.nature.com Internet Source	1%
3	espace.library.uq.edu.au Internet Source	<1%
4	www.biomedexperts.com Internet Source	<1%
5	research.wsulibs.wsu.edu:8080 Internet Source	<1%
6	repository.up.ac.za Internet Source	<1%
7	pure.uva.nl Internet Source	<1%
8	Submitted to Imperial College of Science, Technology and Medicine Student Paper	<1%
9	www.biomedcentral.com	