The pathology of the spleen in lethal canine babesiosis caused by *Babesia rossi*

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Funding information

Funding was provided by Andrew Lambert Leisewitz from a grant held by him from the National Research Foundation (CPRR13080726333).

Abstract

To provide useful information based on the macropathology, histopathology and immunohistochemical investigation in the spleens of dogs with *Babesia rossi* infection. Control spleens were collected from four healthy dogs euthanized for welfare reasons. Nine dogs that died naturally because of a mono-infection with *Babesia rossi* were selected for the diseased group. One haematoxylin-and-eosin–stained section of splenic tissue from each of the infected and control dogs was examined under the light microscope. Immunohistochemical markers were applied to characterize different immunocyte populations. The application of analytic software enabled semi-quantitative comparison of leucocyte subpopulations. Routine splenic histopathology revealed diffuse intermingling of white and red pulp from infected dogs with a clear loss of distinction between these zones. Immunohistochemistry revealed an increase in the proportion of tissue resident and bone marrow origin macrophages in the infected spleens. Apart from a few remnant lymphocytes within the peri-arteriolar lymphatic sheaths and follicles, the majority of the immunocytes redistributed to the red pulp, supporting the observation of white and red pulp intermingling. The majority of our findings are in agreement with histomorphological descriptions of the spleen in a variety of noncanid mammalian hosts with lethal malaria or babesiosis.

Keywords

Babesia rossi; haemoprotozoa; immunohistochemistry; lymphocyte; macrophage; red pulp; white pulp

1 INTRODUCTION

The spleen is the largest secondary lymphoid organ in the body, and therefore, not only does it perform many haematopoietic functions but it also reacts immunologically in response to blood-borne antigens which makes it indispensable in coordinating the immune system's response to blood-borne infection.[1, 2] 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 splenectomized patients.[3-5] 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.[3-5] The immune system's effective response to pathogens is thought to be reliant on the highly organized microarchitecture of secondary lymphoid organs like the spleen.[6] This is supported by evidence that reconstitution of splenectomized mice with whole spleen suspensions did not provide sufficient protection against malaria.[7, 8]

Babesia and malaria are important vector-borne protozoal diseases in humans and animals and have been compared with each other for decades.[9] Both diseases have a similar clinical presentation, and pathology caused both directly by the parasite and as a result of collateral organ damage resulting from the hosts immune response.[9, 10] The clinical description of *Babesia rossi* infection has been reviewed by other.[11, 12] Babesiosis has a worldwide distribution. The three large babesia species of domestic dogs (*Babesia canis, Babesia rossi* and *Babesia vogelli*) have different vectors, are antigenically distinct and differ in pathogenicity and geographic distribution.[13] *Babesia rossi* has a primarily Southern African distribution and is the most virulent/pathogenic specie. Malaria has been extensively studied and although the information regarding its effect on the spleen is limited compared with other organs; there is nevertheless a body of published work describing splenic pathology. There is also some literature describing the splenic pathology associated with equine babesiosis, bovine babesiosis and murine babesia.[14-17] There is nothing published about this aspect of the pathology caused by any babesia species infecting the dog. Maegraith et al (1957) noted prominent splenomegaly in a dog with babesiosis with limited information on the actual splenic histomorphology.[18]

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 blood-borne infection induced multi-systemic diseases. In the present study, the histomorphology of the spleen in *Babesia rossi*-infected dogs was described in detail and a variety of immunohistochemical stains were applied to investigate the role of the spleen in the pathogenesis of this disease.

2 MATERIALS AND METHODS

This was a prospective cohort case-control study. The control dogs (four in number) were euthanized at the Onderstepoort Veterinary Academic hospital, Faculty of Veterinary Science (FVS), for humane reasons. Nine dogs of a variety of breeds, ages, both sexes and body weights that were 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 diagnosed with babesiosis on a stained thin peripheral blood smear. All cases were then proven retrospectively to be positive for a mono-infection of *Babesia rossi* and negative for infection with other *Babesia* species, *Ehrlichia* species, *Anaplasma* species and *Theileria*

species by a polymerase chain reaction and reverse line blot hybridization assay.[19] All animals had complete post-mortems performed, and tissues were collected within an hour of death. A single cubic (1 cm × 1 cm×1 cm), randomly selected splenic sample was collected per dog and placed in ample (at least one part tissue to nine parts formalin) 10% buffered formalin and allowed to fix for a minimum of 12 hours before being processed for histopathology.

Following fixation, samples were processed, embedded, sectioned and stained according to Department of Agriculture, Forestry and Fisheries-accredited standard operating procedures used in the histopathology laboratory, Section of Pathology, Department of Paraclinical Sciences, FVS.[20] The haematoxylin-and-eosin (HE)–stained splenic sections were examined using a light microscope. The immunohistochemical markers applied included CD3 (T-lymphocytes; Dako Catalogue number: A0452), CD20 (mature B-lymphocytes and normal dog plasma cells; Dako Catalogue number: M0755), Mum1 (plasma cells; Dako Catalogue number: M7259), Pax-5 (immature B-lymphocytes; BD Bioscience Catalogue number: 610863), Mac387 (monocyte-macrophages of bone marrow origin; Dako Catalogue number: M0747), CD204 (resident tissue macrophages; Transgenic Inc) and VCAM-1/CD106 (cytokine-inducible surface molecule/vascular cell adhesion molecule; Novusbio Catalogue number: NBP2-03600). A single special stain, Gomori's reticulin impregnation (GRi), was also applied.

Splenic extramedullary haematopoiesis (EMH) was graded on a scale of 0 to 3+ based on the average number of normoblasts over 10 high power fields (HPFs) (400× magnification) per spleen sample as described by O'Keane et al[21] 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 an average of more than 10 such cells per HPF.[21]

The study was descriptive at the microscopic and immunohistochemical level. The tissue sections/slides were scanned on an Olympus scanner (VS120-S6-W slide loader system) for the generation of virtual slide images that allowed the application of analytical software (Olympus cellSens Dimension software—Olympus CellSens V, Olympus, Japan). The software allowed for the semi-quantitative analysis of the specified leucocyte subpopulations in the control and experimental tissues. A statistical software package was used for the statistical analysis (SPSS, version 24, IBM). A nonparametric statistical test (Mann-Whitney *U*) was performed to compare the medians between groups as the sample sizes were small, and the Shapiro-Wilk test criterion for normality was not satisfied.

3 RESULTS

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 dorsoventrally on cross section with sharp edges all round (Figure 1A). The spleens from the babesia-infected dogs were mildly enlarged whilst maintaining an elongated shape (Figure 1B). Splenic enlargement was denoted by an increase in dorsoventral thickness, an increase in length and edges that were rounded. External palpation of the intact spleens revealed a diffusely soft consistency. On cut surface, all the spleens exhibited moderate red pulp (RP) hyperplasia as evidenced by diffuse protrusion of soft, pulpy tissue with no clearly discernible white pulp (WP) areas/tissue and no significant ooze of blood from the cut surface (Figure 1B).

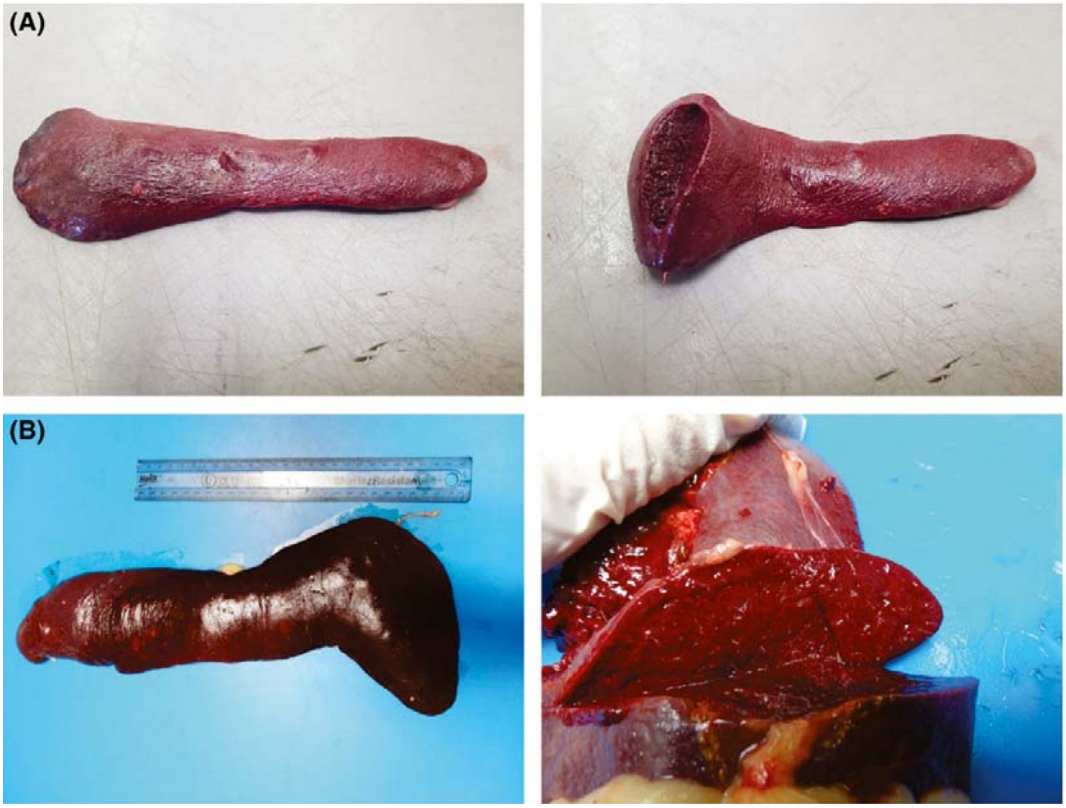

Figure 1. A, Control case number (no) 3. The normal spleens were dark red, elongated, flattened dorsoventrally on cross section with sharp edges and a consistency that resembled normal liver. B, Experimental case no 1. Babesiosis spleens were elongated with mild enlargement characterized by increased dorsoventral thickness and rounded edges. On cut surface, there was moderate red pulp hyperplasia

All of the control cases (CCs) displayed clear demarcation of the splenic tissue into its respective compartments (Figure 2A). In contrast, the splenic histopathology in the experimental cases (ECs) was characterized by diffuse intermingling of the WP and RP zones with a striking loss of distinction between the two compartments (Figure 2B). The majority of the normal spleens displayed variable numbers of plasma cells within the WP and RP. In the WP, plasma cells were observed scattered in the peri-arteriolar lymphatic sheaths (PALS), follicular (germinal centre and mantle zone) and marginal zones (Figure 3A). In the ECs, most plasma cells occurred throughout the splenic RP; they were no longer readily identified within clearly distinguishable WP areas (Figure 3B). Of note, was the lack of significant numbers of neutrophils in both CCs and ECs. Grade 1+ EMH was observed in all the CCs, whereas all of the ECs revealed grade 3+ EMH. In all the CCs and ECs, the GRi stain was largely confined to the trabeculae, the periphery of the intervening blood vessels; minimal staining, without any specific pattern, was observed in the white pulp areas.

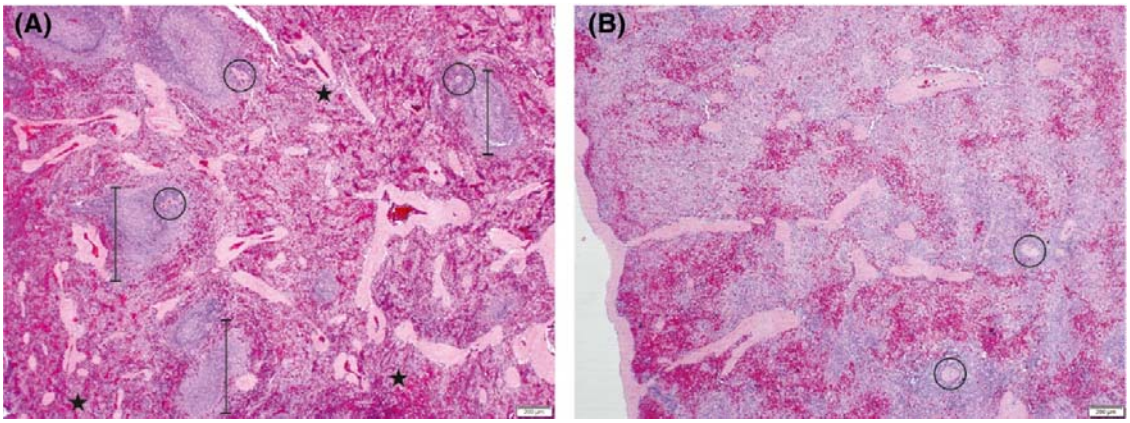

Figure 2.A, Control case no 3, haematoxylin and eosin (HE). There is a clear demarcation of white pulp (brackets) and red pulp (stars). Arteries of the white pulp (central arterioles) are easily observed (encircled). B, Experimental case no 6, HE. No clear demarcation between white and red pulp. Poorly delineated areas of white pulp are indicated by the presence of central arterioles (encircled)

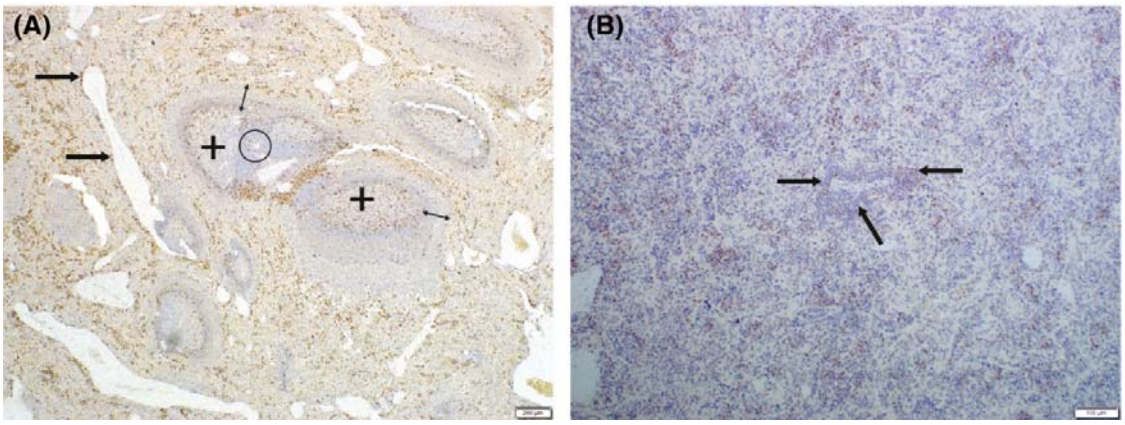

Figure 3. A, Control case no 3, immunohistochemistry for Mum1. Immunoreactive cells are observed within the red pulp, germinal centres (crosses), peri-arteriolar lymphatic sheaths (around the encircled central arteriole) and marginal zone (double arrows). Plasma cells are often observed neighbouring trabeculae (block arrows). Central arteriole (encircled). B, 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)

Within the control spleens, the $Mac387^+$ monocyte-macrophages were mainly confined to the RP with fewer in the marginal zone (Figure 4A). Compared with the CCs, the ECs showed a significant increase in the proportion of $Mac387^+$ monocyte-macrophages ($P = .005$) (Table 1 and Figure 4B). In the ECs, labelling assumed a more diffuse distributed pattern due to WP dissolution. A significant increase in the proportion of $CD204^+$ macrophages was observed in ECs compared with CCs ($P = .045$) (Table 1). $CD204^+$ macrophages were exclusively confined to the RP in both the CCs and the ECs (Figure 5A and B). The location of the $CD204^+$ macrophages within the RP, of especially the ECs, highlighted the WP dissolution by accentuating the decrease in WP size (Figure 5B).

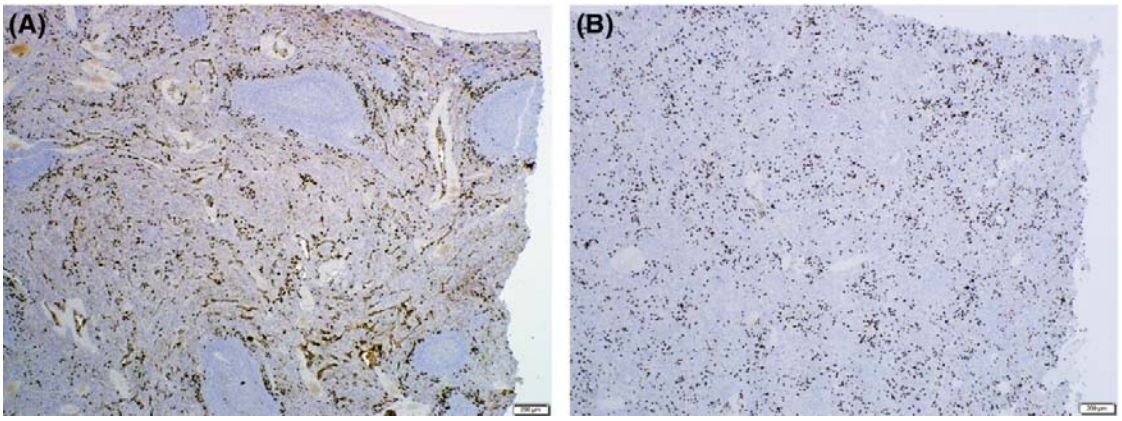

Figure 4. A, Control case no 3, immunohistochemistry for Mac387. Immunoreactive cells are largely confined to the red pulp and marginal zones. B, Experimental case no 7, immunohistochemistry for Mac387. Immunoreactive cells are diffusely scattered throughout the spleen section

Table 1. Mean percentage and comparison across categorical study variables

Case number	CC(1)[a]/EC[b](2)	Mac387[c]	CD204[c]	CD3[c]	CD20[c]	Mum1[c]	Pax5[c]
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		**.005** [d]	**.045** [d]	.064	.643	.217	.064

Note

A nonparametric statistical test (Mann-Whitney *U*) was performed to compare the medians between groups.

[a] Control cases are denoted by the number 1 in the table and include case numbers 1-4.

[b] Experimental cases are denoted by the number 2 in the table and include case numbers 5-13.

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

[d] The *p*-values indicate an appreciable difference in the percentage positive labelling of the respective immunoreactive cells within the experimental cases compared to control cases.

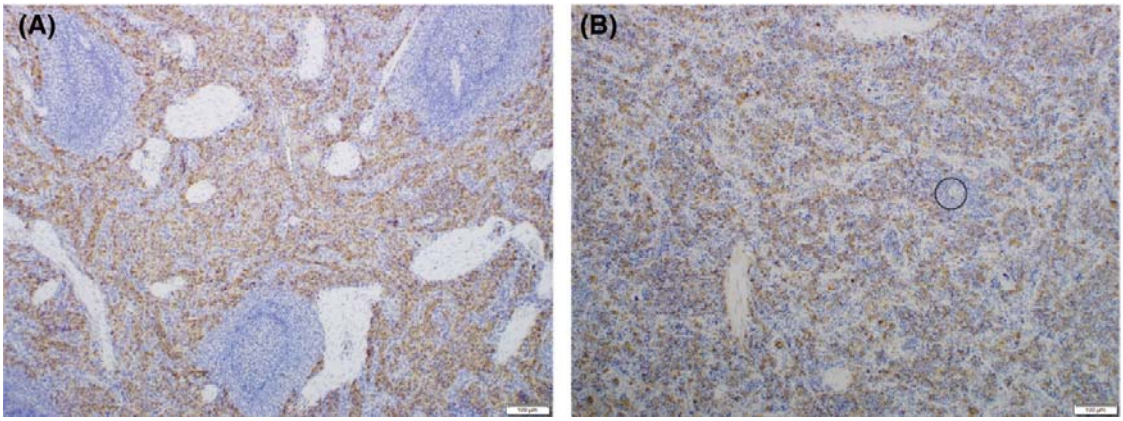

Figure 5. A, Control case no 3, immunohistochemistry for CD204. Immunoreactive cells are largely confined to the red pulp. B, Experimental case no 7, immunohistochemistry for CD204. Immunoreactive cells are largely confined to the red pulp interspersed by disintegrated white pulp zones. Central arteriole (encircled) enables localization of remnant white pulp zone

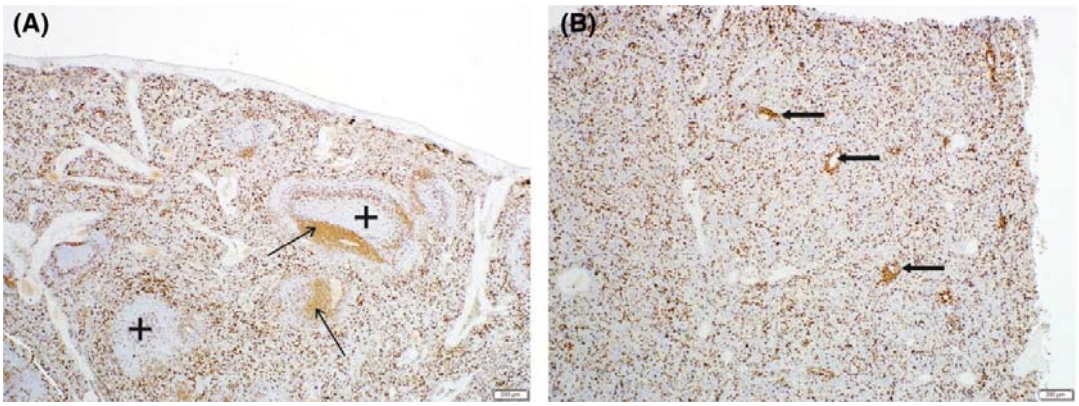

Figure 6. A, Control case no 3, immunohistochemistry for CD3. Immunoreactive T-lymphocytes are seen in the peri-arteriolar lymphatic sheaths (arrows) and scattered throughout the red pulp with rare T cells in germinal centre (crosses). B, Experimental case no 7, immunohistochemistry for CD3. Immunoreactive T-lymphocytes are seen within multifocal small clusters of PALS-associated $CD3^+$ T-lymphocytes (block arrows) amongst a T-lymphocyte-rich red pulp

In the CCs, most $CD3^+$ T-lymphocytes occurred within the PALS, RP and fewer occurred in the follicular germinal centres (Figure 6A). In the ECs, multifocal small clusters of purely PALS-associated $CD3^+$ T-lymphocytes remained in-between what had become a T-lymphocyte-rich RP (Figure 6B). The majority of $CD20^+$ B-lymphocytes in the CCs were confined to the lymphoid follicles and scattered within the marginal zone (Figure 7A). A substantial proportion of $CD20^+$ labelling was also observed within the RP. Multifocal, small, remnant follicular lymphoid tissue consisting of $CD20^+$ B-lymphocytes was retained in all ECs with the most of the remaining CD20 positivity located in the RP (Figure 7B). Pax-5 labelling of immature B-lymphocytes in the CCs corresponded well with the CD20 expression within the WP; the majority of the positive labelling was confined to the lymphoid follicles and marginal zones (Figure 8A). In the ECs, the Pax-5 positive B-lymphocyte areas were dissipated, with greater confluence of WP and RP zones, and this B-lymphocyte population demonstrated a relocation to the RP (Figure 8B). Although in varying proportions, $Mum1^+$ plasma cells were largely confined to the PALS, germinal centres, marginal zones

and RP in the CCs (Figure 3A). Within the RP, Mum1$^+$ plasma cells were often observed in a juxtatrabecular location (Figure 3A). Despite scattered Mum1$^+$ plasma cells within the atrophic lymphoid areas in the ECs spleens, the majority of Mum1$^+$ plasma cells appeared to have relocated to the RP which coincided with the observation of CD20$^+$ plasmacytoid cells in the RP in the HE-stained sections (Figure 3B). There was no significant difference in the VCAM-1 labelling of the ECs versus the CCs.

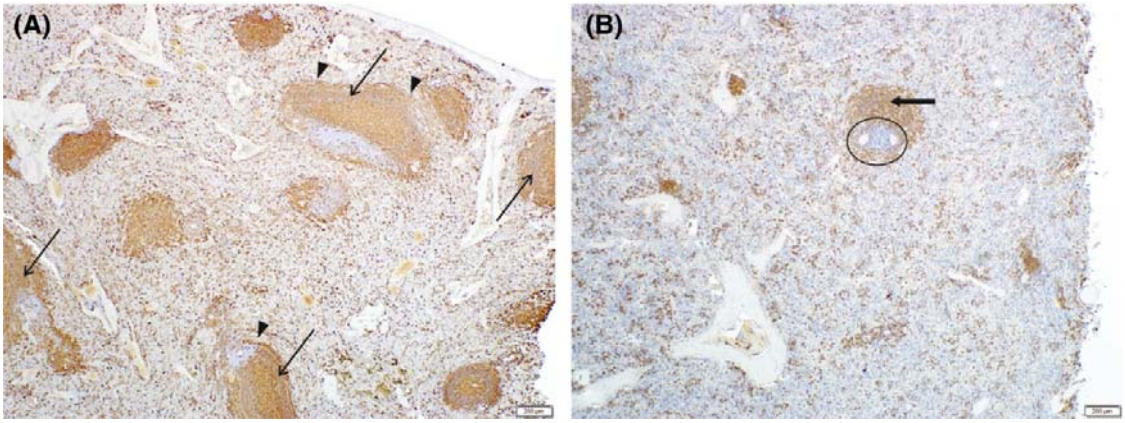

Figure 7. A, Control case no 3, immunohistochemistry for CD20. CD20$^+$ B-lymphocytes confined to lymphoid follicles (arrows) and the marginal zones (arrowheads), with some CD20$^+$ canine plasma cells and fewer B-lymphocytes scattered throughout the red pulp. B, Experimental case no 7, immunohistochemistry 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

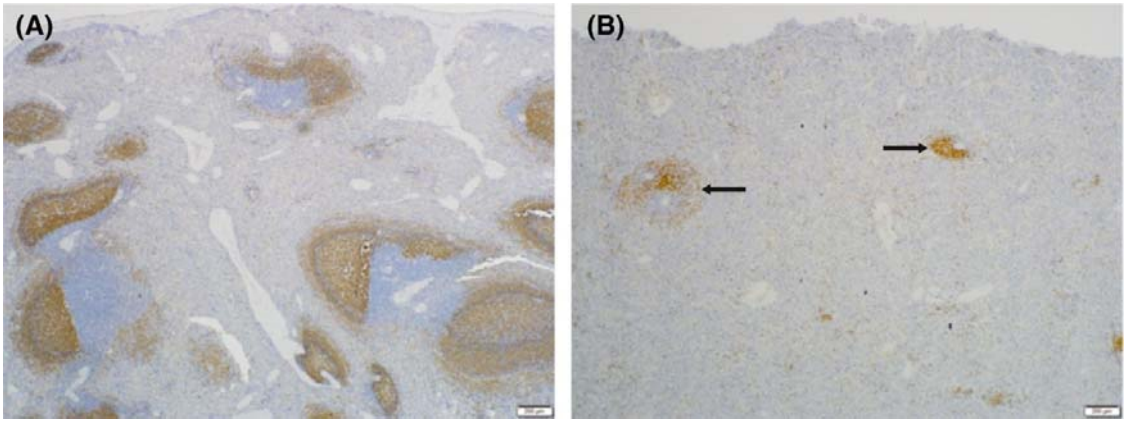

Figure 8. A, Control case no 3, immunohistochemistry for Pax-5. Pax-5 immunoreactivity corresponds with the CD20 immunoreactivity in the white pulp, but far fewer Pax-5$^+$ B-lymphocytes (compared with CD20$^+$ cells) are visualized in the red pulp. B, Experimental case no 7, immunohistochemistry for Pax-5. Compared with 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 DISCUSSION

Splenomegaly is a universal finding in all known haemoprotozoal infections of both humans and animals.[1, 14, 22, 23] The reasons for this are unclear and appear to vary between infecting organism and host. Macroscopically, the spleens of *Babesia rossi*-infected dogs were enlarged with a soft consistency. The splenomegaly in the dogs in the current study can be attributed to increased macrophage densities as well as a significant degree of EMH. 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 specifically is virtually impossible (despite this, the histological and immunohistochemical architecture of a normal spleen can be adequately described and compared with diseased spleens as we did here). Drugs used for euthanasia (normally pentobarbitone) cause severe splenomegaly. Spleens removed surgically are usually pathological and affected by anaesthetic drugs that result in splenomegaly. Spleens collected from dogs that die from noninfectious causes (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 in healthy versus disease dogs.[24] This was however not part of this study but should be considered for a future study.

Although Schneider et al (2011) 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 (large splenic leucocytes) to be the most important contributing factor.[14] Experimental *Babesia microti* infection in mice revealed generalized splenomegaly due to increased numbers of macrophages and haematopoietic precursors.[25] A study on fatal *P. falciparum* infection in humans by Urban et al (2005) suggested that a combined increase in the weight of the WP and RP caused the observed splenomegaly.[23]

The most significant observation in the spleen of *Babesia rossi*-infected dogs was the characteristic loss of distinction between the WP and RP zones due to diffuse intermingling of these two compartments. The intermingling of these zones was not only observed in the spleens of *Babesia divergens*-infected Mongolian gerbils but also in the spleens of *Babesia microti*-infected mice and *Plasmodium falciparum*-infected humans.[17, 23, 25] To the authors' knowledge, this is the first evidence to support a similar observation in the spleen of *Babesia rossi*-infected dogs. *Babesia divergens* in Mongolian gerbils induced a lethal outcome; all of the animals displayed signs of illness on day 5 and were anaesthetized and killed to allow for splenic examination.[17] Microscopic examination revealed the loss of distinction between the WP and the RP.[17] Similar to a study in humans with fatal natural *P. falciparum* infection, the dogs in the current study were only examined once they had died after severe and advanced disease.[23] Thus, WP and RP intermingling may only be a feature of the peak of the disease, when most animals die. Research models, as with the current study, which do not allow for sequential investigation of the spleen during the course of disease deliver a single snapshot in time of the pathology. Research models that do not allow infected animals to recover are unable to elaborate on the capacity of the spleen to recover. The extreme plasticity of this organ may indicate that the normal microanatomy is recoverable in dogs that survive the infection. In the case of malaria, some researchers are of the opinion that the splenic disorganization may prevent the successful development of malarial immunity.[22, 23, 26] A contrasting opinion argues that the changes promote interaction between parasitized erythrocytes, T-lymphocytes and macrophages, forming a blood-spleen barrier, thus protecting developing erythroblasts from the parasite.[26, 27] Schneider et al (2011) suggested that the splenic leucocyte redistribution is central to the acute immune response in naïve

animals but whether previously exposed animals will reveal a similar/dissimilar finding remains uncertain.[14] The impact that splenic disorganization has on the host's capacity to respond to the infection (helpful or harmful) remains undetermined at this point.

Highly organized microarchitecture of especially secondary lymphoid organs is necessary in order for the immune system to mount an effective response to pathogens. [6] The cellular organization within secondary lymphoid organs is responsible for the effective trapping, transport, processing and presentation of antigens.[6] This allows for parasite constraint and induction of a specific immune response. [6] Specific cytokines and chemokines with an attractant effect on various immunocyte populations are responsible for the correct organization and maintenance of splenic architecture and therefore function. The massive disruption of these proteins during haemoprotozoal infection is probably the main contributor to the observed architectural collapse.[28-32] The exact mechanisms and consequences of complete architectural collapse however remain unclear.

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 PALS.[22, 23, 33, 34] The WP dissolution in the current study as well as that of humans naturally infected with *P. falciparum* suggests a possible association between this observation and death. Due to host reliance on the organized microarchitecture of secondary lymphoid organs in the fight against pathogens, it may be that the WP dissolution in babesiosis indicates 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 normalcy was possible. This suggests that apart from species-specific variation in pathogenicity, the retention of some splenic architecture may be required to promote survival. 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 if left untreated or treated late.

The marginal zone is at the interface between the WP and RP, and it plays a crucial immune function in the spleen as it is here that marginal zone macrophages trap blood-borne antigens.[1, 35] Blood flow is arranged in such a way as to ensure that most of the blood entering the organ passes through the marginal zone before filtering through the RP and finally draining into the splenic veins. The specific trapping abilities of marginal zone macrophages are enhanced by the slowed blood flow in this area. This, together with their strategic location, optimizes antigen trapping. The intermingling of WP and RP zones resulted in marginal zone dissolution in spleens from the experimental group. Red pulp macrophages are the main effector cell in the post-trapping phase of the host immune response in the spleen. The current study demonstrated a significantly hyperplastic RP macrophage response, which suggests that marginal zone macrophages either trapped antigen optimally preceding disorganization or that some remnant functionality remained post-disorganization enabling them to trap antigen to such an extent that a subsequent hyperplastic RP macrophage response was possible. It is also possible that RP macrophages either completely or partially take over the function of marginal zone macrophages (ie antigen trapping) enabling subsequent RP macrophage hyperplasia. This hypothesis requires further investigation.

The *Babesia rossi*-infected dog spleens revealed a significant increase in the proportion of $CD204^+$ and $MAC387^+$ macrophages. Apart from the $CD204^+$ resident tissue macrophage population, the normal spleen also contains a large proportion of extravascular monocytes ($CD204^+$ and $MAC387^+$).[36] Increased macrophage densities in the RP were also demonstrated in *Plasmodium falciparum*-infected humans.[22, 23] In *B. divergens*-infected Mongolian gerbils, flow cytometry attributed the observed splenomegaly to increased macrophage densities.[17]

Reasons behind increased macrophage densities in fatal 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 and affect splenic cellular arrangement. The cytokines of macrophage origin (such as TNFa and MCP-1) have also been shown to be elevated in *B. rossi* and are associated with disease severity and outcome.[37] Selective depletion of monocyte-macrophages in mice chronically infected with *Babesia microti* impaired 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.[38] Intra-erythrocytic haemoparasite removal (in a process called 'pitting') is an important function of the macrophage population of the spleen during haemoprotozoal infections.[39] An older study evaluated the rate of erythrocyte loss in splenectomized *B. rodhaini* and *B. microti*-infected rats.[40] The authors demonstrated that splenectomy does not abolish but only delays the loss of erythrocytes suggesting that monocyte-macrophages of liver and bone marrow origin take over this phagocytic function.[40] The spleen does however play a very important role in the clearance of haemoprotozoal organisms as evidenced by the death of splenectomized rats infected with *Plasmodium berghei*.[40] 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.[41] In particular, the specific absence of marginal zone and RP macrophages contributed to the failure to control the parasitaemia in the infected mice.[41]

Subversion of macrophage functions by protozoa (for example *Plasmodium, Trypanosoma*, *Toxoplasma* and *Theileria* spp.) is an important indication of the crucial role macrophages play in host defence.[42-44] Strategies employed by these organisms to avoid macrophage detection include antigenic variation, limited antigen production and altered antigen presentation.[42-44] 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.[45, 46] Alternatively activated macrophages are characterized by lowered production of pro-inflammatory cytokines and increased levels of scavenger receptors, and they promote the killing of parasites.[46]

Alternatively activated macrophages have been shown to suppress T-lymphocyte activation in a variety of infectious diseases, that is trypanosomiasis, toxoplasmosis and pathogenic theileriosis.[47-51] Considering the immune pathogenesis of haemoprotozoal diseases, a significant host T-lymphocyte response would be expected. The current study showed no significant difference between the densities of $CD3^+$ T-lymphocytes in the experimental versus control spleens. The mechanisms underlying the lack of a hyperplastic T-lymphocyte response could be twofold: a proportion or all of the hyperplastic macrophages may be alternatively activated macrophages which could have a suppressive effect on T-lymphocyte activation. Another possible explanation could be that various subpopulations of T-lymphocytes (ie 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.[52] The authors suggested that functional immunosuppression could be an important consequence of this observation.[52] It could be argued that lymphocyte depletion during acute infection results in immunosuppression and contributes to a fatal outcome. Severe disease may also result in acute lymphocytolysis, leading to T-cell population collapse. Lymphocyte accumulation in other organs may also contribute to low spleen numbers through redistribution.

Proportions of $CD20^+$ B-lymphocytes, $Pax\text{-}5^+$ immature B-lymphocytes and $Mum\text{-}1^+$ plasma cells were not significantly different between infected and control spleens. Various studies on protozoal infections have implicated B-lymphocytes and their associated antibodies in parasite clearance with the associated survival of the mammalian host.[53-55] B-lymphocytes originate from the bone marrow and then migrate and mature in the spleen. Protozoa are able to influence B-lymphocyte development and consequently the humoral immune response.[33, 56, 57] Considering the splenomegaly commonly observed in dogs with babesiosis, this may not reflect a real drop in cell number, and 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 follicles.[33] In contrast, Urban and co-workers (2005) found a true B-lymphocyte depletion, without any evidence of lymphocyte relocation in the spleens of humans with fatal natural *Plasmodium falciparum* infection.[22, 23, 33]

The results of our study indicate that *B. rossi* results in B-lymphocyte relocation to the RP. It is possible that *Babesia* parasites, like other protozoans, influence B-lymphocyte development and progression.[33] In a flow cytometric study by Rautenbach et al, it was shown that *B. rossi* infection resulted in a depletion of CD21 positive B-lymphocytes in blood.[52] As such, it does appear that *B. rossi* results in a generalized (even if transient) depletion of important cells of acquired immunity.

The pathology study described here is limited in that it only provided a static snapshot analysis in the terminal stage of fatal disease. An evaluation of the spleen in milder disease may help us understand the role of the spleen in resolving infections which may help discriminate between events associated with death and events associated with disease resolution. This would be potentially important in the spleen which is a highly plastic organ that is capable of remarkable structural rearrangement in response to disease challenge. This study is also limited in that it does not quantify cell numbers. Histology provides an architectural description of an organ but not an accurate quantitative assessment of the cells that comprise the organ. Detailed immunohistochemical characterization of lymphocyte, macrophage (specifically marginal zone macrophages) and dendritic cell subpopulations would also be invaluable particularly if performed in combination with a flow cytometric assessment of immune cell subtypes.

This is the first report on the pathology of the spleen in this important African disease, and novel insights have been provided. The major findings included splenomegaly, loss of a red pulp and white pulp delineation and a significant increase in the macrophage population. The majority of our findings are in agreement with histomorphological descriptions of the spleen in a variety of noncanid mammalian hosts with lethal malaria or babesiosis. The pathogenesis of canine babesiosis is poorly understood.

CONFLICT OF INTEREST

The authors declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

AUTHOR CONTRIBUTIONS

All the listed authors made substantial contributions to this publication. Alischa Henning was primarily responsible for the acquisition of data, data analysis, interpretation and drafting of the manuscript. Both Andrew Leisewitz and Sarah Clift played an important role in formulating the concept and design of the research, revisiting concepts and statements within the drafted manuscript as well as gave final permission for its submission.

REFERENCES

[1]Cesta MF. Normal structure, function, and histology of the spleen. *Toxicol Pathol*. 2006; 34(5): 455- 465.
[2]Ross MH, Kaye GI, Pawlina W. Histology a text and atlas. Histology a text and atlas. Lymphatic system. Philadephia: Lippincott Williams & Wilkins; 2003: 382- 387.
[3]Tsoukas CM, Bernard NF, Abrahamowicz M, et al. Effect of splenectomy on slowing human immunodeficiency virus disease progression. *Arch Surg*. 1998; 133(1): 25- 31.
[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]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.
[6]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. *J Immunol*. 2003; 171(3): 1148- 1155.
[7]Grun J, Long C, Weidanz W. Effects of splenectomy on antibody-independent immunity to Plasmodium chabaudi adami malaria. *Infect Immun*. 1985; 48(3): 853- 858.
[8]Yap GS, Stevenson MM. Differential requirements for an intact spleen in induction and expression of B-cell-dependent immunity to Plasmodium chabaudi AS. *Infect Immun*. 1994; 62(10): 4219- 4225.
[9]Krause PJ, Daily J, Telford SR, Vannier E, Lantos P, Spielman A. Shared features in the pathobiology of babesiosis and malaria. *Trends Parasitol*. 2007; 23(12): 605- 610.
[10]Clark I, Jacobson L. Do babesiosis and malaria share a common disease process. *Ann Trop Med Parasitol*. 1998; 92(4): 483- 488.
[11]Jacobson LS. The South African form of severe and complicated canine babesiosis: clinical advances 1994–2004. *Vet Parasitol*. 2006; 138(1–2): 126- 139.
[12]Leisewitz AL, Goddard A, Clift S, et al. A clinical and pathological description of 320 cases of naturally acquired Babesia rossi infection in dogs. *Vet Parasitol*. 2019; 271: 22- 30.
[13]Schoeman JP. Canine babesiosis. *Onderstepoort J Vet Res*. 2009; 76(1): 59- 66.
[14]Schneider DA, Yan H, Bastos RG, 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.
[15]Hussein HS. Babesia microti and Babesia hylomysci: spleen and phagocytosis in infected mice. *Exp Parasitol*. 1979; 47(1): 1- 12.
[16]Roberts JA, Kerr JD, Tracey-Patte P. Function of the spleen in controlling infections of Babesia rodhaini in mice. *Int J Parasitol*. 1972; 2(2): 217- 226.

[17]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.
[18]Maegraith B, Gilles HM, Devakul K. Pathological processes in Babesia canis infections. *Zeitschrift fur Tropenmedizin und Parasitologie*. 1957; 8(4): 485- 514.
[19]Matjila PT, Leisewitz AL, Jongejan F, Penzhorn BL. Molecular detection of tick-borne protozoal and ehrlichial infections in domestic dogs in South Africa. *Vet Parasitol*. 2008; 155(1): 152- 157.
[20]Bancroft JD, Gamble M. Theory and Practice of Histological Techniques. London; New York: Churchill Livingstone; 2002.
[21]O'Keane JC, Wolf BC, Neiman RS. The pathogenesis of splenic extramedullary hematopoiesis in metastatic carcinoma. *Cancer*. 1989; 63(8): 1539- 1543.
[22]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. *Malar J*. 2007; 6(1): 59.
[23]Urban BC, Hien TT, Day NP, et al. Fatal Plasmodium falciparum malaria causes specific patterns of splenic architectural disorganization. *Infect Immun*. 2005; 73(4): 1986- 1994.
[24]Laman M, Aipit S, Bona C, et al. Ultrasonographic assessment of splenic volume at presentation and after anti-malarial therapy in children with malarial anaemia. *Malar J*. 2015; 14(1): 219.
[25]Djokic V, Akoolo L, Parveen N. Babesia microti infection changes host spleen architecture and is cleared by a Th1 immune response. *Front Microbiol*. 2018; 9: 85.
[26]Weiss L. The spleen in malaria: the role of barrier cells. *Immunol Lett*. 1990; 25(1–3): 165- 172.
[27]Yadava A, Kumar S, Dvorak JA, Milon G, Miller LH. Trafficking of Plasmodium chabaudi adami-infected erythrocytes within the mouse spleen. *Proc Natl Acad Sci*. 1996; 93(10): 4595- 4599.
[28]Kraal G, Mebius R. New insights into the cell biology of the marginal zone of the spleen. *Int Rev Cytol*. 2006; 250: 175- 215.
[29]Mebius RE, Kraal G. Structure and function of the spleen. *Nat Rev Immunol*. 2005; 5(8): 606- 616.
[30]Steiniger B, Barth P. Microanatomy and function of the spleen. *Adv Anat Embryol Cell Biol*. 2000; 151: III- IX, 1–101.
[31]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.
[32]Steiniger B, Ruttinger L, Barth PJ. The three-dimensional structure of human splenic white pulp compartments. *J Histochem Cytochem*. 2003; 51(5): 655- 664.
[33]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. *J Immunol*. 2003; 171(1): 317- 324.
[34]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.
[35]Eurell JA, Frappier BL. Dellman's Textbook of Veterinary Histology. In: JA Eurell, BL Frappier, eds. Dellman's Textbook of Veterinary Histology. Immune system. 6th ed. Oxford, UK: Blackwell Publishing Ltd; 2006: 147- 151.
[36]Swirski FK, Nahrendorf M, Etzrodt M, et al. Identification of splenic reservoir monocytes and their deployment to inflammatory sites. *Science*. 2009; 325(5940): 612- 616.
[37]Leisewitz A, Goddard A, De Gier J, et al. Disease severity and blood cytokine concentrations in dogs with natural Babesia rossi infection. *Parasite*. 2019; 41: e12630.

[38]Li Y, Terkawi MA, Nishikawa Y, et al. Macrophages are critical for cross-protective immunity conferred by Babesia microti against Babesia rodhaini infection in mice. *Infect Immun*. 2012; 80(1): 311- 320.
[39]Anyona SB, Schrier SL, Gichuki CW, Waitumbi JN. Pitting of malaria parasites and spherocyte formation. *Malar J*. 2006; 5: 64.
[40]Todorovic R, Ferris D, Ristic M. Roles of the spleen in acute plasmodial and babesial infections in rats. *Exp Parasitol*. 1967; 21(3): 354- 372.
[41]Terkawi MA, Cao S, Herbas MS, et al. Macrophages are the determinant of resistance to and outcome of nonlethal Babesia microti infection in mice. *Infect Immun*. 2015; 83(1): 8- 16.
[42]Stanisic DI, Barry AE, Good MF. Escaping the immune system: how the malaria parasite makes vaccine development a challenge. *Trends Parasitol*. 2013; 29(12): 612- 622.
[43]Melo MB, Jensen KD, Saeij JP. Toxoplasma gondii effectors are master regulators of the inflammatory response. *Trends Parasitol*. 2011; 27(11): 487- 495.
[44]Kling JC, Körner H. Different regulatory mechanisms in protozoan parasitic infections. *Int J Parasitol*. 2013; 43(6): 417- 425.
[45]Rodrigues V, Cordeiro-da-Silva A, Laforge M, et al. Impairment of T cell function in parasitic infections. *PLoS Negl Trop Dis*. 2014; 8(2): e2567.
[46]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. *Clin Dev Immunol*. 2012; 2012.
[47]Flynn J, Sileghem M. The role of the macrophage in induction of immunosuppression in Trypanosoma congolense-infected cattle. *Immunology*. 1991; 74(2): 310.
[48]Schleifer KW, Mansfield JM. Suppressor macrophages in African trypanosomiasis inhibit T cell proliferative responses by nitric oxide and prostaglandins. *J Immunol*. 1993; 151(10): 5492- 5503.
[49]Sternberg JM. Elevated serum nitrate in Trypanosoma brucei 'rhodesiense'infections: evidence for inducible nitric oxide synthesis in trypanosomiasis. *Trans R Soc Trop Med Hyg*. 1996; 90(4): 395.
[50]Khan IA, Matsuura T, Kasper LH. IL-10 mediates immunosuppression following primary infection with Toxoplasma gondii in mice. *Parasite Immunol*. 1995; 17(4): 185- 195.
[51]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.
[52]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. *Vet Parasitol*. 2017; 241: 26- 34.
[53]von der Weid T, Honarvar N, Langhorne J. Gene-targeted mice lacking B cells are unable to eliminate a blood stage malaria infection. *J Immunol*. 1996; 156(7): 2510- 2516.
[54]Kumar S, Tarleton RL. The relative contribution of antibody production and CD8+ T cell function to immune control of Trypanosoma cruzi. *Parasite Immunol*. 1998; 20(5): 207- 216.
[55]Magez S, Schwegmann A, Atkinson R, 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]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. *J Parasitol Res*. 2011; 2011: 534697.
[57]Weiss GE, Crompton PD, Li S, et al. Atypical memory B cells are greatly expanded in individuals living in a malaria-endemic area. *J Immunol*. 2009; 183(3): 2176- 2182.