

Comparison of histological characteristics and expression of CD3 and CD79a among the hemal nodes, lymph nodes and spleens of yaks (*Bos grunniens*)

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Summary. This experiment compared the histological characteristics and distribution of CD3 and CD79a among yak lymph nodes, hemal nodes and spleen. The three organs from ten clinically healthy newborn and adult yaks were studied using histology and immunohistochemistry. The yak hemal nodes, which consisted of blood sinuses, lymphoid follicles, diffuse lymphoid tissue and lymphoid cords, appeared to share the histological characteristics of the spleen and lymph nodes: the lymphoid follicles of the hemal nodes were much like those of the lymph nodes, which were not surrounded by the central artery and periarteriolar lymphoid sheath. The lymphoid cords of the hemal node, which contained many erythrocytes, were much like the splenic cords. The sinuses of the hemal nodes had a similar structure to the lymph sinuses of the lymph nodes but were engorged with erythrocytes rather than lymph as in the lymph nodes. Interestingly, the splenic sinuses of yak were of two different types: the sinuses with obvious endothelial cells or those consisting of reticular cells. The CD3+ cells were mainly located in the paracortex area and medulla of the lymph nodes, the diffuse lymphoid tissues of the hemal nodes, and the periarteriolar lymphoid sheaths and red pulp of the spleen. Most CD79a+ cells were mainly detected in the lymphoid follicles of all examined lymphoid organs. The

results suggested that although the three organs had specific characteristics, in some respects, they had similar organizational structural characteristics and immune functions. These may be useful to better understand the relationship between the morphology and function of these organs and provide useful references for normal yak lymphoid organs.

Key words: Yak, Hemal node, Sinuses, Lymphoid follicles

Introduction

Yaks (*Bos grunniens*) are native plateau animals that live on the Qinghai-Tibetan Plateau and in adjacent regions (Wiener et al., 2003) and provide the basic resources of livelihood for Tibetans. The yaks are exposed to hypoxia, cold, ultraviolet radiation and other stress factors all year. These stress factors may have a harmful impact on the yaks' physiological function, including their immune function (Mancino et al., 2008). Therefore, basic research on the immune system of this species must be planned.

The lymph nodes and spleen are two important secondary immune organs in the body, and they constitute the main components of peripheral lymphoid tissue in mammals. As the primary sites of interaction between antigenic material and immunocompetent cells, lymph nodes play an essential role in the regional immune response (Luscieti et al., 1980). The histology

of the lymph nodes, which have an outer cortex of lymphoid follicles and an inner medulla of medullary cords separated by the lymphatic sinuses, has been studied in different animal species such as cattle (Casteleyn et al., 2008), goats (Bozkurt et al., 2014), dromedary camels (Zidan and Pabst, 2012) and buffalo (Zidan and Pabst, 2015). In our previous study, we did some research on the yak lymph node (Pu et al., 2017). The spleen is the largest secondary immune organ in the body and functions mainly in the immunological defence of the blood and filtration of old or damaged red blood cells (Cesta, 2006). Mammals present different types of spleens; for example, the storage type (non-sinusal) in horses (Tablin and Weiss, 1983) and the defensive type (sinusal) in man (Polák et al., 2009) and humped camel (Zidan et al., 2010). In the former, blood cells are spread in the capacious pulp space, which is organized by reticular cells and fibres. In the latter, the splenic sinuses are lined with obvious endothelium. In our previous study, we had a simple description of the yak spleen (Kang et al., 2014). Hemal nodes usually exist, especially in ruminants and rats. They are thought to play a role in erythropoiesis, platelet formation, erythrophagocytosis and immunity (Ezeasor and Singh, 1988). In recent years, they have come to the attention of immunologists. The hemal nodes have been studied in various species, including goats (Ozaydin et al., 2012), camels (Zidan and Pabst, 2004) and bovines (Zhang et al., 2012); however, these reports placed emphasis on the distribution and population of immune cells, and information about the morphology of the hemal nodes was rare. Although the morphological characteristics of the hemal nodes in buffalo (Zidan and Pabst, 2010) and roe deer (Bozkurt and Kabak, 2010) have been reported in detail, little information had been mentioned on the fine structure of the blood sinuses in the hemal nodes. At present, there is no detailed description of the morphology of the yak hemal nodes.

The location of immunocompetent cell expression can have important implications for immune responsiveness. T and B lymphocytes are the main cell types in the lymphoid organs. Our research once mainly focused on the expression of T-cell markers CD4 and CD8 α in the lymphoid organs of healthy yaks (Zhang et al., 2017). In this study, anti-CD3 ϵ antibody predicted reacted with cow was used to characterize the T cells, and anti-CD79a antibody reacted with cow was used to characterize the B cells in the hemal nodes, lymph nodes and spleens of yaks.

Immune organ development is mainly based on the morphology. The combination of immune morphology and function is most informative. At present, there are few detailed descriptions of the morphology of the yak hemal node. In particular, no data have been published on the comparative histology of these secondary lymphoid organs in yaks. This study would add new information about the immune system of the yak to our previous studies on the yak thymus (Zhang et al., 2016), spleen (Kang et al., 2014) and palatine tonsils (Sun et al.,

2018). As a native plateau animal, the yak may be useful to study specific, fundamental immunological questions of its immune system and for future immunopathological and comparative immunological studies.

Materials and methods

Animals and tissue collection

Yaks, both male and female (five newborn yaks, 2–5 days old; five adult yaks, 3–4 years old), were obtained from a local farmer in Xining City of Qinghai Province. All procedures were performed according to the Animal Ethics Procedures and Guidelines of the People's Republic of China, and the study was approved by the Institutional Animal Care and Use Committee (IACUC) of the College of Veterinary Medicine of Gansu Agricultural University. All yaks were considered healthy on the basis of results of a physical examination and serum biochemical analysis. The animals were euthanized by means of IV injection of pentobarbital sodium (200 mg/kg) (Sinopharm Chemical Reagent, Beijing, China). Lymph nodes, hemal nodes and spleens were excised from each animal and sampled as follows.

Light microscopy

Lymph nodes (mandibular lymph nodes, bronchial lymph nodes and mesenteric lymph nodes, each part containing five nodes), five hemal nodes (taken from the mesenteric regions and hilus of viscera) and five tissue blocks of spleen, approximately 1 cm³ from each animal, were fixed in 4% neutral paraformaldehyde phosphate buffer (pH 7.3) and processed for paraffin sectioning. For each lymph node, the fixed nodes were carefully cut into two symmetrical halves with a razor blade in a plane perpendicular to their longer diameter, then prepared serial cross-sections (4 μ m). Because the hemal nodes were too small, they were all embedded in paraffin completely and prepared in serial sections (4 μ m). Serial sections (4 μ m) of the spleen were also prepared. Then, all sections were detected with Mayer's haematoxylin and eosin (H&E) stain, Masson trichrome stain, and Gordon and Sweet's silver stain.

Transmission electron microscopy (TEM)

Fresh samples of hemal nodes and spleens, approximately 1 mm³ in size, were fixed immediately in 2.5% phosphate-buffered glutaraldehyde (pH 7.4) at 4°C. Then, the samples were post-fixed in a 1% solution of osmium tetroxide for 2 hours at 4°C, dehydrated in ascending grades of ethanol and embedded in SPION 812. Block orientation was achieved by examining 1- μ m sections stained with toluidine blue. Ultrathin sections (70 nm) were prepared, stained with alcoholic uranyl acetate and lead citrate, and viewed with a JEOL 1230 electron microscope (Tokyo, Japan) at 120 kV.

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Immunohistochemical examination

For immunohistochemistry, the samples were removed, fixed and processed for paraffin sectioning as for light microscopy. The antibodies used were rabbit monoclonal antibodies to CD3+ T cells (SP7) (Abcam, ab16669, Cambridge, England) and mouse monoclonal antibodies to CD79a+ B cells (JCB117 + HM47A9) (Abcam, ab199001, Cambridge, England). The dilution of CD3 and CD79a was 1:100.

The streptavidin/peroxidase complex immunostaining technique was applied to 4- μ m thick sections cut from paraffin blocks. The sections were dewaxed and taken through a graded alcohol series, followed by antigen retrieval in sodium citrate buffer (pH 6.0) in a microwave oven. Then, all sections were pretreated with 3% hydrogen peroxide for 10 min and with normal goat serum for 15 min. Sections were washed in phosphate-buffered saline (PBS, pH 7.2–7.4) three times and incubated with primary antibodies overnight at 4°C in a moist chamber. The next day, sections were incubated with the secondary antibody, and then sections were counterstained with AEC (Solarbio, Beijing, A2010). For negative controls, the primary antibody was replaced with PBS, while all other steps and conditions remained the same.

Results

Anatomical characteristic of hemal nodes, lymph nodes and spleen

The yak hemal nodes were dark red, spherical or oval bodies. All the nodes were randomly distributed along the course of the aorta in the thoracic and abdominal cavities, hilus of the viscera and mesenteric regions (Fig. 1A). They were always embedded in the fat and connected with small blood vessels, and sometimes the hemal nodes were tightly close to the lymph nodes. These nodes apparently differ very much with regard to their location, number and size, seldom being found under mostly similar conditions among individuals. They were very small. In general, their sizes ranged from millet to soybean (1.5–5.8 mm). Particularly in newborn yaks, some hemal nodes were almost the same size as a pinhead.

The yak lymph nodes were pink and usually oval- or soybean-shaped. The diameter of the lymph nodes ranged from 0.5–2.5 cm. The mandibular lymph nodes were situated in the submandibular space and the ventral part of the mandibular gland. The bronchial lymph nodes were distributed on the trachea branches. The mesenteric lymph nodes were mainly spread over the mesentery,

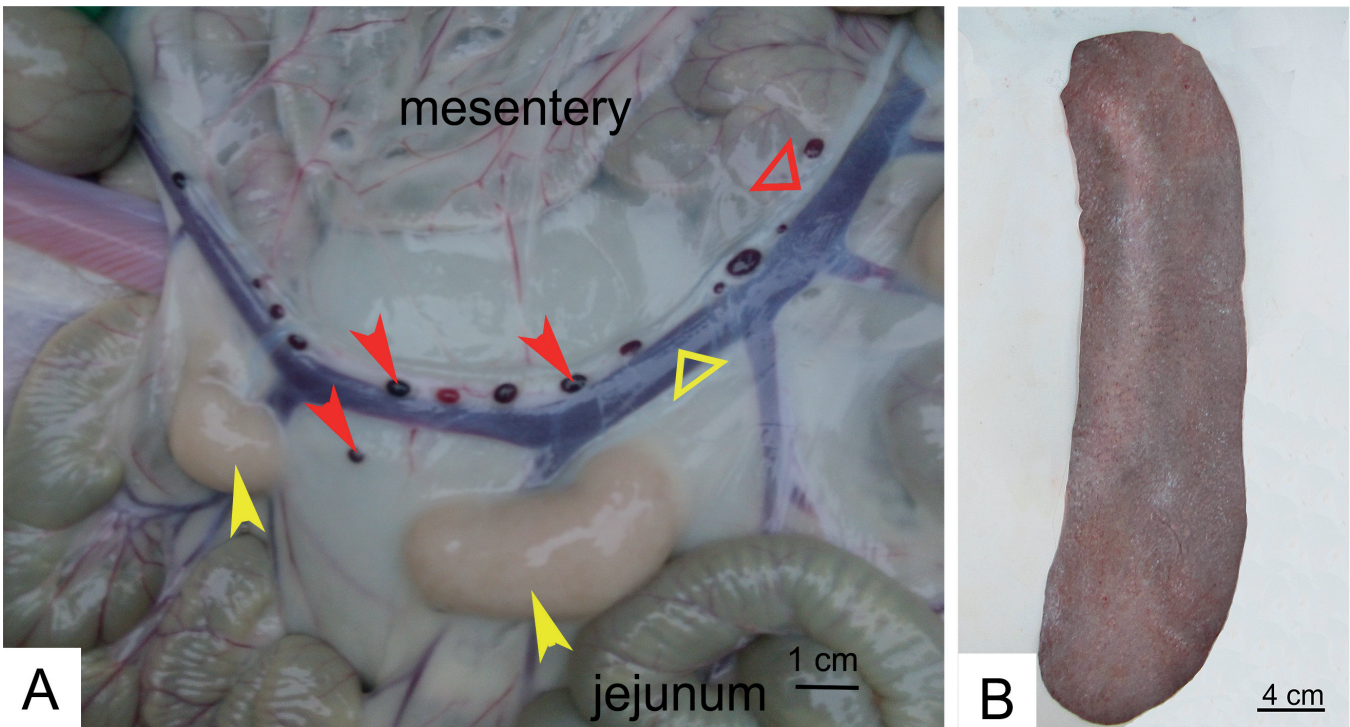


Fig. 1. Gross anatomical observations of the hemal nodes, mesenteric lymph nodes and spleens of adult yaks. **A.** Hemal nodes distribute along the cranial mesenteric artery, portal vein and the mesentery; mesenteric lymph nodes are obvious. Red arrowheads indicate the hemal nodes, red triangle indicates cranial mesenteric artery, yellow arrowheads indicate lymph nodes, and yellow triangle indicates portal vein. **B.** Adult yak spleen.

showing a moniliform distribution (Fig. 1A).

The yak spleen was dark grey and had an elongated, elliptical outline, with both extremities being thin (Fig. 1B). It was located on the left cranial side of the rumen. In adult yaks, average spleen length was 41.80 ± 1.66 cm, average width was 10.96 ± 0.40 cm at its widest, and average thickness was 1.61 ± 0.43 cm at its thickest. In newborn yaks, average spleen length was 13.90 ± 1.41 cm, average width was 3.41 ± 1.83 cm at its widest, and average thickness was 0.79 ± 0.90 cm at its thickest.

The histological structure of hemal nodes, lymph nodes and spleen

Morphological studies revealed that yak hemal

nodes were composed of parenchyma and enclosed with a thin capsule. In adult yaks, the parenchyma was made up of lymphoid follicles, diffuse lymphoid tissues, lymphoid cords and blood sinuses, which included subcapsular sinuses and central sinuses. The lymph follicles were located both in the periphery and centre of the hemal nodes (Fig. 2A). In newborn yak hemal nodes, lymphoid follicles were absent, and lymphoid tissue was very loose and primarily located in the peripheral area, with erythrocytes scattered in it. The inner region was mainly occupied by blood sinuses interpenetrated by many reticular cells (Fig. 2B). The hemal nodes had a hilum through which arteries entered and from which veins left; no afferent and efferent lymphatics were observed (Fig. 2B). Around the secondary follicles,

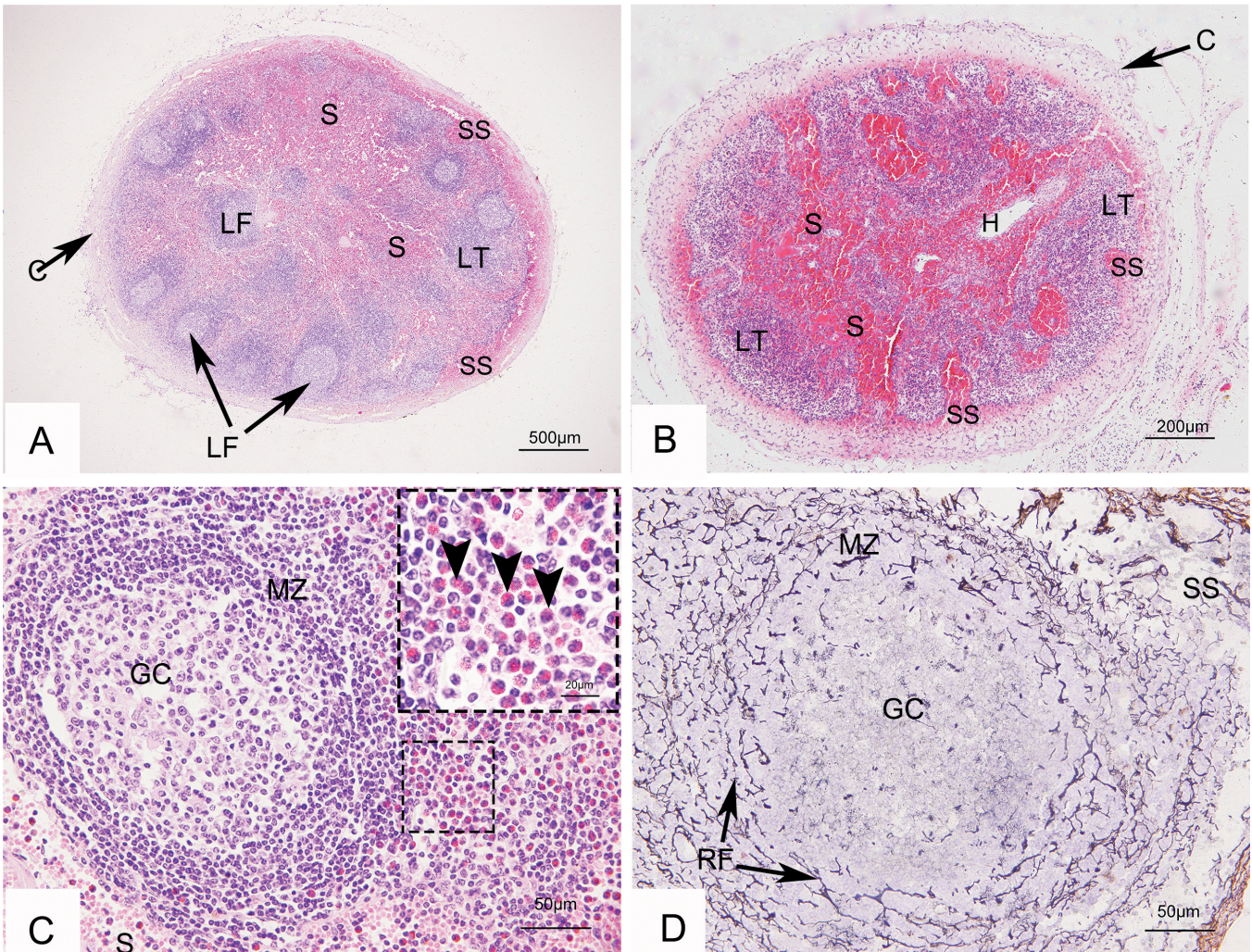


Fig. 2. Structure of the yak hemal nodes. **A.** Basic structure of the hemal nodes in adult yak, H&E stain. **B.** Basic structure of the hemal nodes in newborn yak, H&E stain. **C.** Secondary follicle of the hemal nodes, H&E stain. The dotted square indicates the details of the same area. Arrowheads indicate special eosinophils. **D.** Secondary follicle of the hemal nodes, Gordon and Sweet's silver stain. C, capsule; GC, germinal centre; H, hilum; LF, lymphoid follicles; LT, diffuse lymphoid tissue; MZ, mantle zone; RF, reticular fibres; S, blood sinuses; SS, subcapsular sinuses.

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many eosinophilic granulocyte cells were observed in the diffuse lymphoid tissues (Fig. 2C). The whole hemal node was strongly supported by reticular tissues, but reticular tissues were virtually absent from the germinal centres (Fig. 2D).

The structures of the mandibular lymph nodes, bronchial lymph nodes and mesenteric lymph nodes showed no differences. In the yak lymph node, the cortex was on the outside and the medulla was on the inside (Fig. 3A,B). The cortex was cell rich and formed of lymphoid follicles and diffuse lymphoid tissue. The medulla was made up of medullary cords of diffuse lymphocytes separated by lymphatic sinuses. In adult yak lymph nodes, lymphoid follicles were predominantly in secondary form. Sometimes, some

discrete follicles may extend deep into the cortex, so the paracortical area interdigitated with the cortical area was ill defined (Fig. 3A). In newborn yak lymph nodes, the cortex was thin, lymphoid follicles were mainly presented in primary form and ranged under the capsule (Fig. 3B), and the paracortex was not obvious. By means of argentaffin staining, the parenchyma was observed to be supported by the reticular tissues. The reticular fibres extended through the lumen of the subcapsular sinuses and were rarely observed in the germinal centre (Fig. 3C,D).

In the adult yak spleen, the white pulp was composed of splenic follicles and the periarteriolar lymphoid sheaths. The central artery was located on the side of the splenic follicle. The marginal zone was

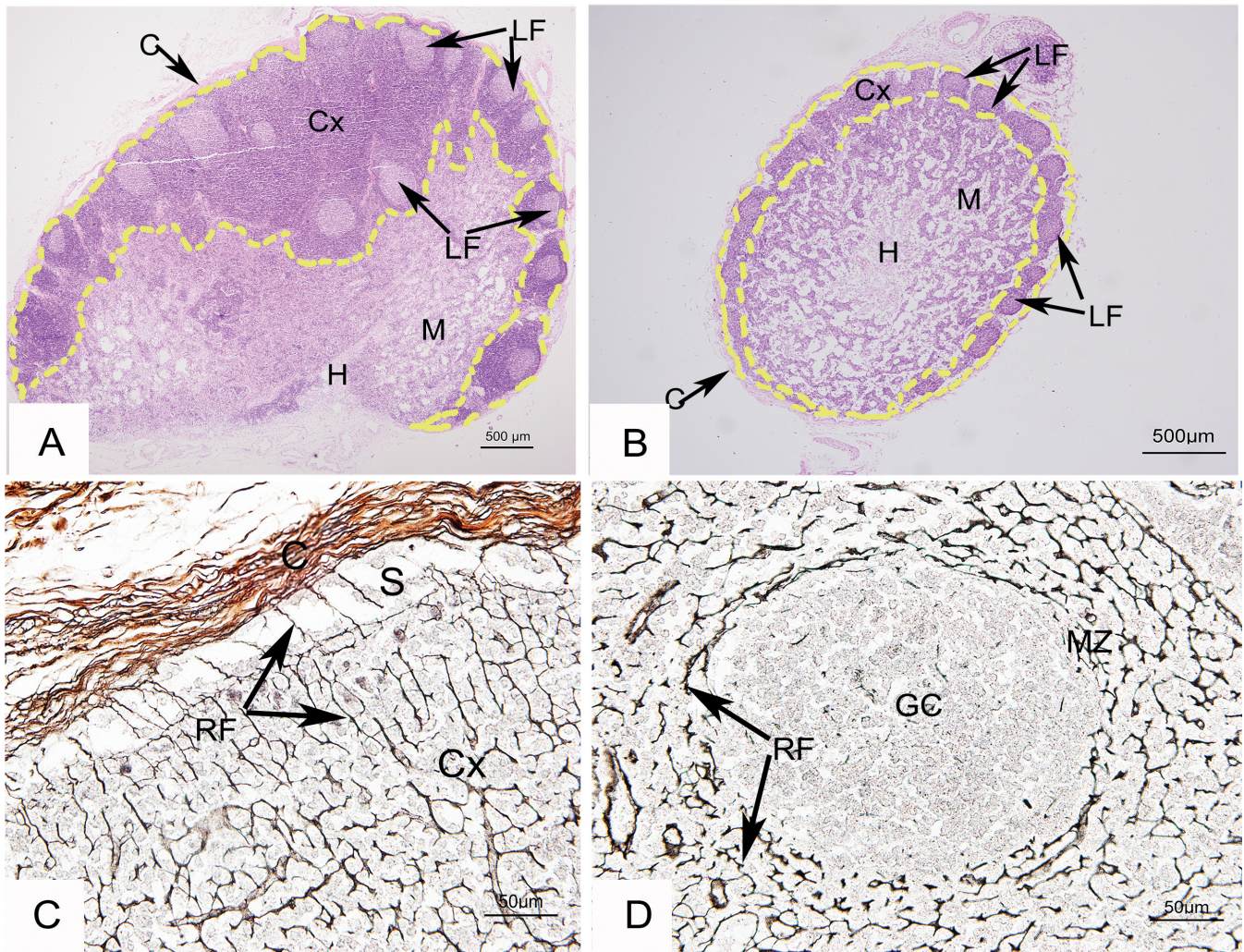


Fig. 3. Structure of the yak lymph nodes. The region surrounded by yellow dotted lines represents the cortical region. **A.** Basic structure of the lymph nodes in adult yaks, H&E stain. **B.** Basic structure of the lymph nodes in newborn yaks, H&E stain. **C.** Subcapsular sinuses of the lymph nodes, Gordon and Sweet's silver stain. **D.** Secondary follicle of the lymph nodes, Gordon and Sweet's silver stain. C, capsule; Cx, cortex; GC, germinal centre. H, hilum; LF, lymphoid follicles; M, medulla. MZ, mantle zone; RF, reticular fibres; S, Subcapsular sinuses.

situated at the interface of the red pulp and the white pulp, with many erythrocytes spread throughout it (Fig. 4A). In newborn yaks, the white pulp was only formed by the periarteriolar lymphoid sheaths, which encircled the central artery, no splenic follicles were observed and the marginal zone was not obvious (Fig. 4B). The splenic follicles appeared in three different shapes: primary splenic nodules, which did not form germinal centres and had darker and denser cells in the centre and lightly stained cells on the edge (Fig. 4C); secondary splenic follicles, which had clear germinal centres with light staining and cells around the germinal centre with denser coloration (Fig. 4A); and transitional splenic follicles, between the two former types, which had an indefinite area of lightly stained cells beginning to appear in the centre. In the serial sections, this region

gradually emerged and expanded, with the whole follicle showing a light staining, then a dark staining, and then a light staining from the inside out (Fig. 4D). The red pulp was composed of splenic cords and splenic sinuses.

The schematic diagrams of the hemal nodes, lymph nodes and spleen are summarized in Fig. 5.

Comparison of sinuses in hemal node, lymph node and spleen

The blood sinuses in the hemal nodes had an irregular shape, and their size and blood content varied greatly. Both the central blood sinuses and subcapsular sinuses were lined with endothelial cells and engorged with erythrocytes; a few macrophages, many lymphocytes, platelets and some eosinophilic

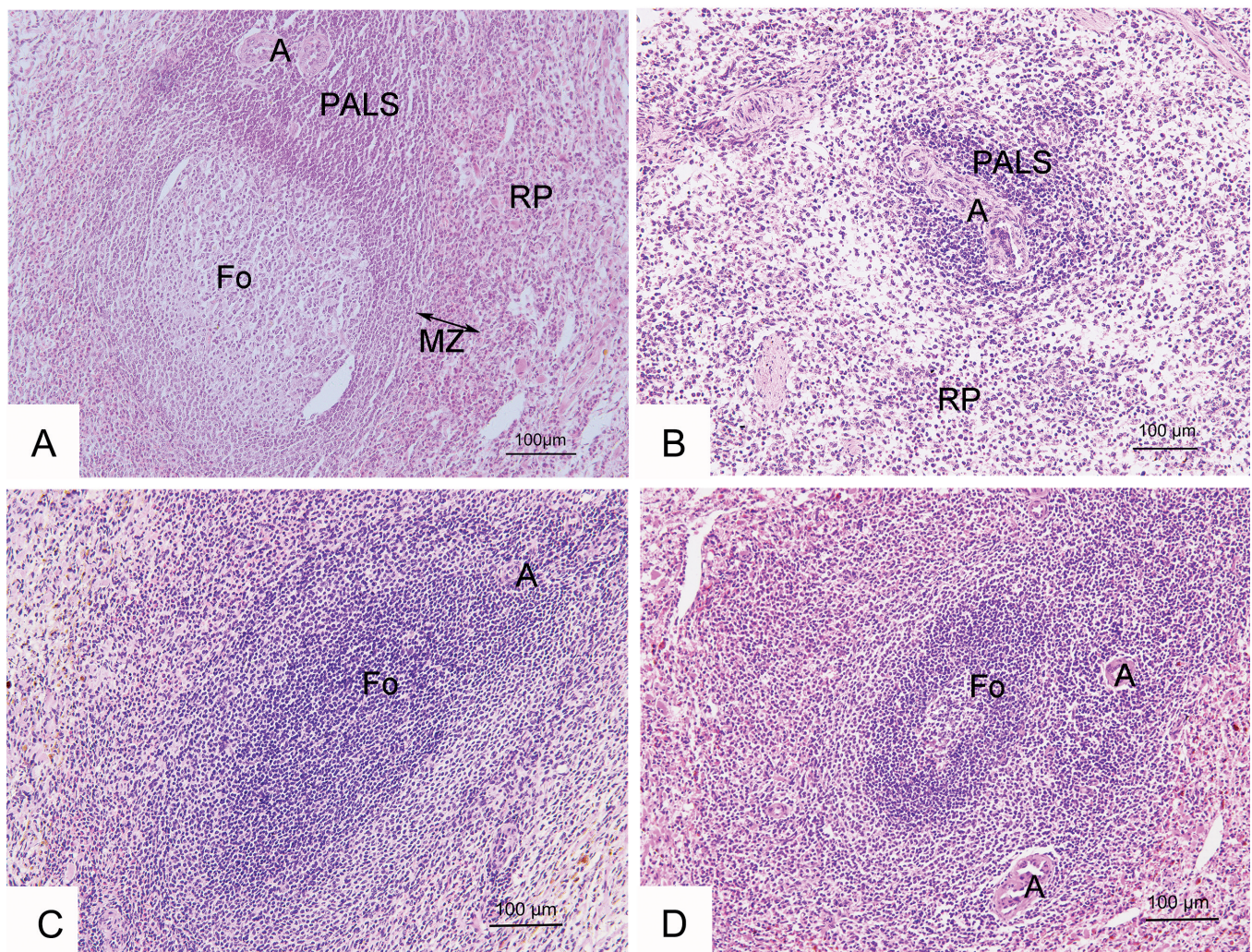


Fig. 4. Histological structures of the yak spleen, H&E stain. **A.** Basic structure of the adult yak spleen. **B.** Basic structure of the newborn yak spleen. **C.** Primary splenic follicle. **D.** Transitional spleen follicle. A, central artery; Fo, splenic follicle; MZ, marginal zone; PALS, periarterial lymphatic sheath; RP, red pulp.

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granulocyte cells were also observed in it (Fig. 6A,B). The structure of the blood sinus wall in the hemal nodes showed no difference between the adult and newborn. In the lymph nodes, the subcapsular lymphatic sinuses extended under the capsule and connected to the trabecular lymphatic sinuses, which surrounded the trabeculae. The medullary sinuses among the lymphoid cords were variable in size (Fig. 6C). All the lymphatic sinuses were lined with endothelial cells. Macrophages, plasma cells and many lymphocytes were seen in the lymphatic sinuses (Fig. 6C). Compared with the hemal node, the splenic sinuses had two different morphological structures both in newborn and adult yaks: in some zones, the walls of the sinuses consisted of obvious endothelial cells (Fig. 6D); in other zones, the sinuses were composed of reticular cells, with erythrocytes wrapped by the reticular cells (Fig. 6D). In the two different types of sinuses, many macrophages were observed.

Under TEM, the endothelial cells lining the subcapsular sinuses and central blood sinuses were obvious in the hemal nodes (Fig. 7A,B). The wall of the two different splenic sinuses were easier to distinguish: in the sinusal sinus, the wall of the sinuses lined with obvious endothelial cells had an unusual discontinuous structure, which was penetrated by erythrocytes, and many erythrocytes were observed both inside and outside of the sinuses (Fig. 7C,D); in the non-sinusal sinus, the sinus was composed of reticular cells and erythrocytes spread over the pulp spaces (Fig. 7E). The electron microscopy showed that the outer wall in the lymph node, the subcapsular sinuses had an intact, continuous endothelium and the endothelial cells were flat and elongated (Fig. 8A). In the subcapsular sinuses lumen, many lymphocytes and a few reticular cells were observed in it. The inner wall of the subcapsular sinuses and the medulla sinuses both were lined by endothelial cells, and deep in the sinus endothelium was a layer of

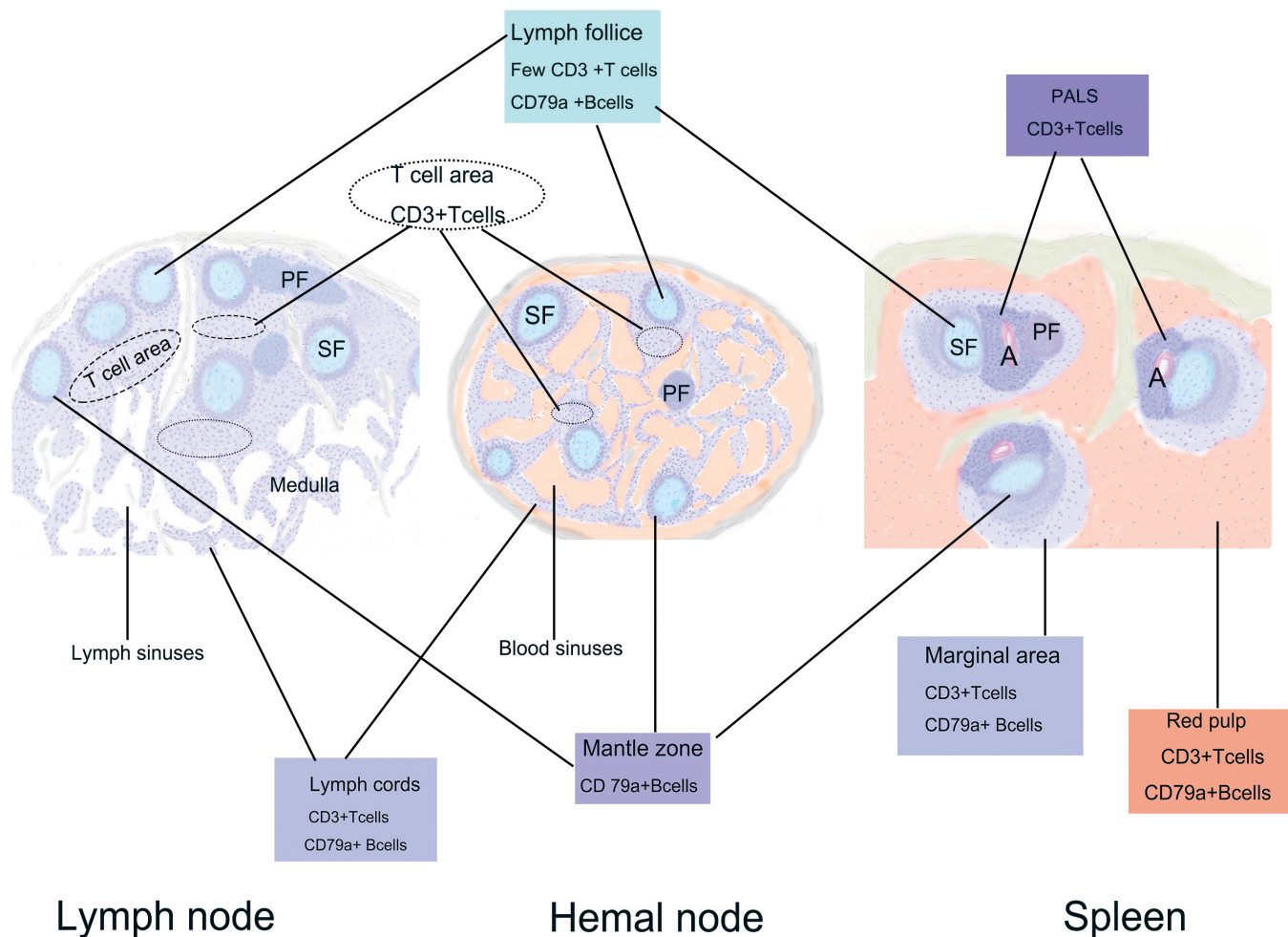


Fig. 5. Schematic diagram showing the structure and distribution of CD3+ T cells and CD79a+ B cells in the yak lymph nodes, hemal nodes and spleen. A, central artery; PF, primary follicle; SF, secondary follicle; PALS, periarterial lymphatic sheath.

connective tissue and a layer of reticular cells (Fig. 8B,C).

In the hemal nodes, a short reticulum may extend either across or only for a short distance into the lumen of the blood sinuses and divide them into small networks (Fig. 9A). In serial sections, the small blood sinuses communicated with each other and formed an open network, such as large blood sinus sets of small blood sinuses. Under TEM, the endothelial cells sometimes lined the reticular cells, separating erythrocytes from each other in the blood sinuses, and between the endothelial cells and the reticular cells were some reticular fibres (Fig. 10A). In addition, sometimes cytoplasmic processes of the reticular cell wrapping the lymphocytes and erythrocytes formed a sinus-like

structure (Fig. 10B). Although the structure of the lymph sinus wall in newborn and adult yak lymph nodes were similar, inside the lymph sinuses there were some differences. In adult yak lymph nodes, reticular fibres were densely distributed in the medullary sinuses (Fig. 9B). However, reticular fibres rarely existed in the medullary sinuses in the newborn yak lymph nodes (Fig. 9C).

Comparison of cords in hemal nodes, lymph nodes and spleen

The structure of the lymph cords in newborn and adult yak lymph nodes showed no difference. In the lymph node, the medullary cords were supported by a

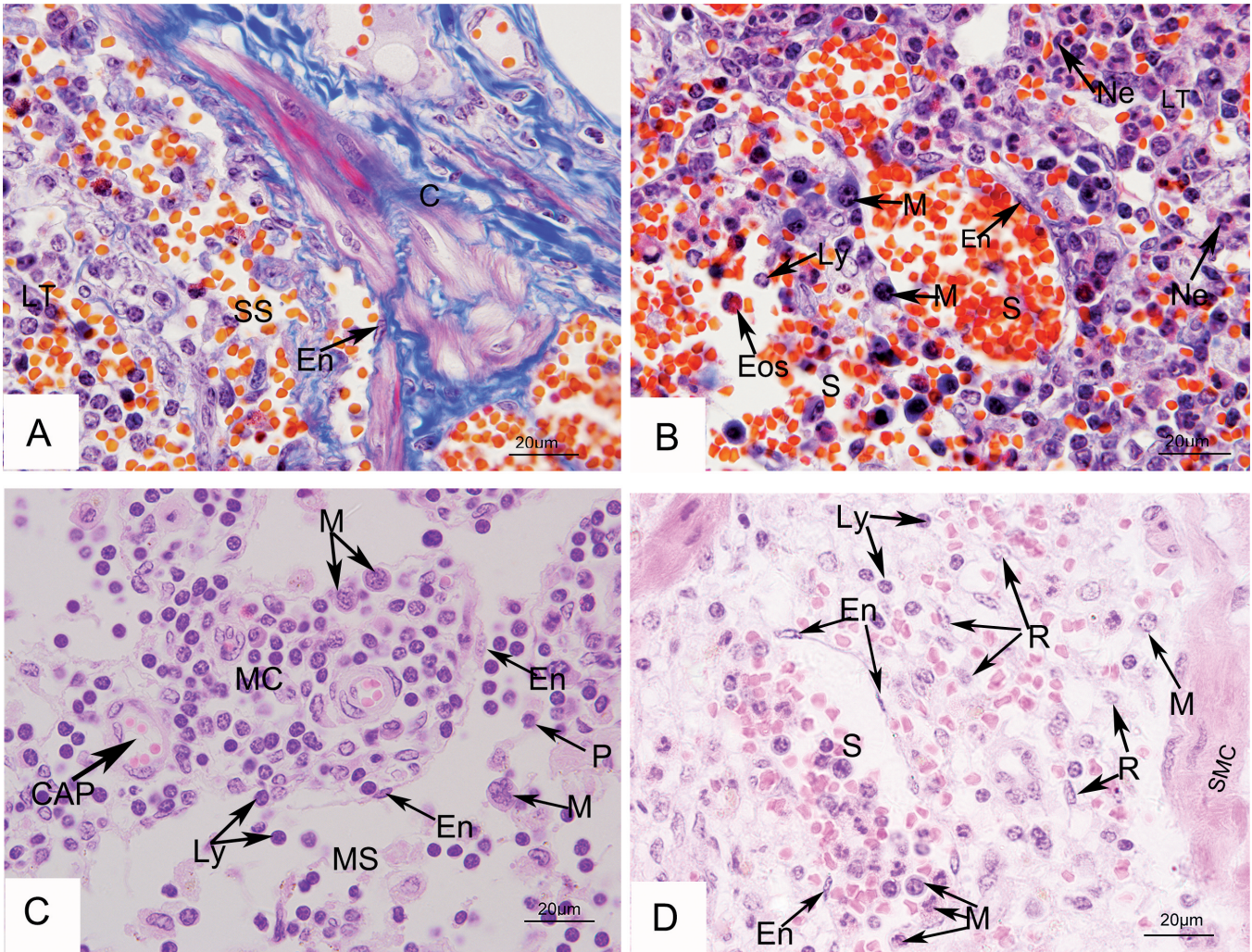


Fig. 6. Sinuses of the hemal nodes, lymph nodes and spleens of yaks. **A.** Subcapsular blood sinuses of the hemal nodes, Masson trichrome stain. **B.** Central blood sinuses of the hemal nodes, Masson trichrome stain. **C.** Medullary lymph sinuses of the lymph nodes, H&E stain. **D.** Blood sinuses of the spleen, H&E stain. C, capsule; CAP, capillary; En, endothelial cell; Eos, special eosinophils; Ly, lymphocyte; LT, lymphoid tissue; M, macrophages; MC, medullary cord; MS, medullary sinus; Ne, neutrophils; P, plasma; R, reticular cell; S, blood sinuses; SMC, smooth muscle cell; SS, subcapsular blood sinuses.

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reticular fibre network and consisted of diffuse lymphocytes and other cells, including macrophages and plasma cells (Fig. 6C). Unlike the lymph nodes, the lymphoid cords of the hemal nodes, which were composed of diffuse lymphoid tissue, have many free erythrocytes. In addition, many neutrophils, eosinophil granulocyte cells and a few megakaryocytes with a large light irregular nucleus and abundant cytoplasm were also observed; the eosinophil granulocyte cells were even more noticeable in adults (Figs. 11, 12). Under TEM, the macrophages with many cytoplasmic processes had a phagosome and a myelin-like figure, and the nucleus of the macrophages showed a deep depression to accommodate the phagosome (Fig. 12B). The plasma cells had many obvious rough endoplasmic reticulum (Fig. 12C). The eosinophilic granulocyte cells had many cytoplasmic granules; these cytoplasmic granules contained many electron-dense, crystalline, thin materials (Fig. 12D,E). In newborn yaks, the

splenic red pulp was sparse, but in adult yaks, it contained more muscle cells and became tighter. The splenic cords with irregular arrangement and without a strip shape were not easy to distinguish under the light microscope. Under TEM, the cords contained large amounts of macrophages, lymphocytes and erythrocytes (Fig. 7C,E).

Distribution of CD3+ T cells and CD79a+ B cells in hemal nodes, mesenteric lymph nodes and spleen

No positive staining was found in the control slides of the three organs. In newborn yak hemal nodes, there were no lymphoid follicles formed, and the lymphoid tissues were sparse; the CD3+ and CD79a+ cells were both observed in the diffuse lymphoid tissues (Figs. 13A, 14A). In adult yaks, the CD3+ cells were mainly located in the diffuse lymphoid tissues, which were around the lymphoid follicles, and they were also

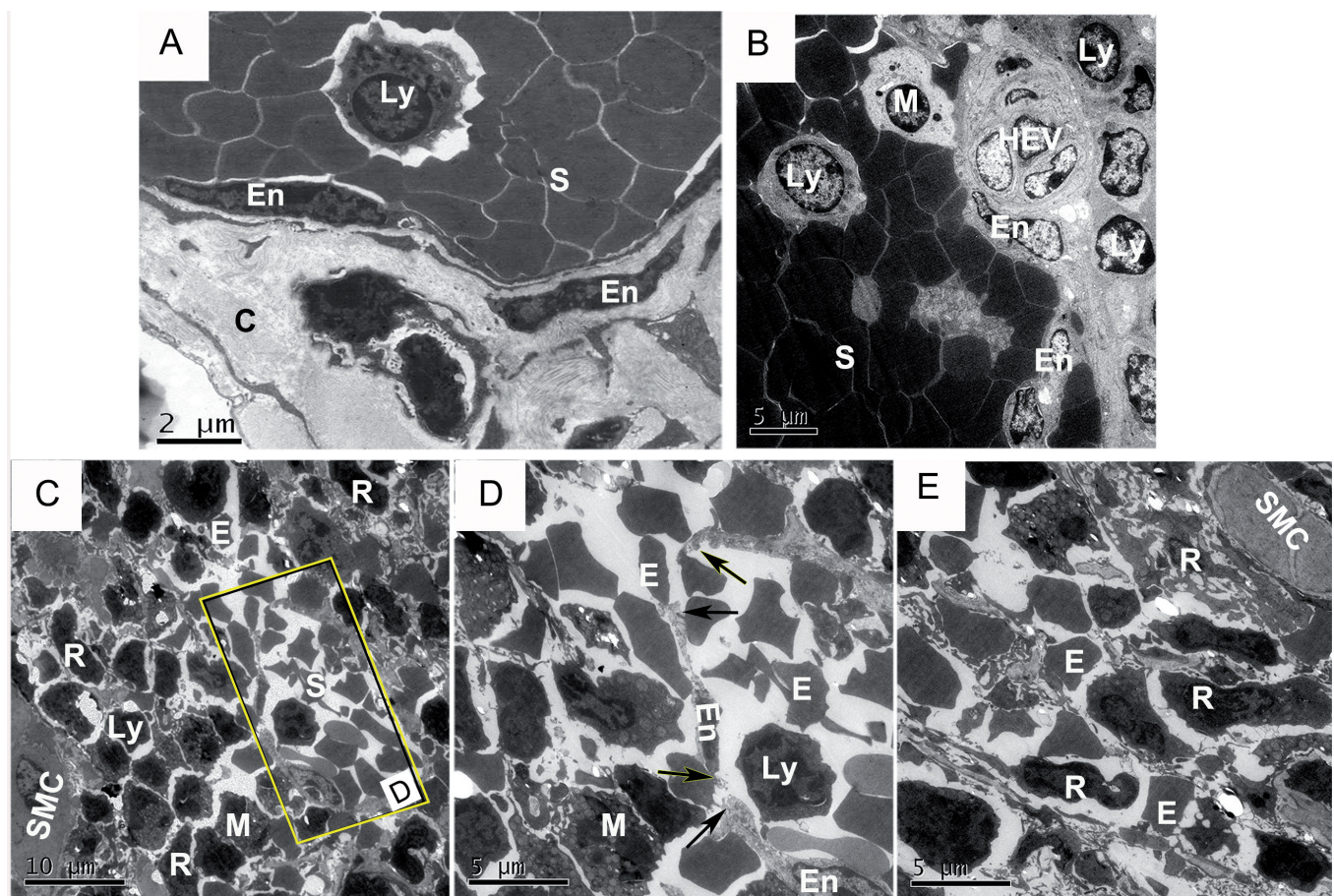


Fig. 7. Transmission electron microscopy of blood sinuses of the yak hemal nodes and spleen. **A.** Subcapsular blood sinuses of the hemal nodes. **B.** Central blood sinuses of the hemal nodes. **C.** The blood sinuses with endothelial cells of the yak spleen. **D.** Higher magnification views of (C), arrows indicate cytoplasmic processes of endothelial cells. **E.** The sinuses were composed of reticular cells of the yak spleen. C, capsule; E, Erythrocytes; En, endothelial cell; HEV, high endothelial venule; Ly, lymphocytes; M, macrophage; SMC, smooth muscle cell; R, reticular cell; S, blood sinuses.

observed in the lymphoid cords, blood sinuses and lymphoid follicles (Fig. 13B). CD79a+ lymphocytes were detected mainly in the lymphoid follicles, especially in the areas bordering the mantle zone. A few CD79+ lymphocytes were observed in the diffuse lymphoid cords and blood sinuses (Fig. 14B).

In the lymph nodes, the CD3+ cells were concentrated particularly in the paracortical and interfollicular areas. Many positive cells were also detected in the medulla, and some positive cells were also present in the lymphoid follicles (Fig. 13C,D). The

CD79a+ cells were mainly located in the lymphoid follicles and the subcapsular region; many cells were also observed in the medulla (Fig. 14C,D).

In yak spleens, cells demonstrating CD3 immunoreactivity were present primarily in the periarteriolar lymphoid sheaths, and some positive cells were also observed in the red pulp and the germinal centres of the splenic follicles (Fig. 13E,F). CD79a staining was mainly detected in the red pulp and in the mantle zone of the splenic follicles; a few positive cells were scattered in the germinal centres (Fig. 14E,F).

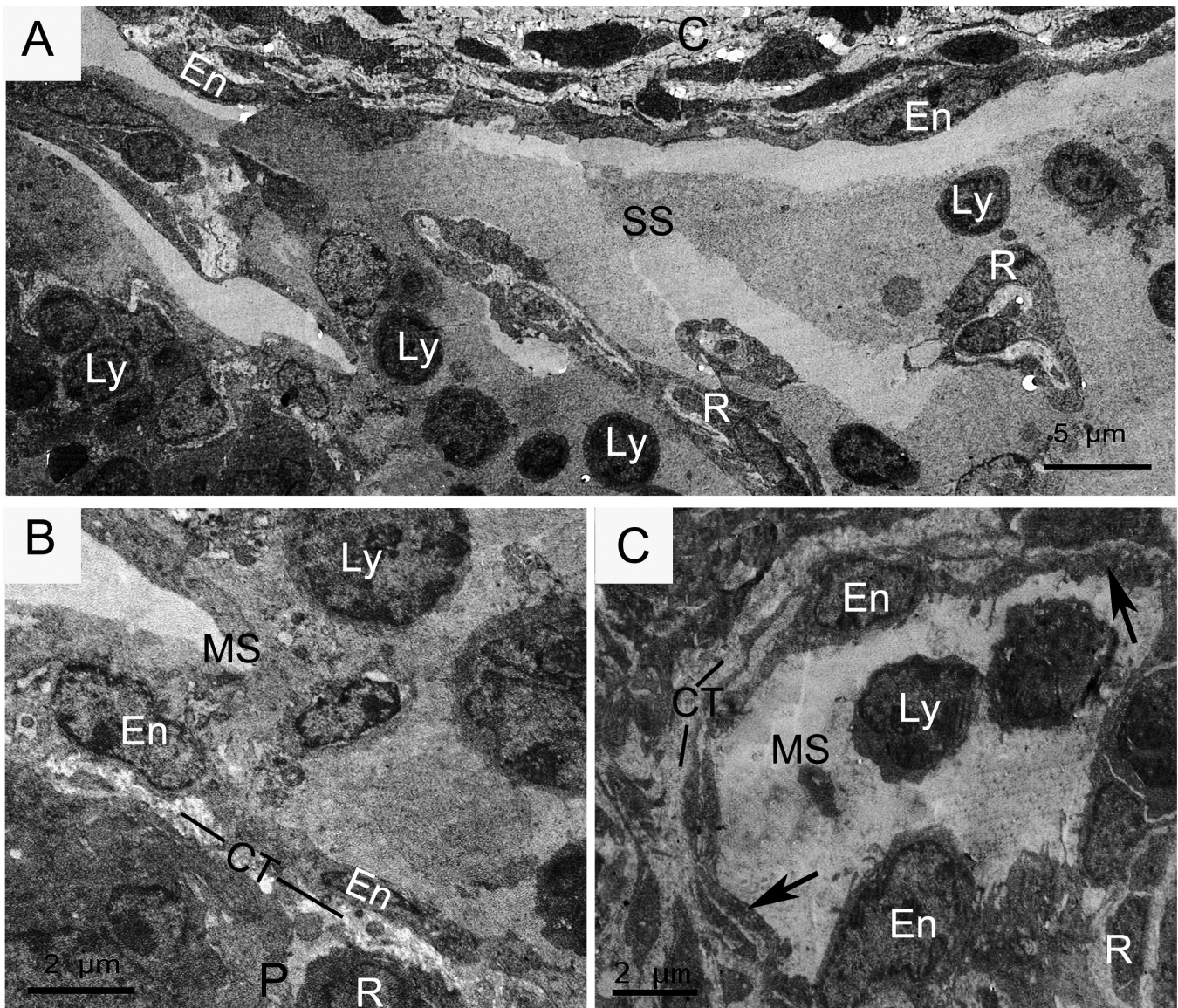


Fig. 8. Transmission electron microscopy of lymph sinuses of the adult yak lymph nodes. **A.** The subcapsular lymph sinus wall on the side of capsule. **B.** The subcapsular lymph sinus wall on the side of parenchyma. **C.** Medullary sinuses. C, capsule; CT, connective tissue; En, endothelial cells; Ly, lymphocytes; MS, medullary sinus; P, parenchyma; R, reticular cells; SS, subcapsular lymph sinus. Black arrows in image (C) indicate the cytoplasmic processes of the endothelial cells.

Expression of CD3 and CD79a in yak (Bos gruniensis) hemal node, lymph node and spleen

Almost no CD79a+ cells were observed in the periarteriolar lymphoid sheaths, while there was a relatively intense background staining of non-lymphoid cells, including smooth muscle and endothelium. The distribution of CD3+ T cells and CD79a+ B cells in the hemal nodes, lymph nodes and spleen are summarized in Fig. 5.

Discussion

This study described the comparative histology of the hemal nodes, lymph nodes and spleen for the first time in the yak. Unlike the lymph nodes, the parenchyma of the hemal nodes could not be divided into cortex and medulla, in keeping with the finding in a

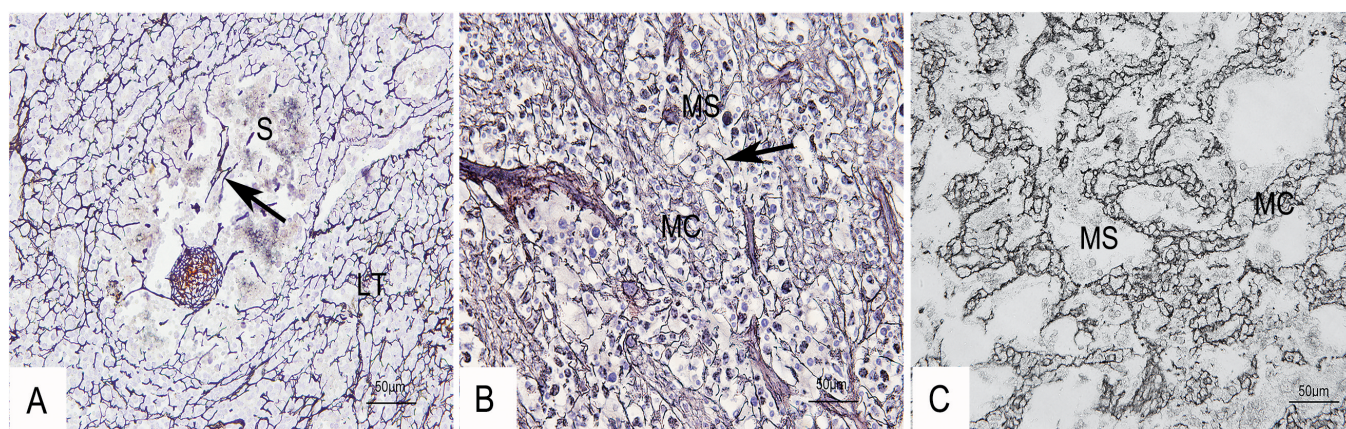


Fig. 9. Reticular network in the yak hemal nodes and lymph nodes, Gordon and Sweet's silver stain. **A.** Blood sinuses in the hemal nodes. **B.** Medullary sinuses in the lymph nodes of adult yak. **C.** Medullary sinuses in the lymph nodes of newborn yak. LT, Diffuse lymphoid tissue; MC, medullary cord; MS, medullary sinus; S, blood sinuses. Arrows indicate reticular fibres.

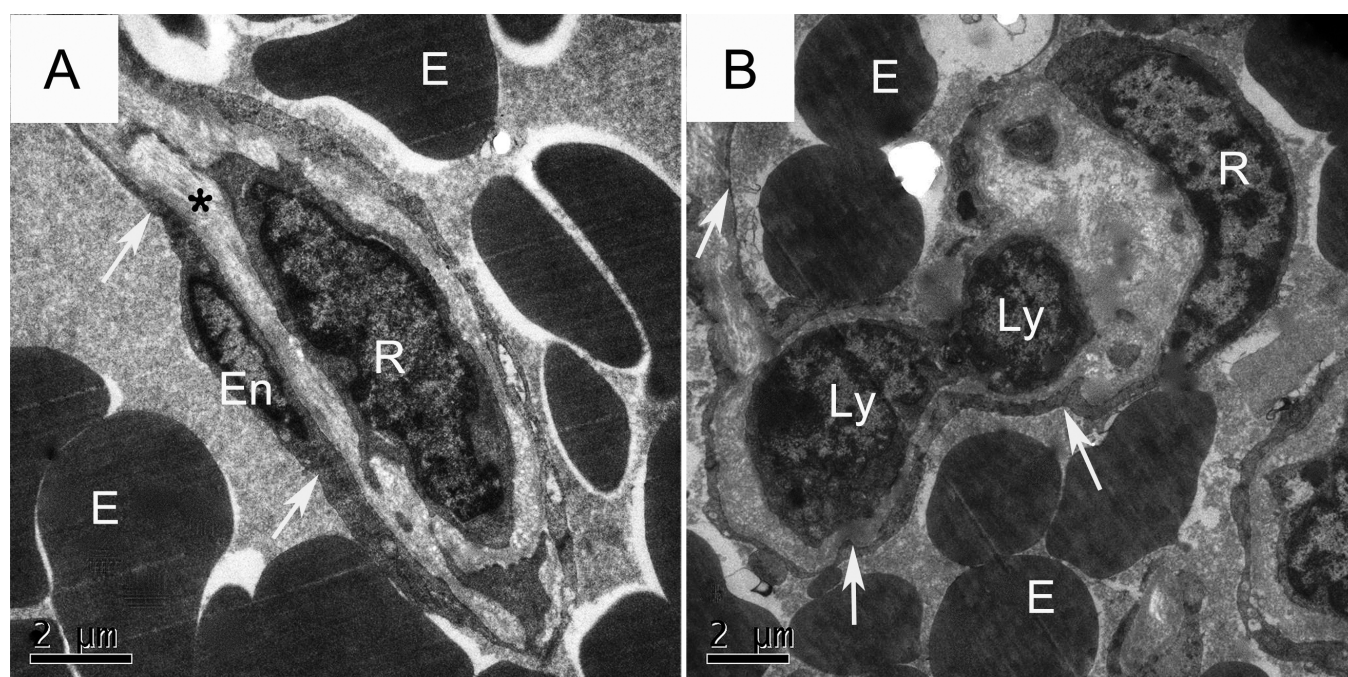


Fig. 10. Transmission electron micrographs of the blood sinuses in the yak hemal nodes. **A.** The endothelial cells lining the reticular cells. **B.** The reticular cell wrapped the lymphocytes and erythrocytes, forming a sinus-like structure. E, erythrocytes; En, endothelial cells; Ly, lymphocytes; R, reticular cells; Arrows indicate cytoplasmic processes, and asterisk indicates reticular fibres.

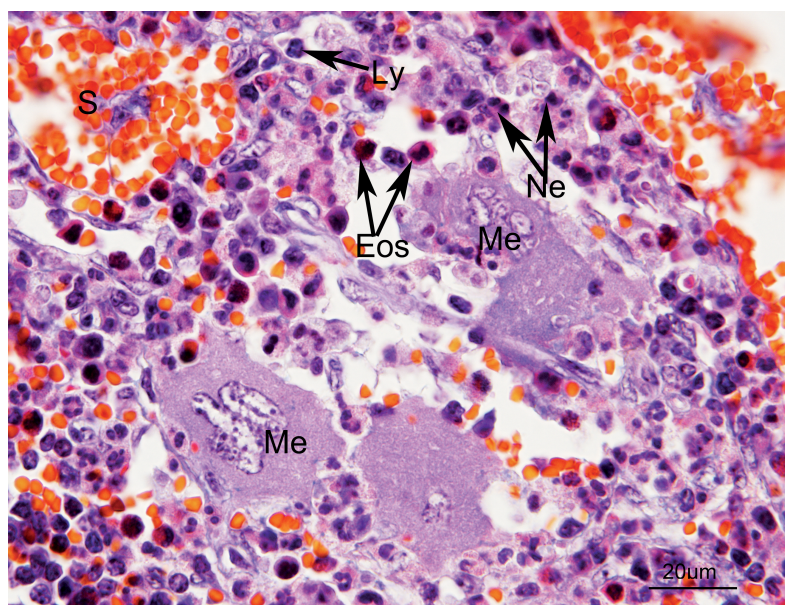


Fig. 11. Lymphoid cord of the yak hemal nodes, Masson trichrome stain. Eos, eosinophils; Ly, lymphocyte; Me, megakaryocytes; Ne, neutrophils; S, blood sinuses.

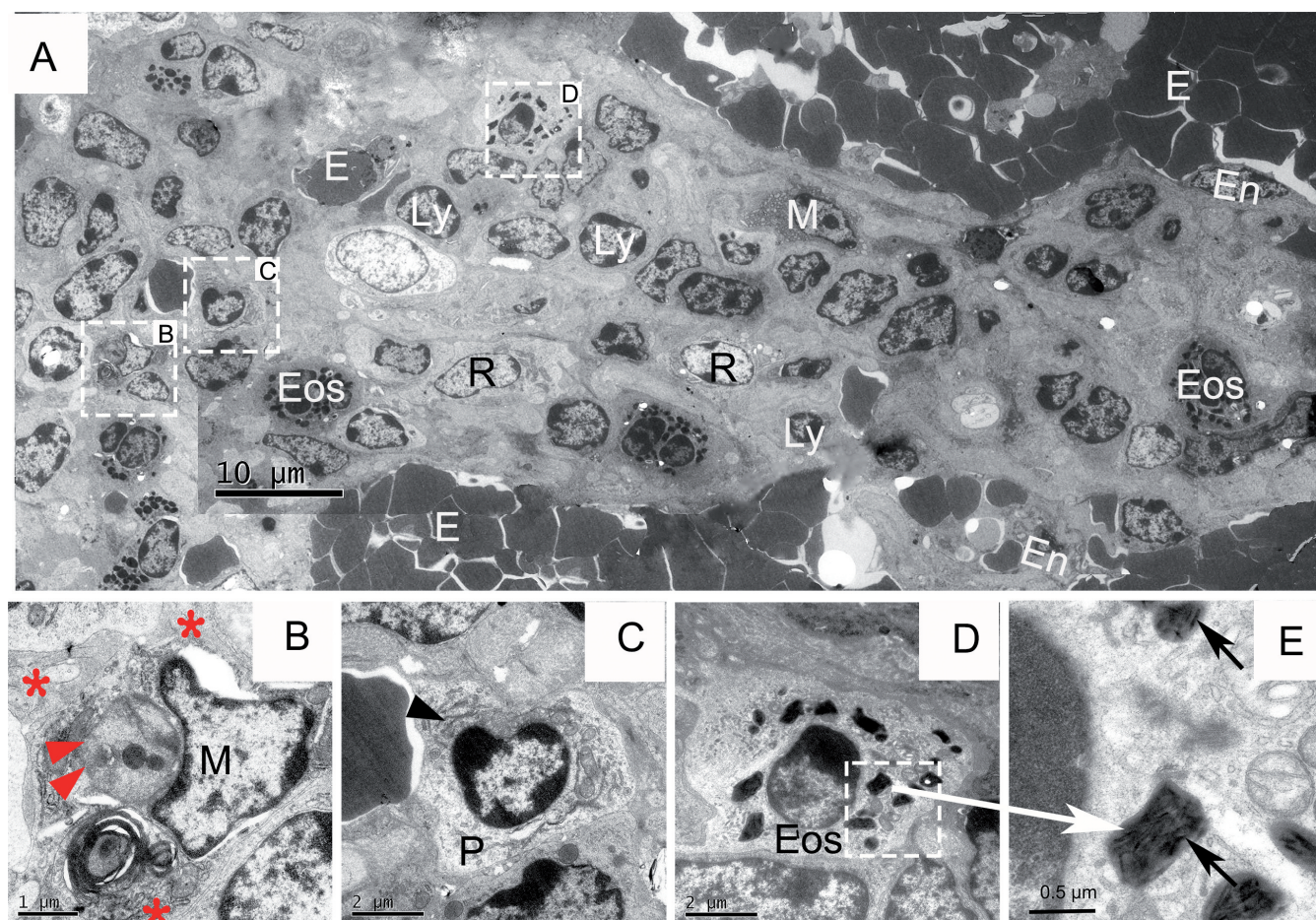


Fig. 12. Transmission electron micrographs of the lymphoid cord in the yak hemal nodes. **A.** Lymphoid cord in the yak hemal nodes. **B.** Macrophage. **C.** Plasma cell. **D.** Special eosinophil. **E.** Granules as seen in image (D) with high magnification. E, erythrocytes; En, endothelial cells; Eos, special eosinophils; Ly, lymphocytes; M, macrophages; P, plasma cells; R, reticular cells; S, blood sinuses. Red asterisks indicate cytoplasmic processes, red triangles indicate phagosomes in the macrophage, black triangle indicates rough endoplasmic reticulum in the plasma cell, and black arrows indicate granules with many electron-dense, crystalline, thin materials in the eosinophil.

Expression of CD3 and CD79a in yak (*Bos gruniensis*) hemal node, lymph node and spleen

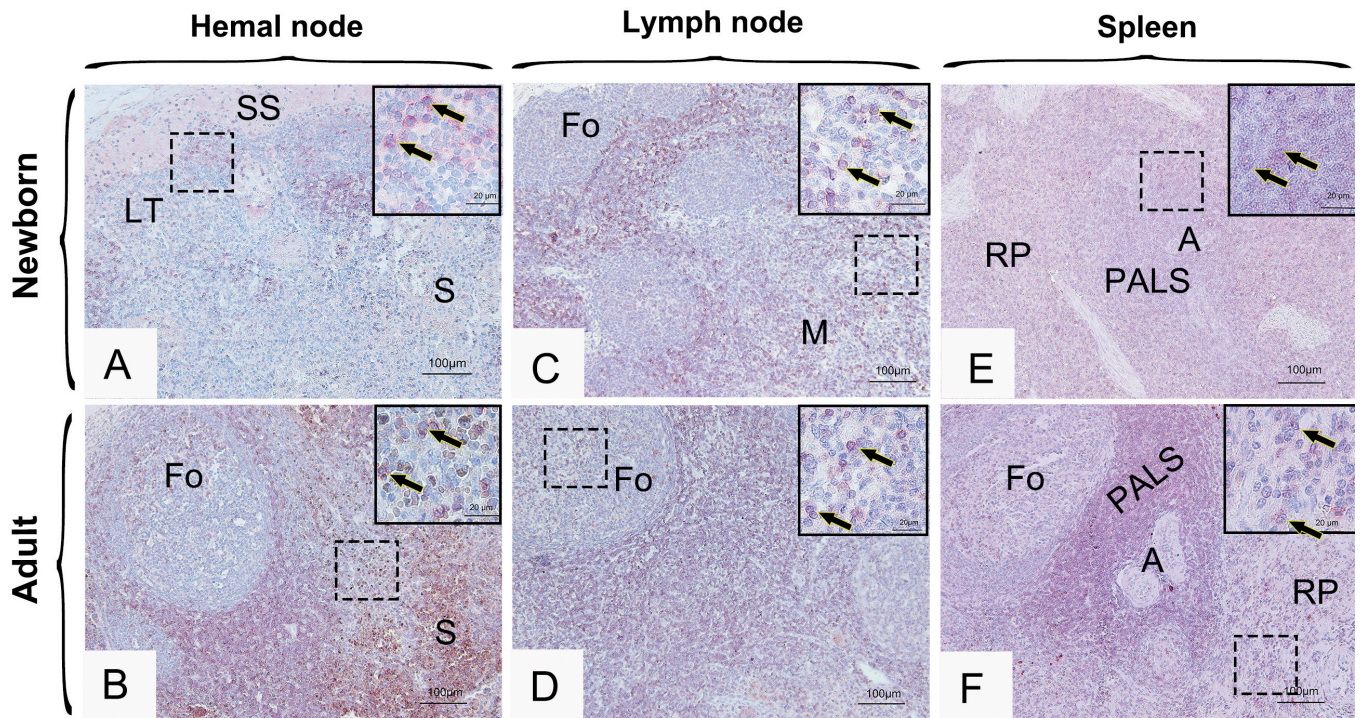


Fig. 13. Immunohistochemical staining of CD3 in the yak hemal nodes, lymph nodes and spleen. The dotted square indicates the details of the same area. A, central artery; Fo, lymphoid follicles; LT, diffuse lymphoid tissue; M, medulla; PALS, periarterial lymphatic sheath; RP, red pulp; S, blood sinuses; SS, subcapsular sinuses. Arrows show the positive cells.

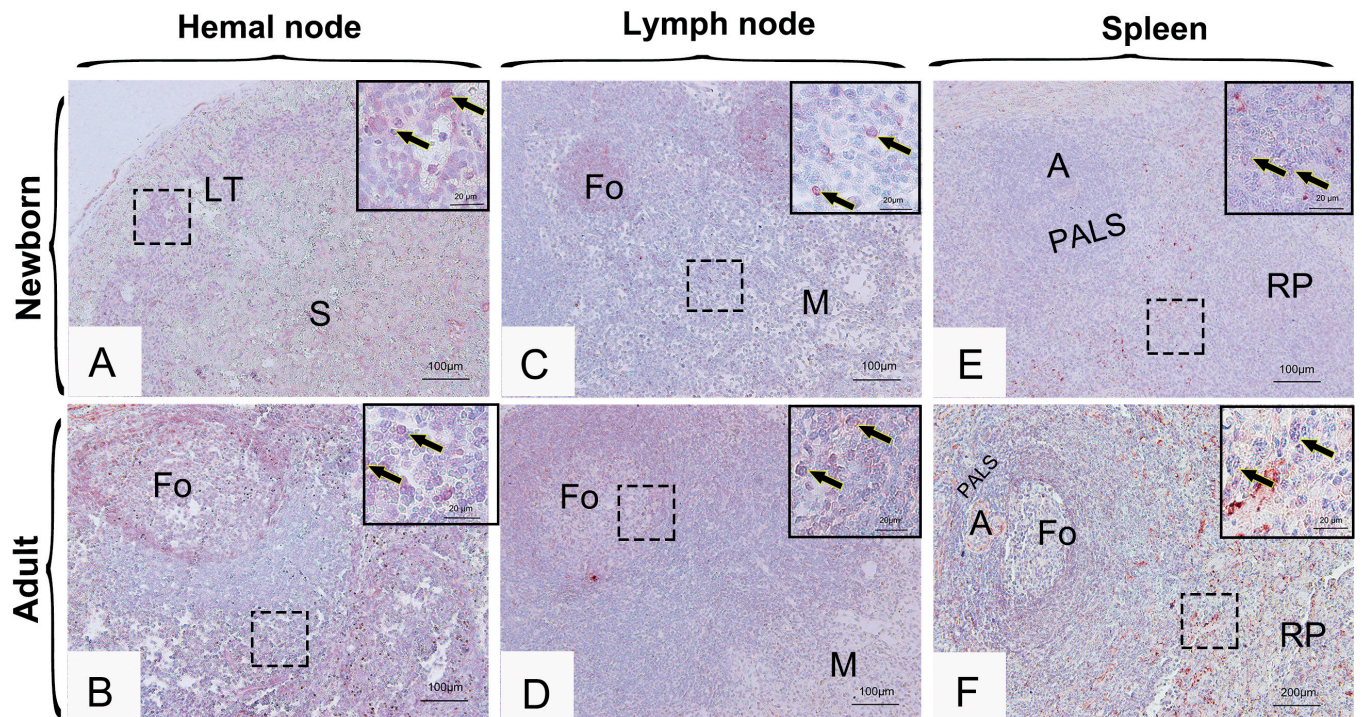


Fig. 14. Immunohistochemical staining of CD79a in the yak hemal nodes, lymph nodes and spleen. The dotted square indicates the details of the same area. A, central artery; Fo, lymphoid follicles; LT, diffuse lymphoid tissue; M, medulla; PALS, periarterial lymphatic sheath; RP, red pulp; S, blood sinuses. Arrows show the positive cells.

previous study on the yak hemal nodes (Kadiralieva et al., 2017). The lymph nodes were in the lymphatic circulation pathway, while the hemal nodes and spleen were in the blood circulation. In adult yaks, the lymphoid follicles, which were surrounded by diffuse lymphoid tissues with many erythrocytes of the hemal nodes, were similar to the white pulp of the spleen but without the central artery and the periarteriolar lymphoid sheath. The morphological structure of the cords in the hemal nodes and lymph nodes was similar; both of them presented in a strip shape. The cords of the hemal node contained many erythrocytes, the same as the observations of the spleen red pulp. The blood sinuses of the hemal node and spleen were distinctly different but showed a similar structure of the lymph sinuses in the lymph nodes, which we shall discuss below.

In newborn yaks, there was no lymphoid follicle formation in the hemal nodes and spleen. Lymphoid tissues in the hemal nodes were very loose and mostly resembled lymphocyte infiltration, as reported previously for 1-month-old goats (Ezeasor and Singh, 1988). The white pulp of the spleen in newborn yaks consisted only of periarteriolar lymphoid sheaths and was without an obvious marginal zone. In newborns, the lymph nodes had a relatively sparse medulla and fewer lymphoid follicles compared with adult yaks. This illustrated that the structure of these organs was not fully developed in the early period after birth but then gradually developed and improved under constant antigen stimulation with age (Patten and Carlson, 2002).

Germinal centres developed in peripheral lymphatic tissue during the primary immune response and played a crucial role in affinity maturation, B cell clonal expansion and somatic hypermutation (Berek et al., 1991; Vitorica and Nussenzweig, 2012). As mentioned earlier, in newborn yaks, there were no lymphoid follicles formed in the hemal nodes and the spleen, but germinal centres had appeared in the lymph nodes. This finding demonstrated that the germinal centres in the hemal nodes and spleen occurred relatively later than those in the lymph nodes in yaks. The lymph nodes might have preliminarily established the humoral immune response in the early period after birth. This phenomenon probably is related to the location of the hemal nodes and the spleen in the blood circulation and the location of the lymph node in the lymph circulation; the antigenic stimulation they receive from the blood and lymph are different. Interestingly, we first observed three different types of splenic follicles: primary, secondary and transitional splenic follicles. The special morphological structures may be due to the different stages of reaction after antigenic stimulation. Even within one individual, the development of secondary lymphatic organs was variable.

We found that the blood sinuses in the hemal nodes and the lymph sinuses in the lymph nodes were both lined with endothelial cells. In the hemal nodes, it is worth mentioning that there were many reticular cells that split the blood sinuses into small networks. Such a

morphological structure may have allowed maximum contact between the blood and the macrophages and reticular cells in the sinuses to the benefit of trapping antigenic material in the blood (Turner, 1969). Moreover, the large number of blood sinuses and many free erythrocytes in the lymphoid tissues indicated the storage capacity of the hemal nodes. In the lymph nodes, the sinus walls were made up of three layers: sinus endothelial cell, connective tissues and reticular cell, from the lumen to the parenchyma, which were in agreement with those of in human (Forkert et al., 1977). Numerous fine reticular trabeculae crossing the lymph sinuses make their lumina labyrinthine and provide a large area for the attachment of various cell types in the lumen. This may help the medullary sinuses perform adequate filtration of the lymph (Lubis et al., 1982). The sinuses of the hemal nodes and lymph nodes showed similar structure and effects in filtration, although they were engorged with different content. The fine reticular trabeculae in newborn yak lymph nodes were seldom observed because they were still not fully developed in newborns. Interestingly, unlike the sinuses of the hemal nodes and lymph nodes, the yak splenic sinuses had two different morphological structure types: in some zones characterized by endothelial cells (therefore, sinusal), the splenic sinuses were covered with a lining of regularly shaped rod and irregularly shaped flat cells, which was consistent with a report in cattle (Abe et al., 1987); in other zones, the splenic sinus wall consisted of reticular cells and erythrocytes spread over pulp spaces (thus, non-sinusal), which was the same as the splenic sinuses in equines (Tablin and Weiss, 1983). Udrouiu (2006) suggested considering these spleens with both sinuses as 'semi-sinusal', as the further diversification of the spleen was probably due to adaptative advantages related to the evolution of mammals. In the next work, the fine structure of the sinuses in the hemal node and spleen will be further investigated.

Generally, the function of eosinophils is associated with parasitic infection or inflammation (Ravin and Loy, 2015). In this study, we found many special eosinophil granulocytes in the parenchyma of the hemal nodes, except the lymphoid follicle. However, they were seldom observed in the spleen and rarely observed in the lymph nodes. In previous studies, the eosinophil granulocytes were referred to less commonly in the hemal nodes. Unlike general eosinophils, these cells had many special eosinophilic cytoplasmic granules. The granules always contained many crystalline, electron-dense materials, which were thin and numerous and were not formed into a crystalline, rod-shaped core. Previous research has shown the role of the eosinophils in phagocytosis and suggested that this was another avenue for erythrophagocytosis in the sheep hemal nodes (Ezeasor et al., 1989). We suggested that these special eosinophil granulocytes may be related to erythrophagocytosis in the yak hemal nodes. In addition, we found that these cells were seldom observed in newborns. Maybe as age increased, erythrophagocytosis

Expression of CD3 and CD79a in yak (*Bos gruniensis*) hemal node, lymph node and spleen

was enhanced and resulted in an increase in these cells. The function and the source of these cells in the hemal nodes will still be our major focus in future studies.

T and B cells were two principal cell types in secondary lymphoid organs. T cells developed within the thymus and then migrated from the thymus to the secondary lymphoid tissues via the blood circulation. In cattle, B-cell development and primary B-cell repertoire diversification occurred mainly in the ileal Peyer's patch (PP), and then the cells migrated from the ileal PP and populated the peripheral B-cell compartment (Yasuda et al., 2006). At present, little is known about the tissue distribution pattern of T and B cells in the secondary lymphoid organs of yaks. The CD3 antigen is associated with the T-cell receptor and is present in all T lymphocytes. The present study showed that the CD3+ T cells were mainly concentrated in the T cell-dependent areas, namely, the paracortical and interfollicular areas of the lymph nodes and periarteriolar lymphoid sheaths of the spleen, in accordance with the results seen in bovines (Casteleyn et al., 2008) and camels (Uhde et al., 2017). In the hemal nodes, the CD3+-stained cells were mainly located in the diffuse lymphoid tissues around the lymphoid follicles. It may be that this area in the hemal nodes resembles the T cell compartment and is comparable to the paracortex of the lymph nodes and periarteriolar lymphoid sheaths of the spleen. The few CD3+ T cells in the follicular centres may act as regulatory cells in B cell-dependent areas (Zidan et al., 2000). The CD79a-labelled cells were mainly distributed in the lymphoid follicles of all these organs, particularly in the mantle zone, which is consistent with the goat (Bozkurt et al., 2014), camel (Uhde et al., 2017) and raccoon (Heinrich et al., 2015). This finding demonstrated that the hemal nodes were also induction sites of antigen-specific B-cell responses as well as other lymphoid organs such as the mesenteric lymph nodes and spleen (Zhang et al., 2012). However, there were non-specific background and positive immunoreactivity of non-lymphoid cells, including smooth muscle and endothelium, as previously described for camels (Zidan et al., 2010) and pigs (Chianini et al., 2001). At present, there are no data on the distribution of CD79a in the hemal nodes.

Conclusion

The most obvious age-related change in the three organs is in the lymphoid follicles. In newborns, the three organs are not fully developed, but gradually, development occurs as age increases. The current study showed that the yak hemal nodes have a blood supply, and the morphology showed a similar structure to both the lymph nodes and the spleen. Their strategic location, blood sinuses and erythrocytes in the lymph tissues suggested that they perform similar functions in the spleen of filtering the blood. The immunohistochemical examination of the yak hemal nodes strongly indicated that they are as immunologically active as the lymph

nodes and spleen. This analysis demonstrated that the hemal nodes share both the morphological and functional characteristics of spleens and lymph nodes, and they were also an important secondary lymphoid organ, though they were very small relative to the spleen and lymph nodes.

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