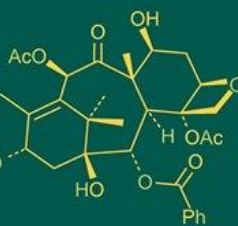
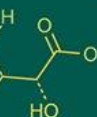
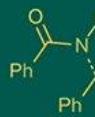


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Microanatomical and cellular characterization of porcine spleen and lymph node as a basis for comprehensive mapping of immune organoid models

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Abstract

The porcine spleen and lymph nodes exhibit distinct structural and cellular features that influence immune responses and make pigs valuable translational models. This study comprehensively characterized the microanatomy and immune cell distribution in porcine spleen and lymph nodes using histology, immunohistochemistry (IHC), and flow cytometry. Haematoxylin and eosin staining revealed a nonsinusoidal spleen with extensive red pulp, poorly defined marginal zones, abundant ellipsoids, and sparsely distributed germinal centres. White pulp was organized into periarteriolar lymphoid sheaths (PALS) rich in T cells, with B-cell follicles located adjacent to PALS. Lymph nodes displayed the characteristic inverted architecture of swine, with central B-cell follicles and paracortex surrounded by peripheral medullary tissue. IHC localized CD3⁺ T cells predominantly to PALS and paracortical areas, while CD79 α ⁺ B cells occupied follicular regions. Flow cytometry confirmed T-cell predominance (60-70% of lymphocytes) and a higher proportion of CD4⁺ helper T cells compared to CD8⁺ cytotoxic T cells, alongside a greater B-cell frequency in lymph nodes than spleen. These findings delineate the structural specialisations and compartmentalised immune architecture of porcine secondary lymphoid organs, providing a foundational reference for immune organoid development, vaccine evaluation, and comparative immunology research.

Keywords: Inverted architecture, histology, immunohistochemistry, flow cytometry, immune organoids

Introduction

The immune system of mammals relies on a network of primary and secondary lymphoid organs that coordinate immune surveillance, antigen recognition, and the generation of adaptive immune responses. Among the secondary lymphoid organs, the spleen and lymph nodes play central roles in maintaining immune homeostasis by facilitating interactions between antigen-presenting cells and lymphocytes (Rothkötter, 2009 and Kaur *et al.* 2022) [12, 13]. The spleen functions primarily as a blood filter, lacking afferent lymphatic vessels, and is uniquely specialized to mount immune responses against blood-borne pathogens. It is divided into red pulp, responsible for erythrocyte clearance and iron recycling, and white pulp, which mediates immune activation through its periarteriolar lymphoid sheath (PALS), lymphoid follicles, and marginal zone (Mark, 2006, Hermann-Josef, 2009 and Mohamed 2021) [15, 10, 16].

In contrast, lymph nodes are interspersed along the lymphatic vessels and serve as the first line of immune response to tissue-derived antigens. They are the only lymphoid organs with both afferent and efferent lymphatics, allowing for efficient antigen filtration and lymphocyte recirculation. Structurally, they are divided into cortex, paracortex, and medulla, each supporting distinct immune cell populations (Dellmann, 2006) [6].

In pigs (*Sus scrofa domesticus*), both the spleen and lymph nodes exhibit species-specific anatomical and immunological features. Notably, porcine lymph nodes demonstrate an inverted architecture, where the cortex and paracortex are located centrally, and the medullary tissue lies peripherally. This contrasts with the conventional structure seen in most mammals and influences lymph flow and immune cell localization (Binns, 1982) [3]. Additionally, porcine lymph nodes feature intra-trabecular lymphatic sinuses and valves, structures not commonly found in other species.

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Similarly, the spleen in pigs presents with less distinctly demarcated marginal zones and sporadically distributed lymphoid nodules, which may impact the organization and function of immune responses (Pabst, 2020) ^[18].

Germinal centres (GCs) are transient microanatomical structures that form within secondary lymphoid organs after B-cell activation. They drive the process of affinity maturation through somatic hypermutation and selection, allowing the immune system to produce high-affinity antibodies and long-lived memory cells. A mature GC has a polarized structure with a dark zone (DZ) rich in proliferating B cells (centroblasts) and a light zone (LZ) where non-proliferating B cells (centrocytes) compete for antigen and T-cell help before re-entering the DZ or differentiating into plasma or memory cells (Mu *et al.* 2024 and Fleischmann *et al.* 2025) ^[17, 9].

Dark Zone (DZ) contains rapidly dividing centroblasts (B cells undergoing somatic hypermutation). Tingible body macrophages (TBMs) reside mainly in the DZ and engulf apoptotic B cells to prevent accumulation of debris and autoantigens. Somatic hypermutation and clonal expansion occur here (Stebegg *et al.* 2018 and Gurwicz *et al.* 2023) ^[20, 10].

Light Zone (LZ) enriched with centrocytes (non-dividing B cells), follicular dendritic cells (FDCs) and T follicular helper (Tfh) cells. FDCs retain antigen-antibody immune complexes for long periods. The zone also contains T follicular regulatory (Tfr) cells and marginal reticular cells (MRCs). Centrocytes test their mutated B-cell receptors by capturing antigen from FDCs and presenting peptide-MHC II complexes to Tfh cells. High-affinity clones receive Tfh help (CD40L, IL-4, IL-21) and either re-enter the DZ or differentiate into plasma or memory cells. FDCs and Tfh cells are essential for positive selection, whereas Tfr cells modulate the response (Stebegg *et al.* 2018) ^[20].

Given the growing utility of pigs as large animal models for studying human and veterinary immunology, a detailed characterization of the porcine immune architecture is crucial. Histology, immunohistochemistry, and flow cytometry offer complementary methodologies to investigate the cellular composition, spatial distribution, and phenotypic characteristics of immune cell populations in lymphoid tissues.

This study aims to comprehensively characterize the microanatomy, immune cell distribution, and phenotypic profile of porcine spleen and lymph node tissues using histological analysis, immunohistochemical localization of specific immune markers, and quantitative flow cytometric profiling. Such an integrative approach is essential for advancing our understanding of porcine immune responses and enhancing their application in translational research, including vaccine development and disease modelling.

Materials and Methods

Sample Collection and Transport

Porcine spleen and lymph nodes were collected from Neeleri slaughterhouse, located near Devanahalli, Bangalore. Tissues were immediately transported to the ICAR-National Institute of Veterinary Epidemiology and Disease Informatics (ICAR-NIVEDI), Ramagondanahalli, Yelahanka, Bangalore under cold conditions for further

processing. For flow cytometry, 5-10 g of spleen and lymph node tissue were collected in 30 ml of ice-cold 1X phosphate-buffered saline in 50 ml conical centrifuge tubes. For histology and immunohistochemistry, approximately 1 cm² of each tissue was collected in dry 50 ml tubes and transported on ice.

Cryopreservation and Sectioning

Tissues were gently blotted using kimwipes to remove residual PBS. The samples were wrapped in aluminium foil and snap frozen by dipping in a metal beaker containing 2/3 volume isopentane, which was pre-cooled in liquid nitrogen up to 1/3 of the beaker height. Frozen tissues were then embedded in Leica optimal cutting temperature (OCT) compound, avoiding air bubbles. Molds were placed on dry ice until solidified. Solidified blocks were either sectioned immediately using a Leica cryostat microtome (at -20 °C) or wrapped in foil, sealed in plastic bags, and stored at -80 °C for later use.

Cryosections of 5 µm thickness were prepared for haematoxylin and eosin (H&E) staining, and 10 µm sections were used for immunohistochemistry. Sections were mounted on positively charged glass slides and stored at -80 °C until staining.

Haematoxylin and Eosin (H&E) Staining

Cryosections (5 µm) were removed from -80 °C storage and fixed in 10% neutral buffered formalin for 20 seconds at room temperature, followed by rinsing in tap water. Sections were stained with Harris haematoxylin for 10 minutes, blued in tap water for 5-10 minutes, and counterstained with eosin for 30 seconds. Slides were dehydrated through graded alcohols (50%, 75%, 90%, and 100%), cleared in xylene, and mounted using DPX mountant. (Bancroft & Gamble, 2008). Histological observations were recorded using an Olympus microscope fitted with a Jenoptik digital camera.

Immunohistochemistry (IHC)

Ten-micrometre cryosections were fixed in cold methanol/acetone (1:1) at -20 °C for 20 minutes. After warming to room temperature, slides were washed twice in PBS and tissue boundaries were outlined using a hydrophobic barrier pen. Blocking was performed using 200 µl of blocking buffer (1X PBS with 4% BSA and 0.05% Tween-20) for 1 hour at room temperature.

For surface marker detection, primary antibodies diluted in washing buffer (1X PBS+2% BSA +0.01% Tween 20) were added and incubated at room temperature for 3 hours. Slides were washed twice in washing buffer and incubated with secondary antibodies for 1 hour (Table.1), followed by another two washing buffer washes. For intracellular staining, permeabilization was done using 0.03% saponin in 1X PBS for 1 hour at room temperature. After permeabilization, sections were washed and stained with intracellular primary and secondary antibodies as above. (Bordet *et al.* 2018) ^[4].

Nuclear counterstaining was performed using ProLong™ Glass Antifade Mountant with NucBlue™ Stain. Sections were imaged using a ZEISS LSM 980 confocal microscope and stored in the dark at 4 °C until further analysis.

Single-Cell Suspension Preparation

For spleen, 8-10 g of tissue was briefly rinsed in ice-cold PBS and trimmed into ~5 mm³ pieces. Tissue was placed on a moistened 100 µm stainless steel tissue strainer and mechanically dissociated using a 2 ml syringe plunger. The homogenate was rinsed with 1X PBS and filtered through a 70 µm nylon cell strainer into 50 mL centrifuge tubes (Kim & Joo, 2016 and Wagar *et al.* 2021) ^[14, 21].

For density gradient separation, 5 ml of cell suspension was layered over 5 ml of Histopaque and centrifuged at 2000 rpm for 30 minutes at room temperature without brake. The buffy coat was collected, diluted in 1X PBS, and centrifuged at 1500 rpm for 7 minutes. Red blood cells were lysed if necessary, using a freshly prepared RBC lysis buffer (9:1 of 0.16 M NH₄Cl and 0.17 M Tris, pH 7.65), followed by quenching with RPMI media. Cells were washed, counted using Trypan Blue, and resuspended in FACS buffer. For lymph nodes, dissociated cells post-70 µm filtration were directly used for flow cytometry.

Flow Cytometry

An aliquot of cell suspension (5×10⁶ cells/ml) was dispensed into 200 µl volumes. Cells were blocked using 100 µl of 2% mouse serum in PBS for 10 minutes at 4 °C. After washing, cells were stained with surface marker-specific primary conjugated antibodies (Table. 2) for 1 hour at 4 °C. Cells were then washed thrice with FACS buffer.

For intracellular staining, cells were fixed and permeabilized with 100 µl of 0.03% saponin in 1X PBS for 1 hour at room temperature. After washing with 0.1% saponin + 1% normal rabbit serum in 1X PBS, cells were stained with intracellular antibodies. Following incubation and washing, final resuspension was done in 200 µl FACS buffer. Data acquisition was performed on a BD FACS Canto II flow cytometer and analysed using FlowJo software version 10.2.

Results

Histological Findings

Histological examination of porcine spleen and lymph node tissues revealed distinct anatomical features consistent with previous reports on porcine immune architecture. Haematoxylin and eosin (H&E) staining of the spleen demonstrated an extensive red pulp occupying the majority of the organ, interspersed with white pulp aggregates. The red pulp comprised splenic cords, ellipsoids, Hemosiderin deposited Macrophages and venous sinuses, reflecting its role in erythrocyte clearance and blood filtration (Fig.2 and Fig.4).

The white pulp was identified as lymphoid aggregates surrounding central arterioles, forming periarteriolar lymphoid sheaths (PALS). Lymphoid follicles were observed adjacent to PALS, and secondary follicles with germinal centres were rarely seen, suggesting a low basal level of antigenic stimulation. The marginal zone separating red and white pulp was poorly demarcated, consistent with reports of limited marginal zone development in pigs (Fig.3).

In contrast, porcine lymph nodes exhibited a characteristic inverted architecture, with central cortex and paracortex and a peripheral medullary region. Lymphoid follicles with germinal centres were located centrally, surrounded by

densely packed lymphocytes. The paracortical zone was prominent and contained high endothelial venules (HEVs) indicative of active lymphocyte trafficking. The peripheral medulla consisted of sinuses and cords with scattered plasma cells and reticular cells (Fig.5).

Immunohistochemistry (IHC)

IHC staining enabled the identification and localization of key immune cell subsets within the spleen and lymph nodes: CD3+ T cells were predominantly located in the PALS of the spleen and paracortical areas of lymph nodes, confirming their role in T-cell-mediated immune surveillance. CD79α+ B cells were localized to follicular regions, particularly within germinal centres of lymph nodes and perifollicular areas of the spleen (Fig. 7&8).

The observed distribution of immune cells supports the functional compartmentalization of both organs, with B-and T-cell-rich zones spatially segregated and supported by a stromal framework.

Flow Cytometry

Flow cytometric analysis of single-cell suspensions from porcine spleen and lymph nodes provided quantitative data on lymphocyte populations with gating pattern (Fig.1): Lymphocyte gating based on forward and side scatter profiles revealed a predominance of T lymphocytes, with CD3+ cells accounting for approximately 60-70% of total lymphocytes in the spleen and slightly higher percentages in lymph nodes. Among T-cells, CD4+ helper T cells were more abundant than CD8+ cytotoxic T cells, consistent with the composition of PALS and paracortex regions. CD79α+ B cells constituted 20-30% of lymphocytes in lymph nodes and 10-15% in spleen samples, reflecting the higher density of follicular structures in lymph nodes.

Viability of cell suspensions was consistently above 95% in fresh preparations, indicating effective processing for downstream applications. These results provide quantitative support for the histological and IHC observations and affirm the utility of flow cytometry in profiling porcine immune cells.

Discussion

In the spleen, haematoxylin and eosin staining revealed a preponderance of red pulp interspersed with discrete white pulp islands. The red pulp forms a three-dimensional meshwork of cords and venous sinuses, a structure that is conserved across mammals and is responsible for filtering blood and clearing senescent erythrocytes. In pigs this meshwork is supported by a thick capsule and heavy trabeculae with interwoven smooth muscle, and the spleen has poorly developed venous sinuses features that classify it as a non-sinusal spleen. These findings were in accordance with (Haley, 2017) ^[11].

The white pulp compartments observed were centred around central arterioles and formed periarteriolar lymphoid sheaths (PALS). PALS contain an inner zone rich in CD4+ T cells and an outer zone with a mixture of T cells, B cells and antigen-presenting cells, (Haley, 2017) ^[11] which matches our immunohistochemical finding that CD3+ T cells dominated the PALS and perifollicular areas while CD79α+ B cells were restricted to follicular regions. The marginal

zone separating red and white pulp was poorly defined, an expected trait in pigs; unlike rodents, pigs possess numerous periarteriolar macrophage sheaths (ellipsoids) and a limited marginal zone. This likely explains the paucity of germinal centres we observed and supports the notion that basal antigenic stimulation in healthy pigs is low.

Our IHC data further illuminate the compartmentalisation of immune cells. Previous studies in pigs have shown that the majority of CD4⁺ and CD8⁺ T cells reside within the PALS and lymphoid nodules, whereas only a small proportion are present in red pulp. Similarly, B cells (CD19⁺/CD20⁺) are concentrated within follicles and the marginal zone and are sparse in red pulp (Dunaievska, 2018) ^[7]. The localisation of CD3⁺ T cells to PALS and CD79α⁺ B cells to follicular regions in our samples aligns with these reports and confirms the functional segregation of T-and B-cell zones in porcine spleen. Although macrophage subsets were not specifically examined here, previous work has shown that CD163⁺CD169⁻/low macrophages populate the red pulp, CD163⁺CD169⁺ macrophages reside in the marginal zone and ellipsoids, and tingible-body macrophages (CD107a⁺) are present in white pulp follicles (Álvarez *et al.* 2023) ^[1]. The presence of distinct macrophage populations likely contributes to the different microenvironments observed histologically.

The lymph nodes studied exhibited the characteristic inverted architecture of swine, with B-cell follicles and paracortex centrally located and the medullary sinuses situated peripherally. In pigs, afferent lymph enters deep in the node and flows outward towards the periphery, and naive lymphocytes exit via high endothelial venules that run parallel to this flow (Bordet *et al.* 2019) ^[5]. Our observation of a prominent paracortical zone with high endothelial venules and central germinal centres is in accord with this

inverted structure. These anatomical and cellular features suggest that pigs may filter antigens centrally and present them to B cells before lymph exits, which has implications for vaccine delivery and pathogen dissemination.

Flow cytometric analysis provided quantitative confirmation of the histological and IHC findings. T cells constituted ~60-70 % of splenic lymphocytes and a slightly higher proportion in lymph nodes, with helper (CD4⁺) T cells being more prevalent than cytotoxic (CD8⁺) T cells. These values are consistent with the dominance of T cells in PALS and paracortical regions and with previous reports of T-cell-rich white pulp in swine (Dunaievska, 2018) ^[7]. B cells accounted for 20-30 % of lymph node lymphocytes and 10-15 % of splenic lymphocytes, in agreement with the relative abundance of follicles in nodes versus spleen. The high viability (>95 %) of single-cell suspensions underscores the suitability of these tissues for downstream functional assays.

Collectively, our study confirms that the porcine immune organs examined display structural specialisations distinct from those of rodents and humans. The robust red pulp and scarcity of germinal centres in spleen, the limited marginal zone and abundant ellipsoids, and the inverted lymph node architecture all likely influence how pigs respond to systemic and lymph-borne antigens. These features should be considered when using pigs as models for human immune responses or when developing veterinary vaccines. Future work could integrate functional assays to evaluate how these anatomical specialisations affect antigen capture, lymphocyte activation and the generation of humoral responses. Such knowledge is crucial for translating findings from porcine models to other species and for improving strategies to manipulate immune responses in pigs.

Table 1: Antibodies used for immunohistochemistry

Marker	Conjugate	Cell Type	Dilution	Marker Localization
Rabbit anti Human CD79a	Unconjugated	B cells	1:100	Intracellular
Goat anti-Rabbit IgG	FITC	—	1:100	—
Mouse anti pig CD3 (PPT3)	Unconjugated	T cells	1:50	Surface
Goat anti-Mouse IgG	Alexa Fluor 647	—	1:1000	—

Table 2: Antibodies used for flow cytometry

Marker	Conjugate	Cell Type	Dilution	Marker Localization
Mouse anti pig CD3	Pacific Blue	T cells	1:10	Surface
Mouse anti pig CD4	FITC	Helper T cells	1:4	Surface
Mouse anti pig CD8	APC	Cytotoxic T cells	1:4	Surface
Mouse anti Ferret CD79a	PerCP-Cy5.5	B cells	1:5	Intracellular
Mouse anti pig CD172a	PE	Myeloid cells	1:2	Surface

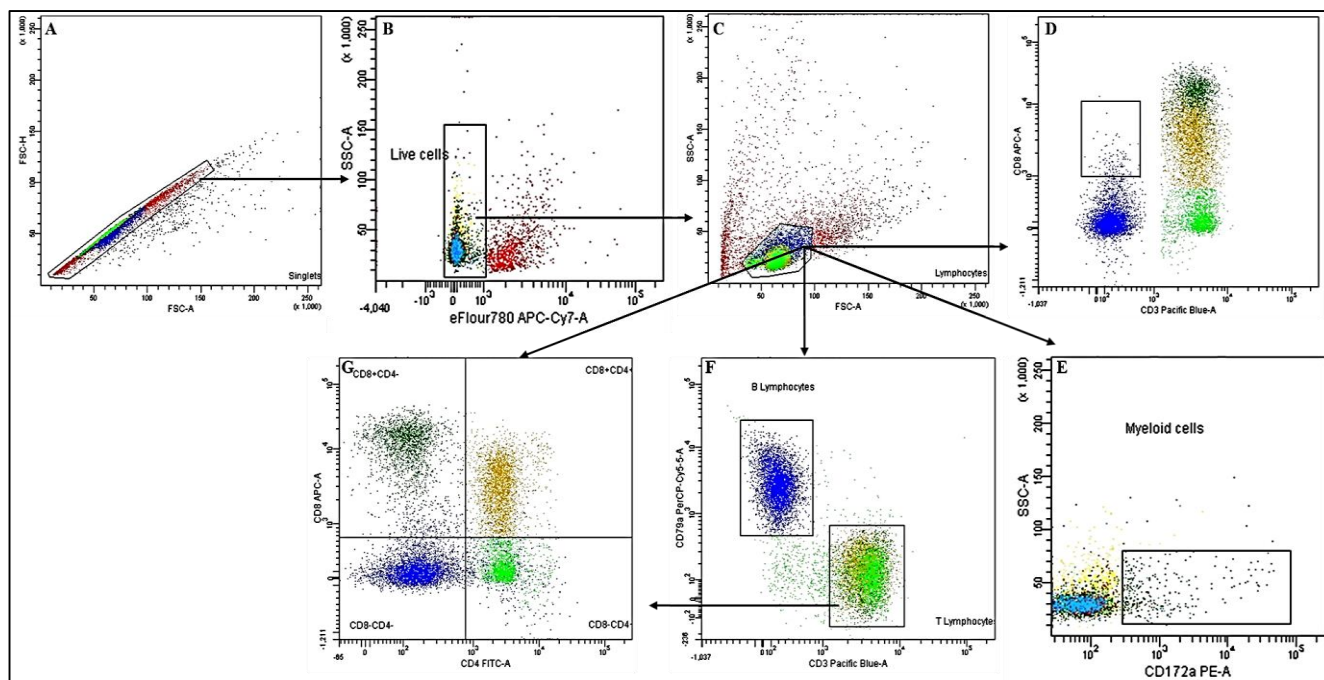


Fig 1: Flowcytometry FSC and SSC Lymphocytes and Myeloid cells gating pattern. A. Singlets (FSC-A against FSC-H), B. Live cells (eFluor780 APC-Cy7), C. Lymphocytes and Myeloid cells (FSC against SSC), D. NK cells ($CD3^-CD8^+$), E. Myeloid cells ($CD172a^+$), F. B cell ($CD79a^+$) and T cells ($CD3^+$) and G. Helper T cells ($CD4^+$) and Cytotoxic T cells ($CD8^+$) and G. $CD4^+CD8^+$ cells.

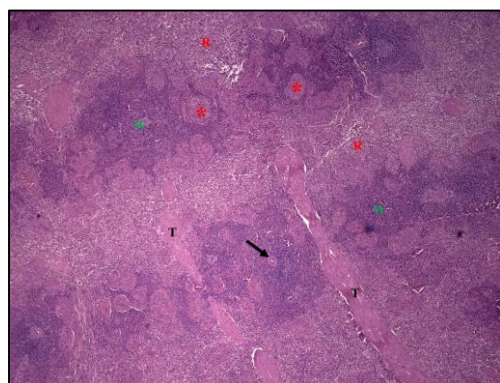


Fig 2: H&E stained section of spleen showing W-White pulp, R-Red pulp, *-Ellipsoids, Black arrow-Central artery and T-Trabeculae. (100X)

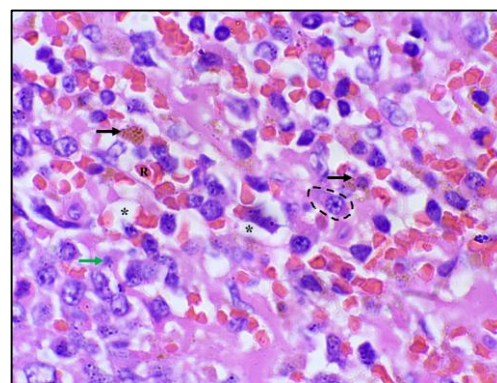


Fig 4: H&E stained section of spleen showing Dotted lines-Plasma cells, Black arrow-Haemosiderin deposited macrophages, Green arrow-Fibroblast, R-RBC's and *-Splenic sinusoids (1000X).



Fig 3: H&E stained section of spleen showing C-Capsule, PALS dotted lines and E-Ellipsoids, Black arrow-Central artery and F-Follicles. (200X)

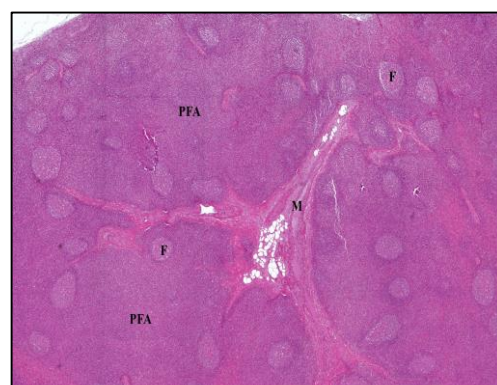


Fig 5: H&E stained section of Lymph Node showing F-Follicles, PFA-Para Follicular Area and M-Medulla (100X).

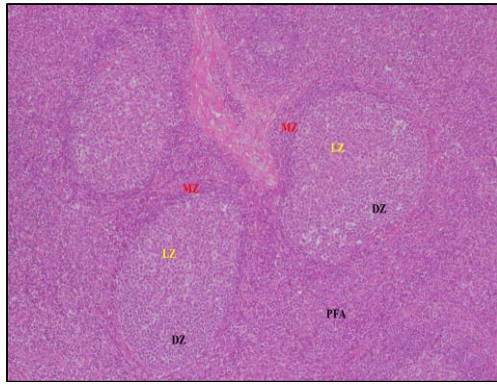


Fig 6: H&E stained section of Lymph Node showing LZ-Light Zone, DZ-Dark Zone, MZ-Marginal Zone and PFA-Para Follicular Area (100X).

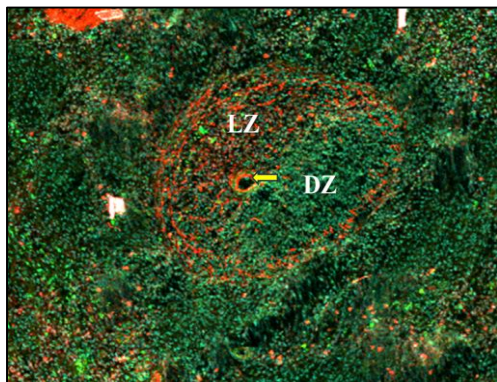


Fig 7: Immunohistochemistry of spleen stained with anti CD3 and anti CD79a antibodies tagged with Alexa flour 640 and FITC respectively. LZ-Light zone with both CD79a+ B cells and CD3+ T cells, DZ-Dark Zone with CD79a+ B cells and few Follicular T cells. (100X)

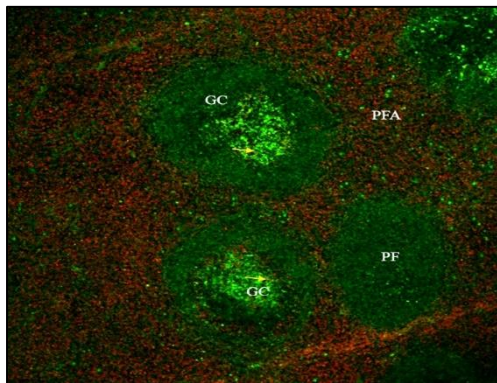


Fig 8: Immunohistochemistry of Lymph Node stained with anti CD3 and anti CD79a antibodies tagged with Alexa flour 640 and FITC respectively. GC-Germinal Center with majority of CD79a+ B cells with few CD3+ Follicular T cells, PFA-Para Follicular Area with CD3+ T cells, PF-Parafollicular area and yellow arrows showing Follicular T helper cells. (100X).

Conclusion

Collectively, our study confirms that the porcine immune organs examined display structural specialisations distinct from those of rodents and humans. The robust red pulp and scarcity of germinal centres in spleen, the limited marginal zone and abundant ellipsoids, and the inverted lymph node architecture all likely influence how pigs respond to systemic and lymph-borne antigens. These features should be considered when using pigs as models for human immune responses or when developing veterinary vaccines. Future work could integrate functional assays to evaluate how these anatomical specialisations affect antigen capture, lymphocyte activation and the generation of humoral responses. Such knowledge is

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