

## **4. STUDY II: Immunohistological study of the immune system cells in paraffin-embedded tissues of conventional pigs**

## **4.1. Materials and methods**

### ***4.1.1. Animals and sampling***

Five, 6 to 10-week-old conventional pigs were used in this study. Pigs were seronegative to PCV2, PRRSV, ADV, transmissible gastroenteritis virus, porcine respiratory coronavirus, swine influenza virus, PPV, *Actinobacillus pleuropneumoniae* and *Erysipelothrix rhusiopathiae*.

Pigs were killed by intravenous injection of sodium pentobarbital and a complete necropsy was performed on all animals. Samples of the following tissues were collected: lungs, tonsil, lymph nodes (mediastinal, mesenteric, inguinal and submandibular), pancreas, spleen, liver, kidney, adrenal gland, ileum and stomach. Tissues were fixed 48 hours in neutral-buffered 10% formalin, embedded in paraffin wax, sectioned at 3µm, and stained with haematoxylin and eosin (HE). All the investigated tissues showed a normal histological structure when stained with HE. For immunohistochemistry, serial 4µm-thick sections of all tissues were cut and placed on silane [3-(triethoxysilyl)-propylamine] coated slides.

### ***4.1.2. Immunohistochemistry***

A total of six primary antibodies anti-CD3, anti-CD79 , MAC387, anti-lysozyme, anti-CD45RA mAb 3C3/9, and anti-SLA-II-DQ mAb BL2H5 (table 3), were used with a standard avidin-biotin peroxidase (ABC)

method. Previously described protocols were used for CD3, CD79 (Tanimoto and Ohtsuki, 1996), MAC387 and lysozyme (Evensen, 1993) immunostaining, whereas standardisation of the protocols for 3C3/9 and BL2H5 antibodies had been performed in the first study. Briefly, tissue sections were deparaffinized with xylene and rehydrated through graded alcohols. Endogenous peroxidase activity was blocked by incubation with hydrogen peroxide 3% in distilled water for 30 min. After pronase, trypsin or microwave treatment, depending on the antibody used (see Table 3), tissue sections were rinsed in 0.1 M Tris-buffered saline (pH 7.6) and incubated with 20% normal goat serum solution in 0.1 M Tris-buffered saline for 1 hour at room temperature. Titration experiments were carried out to obtain the optimal dilution for the commercial antibodies. All antibodies were incubated at 4°C overnight. Biotinylated goat anti-mouse (1/200) and biotinylated goat anti-rabbit (1/400) were used as secondary antibodies, for 1 hour at room temperature. An ABC complex (Pierce, IL, USA) diluted 1/100 in 0.1 M Tris-buffered saline was applied for 1 hour at room temperature. Sections were finally incubated in diaminobenzidine (DAB)-hydrogen peroxide solution for 10 min., counterstained with Harris's haematoxylin, dehydrated, covered with a coverslip and examined microscopically. As negative controls, irrelevant primary antibodies at the same dilution were used in substitution of the specific antibodies.

**Table 3.** Details of the primary antibodies used in the immunohistochemical study

Specificity	pAb/mAb (clone)	Host of origin	Treatment	Dilution	Source
CD3	pAb	Human	Pronase <sup>a</sup>	1/150	Dako (Denmark)
CD79	mAb (HM57)	Human	Microwave <sup>b</sup>	1/25	Dako (Denmark)
L1	mAb (MAC387)	Human	Pronase	1/200	Dako (Denmark)
Lysozyme	pAb (A099)	Human	Trypsin <sup>c</sup>	1/100	Dako (Denmark)
CD45RA	mAb (3C3/9)	Swine	No treatment	Undiluted <sup>d</sup>	INIA Lab <sup>e</sup>
SLA-DQ	mAb (BL2H5)	Swine	Pronase	Undiluted <sup>d</sup>	INIA Lab

<sup>a</sup> Incubation with 0.1% pronase for 10 min at 37°C

<sup>b</sup> Incubation with citrate buffer (pH 7.6) for 12 cycles of 1 min

<sup>c</sup> Incubation with 0.1% trypsin for 2 hours at 37°C

<sup>d</sup> culture supernatant

<sup>e</sup> Kindly provided by Dr. Javier Domínguez, INIA, Valdeolmos, Madrid (Spain).

## 4.2. Results

### 4.2.1. CD3

The anti-CD3 antibody stained the surface and cytoplasm of cells located in T-cell areas of lymph nodes, tonsil, spleen, Peyer's patches and thymus. In lymph node, tonsil and Peyer's patches these areas corresponded to interfollicular areas (Fig. 2a) and, in the spleen, to periarteriolar lymphoid sheaths (PALs) (Fig. 3a). In the thymus, medullary thymocytes stained more intensely than cortical thymocytes. Many positive cells were observed in the lamina propria and in an intraepithelial location in the ileum. A variable amount of positive cells was also observed in the germinal centre of lymphoid follicles of lymph nodes, tonsil, spleen and Peyer's patches and in the subcapsular sinus

of the lymph nodes. In the dome region of the Peyer's patches in the marginal zone of the spleen and in the bronchus-associated lymphoid tissue (BALT) of the lung, scattered positive cells were observed. Furthermore, variable numbers of CD3-positive circulating cells were detected in blood vessels of all the studied tissues.

#### **4.2.2. CD79 $\alpha$**

The anti-CD79 antibody labelled the surface and cytoplasm of small, round cells with a centrally located nuclei and a narrow rim of cytoplasm, localised in B-cell areas. The positive reaction was mainly observed in primary follicles and in the mantle zone of secondary follicles of lymph nodes (Fig. 2b) tonsil, spleen, and Peyer's patches. Most of the cells located in the dome region of Peyer's patches and in the aggregated lymphoid follicles of the gastric tract mucosa were also positive. In the germinal centres of secondary follicles, cells with a larger amount of cytoplasm stained strongly positive. Scattered positive cells were observed in the medulla of thymus. Among non-lymphoid tissues, smooth muscle cells and endothelial cells of small blood vessels gave occasionally a positive reaction.

### **4.2.3. Lysozyme**

Incubation with anti-lysozyme antibody revealed a granular to diffuse cytoplasmic reaction of lobulated or kidney-shaped nuclei cells with an abundant cytoplasm. Scattered positive cells were observed in the germinal centres of lymphoid follicles of lymph nodes tonsil, spleen, and Peyer's patches (Fig. 4b) In the PALs and marginal zone of the spleen isolated positive cells were detected. In the thymus, positivity was observed in scattered cells of the cortical (Fig. 4d) and medullary area. Thymic corpuscles stained also positive. Cells located in the medulla-like subcapsular area of the lymph node were strongly positive (Fig. 4a), while cells in the lumen of the pulmonary alveoli showed a weaker reaction (Fig. 5b) In the liver, cells lining the sinusoids stained strongly positive (Fig. 4c) Among non-lymphoid tissues, renal proximal tubular cells, epithelial lining cells of the tonsillar crypts, epithelial cells of gastric glands and endothelial cells of high endothelial venules in some lymph nodes showed positive reaction.

### **4.2.4. MAC387**

MAC387 antibody reacted strongly with the cytoplasm of circulating polymorphonuclear granulocytes (Fig. 5d) with a variable distribution in the studied tissues, but constantly observed in the red pulp of the spleen (Fig. 5c). Under our conditions, cells with morphology of monocyte-macrophage lineage were not stained with this antibody.

#### **4.2.5. 3C3/9**

The 3C3/9 antibody labelled the surface and cytoplasm of two cellular types localised mainly in B-cell areas. One cellular type had small, round nuclei and a scant amount of cytoplasm, and the other showed more abundant cytoplasm. Positive cells were observed in primary and secondary follicles of the lymph nodes (Fig. 3c) tonsil, spleen and Peyer's patches. In the secondary follicles, the strongest stained area was the mantle zone. In the germinal centre, a small number of cells gave a weak positive reaction. In the Peyer's patches, positivity was present in the corona of the follicles and, in the dome region, a large number of cells were also positive. In the spleen, a variable number of positive cells were observed in the marginal zone and in the PALs. In the aggregated lymphoid follicles of the gastric tract mucosa, many positive cells were distributed in the periphery of the aggregates and only a few positive cells were observed in the centre of the follicles. In the liver, scattered circulating cells stained positive with 3C3/9 antibody.

#### **4.2.6. BL2H5**

Incubation with BL2H5 antibody gave a strong reticular reaction in the surface of two different cell types. One cell type was formed by small round cells with round, central located nuclei and a narrow rim of cytoplasm, while the other was constituted of larger cells with central kidney-shaped nuclei, surrounded by an abundant cytoplasm with or

without cytoplasmic processes. In the mantle zone of lymphatic follicles, all cells stained positive and, morphologically, these cells belonged to the small type group. Larger stained cells were observed in follicular germinal centres, and in interfollicular areas of lymph nodes (Fig. 2d), tonsils and Peyer's patches. Furthermore, in the spleen, positive cells belonging to both types were found randomly distributed in the PALs, in the marginal zone and in the red pulp as isolated cells (Fig. 3d). In the thymus, large positive cells were mainly localised in the medulla (Fig. 4a), whereas in the cortex, only a few cells stained positive. In the dome region of the Peyer's patches and in the aggregated lymphoid follicles of the gastric tract mucosa, a large number of positive cells of both types were observed. In the liver, few small cells lining the sinusoid and some perivascular cell reacted positive to BL2H5. Positivity was also observed in some endothelial cells of small blood vessels in some of the studied tissues.



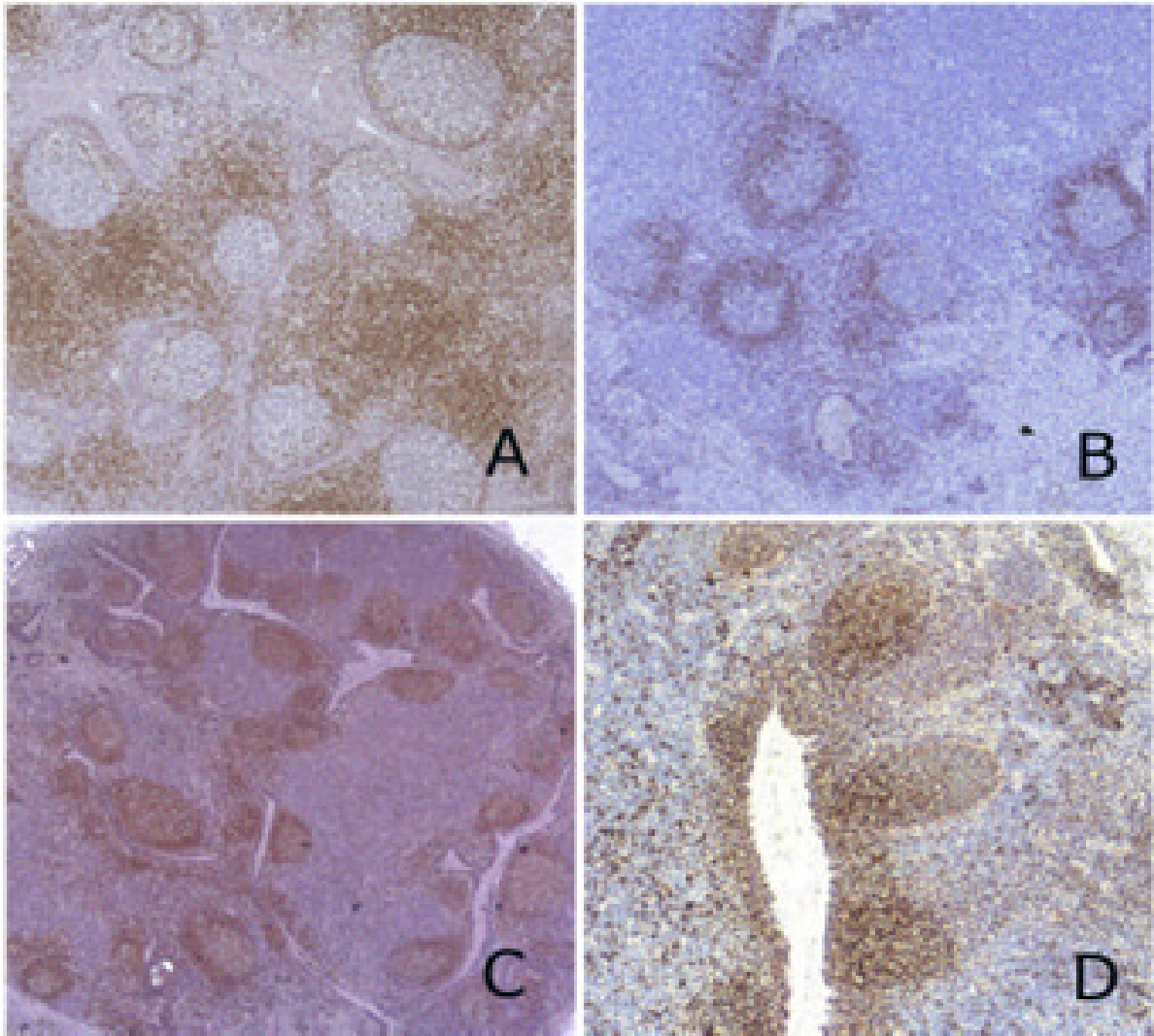


Fig. 2. Immunohistochemical staining of formalin-fixed, paraffin-embedded porcine lymph node sections with anti-CD3 (A), anti-CD79 (B), anti-CD45RA (3C3/9) (C), and anti-SLAII-DQ (BL2H5) (D) antibodies using the ABC method. Mayer's haematoxylin counterstain, x10. T cell areas stain strongly with anti-CD3 (A). Primary follicles and the mantle zone of secondary follicles are depicted with CD79 and 3C3/9 (B and C). The BL2H5 antibody detect lymphoid follicles, and scattered positive cells in the interfollicular area (D).

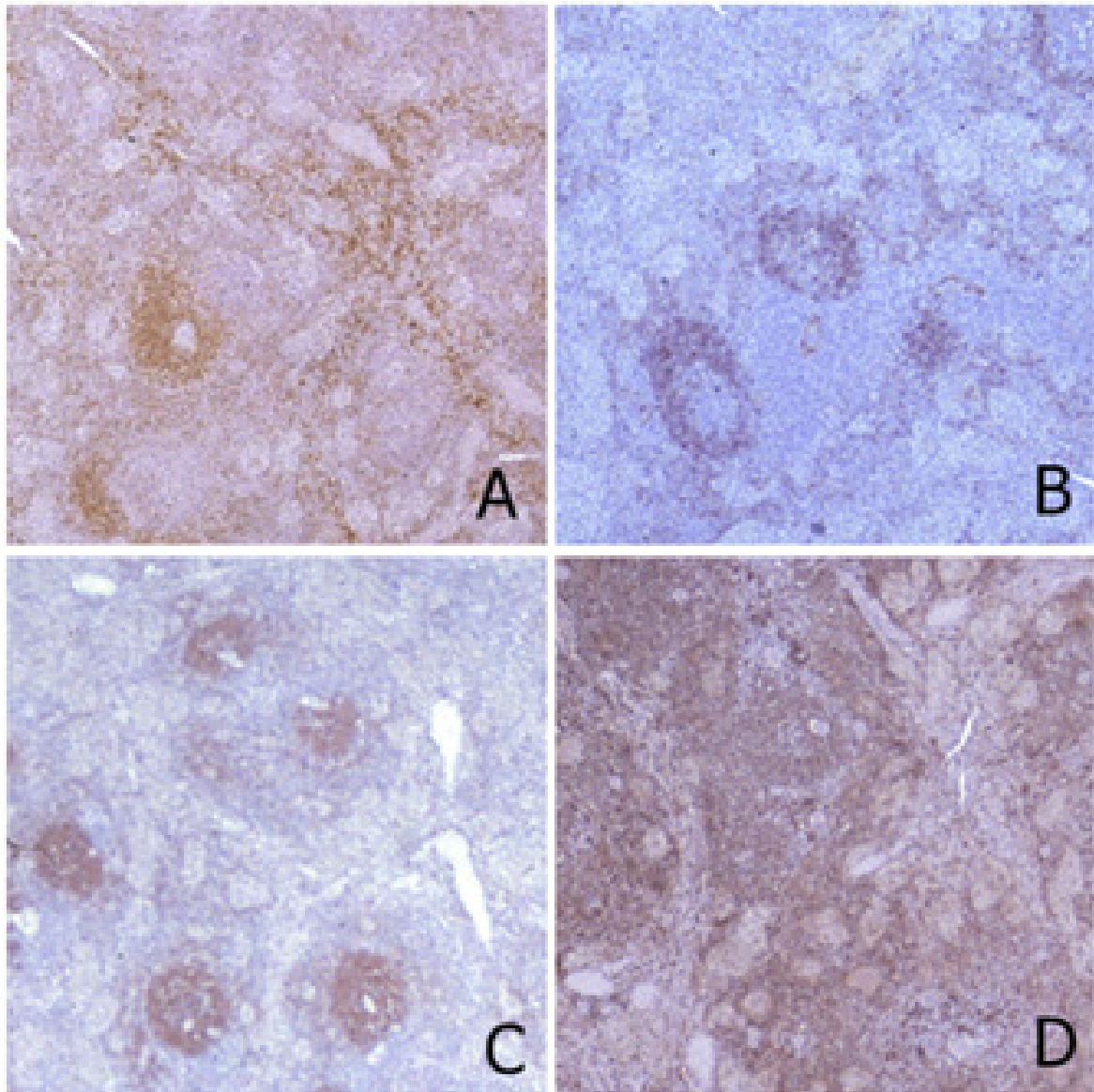


Fig 3. Immunohistochemical staining of formalin-fixed, paraffin-embedded porcine spleen sections with anti-CD3 (A), anti-CD79 (B), anti-CD45RA (3C3/9) (C), and anti-SLAII-DQ (BL2H5) (D) antibodies using the ABC method. Mayer's haematoxylin counterstain, x10. PALs and marginal zones stain strongly with anti-CD3 (A). Follicles and the mantle zone of secondary follicles are depicted with CD79 (B). Follicles are stained with 3C3/9 (C). The BL2H5 antibody detect APC within follicles, marginal zones, red pulp and, occasionally, PALs (D).

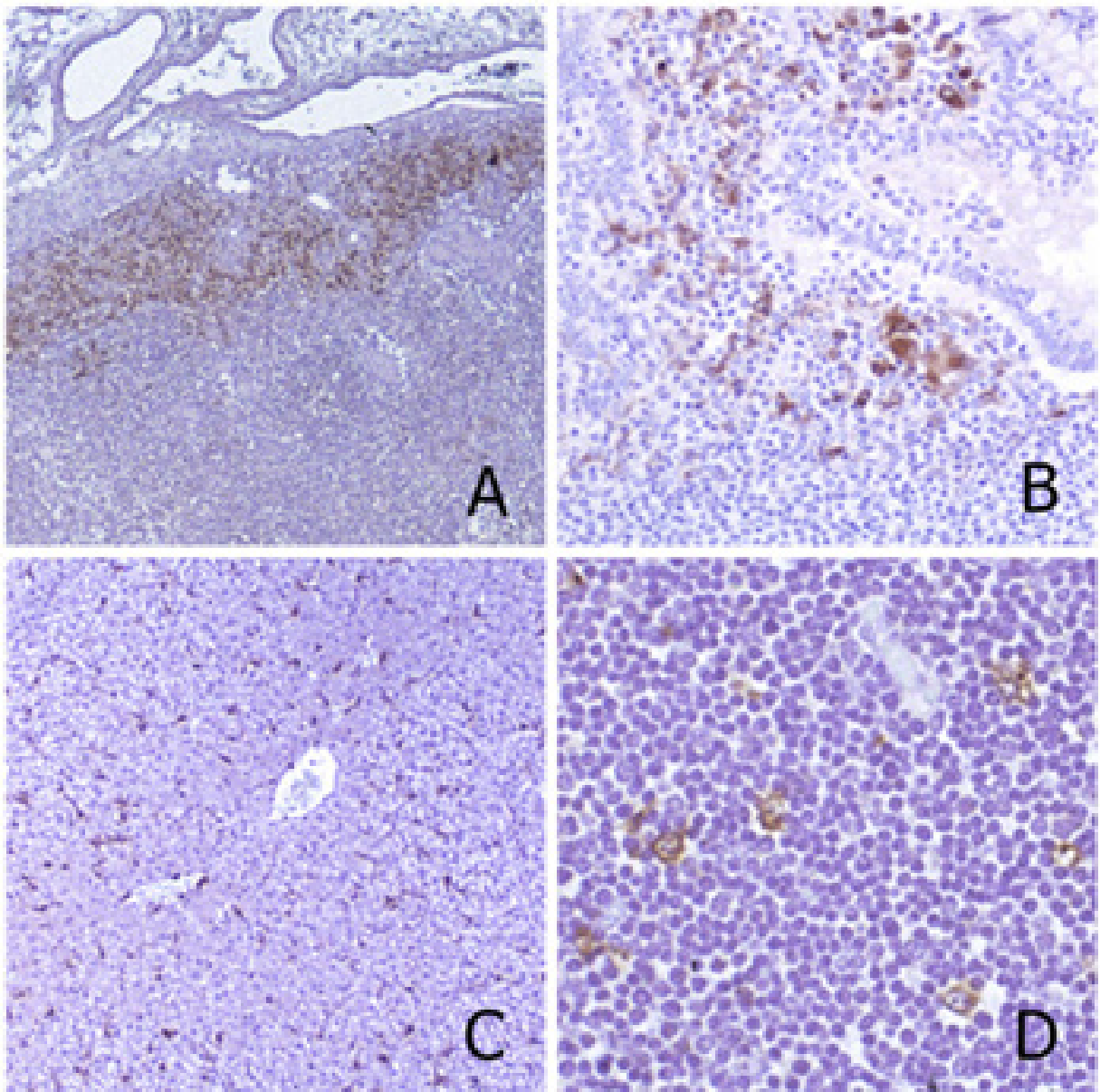


Fig. 4. Immunohistochemical staining of formalin-fixed, paraffin-embedded porcine lymph node (A), Peyer patches (B), liver (C) and thymus (D) sections with anti-Lysozyme antibody using the ABC method. Mayer's haematoxylin counterstain. Strongly positive cells observed in medulla-like subcapsular area, x13.2 (A). Macrophages located in the dome, x50 (B). Cells lining the sinusoids (Kupffer cells) stained strongly positive, x25 (C). Tingible body macrophages within thymic cortex, x50 (D).

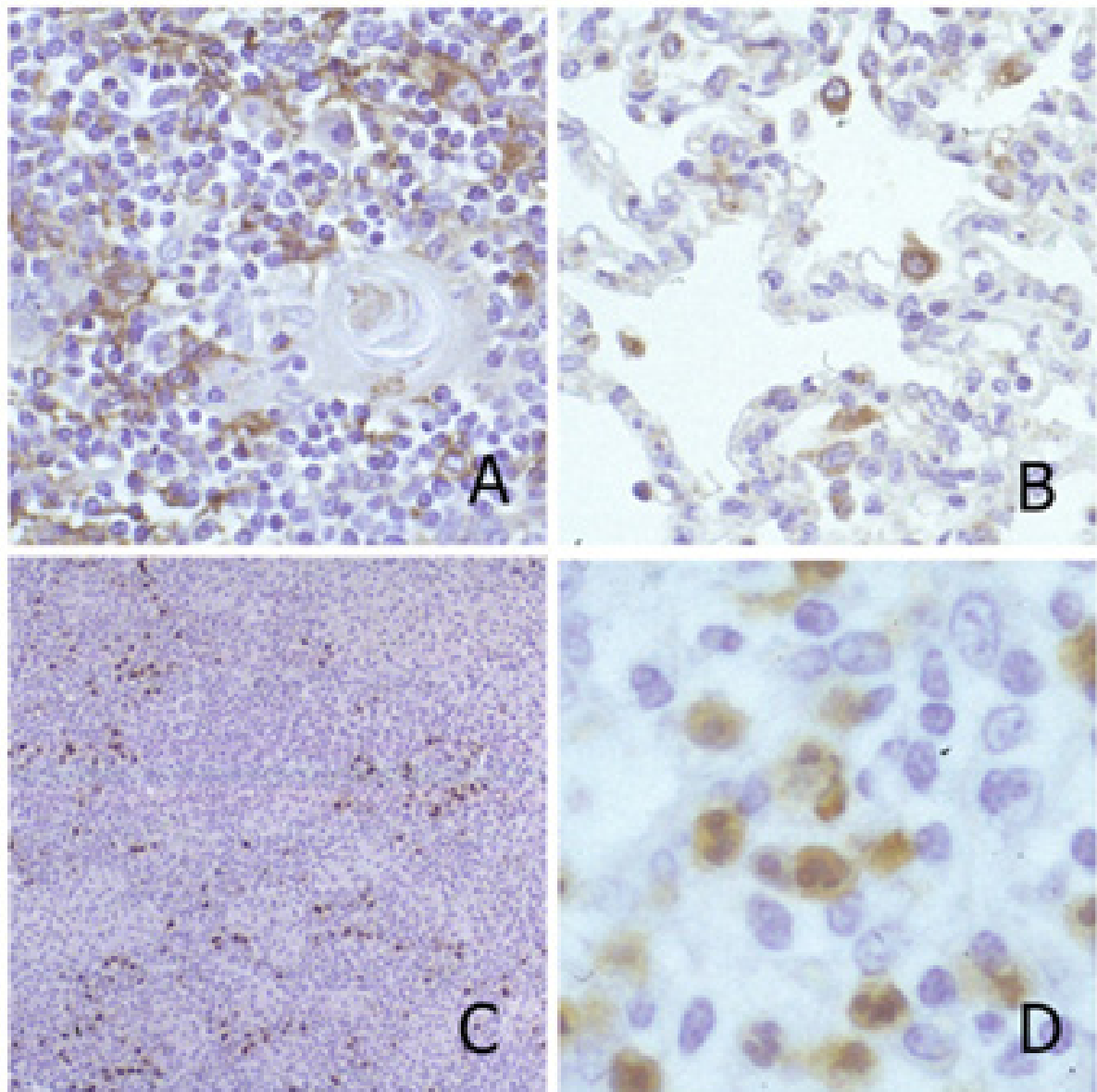


Fig. 5. Immunohistochemical staining of formalin-fixed, paraffin-embedded porcine thymus (A), lung (B), spleen (C) and lymph node (D) sections with anti-SLA-II-DQ (BL2H5) (A), anti-Lysozyme (B) and anti-MAC387 (C and D) antibodies using the ABC method. Mayer's haematoxylin counterstain. Interdigitating cells within the thymic medulla, x100 (A). Alveolar and interstitial macrophages in the lung parenchyma, x132 (B) Strongly positive cells (granulocytes) observed in the red pulp, x25 (C). Polymorphonuclear granulocytes are depicted with MAC387, x250 (D).