3. STUDY I: Development of immunohistochemical techniques to detect immune system cells in porcine paraffin-embedded tissues

3.1. Materials and methods

3.1.1 Tissues

Archival formalin-fixed, paraffin-embedded samples of superficial inguinal lymph node, spleen and liver from three 10-week-old conventional pigs were used to develop different immunohistochemical techniques. Selected pigs came from a high health status farm and were seronegative to PCV2 and PRRSV; their tissues were microscopically normal. Serial 4 µm-thick sections were cut and placed on silane [3-(trietoxysilyl)-propylamine] coated slides.

3.1.2. Antibodies

A total of 15 monoclonal anti-swine antibodies kindly provided by Dr. Javier Domínguez (Instituto Nacional de Investigación y Tecnología Alimentaria y Agraria [INIA], Valdeolmos, Madrid, Spain) were tested (table 2).

Table 2. Details of the primary antibodies used in the immunohistochemical study

mAb	Specificity	Target cells	References
76-12-4	CD4	T cell subpopulations	Pescovitz et al., 1984
76-2-11	CD8	T-cell subpopulations	Pescovitz et al., 1984
BL2F1	CD11a	Adhesion molecules	Alvarez et al., 2000
BL2H5	SLA II DQ	APC	Bullido et al., 1997
BA3A3	CD18	Leukocytes	Alvarez et al., 2000
BL1H7	SWC3	Monocytes/ macrophages	Alvarez et al., 2000
2A10/8	SWC7	B cells, activated T cells	Bullido et al., 1999
2A5	CD45	Leukocytes	Bullido et al., 1997
2F4/11	CD11B	Granulocytes, macrophages	Bullido et al., 1996
3C3/9(B1)	CD45RA	B cells, naive T cells	Bullido et al., 1997
4B7/8	SLA I	Nucleated cells	Bullido et al., 1996
6D8/8	CD46	Lymphocytes, fibroblasts, epithelial cells	Bullido et al., 2000
6D8/8	CD25	T cells	No reference
1H6/8(B1)	CD5	T cells	Pescovitz et al., 1998
2E9/13	SLA II DR	APC	Bullido et al., 1997

3.1.3 Antigen retrieval

Several antigen retrieval procedures were performed to determine suitable conditions for every antibody. Enzyme digestion with 2

different proteases, and microwave heating with different soaking solutions were performed for each antibody.

Protease predigestion was performed with 1% pronase (Sigma chemical, St. Louis, CO, USA) in 0.1 M Tris-buffered saline (pH 7.6), and with 1% trypsin (Sigma chemical, St. Louis, CO, USA) in 0.1 M Tris-buffered saline (pH 7.6). Pronase and trypsin pre-digestion were used at room temperature and at 37ºC. Pronase was applied during 6, 7, or 8 min., while trypsin during 5, 15, 20 min.

For microwave heating, Tris-buffered saline (pH 7.6), 0.1 citrate buffer (pH 6.0), and 0.1 zinc citrate buffer (pH 7.6) were used as soaking solutions. Irradiation cycles of 1 min were applied 5, 8, 10, 12, and 14 times. Samples were allowed to cool during 20 or 60 min after these treatments, in all cases. Sections were washed briefly in Tris-buffered saline (pH 7.6) and immunostained along with untreated sections.

3.1.4. Immunohistochemistry

A standard avidin-biotin peroxidase (ABC) method was performed for all antibodies. 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. All previously described antigen retrieval methods were performed to determine suitable conditions for each antibody. 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. All primary antibodies were used as undiluted culture supernatant. All antibodies were incubated at 4ºC overnight. Biotinylated goat anti-mouse (1/200) diluted in 0.1 M Tris-buffered saline was used as the secondary antibody, for 1 hour at room temperature. An ABC complex (Pierce, IL, USA) diluted 1/100 in 0.1 Tris-buffered saline was applied for 1 hour at room temperature. Sections were finally incubated in 0.1 M Tris-buffered saline with 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.

3.2. Results

Positive immunolabeling was obtained with BL2H5 and 3C3/9 antibodies. All other mAb tested only gave a variable degree of background, independently of the antigen retrieval used. The BL2H5 antibody gave the best result after incubation with 0.1% pronase for 10 min at 37ºC, while the 3C3/9 antibody showed good staining when no antigen retrieval was applied to the tissues.

The BL2H5 antibody gave a strong reticular reaction in the surface of two different cell types, resembling lymphocytes and histiocytic cells, mainly observed in follicular areas of lymph nodes (Fig. 1a, b) and spleen (Fig. 1c). Small round stained cells were observed in liver with a perilobular location (Fig.1d)

The 3C3/9 antibody labelled the surface and cytoplasm of lymphocytic cells and of cells with more abundant cytoplasm, resembling immunoblasts, mainly in B-cell areas of lymph nodes (Fig. 1e) and spleen (Fig. 1f)). Furthermore, stained cells were observed in the marginal zone and in the PALs of the spleen. In the liver, scattered round circulating cells stained positive with 3C3/9 antibody.

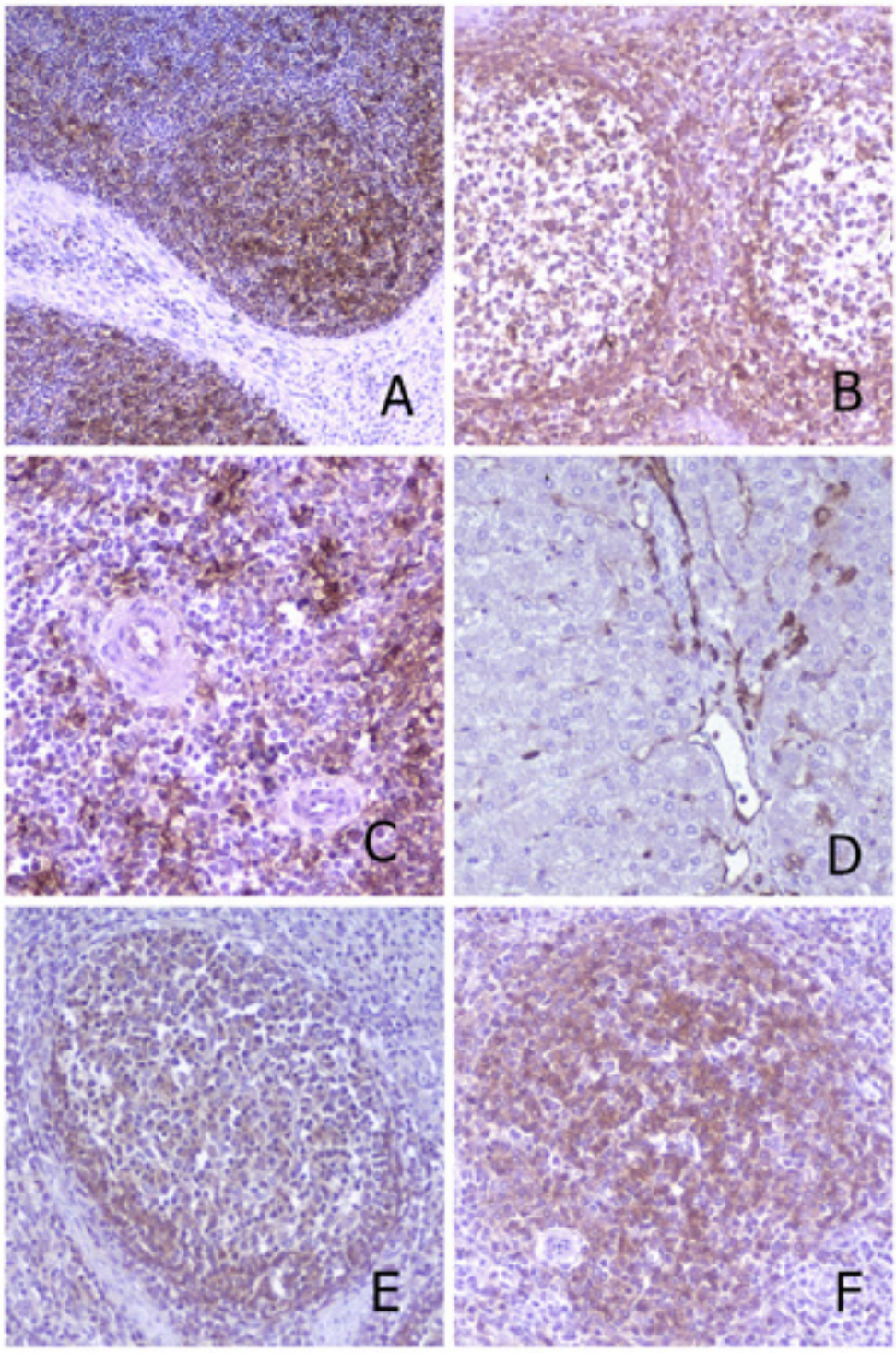

Fig. 1. Immunohistochemical staining of formalin-fixed, paraffin-embedded porcine lymph nodes (A, B and E), spleen (C and F) and liver (F) with antibodies anti-SLA-II-DQ (BL2H5) (A, B, C and D) and anti-CD45RA (3C3/9) (E and F) using the ABC method. Mayer's haematoxylin counterstain. APC in follicular and interfollicular areas, x25 (A) and x50 (B). APC cells in PALs, x50 (C). Small cells lining sinusoid and perivascular cells reacting with mAb BL2H5, x50 (D). Mantle zone and germinal centre of a secondary (E) and a primary, x100 (F) follicle with reactivity anti-CD45RA, x100.