Characterisation of immune system cell subsets in paraffin-embedded tissues from alpine chamois (*Rupicapra rupicapra*)

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Summary

Immune system cell subsets of lymph nodes and spleen from chamois (*Rupicapra rupicapra* subspecies *rupicapra*) living in the Italian Alps, have been characterised by immunohistochemical techniques. A total of seven primary antibodies (against human CD3, CD79αcy, CD68, or ovine CD4, CD8, CD21, and γδ TCR epitopes) were tested on tissues fixed either in formalin or in zinc salts (ZS) and a good cross-reactivity with chamois immune cell epitopes has been detected. ZS fixation was used to allow a wider identification of immune cells, without requirement of antigen retrieval treatments, and CD4 and CD21 cells showed labelling only in ZS fixed tissues. However, antibodies raised against human CD3, CD79 and CD68 antigens, successfully reacted with chamois immune cells, both in ZS fixed tissues and in formalin. The reactivity and distribution of immune cells in lymph nodes and spleen were similar to those described in other domestic and wild ruminants. Results from this study may allow future investigation of the immune response and pathogenesis of chamois diseases.

Keywords: chamois; immune system; immunohistochemistry; zinc salts.

Alpine chamois (*Rupicapra rupicapra* subspecies *rupicapra*, Linnaeus 1758) is a wild ruminant living in the eastern Alps. Infectious diseases in chamois have raised the interest of scientists because they may represent a threat for the conservation of wildlife species and biodiversity (Daszak et al. 2000; Pioz et al. 2008). Moreover, chamois could represent a useful animal model to evaluate the complex balance between host and pathogens (especially parasites, but also virus and bacteria). Population dynamics of chamois are influenced by pathogen-derived epidemics and investigations on polymorphism of major histocompatibility complex (MHC) class II gene, were shown to be useful to study the adaptive processes and pathogen-mediated selection (Cavallero *et al*., 2012; Mona *et al*., 2008). However, evaluation of inflammatory infiltrates in

tissues may be crucial to understand the pathogenesis of infectious diseases in this species. An immunohistochemical study on formalin fixed specimens of healthy and mange infected chamois, failed to demonstrate reactivity of immune cells with a panel of anti-human antibodies (Rode et al. 1998). Indeed, no specific antibodies are available against chamois immune cells epitopes. However, immunohistochemistry results are dependent on adequate fixation of the tissue. Routinely, tissues are fixed in 10% neutral-buffered formalin that inhibits cellular processes and prevents tissue degradation, but could represent a limiting factor for immunohistochemistry as it cross-links antigens and masks epitopes (Webster et al. 2009). Zinc salts (ZS) fixative is a non-aldehyde solution, composed of zinc acetate and zinc chloride in Tris-Ca acetate buffer, considered to preserve sensitive antigens comparably to frozen methods, with a morphological preservation comparable to formalin (Beckstead 1994). ZS fixation has been successfully used in immunohistochemical studies such as labelling of ovine and cervine immune cell surface epitopes (González et al. 2001; Dagleish et al. 2012).

The aims of this study were: (1) to determine the cross-reactivity of anti-human and anti-ovine monoclonal antibodies with chamois immune cells on lymph nodes and spleen, and (2) to evaluate differences and effectiveness of immune cell labelling in chamois tissues fixed either in ZS or in formalin.

Retromandibular lymph nodes and spleen were collected from 10 alpine chamois (7 females and 3 males), aged between 1 to 14 years, which were shot during the hunting season (from September to December 2013). A thin slice (4-5 mm thick) of each tissue sampled was immediately placed in freshly prepared ZS fixative for 24-48 hours (Beckstead 1994), and a duplicate sample placed into 10% neutral buffered formalin for 48-72 hours. All samples were processed routinely for paraffin embedding and stained with haematoxylin and eosin. Serial sections were mounted on treated glass slides (Superfrost Plus; Menzel-Glaser, Germany) for immunohistochemistry. Different antigen retrieval methods were used for formalin-fixed tissues as shown in Table 1. A

panel of monoclonal antibodies (Table 1) was applied and incubated overnight at 4°C. Antibody binding was detected by the EnVision Plus System-HRP (DAB) (code K4007; Dako UK Ltd., Ely, UK). Substitution of the primary antibody with unrelated matched primary antibody, was used to provide a negative control. Ovine lymph node sections were used as positive controls. Bright field images were acquired at x20 magnification with a Leica Microsystem DFC490 digital camera mounted on a Leica DMR microscope. For each animal, five 10,000 μm^2 fields of view of the following areas, were randomly selected for counting positive cells: germinal centre and mantle zone of the lymphatic follicles, interfollicular/paracortical area and medullar cords of lymph nodes, germinal centre and mantle zone of the lymphatic follicles, periarterial lymphatic sheaths, marginal zone and red pulp of the spleen. The number of total cells and CD3, CD79, CD68, CD21, CD4-positive cells within a field were counted using a semi-automatic analysis system (LASV 4.3, Leica, Germany). The mean percentage of positive cells and a 95% confidence interval computed by the Agresti-Coull method were obtained for each marker by area and fixation method. A generalized linear mixed model (GLMM) was fitted to test for statistically significant differences in mean percentage of positive cells detected between the two fixation methods (formalin and ZS) for each organ, area and marker separately. Note that only markers detectable by both techniques were modelled. The GLMMs were fitted by maximum likelihood to the number of positive cells out of the total number of cells, with logit link function and binomially distributed errors, and using Laplace approximations to calculate log-likelihoods. Fixation method was included as a fixed effect and animal identification as a random effect in order to account for both within- and between-animal variability. The statistical significance of the fixation method effect was assessed using *p*-values derived from the likelihood ratio test at the usual 5% significance level. The data analyses and graphics were produced using the R system for statistical computing v3.2 (R Core Team 2015).

Good tissue morphology was evident in lymph nodes and spleen using either formalin or ZS fixation. Antibodies raised against human CD3, CD79 and CD68 epitopes, successfully reacted

with chamois immune cells, both in formalin and ZS fixed tissues. Antibodies raised against ovine CD4 and CD21 labelled chamois cells only in ZS fixed tissues. The clone 7.2.38 of anti-CD3 antibody, labelled the surface and cytoplasm of cells located mainly in T cell areas, namely interfollicular/paracortical areas of lymph nodes (Fig. 5A) and periarteriolar lymphoid sheaths of the spleen (Fig. 6A). A prevalence of CD3-positive cells was evident in the marginal zone of the spleen and medullary cords of lymph nodes (Fig. 3 and 4). A moderate number of CD3-positive cells were present in the mantle zone both in the lymph node and spleen and few CD3-positive cells were seen in germinal centres of the follicles. No statistically significant mean differences were detected in CD3 immunolabelling between formalin and ZS fixed lymph node (Fig. 1). In the spleen statistically significant differences were found only in the marginal zone where ZS showed a higher average percentage of positive cells when compared to formalin fixation (64.65% vs. 41.94%; p= 0.024) (Fig. 2). Specific immunolabelling with anti-CD4 monoclonal antibody was observed only in ZS fixed tissues (Fig. 5B and 6B). CD4-positive cells were small round cells that mirrored the distribution of CD3-positive cells, although with a less intense labelling. The highest mean percentages of CD4-positive lymphocytes were in the interfollicular/paracortical areas of lymph node (69.66%) and periarterial sheath of the spleen (60.97%) (Fig. 3 and 4). CD79-positive cells were mainly localized in germinal centres and mantle zones of follicles both in lymph nodes and spleen (Fig. 5C and 6C). Multifocal CD79-positive lymphocytes were evident also in the medulla of lymph nodes and in the marginal zone of spleen. In the medulla, positive cells were mainly localized around small blood vessels and multifocal large cytoplasmic-positive cells were evident. Smooth muscle cells and endothelial cells of small blood vessels occasionally reacted positively with anti-CD79 monoclonal antibody. Results of immunolabelling were variable between different areas of lymph node and spleen depending on the fixative (Fig. 1 and 2). The mean differences were statistically significant, with formalin fixation detecting on average a significantly higher percentage of CD79-positive cells in the germinal centres of both lymph nodes (87.47% vs.

68.25%; $p < 0.001$) and spleen (91.38% vs. 79.56%; $p < 0.001$); medullary cords of lymph node (10.92% vs. 4.42%; $p = 0.016$) and periarterial sheath (3.90% vs. 1.17%; $p < 0.001$) as well as in the red pulp (4.36% vs. 1.82%; $p = 0.005$) of the spleen. Conversely, ZS fixation revealed the highest percentages of CD79-positive cells in the interfollicular/paracortical area (4.25% vs. 2.27%; $p = 0.003$) and mantle zone (46.46% vs. 38.64%; $p = 0.011$) of lymph node. No statistically significant differences in the mean number of positive cells were observed in the mantle and marginal zones of the spleen. Anti-CD21 antibody stained small round cells and mainly, cells with a reticular pattern, in the light zone of the germinal centre and mantle zones of lymph node and spleen follicles (from 99.66% in the mantle zone of spleen to 97.39% in the mantle zone of lymph node) (Fig. 5D and 6D). Distribution of CD21-positive cells was similar to CD79-positive cells. However, in all considered areas, the percentages of CD21-positive cells were higher, on average, than CD79-positive cells (Fig. 3 and 4).

An intracytoplasmatic granular pattern was observed for cells consistent with macrophages using monoclonal anti-human CD68 antibody (Fig. 5F and 6F). CD68-positive cells were localized mainly in the medullary cords of lymph node, marginal zone and red pulp of the spleen (Fig. 5E and 6E). ZS fixation provided the highest average percentage of CD68-positive cells in the germinal centre (12.53% vs. 10.77%; $p = 0.040$) and interfollicular/paracortical area (7.62% vs. 1.40%; $p < 0.001$) of the lymph node and in the germinal centre (11.11% vs. 4.38%; $p < 0.001$) and the marginal zone (44.59% vs. 35.43%; $p = 0.042$) of the spleen. Conversely, formalin fixation revealed statistically significant higher mean percentage of CD68-positive cells in medullary cords (32.29% vs. 20.41%; $p = 0.029$) of the lymph node and the red pulp (62.45% vs. 49.46%; $p = 0.028$) of the spleen. In the other areas (mantle zone of lymph node and mantle zone and periarterial sheath of spleen), no statistically significant differences in mean percentages of positive cells between fixatives were observed (Fig. 1 and 2). No specific labelling was detected using several clones against CD8 and γδ TCR antigens in ZS or formalin fixed tissues with or without antigen retrieval.

Control ovine lymph node showed that all antibodies labelled the expected cell populations, including CD8-positive Tcells and γδ TCR-positive cells. No labelling was detected in negative control slides.

This study is the first description of the immune system cell subsets in chamois lymph nodes and spleen. Histology of chamois lymph node and spleen was similar to other animals (Cesta 2006; Drayton et al. 2006; Willard-Mack 2006; Navarro et al. 1996; González et al. 2001; Galeotti et al. 1993; Dagleish et al. 2012). The reactivity and distribution of immune cells in lymph nodes and spleen were similar to those described in goat (Navarro et al. 1996), sheep (González et al. 2001), cattle (Galeotti et al. 1993) and deer (Dagleish et al. 2012). Several monoclonal anti-human antibodies, CD3, CD79 and CD68, showed a good cross-reactivity with chamois antigens in lymph nodes and spleen, with both fixatives. Antigen-retrieval methods, which can be difficult to standardise and time consuming, were necessary on formalin fixed tissues to reverse the detrimental effects of fixation. As reported in previous immunohistochemical studies carried out on ovine and cervine tissues (Dagleish et al. 2012; González et al. 2001), ZS fixation allowed a good preservation of tissue morphology and a wider detection of cell surface epitopes compared to formalin fixation. These results could be related to the efficiency of ZS fixative that does not cause conformational changes in the tertiary and quaternary structures of proteins, leaving unchanged the surface receptors for the interaction with Fab (Fragment, antigen binding). Secondary changes of the tissues after antigen retrieval treatment, that can cause rarefaction and detachment of the cells from the slides, should be taken into account in assessing the differences of immunlabelling between formalin and ZS fixation. The absence of reaction with monoclonal antibodies anti-CD8 and anti-γδ TCR was similar to results seen in cervine tissues (Dagleish et al. 2012). These antigens seem to be extremely sensitive to fixation and also to heating (Dagleish et al. 2012; Tingstedt et al. 2003).

In conclusion, we found ZS to be an effective fixative that allows good preservation of surface receptors and specific immunolabelling. This work increased our understanding of the

distribution of cell populations of the immune system in chamois and represents a preliminary study for future studies on the pathogenesis of diseases and immune response in this species.

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Conflict of Interest Statement

All authors have no conflicts of interest to declare.

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Figure legends

Fig. 1. Lymph nodes: mean percentages (%) of CD3, CD79 and CD68-positive cells detected by area and fixative.

Fig. 2. Spleen: mean percentages (%) of CD3, CD79, and CD68-positive cells detected by area and fixative.

Fig. 3. Lymph nodes: mean percentages (%) of CD3, CD4, CD79, CD21 and CD68-positive cells by area detected in ZS fixed tissues.

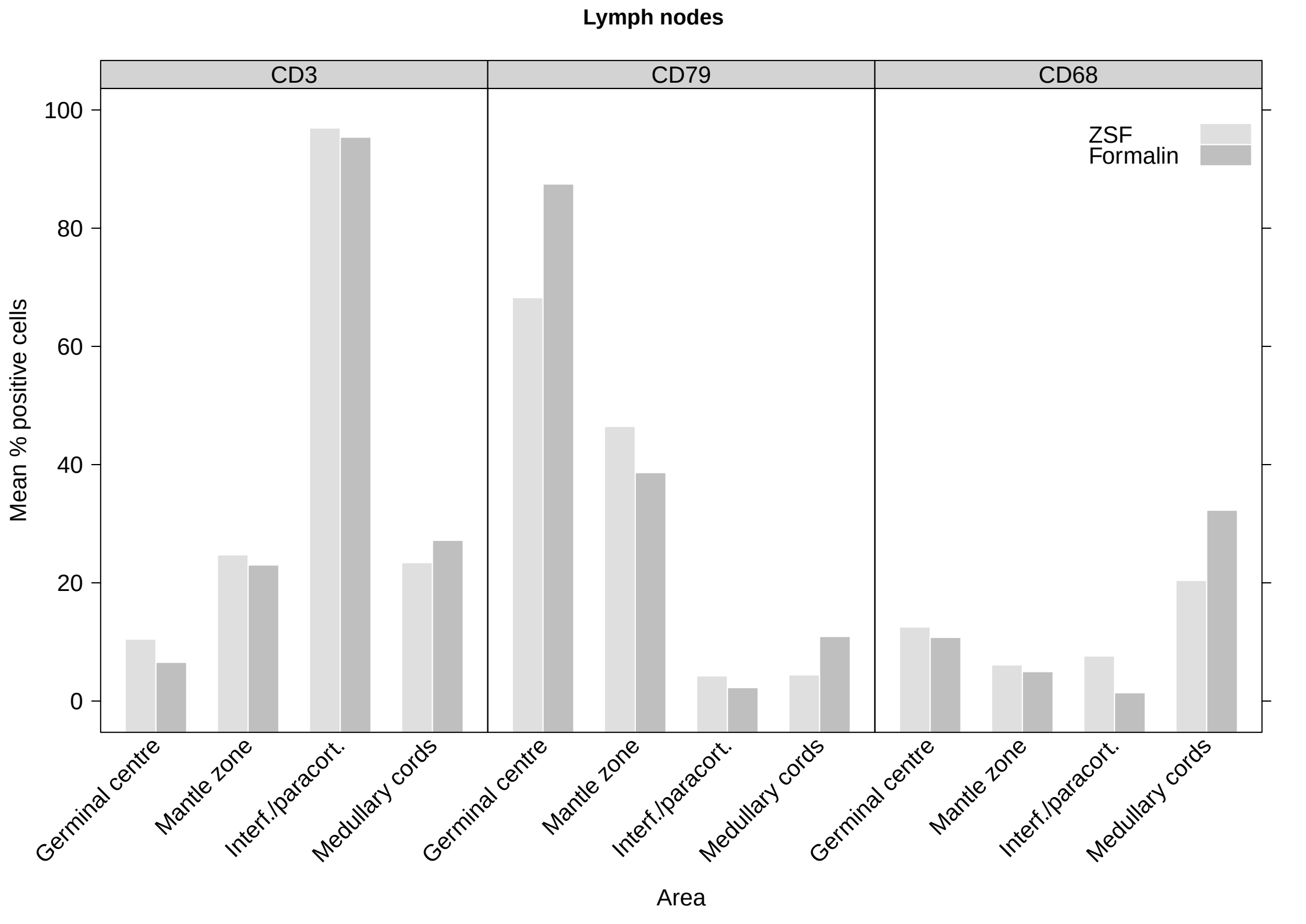
Fig. 4. Spleen: mean percentages (%) of CD3, CD4, CD79, CD21 and CD68-positive cells by area detected in ZS fixed tissues.

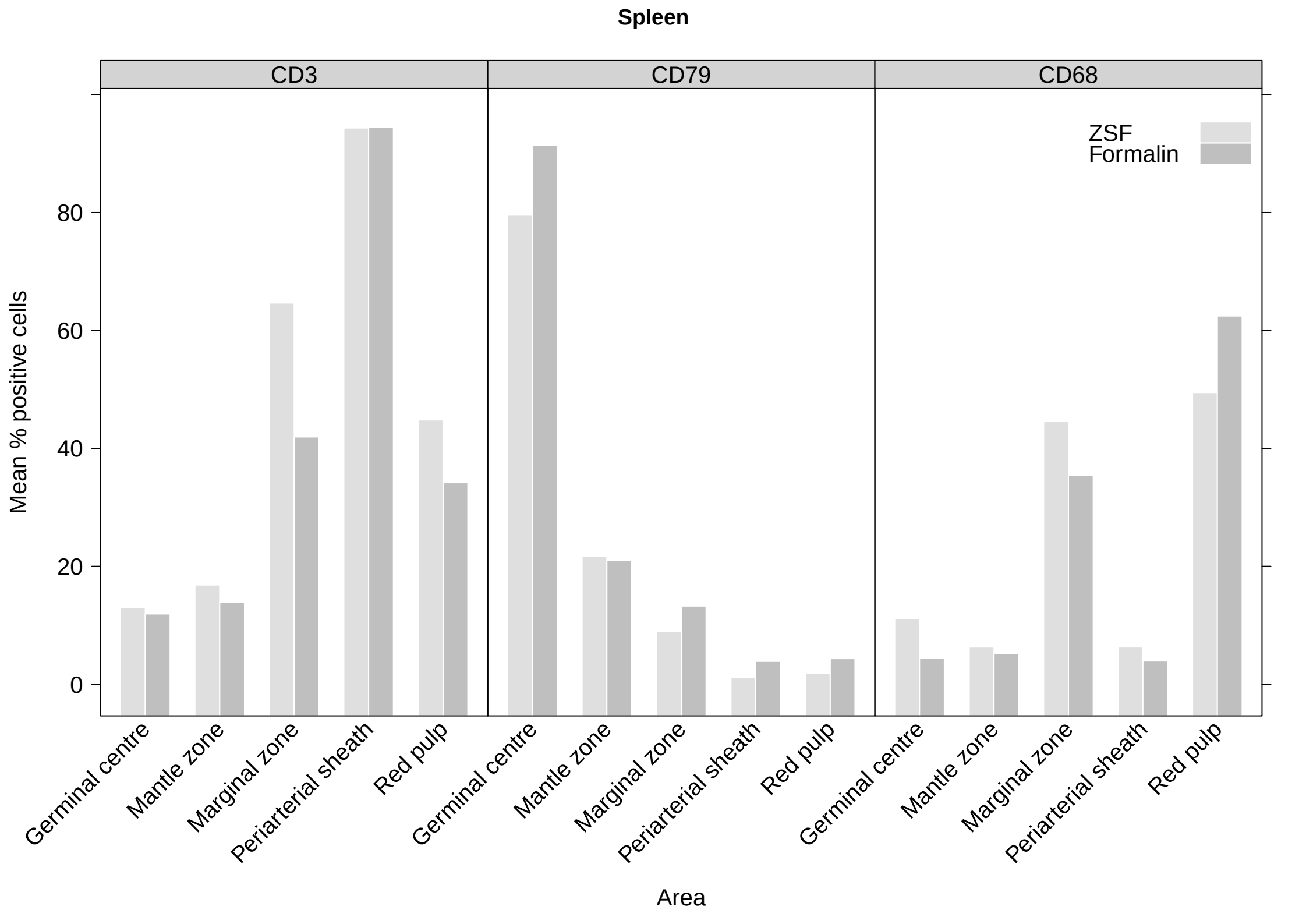
Supplementary material

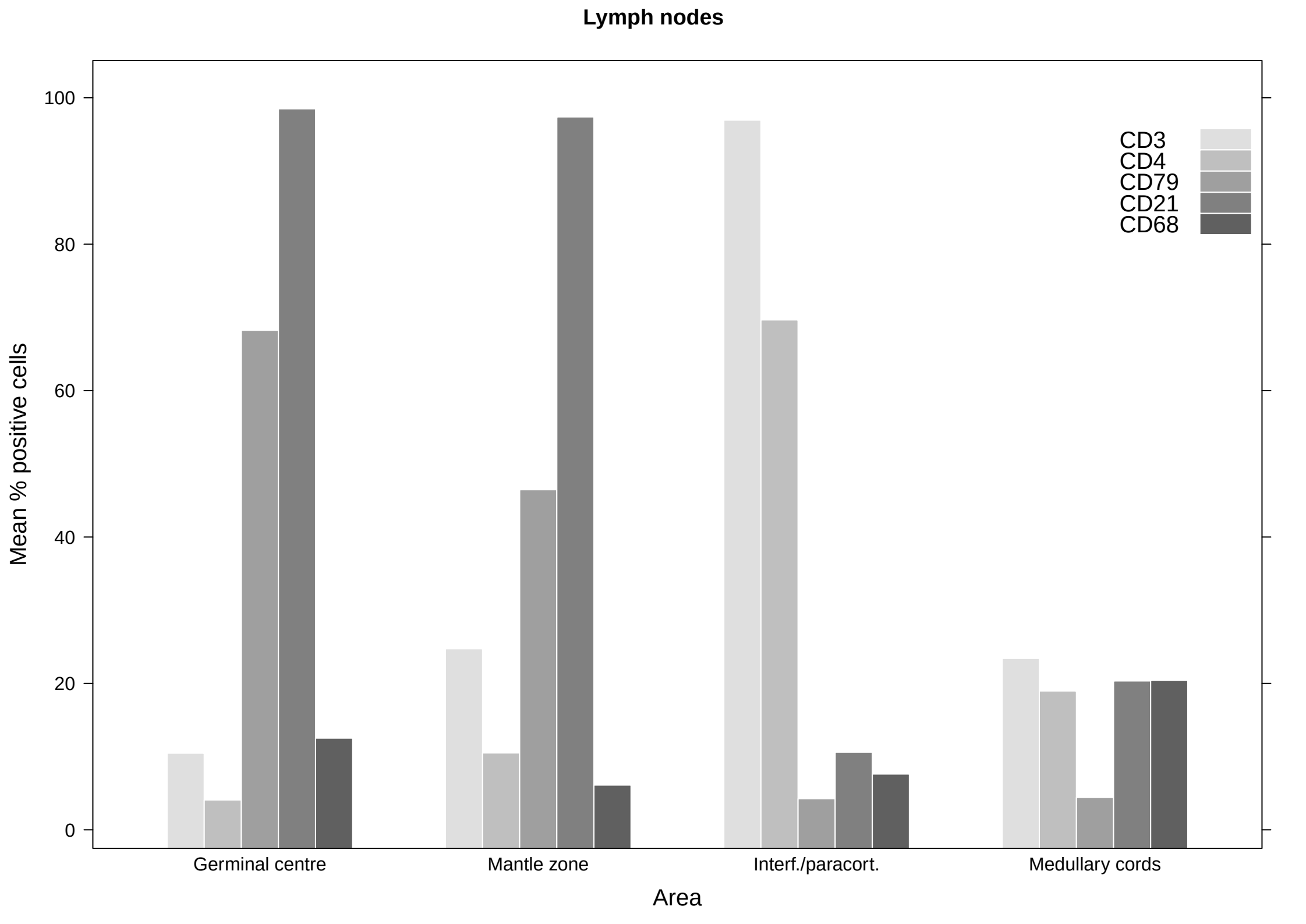
Table 1. Details of the primary antibodies and methods used in the immunohistochemistry study.

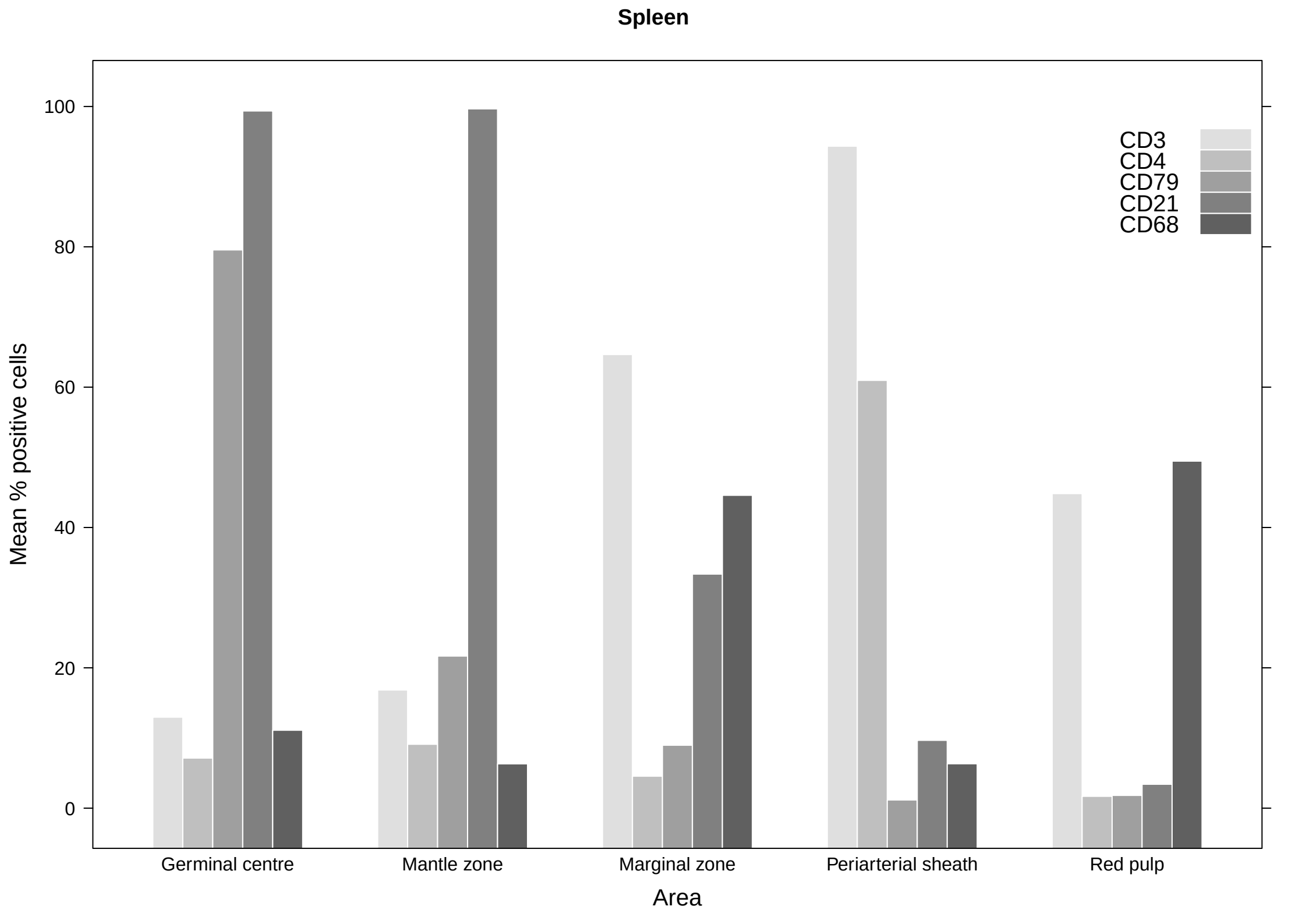
Fig. 5. Immunohistochemical labelling of chamois lymph node with anti-CD3 (A), anti-CD4 (B), anti-CD79 (C), anti-CD21(D) and anti-CD68 (E, F) antibodies. CD3-positive cells were mainly localized in the interfollicular area (A) and CD4 antibody showed a similar pattern (B). CD79 (C) and CD21(D) positive cells (C) in the mantle zone and germinal centre of the follicles. Scattered CD68-positive cells in the germinal centre of the follicle (E). These cells had a large irregular cytoplasm (F). Tissues were ZS (A, B, D, E, F) or formalin (C) fixed. DAB chromogen and haematoxylin counterstain. A, B, C, D, E: bar = 150 µm. F: bar = 35 µm

Fig. 6. Immunohistochemical labelling of chamois spleen with anti-CD3 (A), anti-CD4 (B), anti-CD79 (C), anti-CD21(D) and anti-CD68 (E, F) antibodies. CD3-positive cells in the periarterial area, germinal centre and mantle zone of the follicle (A). CD4-positive lymphocytes were lower in number, but had a similar distribution than CD3 positive cells (B). CD79 antibody labelled cells in the germinal centre and mantle zone of the follicle (C). Strong reticular labelling obtained with anti-CD21 antibody (D). CD68-positive cells in the marginal zone of the follicle (E). These cells showed a large elongated cytoplasm and a multifocal distribution in the germinal centre (F). Tissues were ZS (A, B, D, E, F) or formalin (C) fixed. DAB chromogen and haematoxylin counterstain. A, B, C, D, E: bar = 150 µm. F: bar = 50 µm


Lymph nodes
CD3
CD79
CD68
ZSF
Formalin
Mean % positive cells
100
80
60
40
20
0
Germinal centre
Mantle zone
Interf./paracort.
Medullary cords
Germinal centre
Mantle zone
Interf./paracort.
Medullary cords
Germinal centre
Mantle zone
Interf./paracort.
Medullary cords
Area


Spleen
CD3
CD79
CD68
ZSF
Formalin
Mean % positive cells
0
20
40
60
80
Germinal centre
Mantle zone
Marginal zone
Periarterial sheath
Red pulp
Area


Lymph nodes
Mean % positive cells
100
80
60
40
20
0
CD3
CD4
CD79
CD21
CD68
Germinal centre
Mantle zone
Interf./paracort.
Medullary cords
Area


Spleen
Mean % positive cells
100
80
60
40
20
0
CD3
CD4
CD79
CD21
CD68
Germinal centre
Mantle zone
Marginal zone
Periarterial sheath
Red pulp
Area

Cell marker	***Cell type recognized***	***Clone(s)***	***Species and isotype***	***Dilution***	***Labelling on ZSF fixed tissue***	***Labelling on formalin fixed tissue***	***Antigen-Retrieval method for formalin fixed tissue***
CD3	T lymphocyte	F7.2.38[1]	Mouse IgG1	1:100	Yes	Yes	Trypsin
CD4	T helper cell	17D[2]	Mouse IgG1	1:400	Yes	No	-
CD79αcy	B-cell	HM57[1]	Mouse IgG1	1:50	Yes	Yes	Microwave, 20 min.; citrate buffer
CD21	Follicular dendritic cell and mature B-cell	CC21[4]	Mouse IgG1	1:500	Yes	No	-
CD68	Macrophage	EBM11[1]	Mouse IgG1	1:50	Yes	Yes	Proteinase K
CD8	T cytotoxic cell	7C2 and ILA51[3]	Mouse IgG1	-	No	No	-
γδ TCR	γδ T-cell	86D[2]	Mouse IgG1	-	No	No	-

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