Journal of Advanced Veterinary Research

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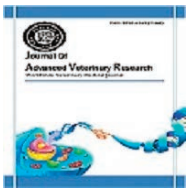

Immunohistochemical Distribution of Immunocompetent Cells in Water Buffalo Spleen (*Bubalus bubalis*)

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ARTICLE INFO

Original Research

Received:
14 October 2020

Accepted:
25 December 2020

Keywords:

Water buffalo spleen, CD68, CD56, Immunocompetent cells, B lymphocytes, Follicular dendritic cells.

ABSTRACT

Spleen holds critical attention among lymphoid tissues inside the animal body. Its physiological organization allows it to host a wide range of substantial functions, about red blood cells and the immune system. There are no sufficient reports dedicated to immune cells in spleen of buffalo. For this reason, the current study focused on recording the reactivity and distribution of certain antibodies to immunocompetent cells in water buffalo spleen. Twenty spleens of both sexes, 4.0±0.5 years, and 400.0±50.0 kg weight units were appointed indiscriminately from apparently healthy animals. Immunohistochemical technique was performed to investigate the binding specificity towards certain antibodies and area % was evaluated by the aid of R- program. The obtained findings revealed that CD5+, CD19+, CD20+, and IgM+ were expressed in splenic cells with insignificant difference area%, while CD21+, CD79A+, and IgG+ showed significant differences in area%. The latter antibodies showed its highest concentration in the marginal zone, few in the lymph node, and moderate in the red pulp. Natural killer cells, macrophages, and follicular dendritic cells highlighted a significant cytoplasmic difference utilizing CD56+, CD68+, CD1A+, respectively. Excessive expression of muscle and myofibroblast cells to alpha SMA antibody was observed. Plasma cells responded to the CD138 antibody without variation along buffalo spleen. This study aspired to present a full quantitative analysis of the normal distribution of immunocompetent cells toward water buffalo spleen. Based on that, chances will be opened for valuable designation to assort alterations in the distribution of these cells.

J. Adv. Vet. Res. (2021), 11 (1), 24-35

Introduction

Water buffaloes fit an excellent profit in animal and industrial production at the native and international level. They're able to participate powerfully in the profitable animal production by their ability to resist diseases, capability to adapt to numerous harsh climate, high edibleness of poor pasture, rapid growth, and high weight gains (Naveena and Kirant, 2014; Rashad *et al*., 2017).

As it is thought, the immune system encompasses lymphoid organs as well as a vast set of lymph vessels. They act as a reservoir of lymphoid cells that ceaselessly recirculate among the vascular system as well as the bloodstream (ILRAD, 1985). Spleen sorts as a secondary lymphoid organ. It embraces an encapsulated compact organ that merges into the stroma and parenchyma. Splenic parenchyma encloses of two main pulps: red and white ones. The red pulp behaves chiefly like a filter for any foreign materials and broken, or old erythrocytes appearing in blood. Additionally, the red pulp is believed to be a storage site for iron, erythrocytes, and platelets. The white pulp lies an effective immune initiator that has a plentiful immune reaction against any attacking antigens (Aichele *et al*., 2003; Cesta, 2006; Schneder *et al*., 2011; Rashad *et al*., 2020).

The architectural design of splenic immune cells remarks a distinct species variation. The dispersion of B, T lymphocyte, follicular dentritic cells, plasma cells, macrophage, natural killer cells and myofibroblast cells are differed corresponding to their role in a certain location. There is growing alertness to the significance of immunocompetent cells in splenic tissues. For instance, lymphocytes involved with innate-like functions in tissue homeostasis, immune regulation, and infection control (Zhang, 2013). The specific detection of certain receptors and their isoform can contribute to the further elucidation of the progression and pathogenesis of malignant neoplasms (Gattenlohner *et al*., 2009), and this achieved potentially to mobilize its catalytic capacity against tumor cells and control their lymphatic dissemination (Ferlazzo *et al*., 2004).

Immunohistochemistry formulates a thorough assessment of the distribution of proteins in the tissue, and the specific association between the cells that express them and other types of cells and tissues. Though, healthful tissue morphology and antigenicity are a necessity (Gonzalez *et al*., 2001). The marker panel enables the proof of identity by immunohistochemistry antibodies to distinguish the forms of white blood cells on diverse phases of maturity, in addition to several important molecules on cell surfaces. Wherever practicable, antibodies have been elevated to bovine antigens or considered as bovine cross-reactive were used, if no documentation on bovine reactivity has been raised (Niku *et al*., 2006). The latest

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ISSN: 2090-6277/2090-6269/

innovations in these procedures allow for quantitative recognition of various immune factors, which in turn will renovate diagnosis and prognosis plans. This allows the enumeration of cell subsets within immune environments, as well as the study of their spatial distribution (Feng *et al*., 2016), particular, attention will be paid to this panel as it was designed as a representative tool in understanding the involvement of the immune system in pathogenic diseases of bovine animals. Also, this approach increases the flexibility of routine diagnosis (ILRAD, 1985; Keresztes *et al*., 1996).

Since the spleen of water buffalo is still poorly defined in terms of its immune cells, its adequate role in different diseases and few available literature was applied on this topic. The present study will hopefully add valuable guides upon immunoexpressing antibodies of bovine animals using the immunohistochemical techniques. Consequently, histologists, immunologists, and pathologists can elucidate immune response background and help to find any splenic alterations in this species.

Materials and methods

Sample collection

The splenic tissues used in this study were gathered from AL-Munib Municipal Abattoir, Giza governorate from adult apparently healthy slaughtered water buffalos, that did not show any symptoms of sickness, 20 spleens of both sexes (10 Male and 10 Female), elderly 4.0±0.5 years and weighing, and 400.0±50.0 kilograms, were selected. The samples were randomly collected and transported through ice packs to the histology laboratory in the Faculty of Veterinary Medicine at Cairo University, Giza, Egypt.

The splenic samples were gently rinsed under tap water and then fixed in 10% neutral buffered formalin (10% NBF). Tissues were maintained 24 h in fixative at room temperature (RT). After that, dehydration and clearing of the fixed splenic tissues were performed by ethanol (70%, 80%, 90%, 95%, and 100%) (32205-M, ALDRICH, Sigma) and 1,4-Bis (trifluoromethyl) benzene (2 changes, each one 10 min) (MFCD00000402, ALDRICH, Sigma), respectively. Later, they were embedded in paraffin wax (MKCL2854, ALDRICH, Sigma). Paraffin sections were cut in a thickness of 4-6 µm using Leica RM2235 microtome. Sections were stained using the peroxidase- anti peroxidase (PAP) method (Gnanadevi *et al*., 2019).

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Ethical approval no. CU II F C 123 18 from the Institutional Animal Care and Use Committee (IACUC).

Staining protocol

For immunohistochemistry protocol, splenic tissue sections were exposed to deparaffinization and rehydration steps. Then, sections were washed twice by using a buffer. However, some required incubation of splenic tissue in a digestive enzyme (others appropriated pretreatment) then sections were washed four times using a buffer. To lack non-appreciate background staining owing to endogenous peroxidase, the slides were incubated in Hydrogen Peroxide Block for ten minutes, after that slides were washed two times for 5 min in TBS plus 0.025% Triton X-100 with gentle agitation. Antigen retrieval step was performed to unmask the antigenic epitope by applying 300 ml of 10 mM citrate buffer, pH 6.0 into the staining container of the slides and then were incubated at 95-100°C for 10 min. After that slides were cooled at room temperature for 20 min and rinsed in 300 ml PBS for 2 changes, 5 min each.

The splenic slides were kept until draining for a few seconds and then were wiped with tissue paper. Each primary antibody was diluted to the manufacturer's recommendations and then applied to splenic slides. In this study, splenic tissue sections were evaluated for:

CD5 Monoclonal antibody (4C7), Mouse Anti Human/Bovine (cross-reactivity), RTU, (Thermo Fisher Scientific Cat# MA1-21195, RRID: AB_558225).

CD19 Monoclonal Antibody (LE-CD19), Mouse Anti Human/Bovine (cross-reactivity), (1:100), (Thermo Fisher Scientific Cat# MA1-81723, RRID: AB_927800).

CD20 Monoclonal Antibody (L26), Mouse Anti Human/Bovine (cross-reactivity), (1:20), (Thermo Fisher Scientific Cat# MA1-22864, RRID: AB_558114).

CD21 Monoclonal Antibody (1F8), Mouse Anti Human/Bovine (cross-reactivity), (1:10-1:25), (Thermo Fisher Scientific Cat# MA1-27120, RRID: AB_779640).

CD79a Monoclonal Antibody (JCB117), Mouse Anti Human/Bovine (cross-reactivity), (1:50-1:100), (Thermo Fisher Scientific Cat# MA5-11636, RRID: AB_10986243).

IgM Polyclonal Antibody, Rabbit Anti-Human/Bovine (cross-reactivity), RTU, (Thermo Fisher Scientific Cat# PA1-29094, RRID: AB_2540051).

IgG Polyclonal Antibody, Rabbit Anti-Human/Bovine (cross-reactivity), RTU, Thermo Fisher Scientific Cat# PA1-29093, RRID: AB_2540050).

CD138 Monoclonal Antibody (MI15), Mouse Anti Human/Bovine (cross-reactivity), (1:10-1:20), (Thermo Fisher Scientific Cat# MA5-12400, RRID: AB_10987019).

CD56 Monoclonal Antibody (123C3), Mouse Anti Human/Bovine (cross-reactivity), RTU, (Thermo Fisher Scientific Cat# MA1-35250, RRID: AB_1073118).

CD1a Monoclonal Antibody (O10), Mouse Anti Human/Bovine (cross-reactivity), RTU, (Thermo Fisher Scientific Cat# MA1-34371, RRID: AB_1955137).

CD68 Monoclonal Antibody (KP1), Mouse Anti Human/Bovine (cross-reactivity), (Thermo Fisher Scientific Cat# MA1-26265, RRID: AB_779735).

Smooth Muscle alpha Actin Monoclonal Antibody (1A4), Mouse Anti Human/Bovine (cross-reactivity), (Thermo Fisher Scientific Cat# MA1-82032, RRID: AB_2223639).

All sections were incubated according to the manufacturer's recommended protocol of each antibody and were washed four times in buffer. Later, the primary antibody enhancer was added, incubated for 10 minutes at room temperature, and washed four times in PBS buffer. Secondary antibodies not used in this methodology to avoid the non-specific and undesirable reactions, that related to the histological nature of the splenic tissue. Sections then were manipulated to horseradish peroxidase polymer (HRP Polymer), incubated for 15 minutes at room temperature, and washed four times in PBS buffer. One drop of (40 µl) 3,3' Diaminobenzidine (DAB) Plus Chromogen were added to 2 ml of DAB Plus Substrate, mixed by swirling, next we applied them to the splenic tissue sections and incubated for 5 minutes. The tissue sections were rinsed four times in distilled water. Finally, sections were counterstained and covered by coverslips using permanent mounting media. Negative control of splenic tissue sections was raised beneath the equivalent conditions with a low amount of primary antibody (Petrosyan *et al*., 2002). Microscopic observations along with photographing shots were performed by a Leica microscope (CH9435 Hee56rbrugg) using different magnification powers.

Evaluation of immunohistochemistry "Area Percentage" (Specific area/ Antibody)

Cell numbers were calculated as the number of cells per cross-sectional area using some features (cell counter/ color

intensity/ color threshold) and IHC plugin of the Image J program (Schneider *et al*., 2012). They were used for evaluation of area percentage (Area%) of each antibody in different spleen compartments as white pulp (WP), marginal zone (MZ), and red pulp (RP).

Statistical analysis

Data related to area percentage was presented as Mean and Standard Error "SE". Variance and multiple comparison test; ANOVA was performed using R codes (DescTools, ggpubr) and Shapiro-Wilk normality test for measuring P-value (R, 2013). This package is available at CRAN (https://cran.r-project.org/). P-values less than 0.05 were regarded as statistically significant.

Results

Immunohistochemistry

B lymphocyte is one of the immunocompetent cells that have a certain function in immune stability. On the surface of this cell, receptors are found to control its activity. In this study CD5, CD19, CD20, CD21, CD79A, IgM, and IgG antibodies were used to test the reactivity of them in the spleen of water buffalo.

Findings from this study revealed positive immune expression of water buffalo splenic tissue to the CD5 antibody. The reaction appeared in the surface of immunocompetent cells throughout all parts of splenic tissues: the least in lymph nodule (Fig.1A), moderate in the marginal zone (Fig.1B) and the highest along red pulp area (Fig.1C). However, there was no difference between the reaction of these regions. Splenic cells were reacted positively to the CD19 antibody in a least amount inside LN of water buffalo. Meanwhile, CD19 expression was moderate in MZ and the highest in RP area. Inside the lymph nodule, reactivity was observed along the plasma membranes (Fig.1D). On the other hand, most expressions acquired a regular and round form in the marginal zone (Fig. 1E), and red pulp (Fig.1F).

Another marker used was CD20. Inside the lymph nodule, splenic cells were reacted to this antibody in a least amount; some of them were immune-positive reacted along their plasma membrane forming circle-like shape, while others revealed intense and irregular expression (Fig. 2A). Moderate expression of CD20 antibody was noticed in MZ area, unlike the highest ones in RP area. Most CD20+ splenic cells which were detected in the marginal zone and red pulp areas of water buffalo spleen showed polygonal surface reactions (Figs. 2B & 2C). CD21 antibody in water buffalo spleen revealed its significant difference throughout water buffalo spleen. Inside the lymph nodule, only a few membranous expressions were observed in splenic cells (Fig. 2D). In the marginal zone, the highest surface expression was detected clearly in splenic cells (Fig. 2E). Meanwhile, moderate expression in red pulp was represented in two forms; one appeared on the surface of splenic cells. The other highlighted in arcs like form (Fig. 2F).

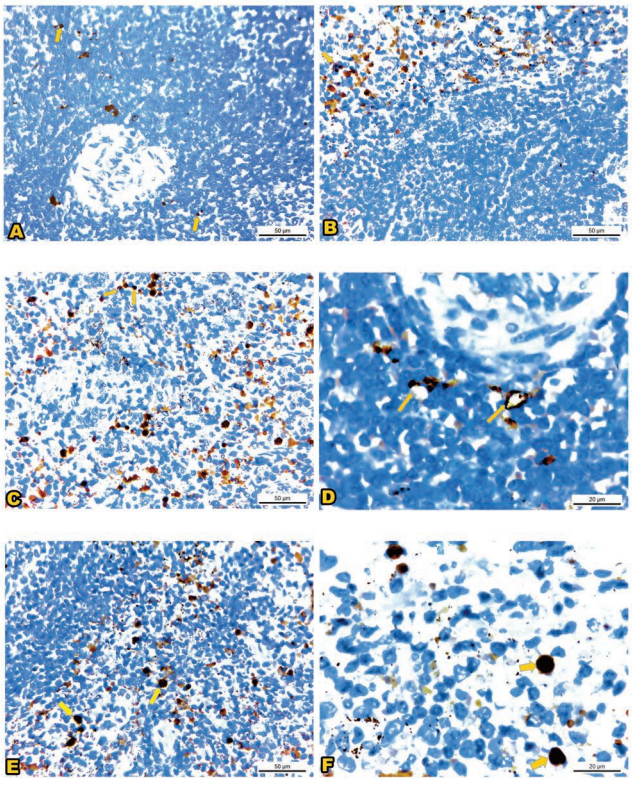

Fig. 1. Photomicrograph in spleen of water buffalo highlighted. (A:C) The immune positively surface expression of CD5 along the splenic cells (arrows) presented as: (A) Least reactivity in lymph nodule. (CD5 X1000). (B) Moderate expression in the marginal zone. (CD5 X400). (C) Red pulp area with the highest reaction. (CD5 X400). (D: F) The expression of CD19 in the splenic cells showed: (D) Splenic cells inside the lymph nodule reacted positively in a less amount to CD19 along their plasma membrane (arrows). (CD19 X1000). (E) Moderate surface reaction of CD19 on the splenic cells highlighted in a regular and round form (arrows) in the marginal zone area. (CD19 X400). (F) Splenic cords of the red pulp marked the same forms of reactivity of CD19 in MZ area but in a highest amount (arrows). (CD19 X1000).

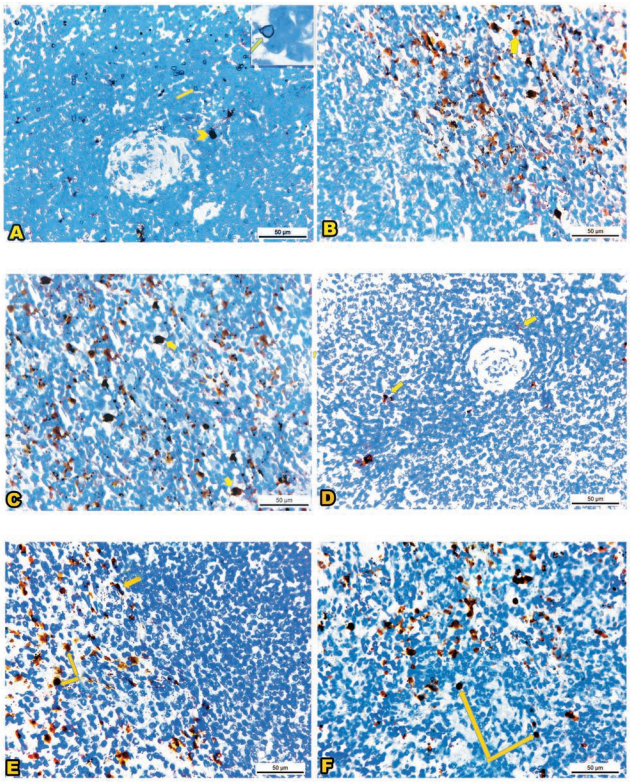

Fig. 2. Photomicrograph in the spleen of water buffalo outlined: (A:C) The reaction of splenic cells to CD20 antibody showed: (A) Lymph nodule area demonstrated the lowest reaction of CD20 antibody along the plasma membrane of some cells (arrow) or as immunopositively intense and irregular reaction in others (arrowhead). (CD20 X400). Inside Cube (CD20 X1000). (B) Marginal zone area showed the moderate surface expression of CD20 (arrow). (CD20 X400). (C) Splenic cords of red pulp represented the highest immunopositively surface expression of CD20 (arrows). (CD20 X400). (D: F) The expression of CD21 antibody in the splenic cells showed: (D) Lymph nodule cells showed few expressions to CD21 on its surface (arrow). (CD21 X1000). (E) Marginal zone clarified the highest surface reaction of CD21 in the splenic cells (arrows). (CD21 X400). (F) Splenic cords of red pulp highlighted a moderate reaction of CD21 either in regular surface expression (Long arrows) and arcs like one (short arrows). (CD21 X400).

This study evaluated also the CD79A antibody in the spleen of the water buffalo. In the lymph nodules, few CD79A reactions were obtained on plasma and nuclear membranes (Fig. 3A). The expression marked its highest level in the marginal zone (Fig. 3B), while in red pulp it was moderate (Fig. 3C). IgM immune-positive cells were recognized in their reaction

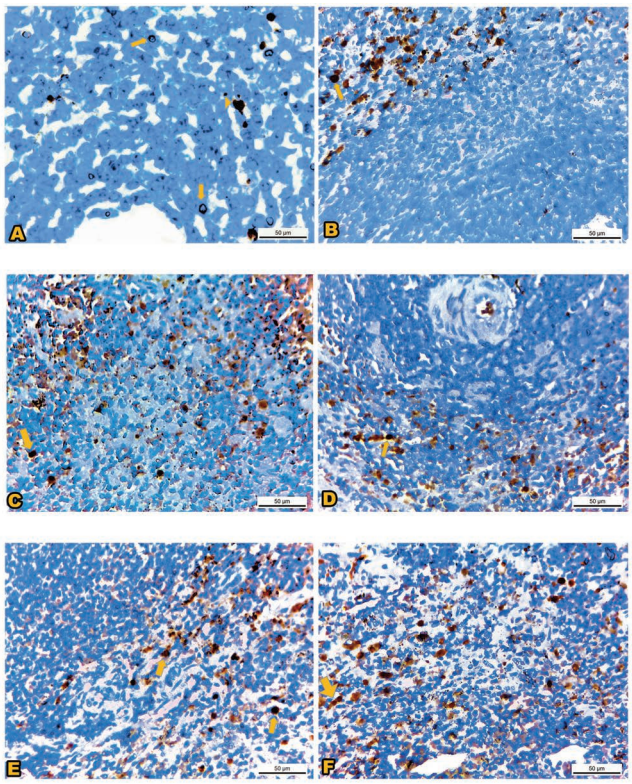

Fig. 3. Photomicrograph of water buffalo spleen explained: (A:C) The expression of CD79A in the splenic cells showed: (A) Lymph nodule represented the few immunopositively reaction of CD79A antibody to splenic cells as plasma membrane reaction (arrowhead) in some cells and nuclear membrane reaction (arrows) in others. (CD79A X1000). (B) Marginal zone area demonstrated the highest surface reaction of CD79A (arrow). (CD79A X400). (C) Splenic red pulp clarified the moderate immunopositively surface expression of CD79A (arrow). (CD79AX400). (D: F) The surface expression of IgM along the splenic cells (arrows) showed in: (D) White pulp presenting the least in lymph nodule area. (IgM X400). (E) Marginal zone area with a moderate reactivity. (IgM X400). (F) Red pulp splenic cords as a highest reactive area. (IgMX400).

as a membranous expression in all splenic compartments; few in lymph nodules (Fig. 3D), moderate through marginal zone (Fig. 3E), and the highest along splenic cords and sinuses of the red pulp (Fig. 3F). Furthermore, splenic cells were also identified by the IgG antibody. The least distribution of these cells was found inside the lymph nodules (Fig. 4A), while the highest was noticed in the MZ (Fig. 4B). On the other hand, higher distribution was focused on splenic cords of the red pulp of water buffalo spleen (Fig. 4C). In all areas, they were expressed as a surface reaction.

In supporting the view of the presence of other important immune cells in the spleen rather than B lymphocytes, this research highlighted some cells including natural killer cells, macrophage cells, and dendritic cells. Natural killer cells were detected by using the CD56 antibody. Positive surface expression of few cells was noticed inside the lymph nodule (Fig. 4D). Moderate distribution of natural killer cells was observed as a surface expression in the marginal zone (Fig. 4E), while the highest expression was recorded in the red pulp (Fig.4F) of water buffalo spleen.

CD68 antibody pointed out the distribution of macrophage cells along water buffalo splenic tissue. In the

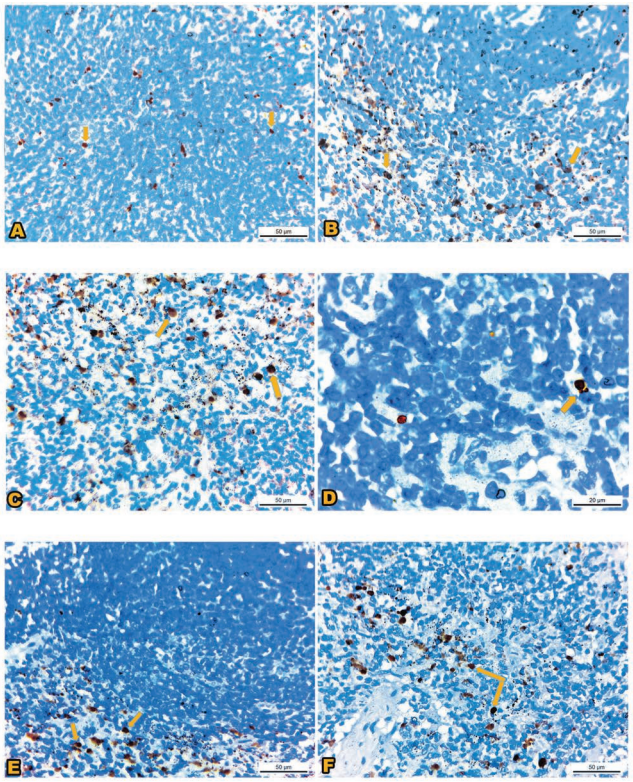

Fig. 4. Photomicrograph of water buffalo spleen demonstrated: (A:C) The distribution and expression of IgG+ splenic cells showed positive immune reaction on their surface (arrows) along : (A) Splenic lymph nodule with least distribution. (IgGX1000). (B) Marginal zone with highest distribution. (IgGX400). (C) Higher distribution in the splenic red pulp. (IgG X400). (D: E) The reaction of natural killer cells to CD56 antibody highlighted immunopositivity surface expression (arrows) which was (D) Few inside lymph nodule (CD56 X1000). (E) Moderate in marginal zone area. (CD56 X400). (F) Highest in red pulp area. (CD56 X400).

lymph nodule, around the central arteriole, the CD68+ macrophage cells were observed few with surface immune positive expression (Fig. 5A). Many CD68+ macrophages were determined with immunopositivity surface expression in the marginal zone (Fig. 5B) and the red pulp (Fig. 5C). Dendritic cells were identified by using the CD1A antibody. Surface immune expression of dendritic cells was recognized few in PALS (Fig. 5D), higher in the marginal zone as regular and irregular forms (Fig.5E), and the highest in the red pulp (Fig. 5F) of water buffalo spleen.

Plasma cells in water buffalo spleen were identified by their positive immune reaction to CD138. The plasma cells were found with a least surface reactivity in the lymph nodule (Fig. 6A), moderate in marginal zone (Fig. 6B), and the highest ones homing red pulp areas (Fig. 6C). Water buffalo spleen was characterized by high muscular tissue in its composition. αSMA antibody was contributed for easy recognition of smooth muscle inside splenic tissue. The spleen of water buffalo is composed of a thick fibromuscular capsule which gave intense expression to αSMA. From its thick muscular trabeculae emerged, which were identified as primary and secondary trabeculae (Fig. 6D). In the lymph nodule, the smooth muscles were detected surrounding the central arteriole and as a discrete line bordering lymph nodule area (Figs.6E&6F). Positive αSMA myofibroblast cells were highlighted between the immune cells along the marginal zone (Fig. 6E) and red pulp (Fig. 6F).

Evaluation of immunohistochemical results

ANOVA test showed a highly significant difference in the

area % values of IgG+ cells, significant differences were obtained in case of using CD21 & CD79A antibodies and no significant differences in CD5+, CD19+, CD20+ & IgM+ cells. Other splenic cells proven a significant difference in the area % values by using CD56, CD68 & CD1A antibodies for natural killer cells (NKCs) cells, macrophage & follicular dendritic cells, respectively. Meanwhile, the CD138+ plasma cells and alpha smooth muscle actin (αSMA) revealed no significant differences in their area % values (Table 1 and Fig. 7).

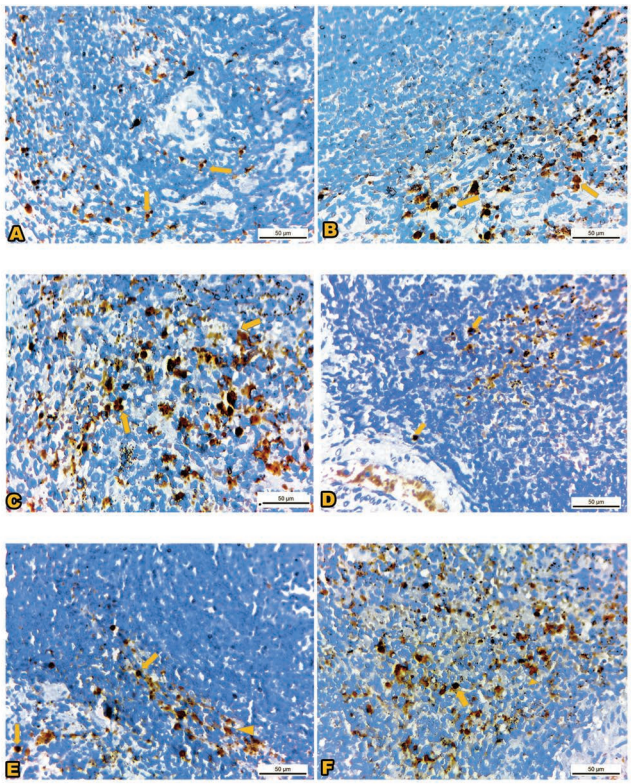

Fig. 5. Photomicrograph of water buffalo spleen showed: (A:C) The reaction of macrophage cells to CD68 antibody showed: (A) Positive immune reactivity of the CD68 antibody in the surface of macrophage cell around the central arteriole in the lymph nodule (arrows). (CD68 X400). (B) Marginal zone remarked the reaction of many macrophage cells to the CD68 antibody. Macrophage cell presented strong positive surface reaction (arrows). (CD68 X400). (C) Red pulp splenic cords were characterized by strong positive surface expression of macrophage cell to CD68 (arrows). (CD68 X400). (D: F) The expression of dendritic cells to CD1A antibody showed: (D) PALS in white pulp clarified the positive reactivity of the follicular dendritic cells to CD1A antibody that appeared as surface reaction (arrows). (CD1A X400). (E) Marginal zone area outlined a higher positive surface expression of CD1A+ dendritic cell in a regular (arrow) and irregular (arrowhead) forms. (CD1A X400). (F) Red pulp showed the highest reaction and the same surface expressions in MZ area. (CD1A X400).

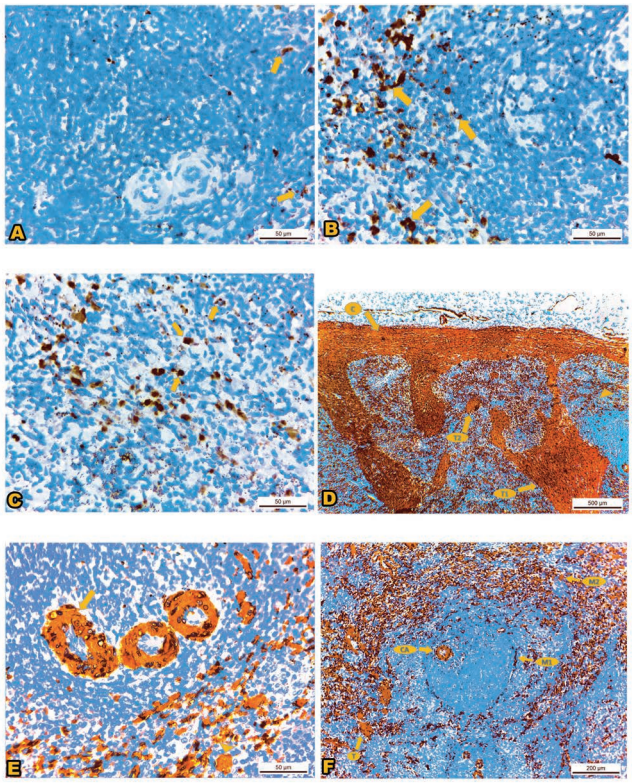

Fig. 6. Photomicrograph in water buffalo spleen outlined: (A:C) The reaction of plasma cells to the CD138 antibody showed surface immunoreaction (arrows): (A) In the lymph nodules. (CD138 X400). (B) Marginal zone area. (CD138 X400). (C) Red pulp. (CD138 X400). (D:F) Positive immune reaction of the smooth muscles to αSMA showed: (D) Splenic capsule remarked the positive immunoreaction of smooth muscle to αSMA, highly muscular capsule (C) gave thick muscular trabeculae which emerged either as primary trabeculae (T1) or secondary trabeculae (T2). Positive expression of myofibroblast to the αSMA antibody was noticed along splenic tissue (arrowhead). (αSMAX40). (E) Positive immunoreaction of smooth muscle to αSMA was observed inside the central arteriole (arrow) and the myofibroblast between marginal zone cells (arrowhead). (CD4 X400). (F) Lymph nodule in the white pulp area exhibited strong positive reaction of smooth muscle to αSMA. Notice that smooth muscle inside the central arteriole of lymph nodule (CA), secondary trabeculae (T), bordered lymph nodule (M1) and myofibroblast was reacted to αSMA between immune cells inside red pulp and splenic tissue (M2). (αSMAX100).

Discussion

The chief component of the spleen is its immune cells, which play the primary role in counteracting any infection. These cells provide the safeguard through donating either

Table 1. Area (%) values of splenic cells markers

	White Pulp	Marginal Zone	Red Pulp	*P*-Value
CD5	0.77±0.21^{c}	5.47±0.24^{b}	18.49±1.51^{a}	0.06
CD19	2.59±0.76^{c}	6.96±1.08^{b}	8.55±1.08^{a}	0.8
CD20	1.48±0.44^{c}	5.61±0.53^{b}	8.86±0.7^{a}	0.5
CD21	0.89±0.3^{c}	11.36±0.2^{a}	8.42±0.48^{b}	0.04*
CD79A	0.65±0.22^{c}	6.33±0.05^{a}	4.9±0.43^{b}	0.03*
IgM	0.88±0.1^{c}	5.53±0.24^{b}	12.54±0.26^{a}	0.07
IgG	0.98±0.03^{c}	13.36±0.17^{a}	12.54±0.26^{b}	0.0007**
CD56	0.14±0.07^{c}	5.38±0.31^{b}	6.41±0.3^{a}	0.006*
CD68	0.78±0.1^{c}	5.17±0.22^{b}	6.15±0.11^{a}	0.006*
CD1A	1.76±0.25^{c}	7.54±0.37^{b}	9.27±0.27^{a}	0.03*
CD138	1.42±0.37^{c}	5.01±0.07^{b}	7.1±0.15^{a}	0.1
Alpha Smooth Muscle Actin (αSMA)	Capsule 29.31±1.3^{b}	21.07±0.47^{c}	29.95±1.03^{a}	0.4

Values are expressed as Mean±Standard error; *P- Values ≤ 0.05 are significant; **P- Values ≤ 0.001 are highly significant

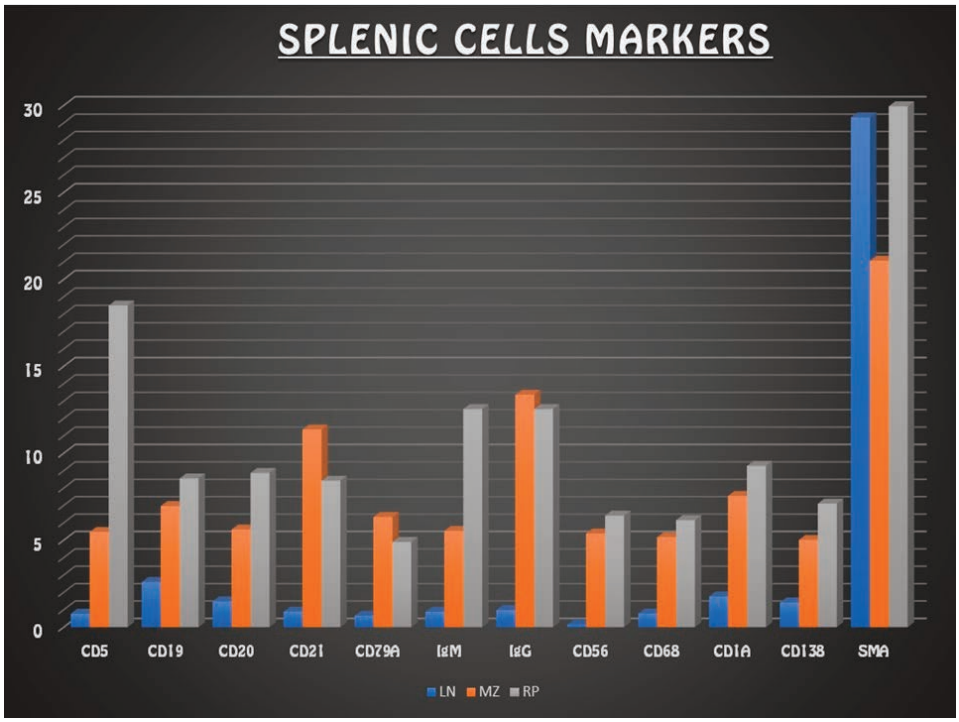

Fig. 7. Area percentage values (Mean ± Standard Error) of splenic cells markers.

non-specific or specific acquired response against any foreign bodies (Goffa *et al*., 2010). Whichever alterations arise to these cell populations regarding their normal structure or location, the result will come up with vital implications around immune reaction after certain disorders (McCulloch *et al*., 2017; Zhang *et al*., 2017). In the current study, authors have detailed the distribution of immunocompetent cells in the spleen of water buffalo species through the application of specific antibodies that react to the receptors of these cells. The panel of immunocompetent cells in this study focused on B-lymphocytes, plasma cells, natural killer cells, macrophages, and follicular dendritic cells with special reference to myofibroblast and their immunological role.

Recent literature proved that the CD5, CD19, CD20,CD21, CD79A, IgM and IgG receptors reacted mainly to B lymphocyte but may express with another immunocompetent cell to a lesser extent. Hence, we marked their expressions as splenic cell reaction not B cell reaction.

In this study, CD5 reacted positively throughout all parts of water buffalo splenic tissues. The expression was along their surface with a least distribution in a LN, moderate in MZ, and the highest one in RP. However, there was an insignificant difference between the reaction of these regions. Furthermore, results distinguished CD5 expression to B lymphocyte. The variation in allocation may reveal the ability of CD5 to act as an inhibitory or stimulatory molecule on B cells (Lozano *et al*., 2000). For instance, it inhibits Ig-mediated signaling in B-1 cells (Bikah *et al*., 1996) and autoimmune responses through negative regulation of Ig receptor signaling in anergic B cells (Hippen *et al*., 2000). Moreover, preferential secretion of IgM by CD5+ B cells (Werner-Favre *et al*., 1989). Lozano *et al*. (2000), revealed that the CD5 antibody expressed in three types of cells: T lymphocyte, B lymphocyte, and macrophage cell. The reason for their existence on more than one cell is owing to CD5 ability to interact in diverse ways according to the cell type and phase of maturation.

The obtained findings matched the literature published by Azzam *et al*. (1998). The distribution of CD5 in T cells proves their critical role to these cells which appears during the development of T regulatory cells (Ordonez-Rueda *et al*., 2009; Henderson *et al*., 2015), in the differentiation of Th1 and Th2 (Imboden *et al*., 1990), and by an improvement of T cell receptor signaling transduction. Consequently, CD5 capable to control the immunological tolerance of T-cells through regulation of their response and energy (Gary-Gouy *et al*., 2002; Axtell *et al*., 2004; Sestero *et al*., 2012).

On the other side, the expression of CD5 in macrophage cells was versus Naoki *et al*. (2002) findings who mentioned that CD5+ B cells are transformed into macrophage-like cells. The explanation of this difference belongs to their deal with most of the CD5+ cells as outgrowing of CD5 cells in infection, and hence CD5 behaves like an activation marker (Freedman *et al*., 1987).

In the current investigation, splenic cells were reacted positively to the CD19 antibody in all tissue compartments of water buffalo. Least distribution was marked in LN, meanwhile, marginal zone was noticed in a moderate reactivity and red pulp with the highest one. Despite of that, this expression showed nonsignificant differences regarding its location. In CD19+ cells, immunoreactivity observed along their plasma membranes inside LN of water buffalo spleen. These observations disagree with Feng *et al*. (2016), who noticed in mice that the majority of CD19+ B cells were located inside the follicles, with the cytoplasmic distribution. On the other hand, most of the surface expressions acquired a regular and round form in the marginal zone, and red pulp in this work. This may claim to the requirement of CD19 for effective B cell receptor (BCR) signaling. CD19 modulates this signal transduction of the BCR by different mechanisms (Wang *et al*., 2012; Li *et al*., 2017). Like its ability to control the differentiation of marginal zone precursors to marginal zone B cells by regulating ADAM28-mediated Notch2 cleavage as what is reported in the cow (De Rie *et al*., 1987). Also, CD19 can regulate calcium flux and activate Mitogen-activated protein kinase (MAP kinase) (Li *et al*., 2017). Through these mechanisms, CD19 possesses a crucial role in B cell development, activation, and differentiation. Thus, it is essential for developing an adequate immune response (Engel *et al*., 1995; Rickert *et al*., 1995). Consequently, any reduction of CD19+ B cells may play a precious pathogenic role in genetic abnormalities, multiple sclerosis (MS), Systemic Lupus Erythematosus, and diffuse large B cell lymphoma. Moreover, its increase is strongly associated with

fasting insulin secretion, skin fibrosis, autoimmunity in the tight-skin mouse, and acute lymphoblastic leukemia (De Rie *et al*., 1987).

De Rie *et al*. (1987) recorded a weak immunoreactivity of CD19 to dendritic cells. The expression is outstanding to a unique phenotype CD19 (hi) Fc gamma IIb (hi) on regulatory dendritic cells which can inspire splenic B cells to be distinguished into a definite subtype of IL-10-producing regulatory B cells.

Another marker used in this study was CD20. CD20+ cells were noticed inside the LN of water buffalo spleen with insignificant difference; some of them were remarked by least immunopositivity reaction along their plasma membrane and other with intense and irregular surface expressions, while most of CD20+ cells were detected with polygonal reactions in a moderate amount to the marginal zone and a highest distribution to red pulp areas. The significance value of CD20 on B lymphocyte can be described in its ability to downstream the BCR, and its function is bound to the expression of this receptor (Li *et al*., 2003; Uchida *et al*., 2004; Polyak *et al*., 2008; Walshe *et al*., 2008). In this way, B cell activation is controlled by CD20 (Golay *et al*., 1985). Moreover, Kanzaki *et al*. (1995) verified that CD20 can also regulate the cell growth of B cell through adjusting calcium cytoplasmic levels and so prove the accuracy of the calcium influx. (Kanzaki *et al*., 1995; Li *et al*., 2003). The former authors mentioned that the dysregulation in the function of this receptor may be an indicator of certain disorders like; Reed-Sternberg cells of Hodgkin's disease and leukemia.

By applying the CD21 antibody in the present study, positive immune expression highlighted with a significant difference throughout water buffalo spleen. Inside the lymph nodule, only a few membranous expressions were observed in splenic cells. Identical results were recorded by Keresztes *et al*. (1996) and Zhang *et al*. (2012) in bovine animals suggesting an extremely specific role for the recognized protein. On the contrary, reports of Herzenberg and Kantor (1993) and Gupta *et al*. (1998) conveyed that 75% of CD21+ B cells in the ovine animals are found within the splenic follicles. The variation in the ratio of CD21 inside follicles is thought to be associated with the degree of CD21 participation in the development of B memory cell regarding that follicles is the site of somatic hypermutation, selection, and differentiation during immune responses (Leanderson *et al*., 1992; Grandien, *et al*., 1994).

Nevertheless, within MZ had the highest surface expression of CD21 in the spleen of water buffalo. This finding is contrary to reports of Herzenberg and Kantor (1993) and Gupta *et al*. (1998) in sheep, who stated that CD21 was absent from the marginal zone. Authors of this research support the suggestion of Rickert *et al*. (1995) and Lopes- Carvalho and Kearney (2004) whose revealed that, even if CD19 is important for the development of MZ B cells, the absence of the complement receptor CD21 will participate in high numbers of MZ B cells (Cariappa *et al*., 2001). This explains how these activation signals can stimulate other cells reflecting effective cooperation between the various cells in the marginal zone in their effort to control homeostasis (Kraal and Mebius, 2006). Moreover, inside the red pulp, the present study outlined with moderate positive cytoplasmic reactivity to CD21. ILRAD (1985) clarified that the initial production of an antibody during a humoral immune response did not occur within the follicles, but in the splenic cords before germinal center formation. The explanation of this result refers to that MZ B cells are also found associated with myeloid DCs (M-DCs) as plasma cells in the red pulp. After migrating towards the T cell area of the spleen, MZ B cells can either transport the whole antigen to follicular dendritic cells (FDCs), depositing it on their surface by cleaving the CD21–IC complex (Pozdnyakova *et al*., 2003; Ferguson *et al*., 2004; Whipple *et al*., 2004); or process antigen and present it to CD4+ T cells and NKT cells on CD1d (Sonoda and Stein-Streilein, 2002; Attanavanich and Kearney, 2004; Kobrynski *et al*., 2005).

The previous literature in cattle and sheep, recommended CD21 reactivity to mark follicular dendritic cells (Keresztes *et al*., 1996; Gonzalez *et al*., 2001). Which confirmed the result in this investigation that was obtained in water buffalo spleen using this antibody; some expression in red pulp was represented in arcs like form. We support the postulation of Fang *et al*. (1998), Barrington *et al*. (2002), and Brockman *et al*. (2006) to CD21 function in the retention of antigens in the membrane of follicular dendritic cells. This process is vital as the antigens exposed by follicular dendritic cells should be acquired by B cells during the germinal center reaction (Attanavanich and Kearney, 2004; Ferguson *et al*., 2004; Cinamon *et al*., 2008; Oropallo and Cerutti, 2014).

Regarding the CD79A antibody, in LN, few CD79A expressed on plasma and nuclear membrane. Similar findings recorded by Mason *et al*. (1991) and Niku *et al*. (2006) in bovine animals, Bozkurt *et al*. (2014) in goat, Heinrich *et al*. (2015) in raccoon, Uhde *et al*. (2017) in camel and Huang *et al*. (2018) in a yak. On the contrary, the highest surface reaction of CD79A+ cells demonstrated in MZ and the moderate ones detected in RP of water buffalo spleen. It agrees with Bannish *et al*. (2001); Fuentes-Pananá *et al*. (2004) and Pike *et al*. (2004) who indicated the period during the plasma cell stage, CD79A presented as an intracellular component. The surface position of CD79A provide this antibody a specialty to be selected as one of the true pan-B markers, essentially in all B-cell differentiation stages from the earliest progenitors to fully mature immunoglobulin-secreting cells (Faldyna *et al*., 2007).

Generally, splenic MZ B cells are focused to screen and arrest blood-borne pathogens, that activate innate-like rapid antibody response. After this, they either respond quickly by distinguishing into IgM-producing plasma cells mostly during the early stages of infection (low IgG) or gain the capacity to represent as antigen-presenting cells (APCs). In the latter, they transport the antigen to lymphoid follicle where adaptive immune responses take place (Kruetzmann *et al*., 2003; Lopes-Carvalho and Kearney, 2004; McCulloch *et al*., 2017). Therefore, in the present study, the reactivity of IgM and IgG antibodies was examined in the splenic tissue.

In respect to the obtained results, the response of splenic tissue to IgM revealed with few positive surface's immune expression along with the splenic lymphoid follicles of water buffalo spleen. The existence of IgM in lymphoid follicles enriches the immunogenicity of immune complexes (ICs) by concentrating IC deposition on FDCs, resulting in optimum germinal center formation (Pozdnyakova *et al*., 2003). On the other hand, IgM antibody in MZ and RP elaborated their immunoreactivity along the cellular surface in water buffalo spleen with a moderate reactivity to the first and a highest reactivity to the latter. Comparing with other reports in sheep, IgM+ B lymphocytes are limited to the MZ area in this animal and had completely definite developmental characteristics (Gupta *et al*., 1998). From this study, variance in IgM distribution between animals may return to species-specific reasons. For instance, it can belong to the exact role of this antibody in these animals and especially in its contribution to MZ B cells in the cellular transport of immune complexes (Whipple *et al*., 2004).

In concerning to IgG+ cells of water buffalo spleen in the current study, they highlighted as a positive immune expression in the least distribution inside the lymph nodule with surface expression, while the highest distribution was detected in the marginal zone. On the other hand, the higher one was concentrated in splenic cords of the red pulp. The high

amount IgG in the spleen of ruminant can represent their critical role in defense against ubiquitous microorganisms as well as in the generation of abroad-specificity germ-line antibodies (ILRAD, 1985 and Gonzalez *et al*., 2001). Therefore, any lack in MZ B-cell, IgM, and IgG antibody will raise the susceptibility to encapsulated bacterial infections (Subramaniam *et al*., 2010 and Neven *et al*., 2014).

This study tended the focus also on other immunocompetent cells like a natural killer cell, macrophage, follicular dendritic cells, and fibroblast through CD56, CD68, CD1A, and αSMA antibodies, respectively.

Throughout the current study, CD56 revealed a few positive surface reactions to natural killer cells inside LN. The moderate distribution of these cells was observed as a surface expression in the marginal zone and highest one in the red pulp of water buffalo spleen. Identical to what reported by Moreira *et al*. (2017) in dogs. The evidence we found points to the great value of natural killer cells in splenic tissue which can be listed in their defensive role against injuries, as they represented cytotoxic lymphocytes fighting viral infections and tumors without prior stimulation (Trinchieri, 1989; Luo *et al*., 2000). Besides, their ability to increase the activation state of mononuclear phagocytes which primes T-cells in the acquired response (Van kayalapati *et al*., 2004) and this explain their precious position in inflamed and cancer tissues (Dalbeth *et al*., 2004; Carrega *et al*., 2014). Moreover, previous literature found that NK cells exhibit a characteristic editing function of antigen-presenting cells (APCs) (Moretta, 2002) and can express inhibitory signals, that intended to avoid healthy cells from lysis (Murphy *et al*., 2008; Shivam *et al*., 2015). Depending on the previous contributions of NK cells, we support the explanation that considers CD56 as a signaling receptor and hence they can influence cellular adhesion, migration, proliferation, apoptosis, differentiation, survival, and synaptic plasticity (Amoureux *et al*., 2000; Ronn *et al*., 2000).

Concerning bovine diseases, NK cells respond to a variety of pathogens that trigger economically critical disorders, involving Mycobacterium Bovis (Olsen *et al*., 2005; Denis *et al*., 2007; Siddiqui *et al*., 2012; Siddiqui and Hope, 2013; Maupome, 2019), Neospora (Boysen *et al*., 2006; Maley *et al*., 2006; Klevar *et al*., 2007), Babesia (Goff *et al*., 2006) and Theileria (Connelley *et al*., 2014). This response fits in their distinctive talent to be not just "killers" that lyse infected or transformed cells but also in modulation of immune responses due to the secretion of immunoregulatory cytokines (e.g., IFNγ and TNF-α) and chemokines (e.g., CCL3 and CCL4) (Alvarez *et al*., 2009; Portevin and Young, 2013; Melsen *et al*., 2016). So, NK cells have major roles in the induction or provision of protective immunological memory and represent potential new targets in vaccination strategies (Cerundolo *et al*., 2009; Le Bourhis *et al*., 2010; Sun *et al*., 2011).

The splenic structure is likely fundamental for appropriate localization and function of macrophages cells (Borges da Silva *et al*., 2015). These cells simulate like defender cells which evolve a pro- or anti-inflammatory reaction according to the nature of the interaction as documented in camels (Geijtenbeek *et al*., 2002; Sandikci *et al*., 2005). Focusing on macrophages cells in the present study, the CD68 antibody displayed immune positive surface reaction to these cells around the arteriole of the lymph nodule. This points us to the assorted capability of splenic macrophages in controlling blood-borne infections that outline several aspects of innate and adaptive immune responses (Borges da Silva *et al*., 2015).

Moreover, we found many CD68+ macrophages in the MZ of water buffalo spleen. Marginal zone macrophages (MZM) claimed a vital role in viral protection as they are the source of interferons (Eloranta and Alm, 1999). Furthermore, they exhibit direct bactericidal effector functions (Aichele *et al*., 2003) and collaborate in cytotoxic T-cell activation (Backer *et al*., 2010). Consequently, in the absence of MZ macrophages, trapping of particulate Ag in an MZ is lost and bacteria will scatter into RP with high susceptibility to bacterial and viral infections (Oehen *et al*., 2002). From previous observation, we support their use in therapeutic strategies for the development of anti-tumor immunity.

Similarly, CD68 in the current study determined many macrophages in RP areas of water buffalo spleen. They marked with immunopositivity surface expression. Which is a piece of evidence to support the hypothesis that RP macrophages are required to maintain tissue and iron homeostasis (Fritsche *et al*., 2003; Flo *et al*., 2004; Mebius and Kraal, 2005; Kovtunovych *et al*., 2010), in phagocytosis of blood-borne pathogens, in the uptake of aged or apoptotic RBCs (Kohyama *et al*., 2009), in the induction of T-regs by IL-10 production (Salcedo *et al*., 2001; De-Jesus *et al*., 2008; Kirby *et al*., 2009), and limitation of autoimmunity (IL-10 and TGFβ in a resolution of inflammation) (Kurotaki *et al*., 2011). Moreover, their probability to be substituted by inflammatory phagocytes as well as by macrophages derived from inflammatory monocytes (Borges da Silva *et al*., 2015).

On account of the former functions of macrophages along with splenic tissue, CD68 can be utilized to evaluate certain pathogens like Salmonella typhimurium (Salcedo *et al*., 2001), Cryptococcus neoformans (De-Jesus *et al*., 2008) and Streptococcus pneumonia (Kirby *et al*., 2009). Additionally, CD68 occupies a considerable insight to distinguish diseases of similar appearances, such as the monocyte/macrophage and lymphoid forms of leukemia, malignant histiocytosis, histiocytic lymphoma, Gaucher's disease and stroke (Kassner *et al*., 2009; Toka *et al*., 2019).

In the present work, dendritic cells identified by using the CD1A antibody. Few surface immune expressions of these cells were recognized in PALS of water buffalo spleen. Schneder *et al*. (2011) in bovine animals previously approved that splenic dendritic cells (DCs) were mostly organized as sparsely cells that are located occasionally within the PALS. In addition, surface immune expression of DCs is recognized in MZ and RP of water buffalo spleen. The obtained results reinforce that DCs are specialized for antigen capture and presentation to T lymphocytes which in turn initiate T cell-dependent immune response (Goffa *et al*., 2010; Nguyen *et al*., 2015). Furthermore, they help macrophages in IL12-IL10 against infection with Babesia Bovis (Goff *et al*., 2002; Hope *et al*., 2004).

It is assumed that blood-borne DC resides in MZ for longer times, and activation occurs upon pathogen encounter (Leisewitz *et al*., 2004) or by uptake of apoptotic cells (Morelli *et al*., 2003) that will trigger them to actively migrate into T cell zone of WP to present the processed antigens (Kraal and Mebius, 2006). This study supports the previous suggestions in bovine animals that initial crosstalk between T and NK cells and immature DCs occurs within MZ. In it, immature DCs is first redirected to encounter pathogens as they entered the spleen and become mature when they processed antigen and migrated to T-cell rich areas (Schneder *et al*., 2011).

It is interesting to note that CD1A participated in bee and wasp venom allergy and antitumor immune responses (La Rocca *et al*., 2004; Subramaniam *et al*., 2016). Furthermore, major of the identified CD1A presented antigens exist from the mycobacterial origin. DCS represented special phagocytes as they can ingest and harbor mycobacteria. Bovine species (Bos Taurus) including buffalo is the natural host of several pathogenic mycobacteria. These pathogens participated as a zoonotic threat, and subsequently lead to economic losses worldwide. Consequently, water buffalo represent vital target species for vaccine development, as well as an alternative model to study the role of CD1A in protection against my-

cobacterial diseases (Van Rhijn *et al*., 2006; Nguyen *et al*., 2015).

Owing to immunohistochemical staining of CD138 enlisted as a plasma cell marker (McCulloch *et al*., 2017), research applied to this antibody. The findings differentiated plasma cells in a positive immune reactivity within water buffalo spleen. In LN, they were few with surface reactions, while they observed with high expressions in the marginal zone and red pulp. Various forms of expression may illustrate the several tasks they are contributed. Gattenlohner *et al*. (2009) and McCulloch *et al*. (2017) in mice described that plasma cells are essential effector cells that are implicated in cell adhesion, endocytosis, wound healing, humoral immune response, and immunological memory. Moreover, their key role in cancers, mainly in multiple myeloma and breast cancer.

The present study provided detailed info regarding smooth muscle and myofibroblast cells along with water buffalo spleen via alpha-smooth muscle actin antibody (αSMA). The findings highlighted a huge amount of muscular tissue that upholding the spleen of water buffalo. They are visualized as a thick fibromuscular capsule which gave intense expression to αSMA. The capsule emerged thick muscular trabeculae which identified as primary and secondary trabeculae. In lymph nodule, smooth muscle detected surrounding central arteriole and as discrete line bordering lymph nodule area. In coincidental to the findings of Lokmica *et al*. (2008), positive myofibroblast cells were highlighted between immune cells along the marginal zone area in this study. Pinkus *et al*., (1986) added that staining might show a partial barrier of cells that separated red from the white pulp. Within the red pulp, the myofibroblasts were immunoreactive for smooth muscle actin (SMA) (Pinkus *et al*., 1986; Mills, 2012). The same finding was noticed in the present study of water buffalo spleen and consequently confirm the patterns of localization of smooth muscle myosin; the latter are correlated with anatomic structures and possible tissue functions (Pinkus *et al*., 1986). Authors of this work support the suggestion of Alshamarry (2010) in camel that the thickness of the capsule, the trabeculae, and the concentration of the smooth muscles are rather significant investigators for a powerful contraction once the body requires blood and they can also contribute to the immune response.

Conclusion

Considerable insight has been gained about immunocompetent cells along water buffalo spleen. CD21+, CD79A+, and IgG+ are concentrated highly in the marginal zone, few in the lymph node, and moderate in the red pulp of splenic cells with significant differences in area percentage. Natural killer cells, macrophages, and follicular dendritic cells highlight a significant cytoplasmic difference utilizing CD56+, CD68+, CD1A+, respectively. The buffalo splenic cells mark insignificant difference of area% to CD5+, CD19+, CD20+, and IgM+. Excessive expression of muscle and myofibroblast cells to alpha SMA antibody are recognized. Plasma cells react to the CD138 antibody without difference along buffalo spleen. Such significant result will aid in the basis of receptor distribution through water buffalo spleen and represent potential new targets in diagnosis strategies. Authors of this study recommended that further mixed immunohistochemistry studies are required for differentiation of CD5, 19, 20, 21, 79A, IgM and IgG antibodies with the different splenic cells.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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