Characterization of Cellular Immune System at Different Ages in Water Buffalo (*Bubalus bubalis*)

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Abstract: In recent years, there has been an increased interest in understanding the immune system of the water buffalo due to the increased economic impact of this species. The study aimed to perform an in-depth evaluation of lymphoid and myeloid cells in water buffalo of different ages. We assess three multicolor panels of antibodies to evaluate by flow cytometry the percentage of the $CD3^+$ $CD4^+$, $CD8^+$, and γδ T lymphocytes; $CD79^+$ and $CD21^+$ B lymphocytes; monocytes and cM, intM, and ncMsubsets; NK cells, granulocytes, and peripheral blood mononuclear cell (PBMC). Seventy-eight animals from three different farms were divided into three groups by age (26 in each group): 80-100 days old calves, 16-18 months old heifers, and 4-6 years old cows. Significant differences by Kruskal-Wallis test were found between age groups in the percentage of $CD4^+$, $CD8^+$, γδ T lymphocytes, NK cells (*P*=0.0001), total monocytes (*P*=0.0008), granulocytes (*P*=0.0358) and PBMC (*P*=0.0056). Between the farms, the adult animals showed differences in the percentage of$CD3^+$ (*P*=0.0152), $CD4^+$ (*P*=0.0047), $CD8^+$ (*P*=0.0019), CD4:CD8 ratio (*P*=0.0033) and γδ (*P*=0.0013) T lymphocytes; $CD21^+$ B lymphocytes (*P*=0.0007);total monocytes (*P*=0.0100), cM and ncM subsets (*P*=0.0320;*P*=0.0252), granulocytes (*P*=0.0030) and PBMC (*P*=0.0120). The calves showed significant differences in $CD79^+$ and $CD21^+$ B lymphocytes (*P*=0.0141; *P*=0.0049), total monocytes (*P*=0.0010), cM, intM and ncM subsets (*P*=0.0335; *P*=0.0499; *P*=0.0065). The heifers group in $CD21^+$ B subset (*P*=0.0439).In summary, this study provides the composition of lymphoid and myeloid cells in this species for the first time, highlighting large differences between age groups and between different herds.

Keywords: Water buffalo, age, immune system, flow cytometry.

INTRODUCTION

Domesticated water buffaloes (*Bubalus bubalis*) are important dairy animals, ranking second in milk production worldwide and holding more than half of the European buffalo population [1]. In Italy, buffalo farming constitutes an important livestock resource for producing typical Mozzarella cheese, a fresh soft cheese [2]. The role of the immune system in host defense against invading pathogens has been recognized for many years because it plays an important role in ensuring animal health. Recent studies point to a much wider role of the immune system as part of the overall regulatory network linking physiology, pathophysiology, and behavior, placing it directly at the center of overall animal welfare [3,4]. Furthermore, the immune system can be viewed both as a source of biomarkers for monitoring health and well-being and as a means of elucidating the mechanisms that lead to adaptation failure, abnormal behavior, and poor well-being. In immunological studies, flow cytometry is a powerful laboratory technology used to allow an accurate, fast, and multiparametric cell analysis and can achieve simultaneous measurement of multiple surface and intracellular antigens, allowing the characterization and identification of specific cell subtypes within a heterogeneous population. This technology has also been increasingly applied in veterinary medicine due to the commercial availability of specific antibody reagents and the studies on the cross-reactivity of these antibodies between livestock species [5-8], including water buffalo [9,10]. In the last years, there has been an increased interest in understanding the immune system of water buffalo due to the growing economic impact of this species. Recently, many studies have been carried out on the immune response of buffalo species to viral and bacterial infections [11-13]. However, many aspects of the immune system at different ages remain poorly characterized. In this study, to characterize the cellular immune system of water buffalo at different ages, we assess three multicolor flow cytometry panels for in-depth characterization of lymphoid and myeloid cells.

MATERIALS AND METHODS

Animals and Experimental Design

The study was conducted on free-stabling buffalo livestock production management spread across the

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territory. The activities involved 3 free-housing dairy buffalo farms. The buffaloes were maintained in open yards that allowed 15 m^2 for each animal. A total mixed ration consisting of 50% to 55% forage and 45% to 50% concentrate, containing 0.90 milk forage units/kg of dry matter and 15% raw protein/dry matter, was fed daily in a group pen situation. Seventy-eight animals from three buffalo farms located in the Campania region, Southern Italy, were divided into three groups (26 in each group) by age: 80-100 days old calves; 16-18 months old heifers, and 4-6 years old cows in dry status. Whole blood samples were collected from the jugular vein in Li-Heparin test tubes (Vacuette®, Greiner Bio-One, Cassina de Pecchi, Italy).

Ethical Approval

Ethical review and approval were not required for the animal study because retrospective data were collected as part of the routine samples of buffalo dairy farm activities to monitor the health status of animals. Approval was obtained from the Farm's management to use the data and publish the findings of the analysis. Written informed consent was obtained from the owners for the participation of their animals in this study.

Flow Cytometry Analysis

Three multicolour flow cytometric panels were designed to identify different subsets of leukocytes (Table **1**): Panel 1 was assessed to evaluate the percentage of total T ($CD3^+$), T helper ($CD4^+$), T cytotoxic ($CD8^+$) and γδ lymphocytes; Panel 2 to evaluate the percentage of total B lymphocytes ($CD79^+$) and $CD21^+$ subset; Panel 3 to evaluate the percentage of total monocytes and their subsets, classical (cM), intermediate (intM) and non-classical monocytes (ncM), NK cells, granulocytes, and PBMC (peripheral blood mononuclear cell). For panels 1 and 3, 50 µL of whole blood was incubated for 20 min at 4°C in the dark with saturating concentration of each antibody. Then, incubation time, the erythrocytes were lysed with 1 mL of TRIS-buffered ammonium chloride solution (0.87% w/v, pH 7.3) for 10 minutes at room temperature (RT). After a wash with PBS, the cells were centrifuged at 300 x g for 5 min and suspended in 120 µL PBS until the flow cytometric acquisition. For panel 2, it was necessary to permeabilize the cells using the PerFix-NC Kit (Beckman Coulter, Brea, CA, USA) because the CD79a marker is localized inside the cell, and the cell labeling was conducted as previously described by Petrini *et al*. [14]. All samples were immediately collected on CytoFLEX flow cytometer (Beckman Coulter, USA), and the data were analyzed using Kaluza software v. 2.1 (Beckman Coulter, USA). A matrix of compensation was created for each panel of antibodies using the VersaComp antibody Capture beads kit (Beckman Coulter, USA) to correct the emission spectra overlap of the fluorochrome, removing the signal of any given fluorochrome from all detectors except the one devoted to measuring that dye. For each panel of antibodies, we applied a specific gating strategy to identify the subsets of lymphoid and myeloid cells (Figure **1**).

Statistical Analysis

All parameters were summarized by median and range (min, max). The Shapiro-Wilk test was used to test the normality of the parameter's distribution. The differences between age groups (adults, heifers, and calves) for all parameters were tested by ANOVA or Kruskall-Wallis when appropriate. For multiple comparisons of the couples' age groups, the Mann-Witney test with Bonferroni correction was used. All statistical analyses were performed by STATA Statistical Software, version 16.1 (Stata Corporation, College Station, Texas, USA).

RESULTS AND DISCUSSION

To characterize the cellular immune system (lymphoid and myeloid cells) in buffaloes at different ages, we enrolled buffaloes from three animal farms and grouped them into three age groups. On each collected blood sample, we assessed three multicolor panels of antibodies to evaluate by flow cytometry the percentage of the $CD3^+$, $CD4^+$, $CD8^+$, and γδ T lymphocytes; $CD79^+$ and $CD21^+$ B lymphocytes; monocytes and their subsets (cM, intM, ncM), NK cells, granulocytes, and peripheral blood mononuclear cell (PBMC).

We found differences in the lymphoid and myeloid cells of buffaloes at different ages. Table **2** shows the median value and range of the leukocyte subset son the total number of animals in each age group. We found significant differences between the three age groups in the percentage of $CD4^+$, $CD8^+$, γδ, and NK cells (P=0.0001), total monocytes (P=0.0008), granulocytes (P=0.0358) and PBMC (P=0.0056) (Table **2**).

Moreover, the pairwise differences of age groups were evaluated, and the differences are presented in

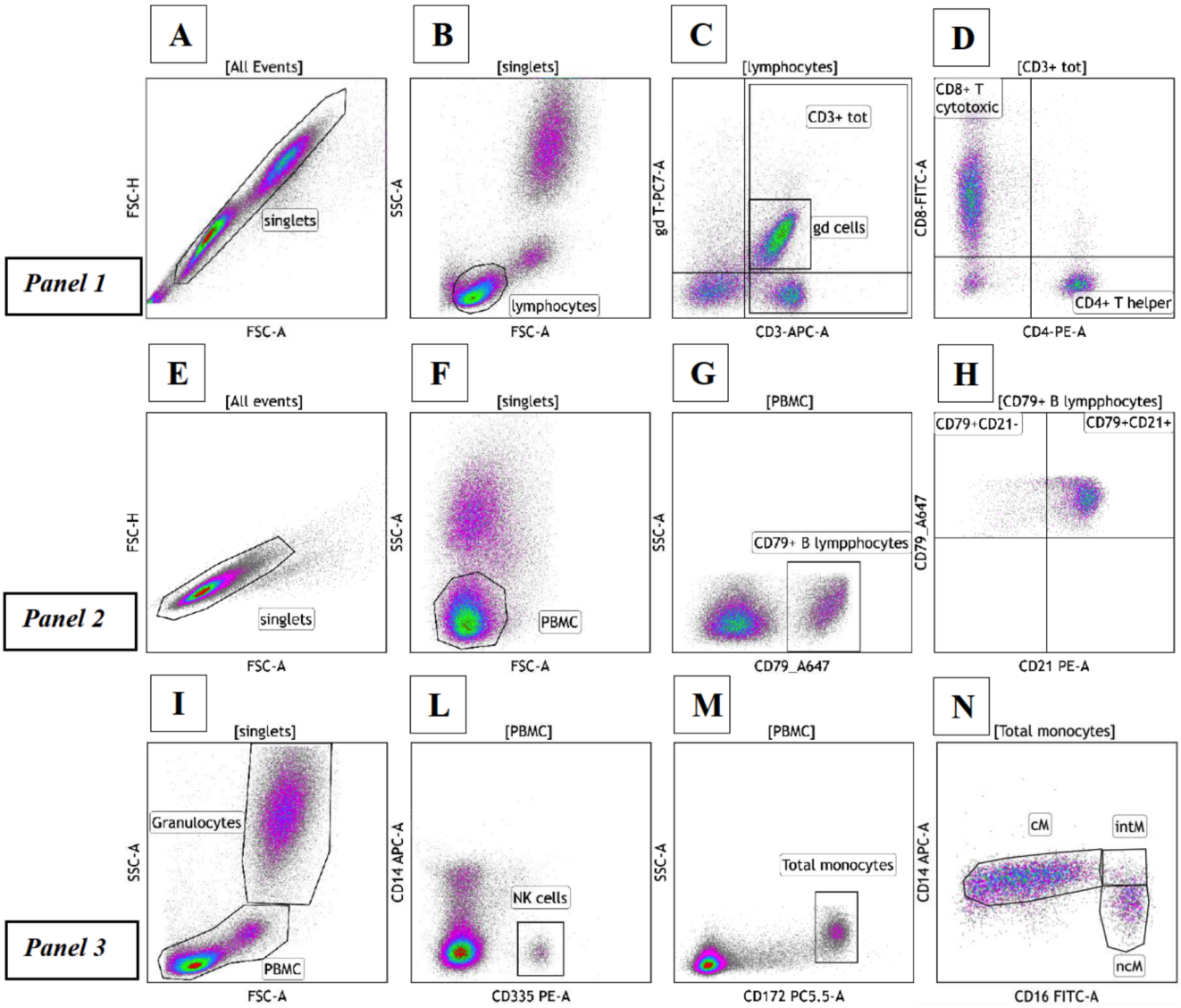

Figure 1: Gating strategy to identify T lymphocytes using *Panel 1*, B lymphocytes using *Panel 2*, and total monocytes, classical (cM), intermediate (intM) and non-classical (ncM), NK cells, granulocytes, and PBMC using *Panel 3*.

Gating strategy used in this study: **(A, E)** A dot plot FSC-A vs. FSC-H on All events was used to exclude doublets, a morphological gate was drawn to highlight single cells (singlets); **(B,F,I)** a dot plot FSC-A vs. SSC-Aon singlets was used to identify leukocyte populations: in ***Panel 1*** a morphological gate was drawn to highlight lymphocytes **(B)**, in ***Panel 2***a morphological gate was drawn to highlight PBMC **(F)**,and in ***Panel 3*** two morphological gates were drawn to highlight granulocytes and PBMC **(I)**; **(C)** a dot plot CD3APC-A vs. γδ TCRPC7-A on lymphocytes was used to identify total and γδ T lymphocytes; **(D)** a dot plot CD4PE-A vs. CD8FITC-Aon $CD3^+$ tot was used to identify the CD4+ helper and CD8+ cytotoxic subsets; **(G)** a dot plot CD79-A647 vs. SSC on PBMC was used to identify $CD79^+$ total B lymphocytes and **(H)** CD21 PE-A vs. CD79 A647-A on $CD79^+$ B lymphocytes to identify the $CD79^+CD21^+$ subsets of B lymphocytes; **(L)** a dot plot CD335 PE-A vs. CD14 APC-A on PBMC was used to identify the NK cell as $CD14^-$ $CD335^+$; **(M)** a dot plot CD172 PC5-A vs. SSC on PBMC was used to identify all monocytes and **(N)** finally the dot plot and CD16 FITC-A vs. CD14 APC-Awas used to caracterize the three subsets of monocytes (cM, intM and ncM).

Table **3**. Significant differences were found between adults and heifers in the median percentage of T helper ($CD4^+$) (55.6 *vs*. 33.3), T cytotoxic ($CD8^+$) (33.1 *vs*. 54.3), CD4:CD8 (1.7 *vs*. 0.6) γδ T lymphocytes (13.3 *vs*. 32.7), total monocytes (6.8 *vs*. 4.3), (P<0.0001), NK cells (5.4 *vs*. 3.5; P=0.0109), granulocytes (19.5 *vs*. 13.2; P=0.0158) and PBMC (76.3 *vs*. 84.6; P=0.0015), between adults and calves in the median percentage of T helper (55.6 *vs*. 27.9), T cytotoxic (33.1 *vs*. 56.1), CD4:CD8 ratio (1.7 *vs*. 0.5), γδ T lymphocytes (13.3 *vs*. 40.4), (P<0.0001), total monocytes (6.8 *vs*. 4.5; P=0.0123) and NK cells (5.4 *vs*. 7.0; P=0.0112) and between heifers and calves in the median percentage of γδ lymphocytes (32.7 *vs*. 40.4; P=0.0002) and NK cells (3.5 *vs*. 7.0; P<0.0001) (Table **3**).

Table 1: Details of Monoclonal Antibodies Used for Flow Cytometry Assay

Panel	Antigen	Antibody Clone	Source	Labeling
Panel 1	CD3	MM1A	WSU MAC[1]	APC (LYNX; Bio-Rad Laboratories)[2]
	CD4	ILA11A	WSU MAC[1]	PE (Zenon™; Invitrogen)[3]
	CD8	CC63	Bio-Rad Laboratories	FITC
	γδ TCR	GB21A	WSU MAC[1]	PE-Cy7(LYNX; Bio-Rad Laboratories)[2]
Panel 2	CD21	LT21	ThermoFisher Scientific	PE
	CD79a	HM47	ThermoFisher Scientific	Alexa fluor 647
Panel 3	CD172a	CC149	Bio-Rad Laboratories	PE-Cy5
	CD14	MM61A	WSU MAC[1]	APC (LYNX; Bio-Rad Laboratories)[2]
	CD16	KD1	Bio-Rad Laboratories	FITC
	CD335	AKS1	Bio-Rad Laboratories	PE

[1]Purchased from Washington State University Monoclonal Antibody Centre, Pullman, WA-USA.
[2]Clone MM1A and MM61A were available only as purified mAb. We used a direct labeling method, and these clones were labeled with LYNX Rapid APC (Allophycocyanin) and LYNX Rapid PE-Cy7 Antibody Conjugation Kits (Bio-Rad Laboratories).
[3]Clone ILA11A was available only as purified mAb. We used a direct labeling method, and this clone was labeled with R-PE (R-Phycoerythrin) Zenon™ Mouse IgG_{2a} Labeling Kit (Thermo Fisher Scientific Inc.).

Table 2: The Median Values and Range (Min, Max) of the Lymphoid and Myeloid Population in Each Age Group (Adults, Heifers, Calves)

	Adults (N=26) Median (range)	Heifers (N=26) Median (range)	Calves (N=26) Median (range)	P-value
$CD3^+$	74.8 (60.9-80.8)	70.7 (60.7-80.6)	74.9 (54.8-83.9)	0.1148
$CD4^+$	55.6 (35.0-65.3)	33.3 (19.8-44.5)	27.9 (18.4-44.3)	0.0001
$CD8^+$	33.1 (18.6-54.6)	54.3 (40.7-71.0)	56.1 (42.5-69.7)	0.0001
CD4:CD8	1.7 (0.7-3.5)	0.6 (0.3-1.2)	0.5 (0.3-1.0)	0.1017
γδ	13.3 (5.7-28.0)	32.7 (20.6-50.3)	40.4 (30.2-55.2)	0.0001
$CD79^+$	15.4 (7.8-29.8)	15.2 (9.2-33.4)	17.1 (8.3-36.3)	0.5581
$CD21^+$	89.1 (68.5-93.6)	84.2 (73.0-94.1)	84.5 (63.7-91.1)	0.2200
Total monocytes	6.8 (3.3-17.1)	4.3 (2.5-11.4)	4.5 (1.4-11.2)	0.0008
cM	68.7 (44.8-81.4)	69.0 (44.1-80.9)	66.1 (38.1-80.9)	0.9248
intM	3.7 (0.7-7.8)	3.0 (0.2-8.0)	3.5 (0.9-7.4)	0.3399
ncM	16.0 (2.5-38.8)	14.5 (7.1-35.6)	13.2 (4.0-28.7)	0.2526
NK	5.4 (1.6-10.6)	3.5 (2.2-5.9)	7.0 (2.5-20.5)	0.0001
Granulocytes	19.5 (7.1-47.5)	13.2 (7.4-45.8)	16.5 1.6-37.8)	0.0358
PBMC	76.3 (39.3-90.6)	84.6 (53.4-90.0)	81.3 (53.2-93.4)	0.0056

In bold, the significant values.

Table 3: The Pairwise Differences of Age Groups in each Leukocyte Subset were Evaluated

Groups	CD3+	CD4+	CD8+	CD4:CD8	γδ	CD79+	CD21+	Total Monocytes	cM	intM	ncM	NK	Granulocytes	PBMC
Adults *vs.* Heifers	0.2204	<0.0001	<0.0001	<0.0001	<0.0001	0.5829	0.1940	<0.0001	0.7437	0.1841	0.8739	0.0109	0.0158	0.0015
Adults *vs.* Calves	0.4050	<0.0001	<0.0001	<0.0001	<0.0001	0.2817	0.0922	0.0123	0.9693	0.5735	0.1467	0.0112	0.1623	0.0292
Heifers *vs.* Calves	0.0402	0.0631	0.4403	0.1468	0.0002	0.6200	0.9885	0.4686	0.7437	0.2962	0.1675	<0.0001	0.4458	0.3051

**P-value*<0.0167 were considered significant by Bonferroni correction. In bold, the significant values.

Table 4: The Median Values and Range of Lymphoid and Myeloid Populations in each Age Group (Adults, Heifers, Calves) and Each Farm

	Adults				Heifers				Calves			
Parameter	**Farm 1**	**Farm 2**	**Farm 3**	***P*-value**	**Farm 1**	**Farm 2**	**Farm 3**	***P*-value**	**Farm 1**	**Farm 2**	**Farm 3**	***P*-value**
CD3+	76.9 (68.8-80.8)	69.4 (60.9-75.9)	71.9 (62.7-80.3)	0.0152	72.4 (67.6-80.0)	72.9 (60.7-80.6)	66.6 (60.7-77.3)	0.3774	76.0 (68.3-80.8)	71.4 (54.8-83.9)	77.3 (65.4-83.4)	0.1061
CD4+	47.3 (35.0-56.9)	55.1 (44.9-62.8)	57.7 (55.4-65.3)	0.0047	34.7 (19.8-44.5)	37.3 (25.7-43.2)	29.4 (21.3-35.0)	0.1139	30.6 (21.2-44.3)	27.1 (18.7-38.4)	29.2 (18.4-41.7)	0.8190
CD8+	40.4 (30.4-54.6)	32.0 (19.0-43.8)	27.8 (18.6-32.4)	0.0019	53.4 (40.7-71.0)	50.5 (44.7-66.2)	58.4 (42.7-66.0)	0.2405	56.1 (43.7-69.3)	55.8 (47.1-69.7)	55.1 (42.5-66.4)	0.9794
CD4:CD8	1.4 (0.7-1.9)	1.7 (1.0-3.3)	2.0 (1.7-3.5)	0.0033	0.7 (0.3-1.2)	0.7 (0.4-1.0)	0.5 (0.3-0.8)	0.2909	0.5 (0.3-1.0)	0.5 (0.3-0.8)	0.5 (0.3-1.0)	0.8941
γδ	17.4 (13.3-28.0)	10.0 (5.7-16.8)	7.9 (6.7-14.9)	0.0013	33.2 (20.6-50.3)	29.8 (22.8-39.5)	37.7 (24.4-42.7)	0.0976	40.4 (33.7-55.2)	39.1 (30.2-48.9)	42.8 (32.0-50.0)	0.7358
CD79+	13.4 (7.8-19.9)	18.8 (8.9-29.8)	15.6 (11.1-22.0)	0.0797	14.4 (12.0-21.6)	18.2 (13.1-29.6)	16.8 (9.2-33.4)	0.3779	13.0 (8.3-17.9)	16.3 (13.3-36.3)	19.7 (17.0-29.5)	0.0141
CD21+	79.7 (68.5-89.7)	87.5 (78.9-93.3)	92.1 (89.1-93.6)	0.0007	83.5 (73.4-89.8)	87.5 (81.7-93.6)	78.1 (73.0-94.1)	0.0439	87.1 (68.1-91.1)	76.5 (63.7-84.7)	87.9 (81.2-90.7)	0.0049
Total Monocytes	6.3 (3.9-10.0)	5.5 (3.3-11.6)	9.3 (6.6-17.1)	0.0100	3.9 (2.5-11.4)	4.8 (3.1-10.7)	4.1 (3.0-6.1)	0.6521	3.2 (1.4-4.4)	5.3 (3.6-11.2)	7.1 (4.5-10.6)	0.0010
cM	63.2 (49.5-75.5)	61.1 (44.8-81.4)	76.3 (64.5-80.5)	0.0320	65.0 (54.5-80.9)	68.3 (44.1-77.1)	72.5 (59.4-80.6)	0.2731	64.2 (38.1-70.9)	68.6 (62.5-72.0)	72.9 (63.6-80.9)	0.0335
intM	4.0 (2.5-5.8)	6.2 (1.3-7.8)	2.6 (0.7-5.4)	0.1413	2.6 (1.9-8.0)	4.2 (1.6-6.1)	2.0 0.2-3.9)	0.0584	4.0 (1.5-5.1)	4.6 (2.9-7.4)	2.4 (0.9-5.0)	0.0499
ncM	17.4 (12.5-38.8)	23.2 (4.9-34.2)	10.2 (2.5-17.2)	0.0252	12.3 (7.1-30.8)	20.7 (11.2-35.6)	14.5 7.3-28.3)	0.1491	16.1 (11.9-28.7)	14.1 (10.7-23.1)	9.2 (4.0-15.0)	0.0065
NK	5.5 (2.8-10.6)	5.5 (2.5-8.0)	5.1 (1.6-10.5)	0.8201	3.5 (2.3-5.5)	4.0 (2.3-5.9)	3.3 2.2-5.3)	0.6534	9.9 (3.6-12.3)	8.0 (2.5-20.5)	6.4 4.8-9.8)	0.2892
Granulocytes	14.1 (7.1-21.6)	19.9 (10.6-47.5)	27.0 (15.6-35.4)	0.0030	17.9 (7.4-45.8)	12.7 (8.4-37.8)	11.4 (8.1-23.7)	0.2716	12.8 (6.1-25.1)	12.2 (5.3-37.8)	21.6 (1.6-30.8)	0.2612
PBMC	82.8 (39.3-90.6)	76.0 (51.3-86.5)	64.8 (54.1-79.7)	0.0120	81.0 (53.4-90.0)	85.2 (61.3-89.1)	85.8 (73.7-88.6)	0.4469	85.3 (74.2-93.4)	85.4 (53.2-92.1)	76.2 (68.0-83.2)	0.0651

In bold, the significant values.

Furthermore, we found several differences in the lymphoid and myeloid populations of buffaloes at different ages between the three farms (Table **4**). Significant differences in the Kruskal-Wallis test were found between the three farms. The adult group showed differences in the median value of $CD3^+$ (*P*=0.0152), $CD4^+$ (*P*=0.0047), $CD8^+$ (*P*=0.0019), CD4:CD8 ratio (*P*=0.0033) and γδ T lymphocytes (*P*=0.0013); $CD21^+$ B lymphocytes (*P*=0.0007), granulocytes (*P*=0.0030), PBMC (*P*=0.0120), total monocytes (*P*=0.0100), cM and ncM subsets (*P*=0.0320; *P*=0.0252). The calves group showed differences in $CD79^+$and $CD21^+$B lymphocytes (*P*=0.0141 and *P*=0.0049 respectively), total monocytes (*P*=0.0010), and cM, intM ncM subsets (*P*=0.0335, *P*=0.0499, and *P*=0.0065, respectively).

The heifers group showed differences only in the $CD21^+$ B lymphocytes subset (P=0.0439) (Table **4**).

Research on the immune system of water buffalo has recently gained particular interest, leading to the characterization of key elements of the immune cells. However, many aspects of the immune system at different ages remain poorly characterized. Although two previous studies reported differences in the percentage or absolute concentration of some leukocyte subsets at different ages [9,15], a comparison with our data is difficult due to the different age group criteria and because the leukocyte subpopulations studied are different. However, the higher percentage of the γδ cells in young buffaloes (calves and heifers groups) compared to old buffaloes (adults group) was confirmed and consistent with observations in cattle [16]. Furthermore, in our study, the different farms of origin of the animals were taken into consideration. Our results highlight that the percentages of leukocyte subsets in the different age classes of animals differ between animal farms. This result could also be useful for evaluating management in future studies.

CONCLUSIONS

In conclusion, this study provides an in-depth phenotyping of the various leukocyte subsets for the first time, highlighting differences in the percentage of $CD79^+$ and $CD21^+$ B lymphocytes, NK cells, and monocyte cells between calves, heifers, and adults. These results, although preliminary, show that the age of the animals and the farm can influence the cellular immune system. Although further investigations are needed, the evaluation of the cellular immune response at different ages could be a useful approach to monitoring the health and welfare status of water buffaloes, and this approach could also be extended to other species of farm animals.

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