Flow cytometry expression pattern of CD44 and CD18 markers on feline leukocytes

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Running head: Panleukocyte markers expression on feline WBC

Abstract. A paucity of specific feline antibodies for flow cytometry (FC) is an ongoing challenge. Flow cytometrists must extrapolate from information from relatively few markers. We evaluated the expression pattern of the panleukocytes markers CD18 and CD44 on leukocyte (white blood cell, WBC) subclasses in the peripheral blood (PB) of 14 healthy cats. The degree of expression of CD18 and CD44 was calculated as the ratio between the median fluorescence intensity (MFI) value of antibody-stained cells and autofluorescence. All samples were acquired with the same cytometer with constant laser setting and compensation matrices. Both molecules were expressed at higher levels on monocytes, intermediate levels on neutrophils (PMNs), and lower levels on lymphocytes. CD18-MFI discriminated well among the 3 populations, whereas CD44-MFI mostly overlapped between monocytes and PMNs. However, CD44-MFI had a lower intra-population variability. Evaluation of CD18 and CD44, together with morphologic parameters, was useful for discriminating among WBC subclasses in healthy cats. This information may be helpful for future studies given that an increase in CD18-MFI may indicate reactive changes, whereas fluctuations in CD44-MFI may suggest neoplasia.

Key words: cat; lymphocyte; median fluorescence intensity; monocyte; neutrophil; pan-leukocyte.

Flow cytometry (FC) in feline veterinary medicine is decades behind human and canine medicine, though our research group has demonstrated that most feline samples are suitable for FC analysis.[9] The diagnostic accuracy of FC in discriminating between neoplastic and non-neoplastic lymphoproliferative disorders has been demonstrated in cats.[8] However feline FC still suffers from a paucity of commercial antibodies that cross-react in this species. Thus, flow cytometrists attempt to gain as much information as possible by analyzing only a few molecules. The degree of expression of panleukocyte markers helps in identifying the white blood cell (WBC) subpopulations in the peripheral blood (PB) from healthy dogs, and to characterize hematologic neoplasms in dogs.[4,5,7]

We describe herein the pattern of expression of 2 different pan-leukocyte markers, CD18 and CD44, on WBC subpopulations in the PB of healthy cats, to provide basic data useful for future studies on diseased animals. We selected these 2 antigens for our study because of their potential diagnostic utility.

CD18 is a component of β2 integrins, which are adhesion molecules involved in leukocyte extravasation. CD18 is expressed on the cell surface of all WBC subclasses, with variable levels of expression according to cellular activation and differentiation status, and is primarily involved in leukocytes rolling on the endothelium and their subsequent diapedesis.[12] Accordingly, analyses of the degree of expression may prove useful in reactive conditions.

CD44 is a hyaluronan receptor that is expressed ubiquitously on the cell surface and is involved in many processes requiring interaction with the extracellular matrix. This molecule is considered a cancer stem cell marker and has been studied extensively because of its role in tumorigenesis and development of metastasis.[11] Thus, analyzing its degree of expression may prove useful in neoplastic conditions.

We analyzed peripheral blood (PB) samples collected into EDTA tubes from 14 healthy cats. All samples were delivered to the laboratory and processed within 2 h of

sampling. All cats were privately owned and sampled as part of routine health examinations at the Veterinary Teaching Hospital of the University of Milan. As per policy at this institution, specific approval from the Ethical Committee is not required for research use of leftover diagnostic specimens (EC decision 29 October 2012, renewed with protocol 02-2016).

A CBC was performed with an automated hematology analyzer (XT 2000-iV; Sysmex) and sample processing for FC was performed according to protocols published previously.[9] One tube served as negative control (unstained cells); 3 other tubes were investigated by means of the following antibody (Ab) cocktails: CD5/CD21/CD18, CD4/CD8/CD18, and CD44/CD18. Propidium iodide (PI) (Genetex) was also included in the third tube, to assess cell viability. Ab clones and fluorochromes (Table 1) have already been documented to stain feline samples by FC.[3,9,11] All antibodies, as well as PI, had been titered before use to determine the best working dilutions. All samples were evaluated immediately after staining by means of a flow cytometer (BriCyte E6; Mindray) equipped with 2 lasers and detectors for up to 4 fluorescence channels. The cytometer status was checked and, if needed, calibrated at the beginning of each laboratory session by means of specific controls (SPHERO Supra Rainbow Fluorescent Particles Mid-Range; Spherotech). Laser voltages and compensation matrices were kept constant during the whole experiment. For each tube, 10 x 10^3 nucleated cells were evaluated.

Analyses were performed by means of specific software (MRFlow; Mindray) by a single operator (V Martini) in a single session. The percentage of PI-positive (necrotic) cells was regarded as an index of sample viability. Outlier samples with an excessively high percentage of PI-positive cells were excluded from the study. The Dixon method was used to make this determination.[6]

For each sample, the median fluorescence intensity (MFI) of unstained cells (FL-1 and FL-4 channels), CD18, and CD44 was recorded separately for polymorphonuclear cells

(PMNs), monocytes, and lymphocytes, via a back gating strategy based on cellular morphologic properties (Forward Scatter – Height (FSC-H) versus Side Scatter – Height (SSC-H)). PMNs were considered as a whole, without sub-grouping into neutrophils, eosinophils, and basophils, because morphologic or phenotypic properties were not available to distinguish the 3 subpopulations by FC. CD18-MFI and CD44-MFI were calculated for each population by dividing the MFI value of Ab-stained cells for the MFI value of unstained cells, in the corresponding fluorescence channel.[1,7] Statistical analyses performed included a Shapiro-Wilk test to assess normal distribution of data, a Friedman test to assess possible differences among WBC subclasses in CD18- and CD44-MFI, and a Wilcoxon signed-rank test for post-hoc analyses. Significance was set at $p \leq 0.05$.

Both CD18- and CD44-MFI significantly varied among WBC subclasses ($p<0.001$ for both analyses; Table 2). Monocytes had the highest level of expression of both CD18 and CD44; lymphocytes showed the lowest for both antigens. CD18-MFI was 5-fold higher in monocytes than in PMNs (mean CD18-MFI ratio between monocytes and PMNs = 5.6 ± 3.7; $p = 0.001$), 8-fold higher in PMNs than in lymphocytes (mean CD18-MFI ratio between PMNs and lymphocytes = 8.4 ± 7.7; $p = 0.001$) and 34-fold higher in monocytes than in lymphocytes (mean CD18-MFI ratio between monocytes and lymphocytes = 34.1 ± 30.5; $p = 0.001$). CD44-MFI did not differ between monocytes and PMN (mean CD44-MFI ratio = 1.1 ± 0.3; $p = 0.196$) and was 2-fold higher in PMNs and in monocytes than in lymphocytes (mean CD44-MFI ratio = 2.7 ± 1.2 and 2.9 ± 0.9, respectively; $p = 0.001$ for both analyses).

Although both markers stained all leukocytes, analysis of the level of expression in the different cell populations allowed differentiation among the leukocyte groups. Both proteins are expressed at higher levels on monocytes than on PMNs and lymphocytes. However, CD18-MFI allows better discrimination than CD44-MFI among the 3 subclasses, as documented by the higher ratios obtained when coupling CD18-MFI on monocytes with

either CD18-MFI on PMNs or lymphocytes. Despite the higher mean and median CD44-MFI shown by monocytes compared with PMNs, differences were not significant, thus complicating the discrimination between these 2 classes based on fluorescence level. As a result, lymphocytes are easily identified in a dot plot coupling the intensity of fluorescence of CD18 and CD44 as a discrete population with low intensity of fluorescence of both antigens. Conversely, monocytes are located at the edge of a smear with homogeneous CD44-MFI, without a clear separation from the PMN cloud (Fig. 1). Monocytes and PMNs are more easily discriminated by coupling CD18-MFI with a complexity index (SSC-H); this type of scattergram seems to be the most appropriate to distinguish among WBC subclasses in cats. Unfortunately, CD18-MFI suffers from a great variability within each WBC subclass, as documented by our results; monocytes had the lowest CV for CD18-MFI (> 50%), and a peak of >100% was reached for lymphocytes, whereas the CV for CD44-MFI was consistently < 40%.

Unfortunately, we were not able to assess possible differences in pan-leukocyte marker expression among different lymphoid subclasses, because of conflicting combinations of antibodies and fluorochromes (CD44 was –FITC labelled, as well as CD5 and CD4, thus preventing concomitant assessment of these molecules) and because of the low number of cells within each lymphoid subclass. Interestingly, CD expression on lymphocytes was normally distributed, with a single peak on histogram (Suppl. Fig. 1); different levels of expression between B and T -cells is therefore unlikely because 2 different peaks would have resulted.

Limitations of our study are the small number of samples analyzed and the lack of isotypic controls. Most studies have relied on isotypic controls to set fluorescence background; however, this approach has recently been demonstrated to be misleading.[2]

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Declaration of conflicting interests

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168 **Table 1:** Antibodies used for flow cytometric analysis of peripheral blood samples from 14

169 healthy cats

Antibody	Conjugation	Clone	Source	Specificity
CD5	FITC	f43	SouthernBiotech	T cells
CD4	FITC	3-4F4	SouthernBiotech	T-helper cells
CD8	PE	fCD8	SouthernBiotech	T-cytotoxic cells
CD21	PE	CA2.1D6	Bio-Rad	B cells
CD18	AlexaFluor647	CA1.4E9	Bio-Rad	All leukocytes
CD44	FITC	IM7	BD Pharmingen	All leukocytes

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172 **Table 2:** Expression of CD18 and CD44 antigens on the cell surface of circulating leukocyte subclasses from 14 healthy cats, as determined by
173 fluorescence analyses on flow cytometry. CD18-MFI and CD44-MFI were calculated for each population by dividing the MFI value of Ab-
174 stained cells for the MFI value of unstained cells, in the corresponding fluorescence channel.

		Median fluorescence intensity (MFI)									
		FL-1 channel					FL-4 channel				
		Mean	Standard deviation	Median	Range	CV	Mean	Standard deviation	Median	Range	CV
Unstained cells	PMNs*	582	119	555	438-845	20	11	4	12	5-16	34
	Monocytes	528	114	471	426-710	22	8	3	8	5-14	37
	Lymphocytes	314	70	322	202-442	22	6.8	4	7	2-16	53
		CD44-MFI					CD18-MFI				

		Mean	Standard deviation	Median	Range	CV	Mean	Standard deviation	Median	Range	CV
Stained cells	PMN*	312	68	319	185-420	22	524	362	424	133-1050	69
	Monocytes	361	104	375	195-483	29	2030	1150	1830	1060-5610	56
	Lymphocytes	132	50	125	35-243	38	107	125	77	12-183	117

175 PMNs = polymorphonuclear cells, including neutrophils, eosinophils, and basophils.

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Figure 1. Flow cytometric analysis of a peripheral blood sample from a healthy cat. A gate (P1) was set to exclude platelets and debris in a morphologic scattergram ((Forward Scatter – Height (FSC-H) versus Side Scatter – Height (SSC-H), Suppl. Fig. 1)). **A.** Only P1 cells are shown; 3 gates were set to include lymphocytes (P2, green dots), monocytes (P3, blue dots) and polymorphonuclear cells (PMNs, including neutrophils, eosinophils and basophils; P4, violet dots). **B,C,D.** Scattergrams showing CD18 and CD44 expression in the 3 WBC subclasses; color code is the same as Fig. 1B. **B.** Fluorescence level of CD44 (FITC-A) versus CD18 (APC-A). **C.** Fluorescence level of CD18 (APC-A) versus cellular complexity (SSC-H). **D.** Fluorescence level of CD44 (FITC-A) versus cellular complexity (SSC-H).

Supplementary Figure 1. Flow cytometric analysis of a peripheral blood sample from a healthy cat. **A.** A gate (P1) was set to exclude platelets and debris in a morphologic scattergram (Forward Scatter – Height (FSC-H) versus Side Scatter – Height (SSC-H)). **B.** Only lymphocytes are shown, based on CD18 expression (red line), overlay with unstained cells (green line). **C.** Only lymphocytes are shown, based on CD44 expression (red line), overlay with unstained cells (green line).