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Immunophenotyping pattern characterization in canine blood: towards a clinical application

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Abstract

Immunophenotyping is a widely used method for a precise diagnosis and classification of haematopoietic neoplasia in human beings and also in dogs. The gold standard for cell preparation is density gradient centrifugation of mononuclear cells. Alternatively, another way to separate human leukocytes is carrying out whole blood lysis. The aim of this study was to validate whole blood lysis as an alternative method in clinical veterinary procedures using an immunophenotype panel of leukocytes designed by our group. Flow cytometry study of adult canine leukocytes subset groups, using whole blood lysis or mononuclear cells tested against an array of canine leukocyte antibodies were done. Besides differential white blood cell counts were done. Also immunophenotyping studies in whole blood samples stored at 4 °C for 48 h were performed. The Coefficient Variation values were less than 20%, for most of the comparison. Consistent results were observed in phenotyping canine peripheral blood leukocytes. Stability results indicated that whole blood samples might be stored for 48 h without a significant difference in the data compared to samples processed immediately after blood collection. This study shows that whole blood lysis represents an efficient and quick alternative for canine leukocyte preparation. In addition, samples can be analysed immediately or stored for 48 h without a significant difference between them. This is relevant for veterinary medicine considering the lack of facilities in many laboratories to process samples.

Flow cytometry, peripheral blood, leukocyte subsets, long-term stability

Immunophenotyping is a widely used method, complementing morphology, cytochemistry, clonal assays and karyotype study to make more precise diagnosis and classification of haematopoietic neoplasia in animals as well as humans. More precisely, dogs are relevant for immunological investigation since spontaneous immune-mediated and autoimmune diseases occur at high prevalence in canine population (Kolb et al. 1997).

Flow cytometry is a powerful tool used for diagnostic purposes. Evaluation of surface and intracellular antigens with a panel of antibodies allows identification of specific cell lineages as well as the maturation stage in malignant diseases (Weiss 2002). However, in contrast to humans, the small number of commercial mAbs available for dogs makes the standardization studies for specific diseases more difficult. Good Laboratory Practices recommend validation of sample preparation by flow cytometry (Zeigler et al. 2013). Peripheral blood, bone marrow, or tissue specimens should be processed to contain a cell suspension, eliminating erythrocytes, to give an optimal cell concentration for monoclonal staining (Owens et al. 2000).

The gold standard for mononuclear cell preparation is density gradient centrifugation (Van Dogen et al. 2003). Alternatively, another way to separate human leukocytes is

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carrying out whole blood lysis (Schwonzen et al. 2007). This method is more appealing because it is less time consuming, cheaper and requires less sample volume. The percentages of canine lymphocyte subsets obtained by these two methods did not show significant differences (Faldyna et al. 2001) and previous observation did not find significant differences between the two methods (De Paoli et al. 1984; Mansour et al. 1990).

Canine Leukocyte Antigen Workshop (CLAW), examined lymphocyte populations in healthy dogs. Many similarities between the dog and other animals have been demonstrated, establishing canine antibody equivalents of CD3, CD5, CD4, CD8 used to label lymphocyte T-cell subsets from peripheral blood (McSweeney et al. 1998; Hardy et al. 2013). Furthermore, in healthy beagle dogs over 8 years old, where tumour incidence becomes higher, the immune status of CD3, CD4, CD8 and CD21 could predict future morbidity and mortality (Watabe et al. 2012). By turn, the discovery of CD34 as an immature haematopoietic cell surface antigen and the production of anti CD34-monoclonal antibodies provided an important tool to study haematologic malignancies (Cobbold et al. 1994; McSweeney et al. 1998; Ostronoff et al. 2008; Williams 2008). In addition, CD4, CD5, CD8, CD21 and CD34 have been utilized as markers to study canine lymphoproliferative prognosis (Krause et al. 1996).

The goal of this study was to establish an immunophenotype panel of leukocytes (CD3, CD4, CD8, Dog10(CD8), Dog17(Pan T), CD21, CD34) from canine mononuclear cells (MWBC) and total white blood cells (TWBC) of clinically healthy dogs aged between 6–8 years. The verification of both methods will be beneficial for the analysis in haematological diseases in animals. A secondary aim was to validate Dog 17 (panT) and Dog 10 (CD8) as canine antigens. Future studies will include these specific markers, which will ultimately be used for diagnosis and prognosis of canine leukaemia or malignant lymphoma.

Materials and Methods

Animals

10 healthy Beagle dogs (5 males and 5 females) within the age range of 6–8 years were selected for this study. All dogs were clinically healthy and presented no abnormalities in haemogram and serum chemistry. All serology were negative against Ehrlichia and Leishmania spp. This study was approved by the Institutional Animal Utilization Committee for Investigation of the Complutense University of Madrid, and was conducted in accordance with the Spanish legislation for the care of experimental animals (RD 53/2013). All animals were housed and cared for at the animal research facilities of the Veterinary Teaching Hospital of the Complutense University of Madrid.

Collection and processing of blood specimens

Blood samples were drawn in the morning from fasted animals by venipuncture of the jugular/cephalic vein and were collected in EDTA tubes for immediate immunophenotyping and flow cytometry analysis or stored for 48 h at 4 °C for the stability study.

Cell preparation

Blood samples were processed after surgery to obtain the mononuclear white blood cells (MWBCs) and the total white blood cells (TWBCs) free of erythrocytes. In both cases, only cellular suspensions with > 95% of viable cells were used.

MWBCs were obtained by blood centrifugation with Ficoll-Paque™ (d = 1.077 g/cm^3) (GE Healthcare, Uppsala, Sweden). Further, mononuclear cells were collected and washed with 10 ml of MACS buffer (Miltenyi Biotech, Galdabach Germany). Finally, the cell pellet was suspended in 1 ml of MACS buffer.

TWBCs were obtained by erythroid lysis. Well-mixed blood (1 ml) was placed in a sterile tube where 10 ml of erythrocyte lysis buffer (e-Bioscience) were added. After 12 min of incubation at room temperature (RT), tubes were centrifuged (300 *g*, 5 min) and the cell pellet was resuspended in 1 ml of MACS buffer.

Flow cytometry immunophenotyping

We analyzed leukocyte canine antigen expression using monoclonal antibodies (mAb) from AbD (Serotec Oxford, UK): anti-canine CD3(CA17.2A12) conjugated with fluorescein isothiocyanate (FITC), anti-canine CD21(CA2.1D6) conjugated with phycoerythrin (PE). Non-conjugated antibodies CD4(YKIX302.9), and

CD8(YCATE55.9) were supplied by the Institut für Molekulare Immunologie (München, Germany). Antibodies against tissue antigens were provided by E. Kremmer; Dog17(panT), Dog10(CD8) and anti-canine mAb 3B4 (Ostronoff et al. 2008), instead of the commercial CD34 (McSweeney et al. 1998), were incubated at 10 µg/ml before titration.

For the non-conjugated mAbs, 200 µl of MWBCs or TWBCs samples with 1×10^6 cells, were placed in 5 ml tubes and incubated for 30 min at 4 °C adding 10 µl of primary mAbs. Cells were washed with MACS buffer (300 *g*, 5 min) and then suspended in 50 µl of MACS buffer. As secondary Ab PE-donkey anti-mouse-IgG was added to the sample with CD8, and FITC-rabbit anti-rat-IgG to the remaining samples. All samples were incubated for 15 min at 4 °C in the dark, then the cells were washed with MACS buffer (300 *g*, 5 min) and suspended in 600 µl of PBS. For the conjugated mAbs, 100 µl of blood or MWBCs samples (with 1×10^6 cells) 10 µl of each antibody were added into a 5 ml tube and incubated for 30 min in the dark at RT. After that, 2 ml of erythrocyte lysis solution was added and incubated for 12 min at RT. Then, cells were washed with MACS buffer (300 *g*, 5 min) and resuspended in 600 µl of PBS.

Flow cytometry acquisition was performed using a FACScan (Becton Dickinson, San Jose CA, USA). Technician calibrates the FACScan everyday using the appropriate beads. A minimum of 10,000 events was acquired for each sample. Flow cytometry data were analysed using FacsDiva 6.0 software (Becton Dickinson, San Jose CA, USA). Dead cells were discarded adding 1 µg/ml propidium iodide (PI) (SouthernBiotech, Birmingham, USA) to the samples. Non-staining cells were used as negative controls, and secondary antibodies were used as isotype control to prevent unspecified cross staining.

Cytological evaluation

Blood smears and cytocentrifugated preparations of MWBCs and TWBCs were stained with May Grünwald-Giemsa. A minimum of 200 white blood cells was counted by two different pathologists. In flow cytometry we established the number of cells for each subpopulation; using an absolute cell counting (cell number/µl of whole blood), and with the percentage gated, which is the frequency (%) of events in the subpopulation compared with all the acquired events (Watabe et al. 2012).

Statistical analysis

Unpaired two-tailed *t*-test was performed for comparisons between two groups of non-paired data. The coefficient of variation (% CV) was calculated as (standard deviation/mean) × 100. Statistical tests were performed on GraphPad Prism 5.0 software. *P*-values ≤ 0.05 were considered significant.

Results

Comparative analysis between differential counting and flow cytometry in MWBCs and TWBCs

Blood samples from Beagle dogs (5 males and 5 females) were utilized during this study for comparative analysis by differential counting (DC) and flow cytometry (FC) leukocyte cell number populations utilizing two processing methods: TWBCs and MWBCs. Figure 1 (Plate VIII) shows a representative dot plot and microphotographs of both populations. Data in the columns were expressed as percentage (%) of total cell number. The represented populations were granulocytes, monocytes and lymphocytes depending on the isolation method. We compared the number of cells selected by flow cytometry against the differential count.

Results indicated that CV between DC and FC for TWBC were 3.54, 18.92, and 18.21 for granulocytes, monocytes, and lymphocytes, respectively; 18.90 for monocytes and 6.42 for lymphocytes in MWBC. All CV values were under 25 (consensus limit for CV comparative analysis (Herzenberg et al. 2006)).

Immunophenotyping of blood leukocyte subsets in MWBCs and TWBCs

The immunophenotyping analysis was carried out in samples obtained by TWBC and MWBC. We tested the samples against an array of canine leukocyte antibodies: CD3, CD4, CD8, Dog10(CD8), CD21, Dog17(panT), and CD34. Table 1 shows the mean values of leukocyte subsets labelled with the antibodies obtained either for TWBC or MWBC. All CV values in the table were under 25. Moreover, *P*-values indicated that there were no significant differences for the percentage of cells in TWBC and MWBC except for CD21. These data dealt with the main goal in this study, demonstrating that the sample preparation

method, blood lysing (TWBC) or density gradient (MWBC), had no impact on leukocyte subpopulations for immunophenotyping.

Figure 2 (Plate VIII) shows representative dot plots from 80–90 samples of peripheral blood leukocyte subsets from samples labelled with the array of canine leukocyte antibodies for TWBC and MWBC. In the dot plots stained populations were displayed to show SSC (side scatter) vs. FITC or PE fluorescence. Our data revealed good homogeneity between samples indicating good experiment reproducibility.

Table 1. Means of cellular markers and comparative commercial vs. dog specific antibody.

A	MWBC*	TWBC*	CV(n)	*P*-values
CD3**	58.00	66.10	6.53	0.1320
panT**	60.60	71.80	8.46	0.0904
CD4**	37.00	38.75	2.31	0.0592
CD8**	23.00	16.70	15.87	0.0871
Dog10**	16.80	19.50	7.44	0.4295
CD21**	13.65	20.45	19.94	0.0211*
3B4***	0.55	0.91	24.63	0.9191
B				
CD3/panT MWBC				0.9472
CD3/panT TWBC				0.4747
CD8/Dog10 MWBC				0.0766
CD8/Dog10 TWBC				0.6891

A: Mean values of immunophenotype from peripheral blood leukocyte subsets obtained using the two different methodologies, density gradient separation of mononuclear cells and whole blood lysis. CV was obtained after comparative analysis of mean values from MWBC and TWBC for each antibody; all CV values are below 25. Included are the results of the statistical analysis unpaired two-tailed *t*-test for each antibody; there are no significant differences ($P > 0.05$) between leukocyte subsets and ratios, with the exception of CD21.
* Mean values are expressed in %
** Data are referred to lymphocyte population
*** Data are referred to total live cells
B: Comparative results between CD3 versus the tissue antigen Dog 17 (panT) and CD8 versus the tissue antigen Dog10 (CD8).

Storage of samples of canine blood cells

The effects of storage of blood leukocyte samples, depicted in percentages of leukocyte subpopulations and immunophenotyping pattern, were investigated on peripheral TWBC. We carried out a comparative long-term stability analysis at two time points: TWBC immediately processed (0 h) or stored 48 h at 4 °C. Data analysed at 0 h were previously presented in (Plate VIII, Fig 1).

After 48 h of storage at 4 °C, TWMC samples were analysed to determine leukocyte subpopulations using the two methods; FC and DC. Figure 3A shows the leukocyte subpopulation at 48 h of storage. The CV values were 2.60, 21.75, and 0.74 for granulocytes, monocytes and lymphocytes, respectively. Data of cell population were represented as means with an error bar. In (Plate IX, Fig. 3) we analysed subpopulations at two different time points, 0 h vs. 48 h, for FC Fig. 3B and for DC Fig. 3.C as intra-method comparison. In both cases, *P*-values indicated no significant differences between the samples due to storage.

We then carried out an immunophenotype analysis at two time points (Plate IX, Fig. 4). Our data showed no significant differences to any of the Ab used for this study.

Our study was performed with healthy dogs of both sexes. We carried out a comparative analysis between males and females using the cell number in TWBC samples. Our study

showed a CV of 5.86, 5.80, and 0.99 for granulocytes, monocytes and lymphocytes, respectively. Also we evaluated the immunophenotyping of T (pan T) and B (CD21) lymphocytes in both sexes finding a CV of 4.39 for pan T and 19.88 for CD21. The B-lymphocytes showed a 20% of variance. Also, no significant differences were observed between male and female donors, P-values were $P = 0.19$, $P = 0.19$, and $P = 0.15$ for granulocytes, monocytes, and lymphocytes, respectively, and $P = 0.11$ for T-lymphocytes and $P = 0.18$ for B-lymphocytes.

Discussion

Clinical applications of flow cytometry have become common practice to establish ranges for phenotype percentages in healthy animals; this is essential as reference data to monitor alterations associated with disease. In this study, leukocyte subsets were examined in 7.5-year-old Beagle dogs, using whole blood lysis or density gradient centrifugation to obtain cell populations. We determined that those two methods could be equally used for animal diagnosis. To the best of our knowledge and despite the broad usage of the two methods in immunophenotyping, only little comparative information is available to date. Moreover, this is a long-term stability study in terms of information on leukocyte subpopulations and analysis of antibodies. Often, blood samples are collected at one site and then shipped to another site for analysis.

Here we demonstrated that no significant differences were found between samples processed following a whole-blood lysis method and samples processed after density gradient separation (Plate VIII, Fig. 1 and Table 1). It is important also to indicate that our immunophenotyping data of lymphocytes were within the same range as those obtained for 8-year-old healthy dogs described previously (Williams et al. 2008), and for dogs over 5 years of age (Faldyna et al. 2001).

No differences in canine leukocyte subpopulations have been described in the literature between males and females although Faldyna et al. (2001) concluded that perhaps there were sex-related differences in the lymphocyte subsets they studied, even if these differences were examined in Beagles and Dachshunds 1–5 years old. However, our results should be interpreted carefully taking into account the limited number of animals included in each group. The results of this part of the study confirmed our preliminary hypothesis based on literature, relating the lack of differences between cell populations and leukocyte subsets when using donors of different sex.

In our study, we compared results obtained with the commercial antibody CD3 versus the specific Dog17(panT) and data obtained with the commercial CD8 versus Dog10 (CD8), (Table 1B) with no significant differences between values either for MWBC or TWBC. These findings allow us to validate the use of Dog17 as a panT marker, and the Dog10 as CD8 antibody specifics for dogs.

As we indicated previously this paper offers a panel of canine antibodies used in healthy dogs, which will be helpful to estimate levels of antibodies in future studies of canine leukaemia or malignant lymphoma. In that respect, and taking into consideration the amount of B-associated neoplasias, we will plan to amplify the B lymphocyte panel of antibodies, which are critical to identify and classify those diseases.

As far as we know, no methodology has been published focusing on the long-term (48 h) sample storage on canine leukocytes. Our results demonstrated that there are no significant differences at different time points (0 h and 48 h). That indicates a great advantage for laboratory workers or for the processing and also the possibility to store samples without any disturbance in the cell populations, independently of the method used for cell analyses, FC or DC. Furthermore, the same results were observed when we studied antigen expression of samples immediately processed and analysed after blood collection (0 h) and sample

storage for 48 h at 4 °C. Figure 4 shows the *P*-values. Our results indicate that storage at 4 °C for 48 h had no effect on leukocyte subset distribution. In concordance with our data, other authors (Shield et al. 1983; Nicholson et al. 1984; Ponzio et al. 1984; Prince et al. 1986) describe similar conclusions for storage for a shorter period of time, between 2–16 h.

Since Faldyna et al. (2001) described the difficulties in monocyte determination only by their light scatter characteristics in flow cytometry, several new flow cytometry analysis softwares have been released. Here, we gated a monocyte population using flow cytometry with accurate precision when comparing the monocyte flow cytometry data with differential counting data, with CV less than 20.

The outcome of the present study showed that whole blood lysis represents an efficient and quick alternative for canine leukocyte preparation. In addition, samples can be stored 48 h at 4 °C without a significant difference with respect to samples processed immediately after blood collection. This finding is relevant for veterinary medicine considering the lack of facilities in many laboratories to do immunophenotyping studies. These results indicate that whole blood lysis represents an effective, fast and cheap alternative for canine leukocyte preparation.

Conflict of interest

The authors declare no conflict of interest.

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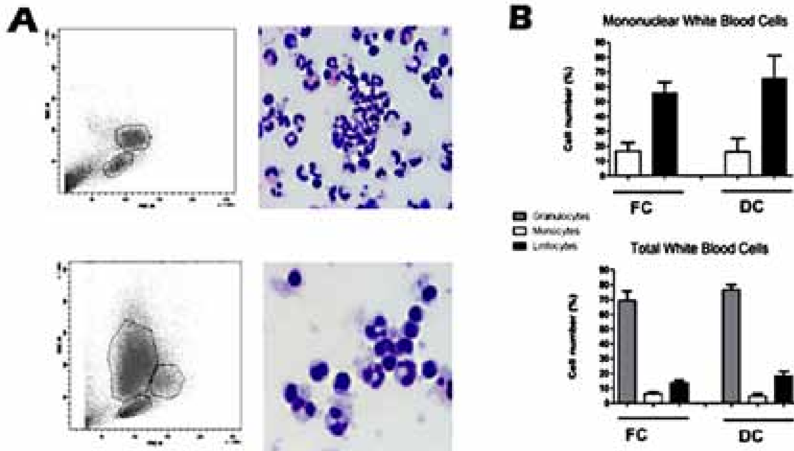

Fig. 1. MWBC and TWBC populations
A: Representative dot plots (85–90 different samples) of cell populations obtained using whole blood lysis or density gradient centrifugation. In parallel with these dot-plots are included microphotographs (× 400)
B: Data from comparative analysis between flow cytometry (FC) and differential count (DC) are represented in the bar graphs. The bars represent the % of the mean and error bar for the different populations.
MWBC: mononuclear white blood cells, (monocytes and lymphocytes); TWBC: total white blood cells, (granulocytes, monocytes, and lymphocytes).
Comparative analysis indicated a coefficient of variation (CV) of 18.90 and 6.42 for monocytes and lymphocytes, respectively, in MWBC, and a CV of 3.54, 18.92, and 18.21 for granulocytes, monocytes, and lymphocytes, respectively, in TWBC.

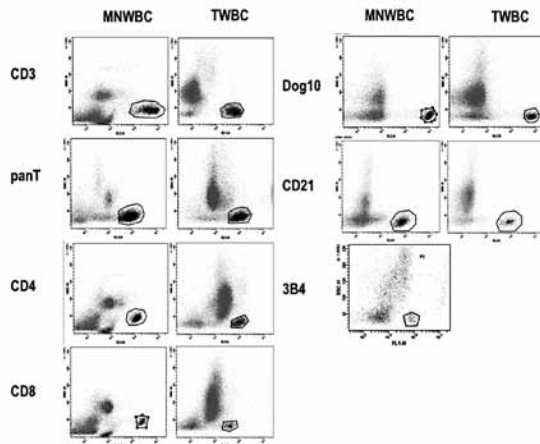

Fig. 2. Representative dot plots of peripheral blood leukocyte subsets.
Representative dot plots (80–90 different samples) of peripheral blood leukocyte subsets. The figure shows flow cytometry analysis for the cell population stained by different antibodies. The gating was made taking in consideration previous data used for this study. 10,000 cells were acquired for each sample. Cell dead discrimination was make by forward and side scatter (FSC/SSC) and PI.

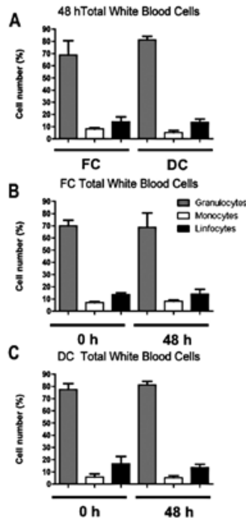

Fig. 3. TWBC time stability
This figure shows long-term stability of total white blood cells (TWBC) at two time points. Data in the column are represented by mean and error bar. A: Comparative analysis was made between flow cytometry (FC) and differential count (DC) at 48 h. B: Analysed subpopulations at two different time points (0 h vs 48 h) using FC as control. C: Analysed subpopulations at two different time points (0 h vs 48 h) using DC. For both methods, FC (Fig. 3B) and DC (Fig. 3C), the *P*-values obtained from comparison of the two time points give no significant differences ($P > 0.05$) in any cell population.

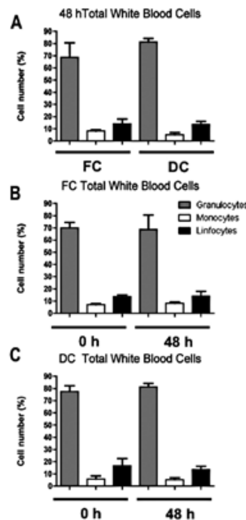

Fig. 4. Population stability: leukocyte subsets data at different time points
The mean and error bar represent data in the column graphs. Mean values of the immunophenotype from total white blood cell leukocyte subsets at two time points 0 h and 48 h. The statistical analysis (unpaired two-tailed *t*-test) for the FC data at the two time points, as well as the DC, data reveal no significant differences ($P > 0.05$) in the percentage of leukocyte subsets (data not shown).