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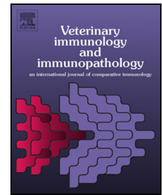

Identification of a suitable internal control for fluorescence analysis on canine peripheral blood samples

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ABSTRACT

Reliable detection of fluorescence intensity (FI) by flow cytometry (FC) is fundamental. FI depends on instrument settings and sample processing procedures: thus, measurements should be done using internal controls with known FI. Commercially available beads-based standards are expensive, thus reducing their usability in the veterinary practice. Cell subsets with stable mean FI (MFI) within the population have been proposed as acceptable surrogates in human medicine. In veterinary medicine, no data exist about stability of antigen expression among different subjects or upon sample storage. The aim of the present study was to evaluate MFI variability of main lymphocytes antigens among the lymphoid cells within each subject, among different subjects, and upon 24-h storage, in order to identify the antigen most suitable as stable internal control in MFI analyses.

Peripheral blood samples from 18 healthy dogs were analysed by FC within 3 h from sampling to assess the expression of CD3, CD5, CD4, CD8, CD21 and cyCD79b using conjugated monoclonal antibodies. Analyses were restricted to the lymphoid population. Fluorescent microbeads were added to each tube, and antigen MFI was calculated as Relative Fluorescence Intensity RFI (CD/beads). Fluorescence histogram CV (fhCV) for each CD was regarded as an index of the variability of expression among lymphocytes within each subject (cell-to-cell variability); whereas the CV of RFI was regarded as an index of inter-subjects variability (dog-to-dog variability). In 11 cases, FC analyses were repeated after 24 h storage at 4 °C and RFI and CVs of fresh and stored samples were compared to assess variability linked to storage. CD4 was identified as the best antigen to be used as an internal control for MFI analyses in canine peripheral blood samples because of low cell-to-cell and dog-to-dog variability, and optimal stability upon 24-h storage. Blood samples from a second group of 21 healthy dogs were labelled only with CD4, in order to assess the influence of breed, sex and age on the expression of CD4 in a larger case series. Based on univariate GLMs, none of these variables influenced CD4 RFI.

Normalizing fluorescence data using lymphoid CD4 MFI as a reference would improve the comparison of results obtained by different laboratories, patients or times in diagnostic and research analyses of FI. Further studies are needed to confirm our results with different FC approaches.

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1. Introduction

Flow cytometry (FC) is frequently used in both human and veterinary medicine to resolve the subpopulations in a cellular suspension. In addition, FC allows a quantitative or semi-quantitative assessment of the expression of the investigated antigens, by means of the mean fluorescence intensity (MFI). This parameter has been used for many different diagnostic and research purposes in human medicine. Reliable assessment of fluorescence intensity (FI) by FC is therefore mandatory, in order to compare results from different laboratories, data from different subjects, or to monitor patients.

MFI is influenced by many factors, including cytometers' variables (model, manufacturer, configuration, setting, filter setup, but also age and overall conditions of the instrument), and non-instrument variables, such as the choice of fluorochrome, antibody, and the ratio of fluorochrome to protein on the conjugated antibody (Maher and Fletcher, 2005). In addition, MFI may vary based on the processing method, since it is affected by pH (Lenkei and Andersson, 1995), lysis agent and fixative (Bossuyt et al., 1997).

Therefore, besides a stringent and standardized instrument quality control, standard fluorescence controls should be used when MFI has to be assessed. Bead-standards are widely used to this

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aim, as they offer the unique opportunity of testing the number of molecules expressed on the tested population, by means of a quantitative FC (Vogt et al., 2000; Maher and Fletcher, 2005; Rossmann et al., 2007). Unfortunately, these methods are quite expensive and almost not affordable in clinical setups.

The use of surrogate, more cost-effective methods to standardize the FI assessment is making way. For example, MFI of the tested antigen may be normalized based on a cell subset with a known stable MFI within the examined population. CD45 and CD4 have been proposed for such a use in human medicine (Bikoue et al., 2002; Davis et al., 1998; Hultin et al., 1998; Wang et al., 2015).

In dogs, FI assessment has been used to identify activated platelets and neutrophils by labelling with P-selectin and CD11b antibody, respectively (Moritz et al., 2005; Maeda et al., 2010) and to characterize normal and neoplastic hematopoietic cell subpopulations by double labelling with CD18 and CD45 (Comazzi et al., 2006a, 2006b). Other studies used MFI values to demonstrate a different degree of CD44 expression between canine acute and chronic leukaemias (Gelain et al., 2014), and the prognostic impact of the degree of class II MHC expression in canine B-cell lymphomas (Rao et al., 2011). Finally, a recent study investigated the effects of storage in a preservative medium on canine peripheral blood samples, reporting the MFI values of the main lymphoid antigens (Cian et al., 2014). Despite this number of published studies, no effort has been made so far to normalize MFI data obtained on canine samples.

Veterinary FC facilities are available only in few referral laboratories. Even if overnight shipping of samples is usually recommended, the time slot between sample collection, processing and analysis may affect results: this is particularly true when MFI is assessed, even in fixed samples (Cian et al., 2014). Thus, FI controls should be introduced, together with strict quality controls. Bead-based standards may be not affordable for veterinary FC facilities, whereas the use of internal biological FI standards might be more accessible. To date, no suitable internal FI control for canine samples has been suggested.

The aim of the present study was to assess the variability of the expression of the main lymphoid antigens among lymphocytes within each subject (cell-to-cell variability) and among different subjects (dog-to-dog variability) in a population of healthy dogs, and secondarily to assess whether 24 h storage at 4 °C does affect this variability. The final aim was to identify the antigen with the lowest variability of expression, to be used as an internal biological FI standard for future MFI analyses.

2. Materials and methods

Samples from the present study were provided to the Laboratory of the Department of Veterinary Sciences (University of Turin, Grugliasco, Turin) for routine haematology analysis. To be included in the study, dogs had to fulfil the following inclusion criteria: (1) absence of clinical abnormalities; (2) no disease report in the clinical history; (3) no vaccination or therapy administered in the last month; (4) no haematological abnormalities.

All dogs were privately owned and sampled for routine haematological controls with the written informed consent of the owners. Thus, a formal approval of the Institution Committee for Animal Care of the University of Turin was not necessary.

Based on the day of collection, dogs were subdivided into two groups. Dogs in group 1 were enrolled from December 2014 to March 2015. Dogs in group 2 were enrolled from September to December 2015.

Peripheral blood (PB) samples (1 ml) were collected from the cephalic vein into EDTA tubes and processed for FC within 3 h from sampling (T0). In most cases, processing for FC and analyses were repeated after 24 h storage at 4 °C in the dark (T24).

Table 1
Antibodies used to label peripheral blood samples from healthy dogs. All antibodies were provided by Serotec (Oxford, UK).

Antibody	Clone	Target species	Volume used (μl/tube)
CD3	CA17.2A12	Dog	10
CD5	YKIX322.3	Dog	2
CD4	YKIX302.9	Dog	2
CD8	YCATE55.9	Dog	10
CD21	CA2.1D6	Dog	2
CD79b	AT107-2	Mouse	5

Prior to labelling, all samples were counted via an automated haematology analyzer (ADVIA 120, Siemens Healthcare Diagnostics, Milan, Italy) to assess cellularity, and underwent RBC lysis with an erythrocytes lysis buffer containing 8% ammonium chloride. After washing, cells were resuspended in RPMI 1640 medium, containing 5% fetal bovine serum. For surface marker labelling, 1×10^6 cells/tube were incubated with one of the following antibodies: CD5-FITC, CD3-FITC, CD4-FITC, CD8-PE, CD21-PE. For intracellular marker staining, a permeabilization procedure was performed using the Leucoperm reagents (Serotec, Oxford, UK) following the manufacturer's instructions; 1×10^6 cells/tube were then incubated with cyCD79b-FITC. All antibodies were provided by Serotec and had been previously titered to determine the best working dilutions (Table 1). After incubation of 20 min at 4 °C in the dark, samples were washed twice in RPMI 1640 and finally resuspended in 500 μl PBS for final acquisition. 15–20 drops of flow-set fluorospheres (Beckman Coulter, Brea, CA, USA) were added to each tube immediately before acquisition, to be used as FI standards. One tube with unstained cells was also prepared for each sample, to serve as negative control.

Samples from Group 1 were labelled with all antibodies, in order to identify the antigen with the lowest cell-to-cell and dog-to-dog variability. Samples from Group 2 were labelled only with the antibody selected based on data from Group 1.

Samples were acquired via a Coulter Epics XL (Beckman Coulter) flow cytometer. For each tube, at least 6000 events in the lymphocytes gate were acquired. Data were analysed via the specific software Expo32 (Beckman Coulter).

Acquired events were shown in a dot plot scatter gram based on forward- (FSC) and side-scatter (SSC). Two gates were set to include only fluorospheres and lymphocytes, respectively, and analyses were restricted to these two populations. A histogram with log values for FI on the x-axis and cell number on the y-axis was used for the analyses (Fig. 1). MFI of fluorospheres, MFI and fluorescence histogram Coefficient of Variation (fhCV) of CD-positive cells were measured. In order to avoid any possible bias due to sample processing or cytometer conditions, the Relative Fluorescence Intensity (RFI) was calculated making a ratio between lymphocytes and fluorospheres MFIs and used for further analyses (Henderson et al., 1998). Finally, since a heterogeneous fluorescence distribution was detected for CD21, analyses were performed including all CD21-positive lymphocytes (CD21tot) and only lymphocytes with higher CD21 expression (CD21high), respectively (Fig. 1).

For each antigen in T0 samples the mean fhCV was regarded as an index of the cell-to-cell variability, whereas the CV of the RFIs was regarded as an index of the dog-to-dog variability.

According to the distribution of data, paired-samples Wilcoxon or *t*-test was performed to compare RFI and fhCV of each antigen between T0 and T24. Influence of experiment (group 1 and 2), time (T0 and T24), sex (male and female), breed (purebred and crossbred) and age (<2 yrs, 2–10 yrs and >10 yrs) on RFI of CD4 was assessed through GLM univariate analysis. The statistical software IBM SPSS Statistics v23.0 for Windows was used for statistical analyses. Significance was set at $p \leq 0.05$ for all tests.

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