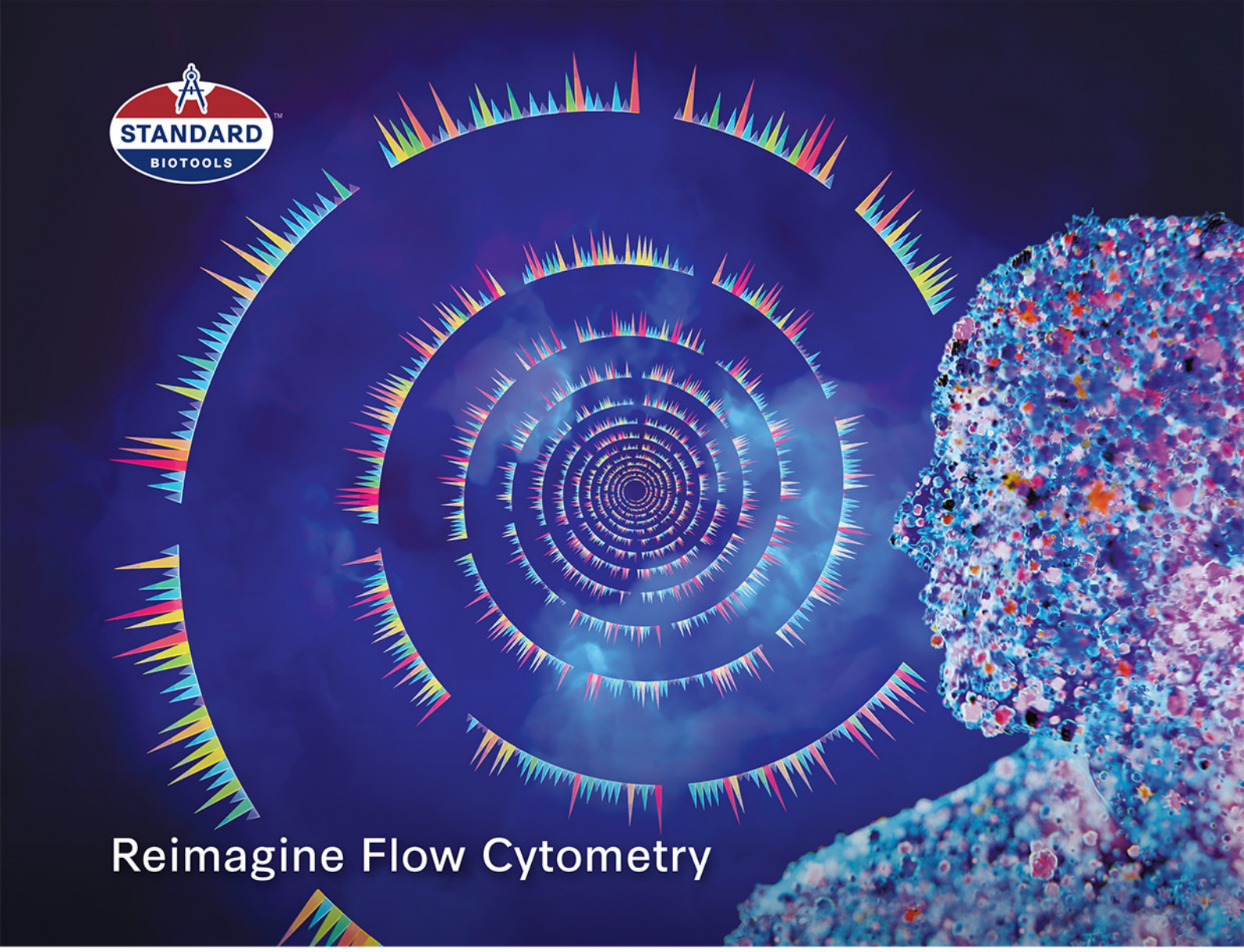

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TECHNICAL NOTE

ISAC CYTOMETRY Journal of Quantitative Cell Science PART A

A method to test antibody cross-reactivity toward animal antigens for flow cytometry

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Abstract

The availability of cross-reacting antibodies and/or of antibodies working in flow cytometry is a major issue in the veterinary field. One of the main problems is the availability of certain positive controls. With this brief communication, we report an method to quickly screen a wide number of products without the need to look for positive biological samples. We propose this approach as a first step to select the best antibodies to test on biological specimens.

KEYWORDS
cross-reactivity, dog, microbeads, PD-L1, veterinary

1 | INTRODUCTION

Dealing with flow cytometric analysis of veterinary samples, either for diagnostic or research purposes, veterinary flow cytometrists must face many difficulties, including above all the paucity of commercially available species-specific or cross-reactive antibodies, and the problematic identification of positive controls. Most often, antibodies are considered cross-reactive if a positive stain is obtained on the same cell types that express the antigen of interest in the primary target species. However, such an approach does not consider inter-species differences (as an example, CD4 molecule is expressed on the surface of neutrophils in dogs, but not in cats), and it does not allow to confirm the specific molecular target to which the antibody binds in the newly tested species.

Thus, we designed a way to test contemporarily the cross-reactivity of the antibodies and their possible use in flow cytometry (FCM), overcoming all the limitations described above.

In particular, we focused on the identification of an antibody binding to the extra-cellular domain of canine PD-L1: such an antibody is still lacking in the market, despite the high number of research groups investigating this molecule in canine samples. Indeed, the antibodies used via FCM in the published studies are not commercially available [1–6].

2 | MATERIALS AND METHODS

Three different antibodies were selected from the market, based on the information included in the manufacturer's datasheets: (1) a rabbit polyclonal anti-human antibody from NovusBiologicals (cod NBP1-76769SS), which is suitable for FCM and recognizes PD-L1 molecule in three different species; (2) a mouse monoclonal antibody from Biolegend (clone 29E.2A3), which had been tested via FCM and recognizes PD-L1 molecule in five different species; (3) a rabbit polyclonal anti-canine antibody from KingFisher Biotech (cod KP1412D-100), which recognizes canine PD-L1 molecule (unknown domain), but had never been tested via FCM.

The extracellular domain of canine PD-L1 was expressed and linked to polystyrene beads, to be used as a positive control via FCM. Briefly, the region encoding the extracellular domain of canine PD-L1 was amplified and cloned into a mammalian expression vector in frame with N′ terminal secretory peptide and C′ terminal monomeric

streptavidin. The HEK293T cell line was transiently transfected into, and the supernatant containing the secreted protein was collected after 48 h. The fusion protein was covalently coupled on carboxyl functionalized fluorescent polystyrene beads (Bio-plex Pro, Biorad) pre-coupled with biotin to link avidin-labeled proteins.

The PD-L1 coated beads were then analyzed via FCM with a Cytoflex flow cytometer (Beckman Coulter). Briefly, 100,000 beads in 100 µl were incubated with each antibody (1, 2, and 1 µg, respectively) in three different tubes. After two washing steps with PBS, the appropriate secondary antibody was added (goat anti-rabbit IgG for antibodies nr 1 and 3 and goat anti-mouse IgG for antibody nr 2, both conjugated with Alexa-Fluor-488, both from Invitrogen). Two control tubes (one for each secondary antibody) were also prepared. After 20 min of incubation, the beads were washed and acquired. The beads were gated on FSC vs SSC scattergram and visualized in histograms plotting FL-1 in the X-axis.

3 | RESULTS

Beads labeled with antibody n 2 were negative. Antibody n 1 reported a partial and dim positivity, whereas the beads stained positive with antibody n 3, showing high fluorescence intensity with a low CV (Figure 1).

Thus, antibodies nr 1 and 3 recognize the synthetic extracellular domain of canine PD-L1 and work via FCM, with antibody n 3 showing the most promising results and being the best candidate for testing on biological samples.

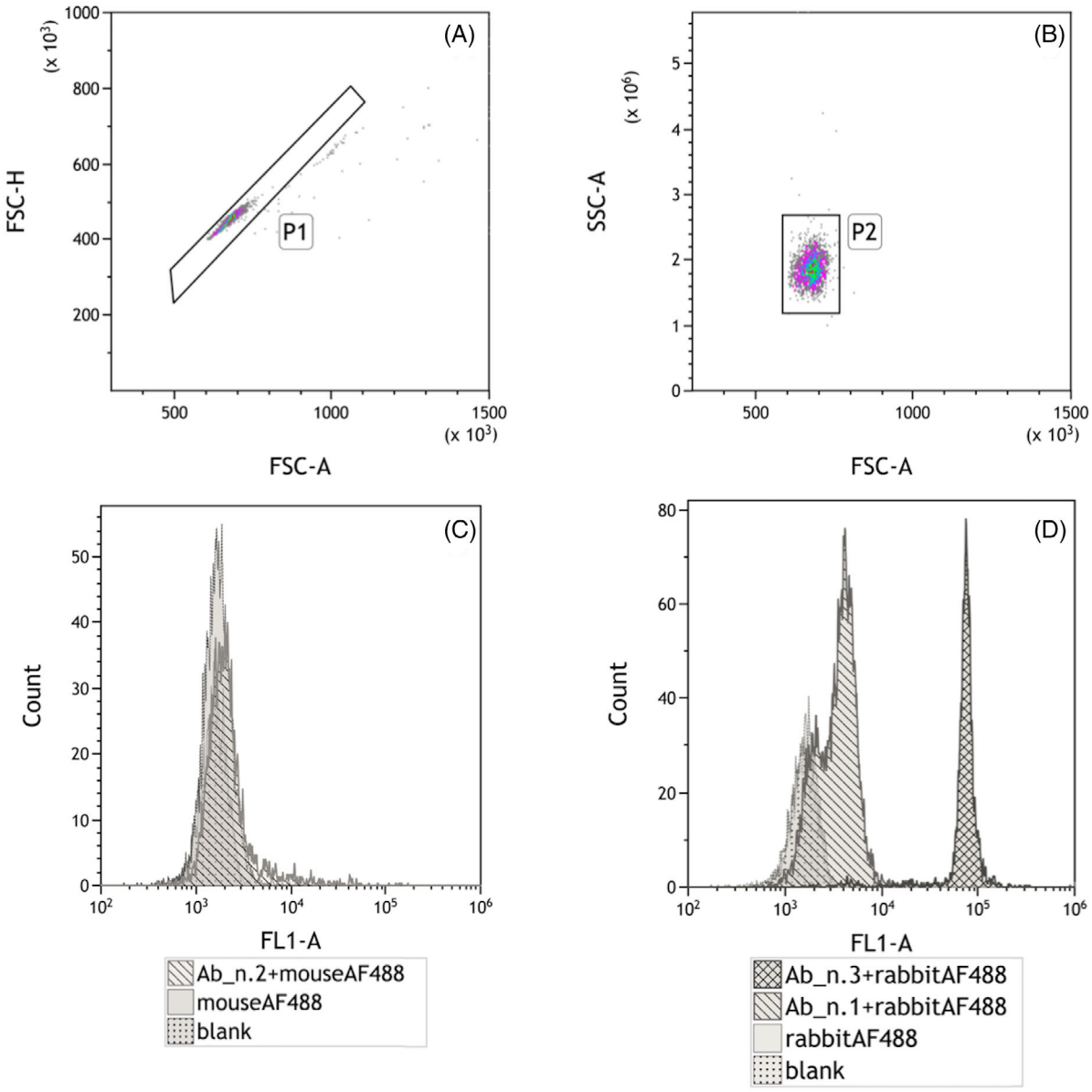

FIGURE 1 Flow cytometric analysis of PD-L1 coated beads. (A,B) Scatter plots with gate of analysis (P2) after doublets exclusion (P1). (C) P2-gated beads: blank, labeled with secondary anti-mouse antibody and antibody nr 2. (D) P3-gated beads: blank, labeled with secondary anti-rabbit antibody, antibody nr 3 and antibody nr 1. MouseAF488: goat anti-mouse IgG-Alexa Fluor 488; rabbitAF488: goat anti-rabbit IgG-Alexa Fluor 488 [Color figure can be viewed at wileyonlinelibrary.com]

4 | DISCUSSION

Herein, we report a protocol suitable to screen a wide number of antibodies for their possible binding toward specific epitopes of target proteins, limiting the use of biological controls to the subset of the best promising antibodies. This is of fundamental relevance in veterinary medicine, where gross inter-species differences may affect the use of biological controls to assess antibody cross-reactivity. The use of protein-coated beads as positive controls allows to assess the results of labelling with different antibodies, determining in a single experiment which antibody recognizes the protein (or epitope) of interest and works via FCM.

It should be noted that, for most cellular markers, specific antibodies are raised using linear synthetic peptides as immunogen, designed on specific target sequence. In such a case, the ability of these antibodies to bind native protein greatly depends on the chance to recognize specific epitope exposed on the surface of the molecule and the assay in which the antibody/antigen complex is detected. The expression of extracellular domain in a mammalian system offers the greatest chance to mimic native conformation for membrane protein and allow selection of antibodies capable to bind surface epitope or conformational epitope useful for FCM analysis. However, we still consider experiments on biological controls mandatory to select the antibody to be used in diagnostic or research activities also because the target protein may assume different conformations (as recently demonstrated for PD-1) [7] that may affect antibody binding. By applying the protocol described herein, however, such experiments would be limited to the best promising antibodies, which have been proven to bind the epitope of interest. In addition, post-translational modifications can occur leading to a false negative result with the described approach and a western blotting should be carried out to demonstrate an actual lack of reactivity of the antibody. However, in the perspective of a rapid screening to find one or more reacting antibodies, the execution of western blotting can be limited to the event that no positive result is obtained from any of the tested antibodies.

AUTHOR CONTRIBUTIONS

Fulvio Riondato and Valeria Martini designed the experiment; Barbara Colitti and Sergio Rosati synthesised the protein and produced the coated beads; Fulvio Riondato and Federica Sini performed flow cytometry analyses; Fulvio Riondato and Valeria Martini wrote the manuscript; Barbara Colitti, Sergio Rosati, and Federica Sini reviewed the draft of the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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