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Elimination of erroneous results related to bovine mononuclear cell immunophenotyping by antibodies binding to Fc receptors

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ABSTRACT

Blocking immunoglobulin G (IgG) binding receptors on leukocytes is an established and highly recommended preventive procedure for immunological assays. Failing to prevent such nonspecific binding can lead to erroneous results. Several studies testing different blocking reagents have been performed in murine or human cells, however, there are no specific studies on bovine cells. Our study aimed to investigate the efficiency of blocking reagents to inhibit the nonspecific binding of mouse monoclonal antibodies (mAbs) to bovine peripheral blood cells. We observed nonspecific interactions of IgG2a and IgG2b negative isotypes with bovine leukocytes, but not IgG1. We found that these nonspecific bindings could be eliminated by blocking with purified mouse IgG, whereas little or no blocking effect was observed when bovine serum or Mouse Seroblock FcR were applied. Moreover, in the absence of an efficient blocking reagent, the percentage of CD335 positive cells was significantly higher than in the group previously blocked with mouse IgG. Based on these results, and due to the lack of specific commercial blocking reagents for bovine cells, our recommendation is to use purified mouse IgG as a blocking reagent for immune assays targeting bovine leukocytes in order to enhance the accuracy of the results.

1. Introduction

Fc receptors (FcRs) are cell surface structures that bind to the Fc region of antibodies. This binding of the Fc part of immunoglobulin G (IgG) antibodies to Fc gamma receptors (FcγR) expressed on the surface of leukocytes, mainly macrophages and monocytes, is crucial for the regulatory and effector functions of immune responses "in vivo", however for immunoassays, such as immunophenotyping by flow cytometry, it is a well-known cause of erroneous results (Biburger et al., 2015; Andersen et al., 2016). In human and mouse, FcRs have been extensively studied and characterised, whereas they remain not fully elucidated for bovine species. As reviewed by Kacskovics (2004), there are four different FcγR in bovines: FcγRI (CD64), FcγRII (CD32), FcγRIII (CD16), and Fcγ2R. The overall similarity of these receptors to their human and mouse counterparts are known, although some studies have described some notable differences. FcγRII seems to bind only to bovine IgG1 and not to IgG2, whereas bovine FcγRIII was described from γ/δ T cells as a molecule similar to human FcγRIIIA, but presenting only a single extracellular domain. Bovine Fcγ2R is the most notable FcγR. It belongs to a novel gene family that includes the human killer cell inhibitory receptor and FcαRI proteins and has a high affinity for IgG2 but not for IgG1 or IgA. These particular species specific differences might also reflect differences in the degree and affinity of FcR binding to the Fc region of monoclonal antibodies (mAbs), and consequently the levels of undesirable nonspecific antibody binding during immunophenotyping by flow cytometry. Another source of undesirable nonspecific binding is due to the fluorochrome molecule that is conjugated to the antibody, which is mostly observed for the cyanine fluorochromes PE-Cy5 and Cy5 alone, which bind to FcγRI, whereas no such binding has been observed for FcγRII or FcαRI (CD89) (van Vugt et al., 1996; Jahrsdörfer et al., 2005). These nonspecific interactions can be eliminated or reduced by blocking Fc-receptors with anti-Fc-receptor antibodies, Fab or F(ab)2 fragments, immunoglobulins, or whole serum (Hulspas et al., 2009). Thus, the effective blocking of nonspecific binding of mAbs is of vital importance in flow cytometry, however, to the best of our knowledge, there have been no previous

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studies evaluating these interactions and the efficiency of blocking reagents for bovine leukocytes. Therefore, in view of the species specific differences of bovine Fc receptors, we formulated this study with the aim to investigate the presence of nonspecific interactions between different IgG isotypes and bovine mononuclear cells, and to quantify the efficiency of blocking reagents at eliminating these interactions.

2. Material and methods

2.1. Blood samples

Blood samples were taken by jugular venipuncture using sterile vacuum tubes containing 7.2 mg EDTA anticoagulant (Cat. 367839, BD, Franklin Lakes, NJ, USA) of five calves reared on the experimental farm of Embrapa Pecuária Sudeste, São Carlos, SP, Brazil. All procedures were approved by the Embrapa Pecuária Sudeste Ethical Committee for Animal Experimentation (CEUA/CPPSE), following the ethical principles and guidelines of animal experimentation adopted by the Brazilian College of Experimentation (process number PRT 02/2017). The samples were stored at room temperature (RT, from 20 to 25 °C) and processed within 4 h.

2.2. Experiment 1: nonspecific staining of mononuclear bovine cells

The nonspecific interactions between mouse mAbs and bovine leukocytes were evaluated by testing five negative isotype controls from three different IgG subclasses (IgG1, IgG2a, and IgG2b). The final concentrations of the negative isotype controls used in this experiment were the same as the concentrations of the respective primary fluorochrome-conjugated mouse anti-bovine mAbs which were previously titrated for optimal staining performance (data not shown). The description and concentration of each negative isotype is as follows: 1 μL (0.05 mg/mL) of IgG1 AlexaFluor (AF) 488 (Cat. MCA928A488, Serotec, Hercules, CA, USA), 2 μL (0.1 mg/mL) of IgG1 FITC (Cat. MCA928 F, Serotec), 2 μL (concentration not provided by the manufacturer) of IgG1 RPE (Cat. MCA928PE, Serotec), 2 μL (0.05 mg/mL) of IgG2a clone OX-34 AlexaFluor647 (Cat. MCA929A647, Serotec), and 1 μL (concentration not provided by the manufacturer) of IgG2b RPE-Cy5 (Cat. MCA691C, Serotec). Briefly, 25 μL of whole blood cells was placed into polystyrene tubes. The negative isotype control antibodies were mixed with Stain buffer containing fetal bovine serum (FBS) and ≤0.09% sodium azide (Cat. 554656, BD Biosciences, San Diego, CA, USA) for a final volume of 50 μL and added separately. The samples were incubated at 4 °C in the dark for 30 min. Then, the erythrocytes were lysed by adding 1 mL of non fixing 1× lysing solution (Cat. 555899, BD Biosciences) and incubating for 15 min at RT. The samples were centrifuged at RT for 5 min at 300 × *g*. The total leukocytes were washed with 500 μL of Stain buffer and centrifuged at RT for 5 min at 300 × *g*. The pelleted stained cells were resuspended in 100 μL of Stain buffer and analysed by flow cytometry (Accuri C6 Plus, BD Biosciences) on the same day. The cytometer settings were calibrated and adjusted each day after running the cytometer setup and tracking using CS&T RUO beads (Cat. 661415, BD Biosciences). At least 30,000 events were acquired and the mononuclear cells (MN) were gated according to the side (SSC) and forward scatter (FSC) dispersion, except for doublet cells (Supplementary information 1). The data were collected in the BD Accuri C6 Plus software version 1.0.23.1 (BD Biosciences) and analysed in the FlowJo software version 10.5.3 (BD Biosciences). The hierarchical gating strategy was applied by gating singlet cells and next the mononuclear cells (Supplementary information 1).

2.3. Experiment 2: efficiency of FcR blocking reagents for IgG2 isotypes

Immediately before staining with IgG2 isotypes (IgG2a AF647 and IgG2b RPE-Cy5), performed as described in Experiment 1, 25 μL of whole blood cells from each of five animals was placed into polystyrene tubes with different blocking reagents (diluted for a final volume of 50 μL) and incubated at 4 °C for 15 min.

The blocking reagents and respective concentrations used in this experiment were: 1.0 μg (0.02 μg/μL) and 5.0 μg (0.1 μg/μL) of Mouse Seroblock FcR (Cat. BUF041B, Serotec); 0.25 μg (0.005 μg/μL), 0.75 μg (0.015 μg/μL), and 1.25 μg (0.025 μg/μL) of purified mouse IgG; and 100% and 50% bovine serum (from adult healthy Canchim breed animals) previously heat inactivated at 56 °C for 45 min and sterile filtered (0.22 μm filter). The data were collected and analysed as described for Experiment 1. For this experiment, the 100% and 50% bovine serum treatments were tested on a different collection of blood samples and also on a different occasion, therefore, the statistical analysis for these treatments was performed separately.

2.4. Experiment 3: interference of mouse IgG with specific monoclonal antibodies staining

The interference of purified mouse IgG as a blocking reagent was investigated by testing five blood samples previously incubated or not with 50 μL of mouse IgG (1.25 μg), stained with two different panels of monoclonal antibodies diluted in Stain buffer. All the monoclonal antibodies were previously titrated and the stain indexes were calculated. Briefly, 25 μL of whole blood cells was placed into polystyrene tubes with 50 μL of purified mouse IgG (1.25 μg) and incubated at 4 °C for 15 min. The first panel was composed of 1 μL (0.05 mg/mL) anti-CD335 AlexaFluor 488 (Cat. MCA2365A488, Serotec) and 1 μL (concentration not provided by the manufacturer) anti-CD172a RPE-Cy5 (Cat. MCA2041C, Serotec). The second panel consisted of 2 μL (0.1 mg/mL) anti-CD21 FITC (Cat. MCA1424F, Serotec), 2 μL (concentration not provided by the manufacturer) CD8β RPE (Cat. MCA1654PE, Serotec), and 2 μL (0.05 mg/mL) CD4 AlexaFluor647 (Cat. MCA1653A647, Serotec). Both panels were diluted to a final volume of 50 μL. The samples were incubated at 4 °C in the dark for 30 min. Then, the erythrocytes were lysed by adding 1 mL of non fixing 1× lysing solution and incubating for 15 min at RT The samples were centrifuged at RT for 5 min at 300 × *g*. The total leukocytes were washed with 500 μL of Stain buffer and centrifuged at room temperature (from 20 to 25 °C) for 5 min at 300 × g. The pelleted stained cells were resuspended in 100 μL of Stain buffer and one drop of cell viability marker 7AAD (Cat. 1351102, Biorad, Hercules, CA, USA) was added to each sample stained with panel 2. All the samples were analysed by flow cytometry (Accuri C6 Plus, BD Biosciences) on the same day. The cytometer settings were calibrated and adjusted each day after running the cytometer setup and tracking using CS&T RUO beads. At least 30,000 events were acquired in the cell gate for panel 2, whereas 50,000 events were acquired for panel 1 due to the low counts of natural killer (NK) cells (identified as CD335+ cells), The data were collected using BD Accuri C6 Plus software and analysed in the Flowjo software version 10.5.3. All data were compensated and hierarchical gating strategy was applied by gating (in the following sequence) singlet cells, live cells (not stained with 7AAD, for panel 2 of mAbs) and mononuclear cells (Supplementary information 1 and 2). Tentative protocols were carried out using Sytox orange dead cell stain (Cat. S34861, Thermofisher, Carlsbad, CA, USA) or MitoStatus Red (Cat. 564697, BD Biosciences) as viability cell markers for the panel 1 of mAbs, though required fluorescence compensation lead to high resolution losses, making not possible to apply these markers (data not shown), therefore, due lack of viability cell marker in the panel 1, all assays were carried out using fresh samples (stained in the same day of collection).

2.5. Statistical analysis

Statistical analyses were performed using SAS software version 2002 (SAS, Cary, NC, USA). MFI (Median Fluorescence Intensity) values were transformed using logarithmic function (log10, n + 1). To compare the treatments, the mixed model was applied, with Tukey´s multiple

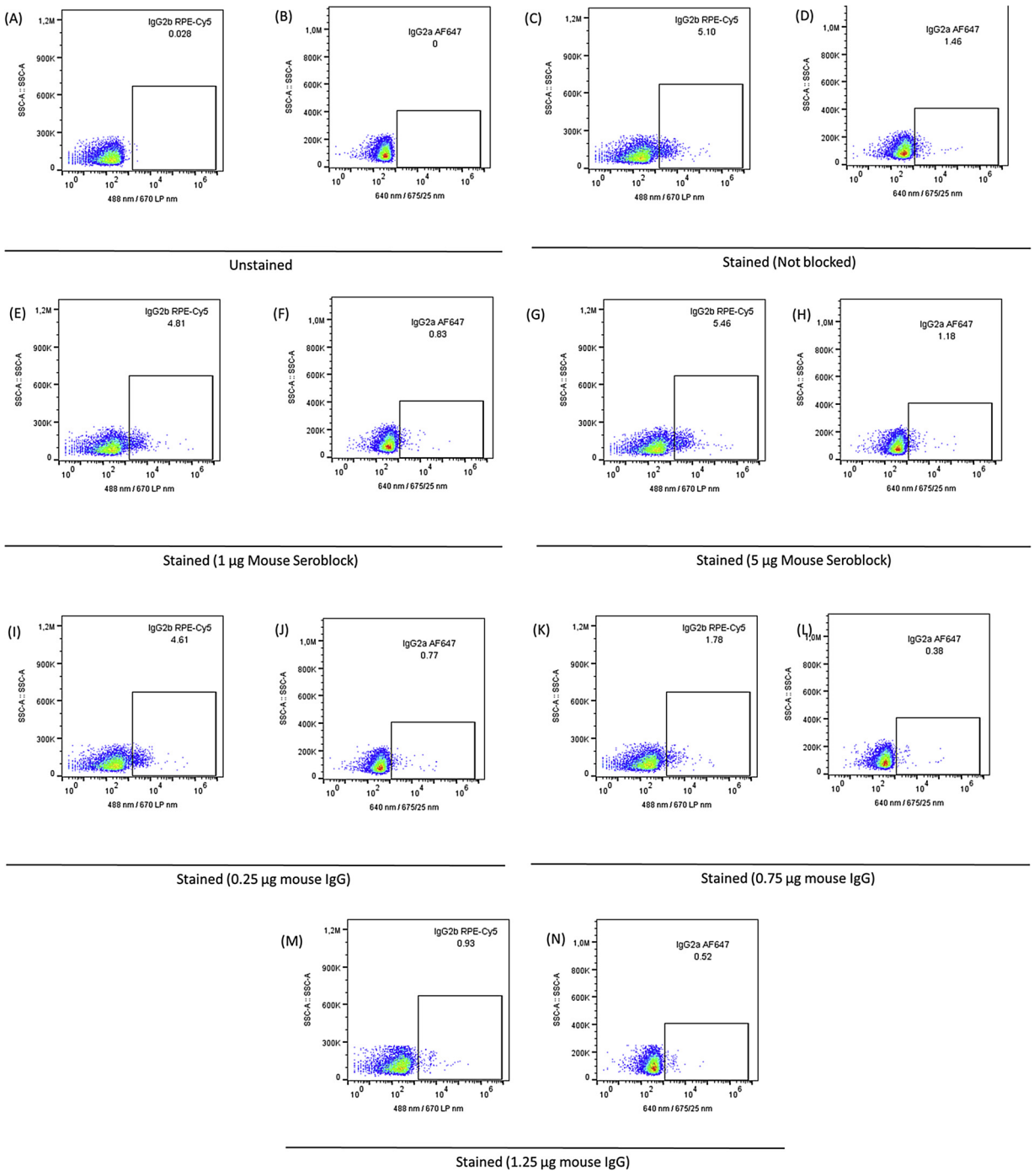

Fig. 1. Mononuclear cells stained with IgG2b RPECy5 (left) or IgG2a AF647 (right) isotypes. (A and B) Unstained cells. (C and D) Stained cells not blocked. (E and F) Cells blocked with 1 µg of Mouse Seroblock and stained. (G and H) Cells blocked with 5 µg of Mouse Seroblock and stained. (I and J) Cells blocked with 0.25 µg of mouse IgG and stained. (K and L) Cells blocked with 0.75 µg of mouse IgG and stained. (M and N) Cells blocked with 1.25 µg of mouse IgG and stained.

comparisons test (for more than two treatments) or unpaired *t*-test (for two treatments). A P value < 0.05 was considered statistically significant.

3. Results

3.1. Experiment 1: nonspecific staining of mononuclear bovine cells

No nonspecific staining was observed for all three IgG1 isotype conjugates evaluated (AF488, FITC, and PE) since there was no significant difference in the percentage of positive cells and the median fluorescence (Supplementary information 3) between the stained (not blocked) and unstained samples. However, nonspecific staining was observed for the IgG2a and IgG2b isotypes, for which the percentage of positive cells and/or the median fluorescence (Fig. 1 and Table 1) in the stained (not blocked) samples were significantly higher than in the unstained samples. Despite intense nonspecific staining for IgG2b isotype, absence of significant difference of MFI was observed in samples of Table 1 (but not for samples in Table 2), thus, the main criteria for analysis of both efficiency of blocking reagents (experiment 2) and interference of mouse IgG staining of specific monoclonal antibodies (experiment 3) were focused in the percentage of positive cells.

Table 1
Means of median fluorescence intensity (MFI) (± SD) values and percentage of positive cells obtained for mononuclear cells unstained and stained with each isotype control mAb, previously treated or not treated with different blocking reagents.

	MFI (± SD)		% Positive cells	
	IgG2b RPE-Cy5	IgG2a AF647	IgG2b RPE-Cy5	IgG2a AF647
Reference tube Not blocked (no isotype controls)	3052.20 ± 1011.53 b	1090.00 ± 65.82 b	0.08 ± 0.05 c	0.02 ± 0.01 c
Staining with isotypes (not blocked)	2652.00 ± 262.58 b	1721.00 ± 407.22 a	2.27 ± 1.53 ab	1.46 ± 0.60 ab
Mouse Seroblock FcR (1 µg)	2862.80 ± 386.99 b	1744.40 ± 467.74 a	2.22 ± 1.44 ab	1.66 ± 1.03 a
Mouse Seroblock FcR (5 µg)	2983.25 ± 525.21 b	1666.40 ± 406.44 a	2.38 ± 1.83 a	1.30 ± 0.36 ab
Purified mouse IgG (0.25 µg)	2484.60 ± 245.97 b	1500.60 ± 211.13 a	2.18 ± 1.46 ab	1.37 ± 0.34 ab
Purified mouse IgG (0.75 µg)	3282.40 ± 807.10 ab	1868.40 ± 586.26 a	1.04 ± 0.53 bc	0.81 ± 0.27 b
Purified mouse IgG (1.25 µg)	4135.20 ± 876.82 a	1737.40 ± 251.27 a	0.78 ± 0.25 c	0.88 ± 0.27 b

Means followed by different letters in the same column differ significantly ($p < 0.05$).

Nonspecific staining for both IgG2 isotypes, but especially the IgG2b isotype, was observed in mononuclear cells and lightly in granulocytes (Supplementary information 4), however, all the results obtained in this study focus on mononuclear cells and all the analyses were performed in the mononuclear cell gate.

3.2. Experiment 2: efficiency of FcR blocking reagents for IgG2 isotypes

Mouse Seroblock reagent and bovine serum were not able to block nonspecific interactions between the IgG2 isotypes and FcRs of bovine mononuclear cells, since no significant differences were observed for percentage of positive cells between the not blocked stained samples and the samples blocked with either of these reagents (Fig. 1 and Tables 1 and 2).

However, purified mouse IgG was able to efficiently block FcR interactions with the IgG2b isotype, since a significant decrease in the percentage of positive cells was observed for the highest mouse IgG dilution (1.25 µg) compared to the stained group that was not blocked (Fig. 1 and Table 1), besides, this blocking treatment presented no significant difference of percentage of positive cells compared to unstained group. Regarding the IgG2a isotype, despite the absence of a significant difference in the percentage of positive cells between the stained group not blocked and the stained groups previously blocked with mouse IgG, the p values were close to 0.05 ($p = 0.0505$ and $p = 0.0770$ for groups blocked with 0.75 and 1.25 µg of mouse igG, respectively) (Fig. 1 and Table 1).

3.3. Experiment 3: interference of mouse IgG with the staining of specific monoclonal antibodies

There was no significant difference for the mean fluorescence intensity or percentage of positive cells between the samples previously blocked with 1.25 µg of mouse IgG or not blocked, and then stained with two different panels of specific monoclonal antibodies (Supplementary information 5 and 6), except for anti-CD335, for which the percentage of positive cells was significantly higher ($p = 0.024$) in group not blocked than the blocked group (Table 3).

4. Discussion

The nonspecific interactions of mouse mAbs in bovine mononuclear cells were evaluated in the present study, wherein the efficiency of three different blocking reagents was also tested. We found that IgG2a/AF647 and IgG2b/RPE-Cy5 isotypes, but not IgG1 (conjugated with FITC, AF488 or RPE), presented nonspecific binding to bovine leukocytes. Our results did not corroborate the results of a previous study using human mononuclear cells, in which strong nonspecific binding of IgG1/AF647 and IgG2a/FITC isotypes but not IgG2b/PE was observed (Andersen et al., 2016). These differences might be due to differences in the conjugated fluorophores or to FcR diversity between human and bovine cells. Cy5 based mAbs are known to bind nonspecifically to FcγRI receptors, however, since commercial blocking reagents specifically recognise epitopes on the extracellular domain of the FcγRIII and FcγRII receptors, they are inefficient at suppressing the binding of Cy5 conjugates to FcγRI (Jahrsdörfer et al., 2005). In our experiment, we observed results consistent with this statement, since Mouse Seroblock reagent did not efficiently block the nonspecific interactions of both IgG2 isotypes. Besides, Mouse Seroblock recognises mouse CD16 and CD32, which may not cross-react with respective homolog receptors of bovine specie. The incomplete block of nonspecific binding of antibodies to monocytes by these commercial blocking reagents has also reported by other studies (Biburger et al., 2015; Kuonen et al., 2010).

Serum is another blocking reagent frequently used in immunophenotyping studies, however, it has been reported that the IgG concentration in serum might be too low for efficient blocking (Stewart and Stewart, 2001). Our findings corroborate this statement; despite the lower percentage of positive cells observed in the groups previously blocked with this reagent, the means were not significantly different to the stained groups that were not blocked for both IgG2 isotypes.

For the IgG2b isotype, the quantity of 1.25 µg of mouse IgG as a blocking reagent presented the best results, since only this treatment presented percentage of positive cells value significantly different to the

Table 2
Means of median fluorescence intensity (MFI) (± SD) values and percentage of positive cells obtained for mononuclear gated cells unstained and stained with each isotype control mAb, previously treated or not treated with 50% or 100% bovine serum.

	MFI (± SD)		% Positive cells	
	IgG2b RPECy5	IgG2a AF647	IgG2b RPECy5	IgG2a AF647
Reference tube Not blocked (no isotype controls)	1270.80 ± 130.52 b	932.60 ± 10.69 b	0.2 ± 0.09 c	0.12 ± 0.04 b
Staining with isotypes (not blocked)	2220.40 ± 110.57 a	1050.40 ± 41.50 a	3.13 ± 0.76 a	1.11 ± 0.35 a
50 % bovine serum	2067.40 ± 189.94 a	1076.00 ± 24.11 a	2.07 ± 0.46 b	0.83 ± 0.23 a
100 % bovine serum	2158.20 ± 304.86 a	1051.80 ± 30.67 a	2.74 ± 0.62 a	0.90 ± 0.22 a

Means followed by different letters in the same column differ significantly ($P < 0.05$).

Table 3
Means of median of fluorescence intensity (± SD) values and percentage of positive cells obtained for mononuclear cells stained with specific monoclonal antibodies (panel 1 and panel 2), previously blocked or not blocked with 1.25 μg of purified mouse IgG.

	MFI (± SD)		% of positive cells	
	Not blocked	Blocked	Not blocked	Blocked
CD172a	6841.80 ± 2038.48 a	6878.80 ± 1382.25 a	7.54 ± 1.52 a	6.60 ± 1.70 a
CD335	4060.00 ± 708.77 a	3953.40 ± 376.12 a	1.67 ± 1.06 a	1.89 ± 1.18 b
CD21	4420.20 ± 1000.27 a	3937.40 ± 897.13 a	38.44 ± 6.06 a	37.12 ± 7.46 a
CD8β	4063.00 ± 1084.50 a	1559.20 ± 31512 b	6.86 ± 1.32 a	6.33 ± 1.88 a
CD4	33500.60 ± 9855.14 a	30332.20 ± 9384.31 b	14.16 ± 2.12 a	14.18 ± 2.32 a

Means followed by different letters in the same line for the same parameter (MFI or % of positive cells) differ significantly ($P < 0.05$).

stained group that was not blocked, and reached the same value of unstained group. However, there were no significant differences between the IgG2a AF647 stained group that was not blocked compared to all the groups blocked with mouse IgG, the absence of significant difference might be due to a lowe level of nonspecific staining observed by this isotype compared to IgG2b. In view of these results, the quantity of 1.25 μg of mouse IgG was selected as the most efficient method to avoid nonspecific interactions mediated by FcR. Similar results have also been reported for human monocytes and macrophages (Andersen et al., 2016). Therefore, this protocol was tested in Experiment 3 to evaluate the interference of blocking with mouse IgG with specific monoclonal antibodies staining. The percentage of CD335 positive cells was significantly higher in group that was not blocked than the group previously blocked with mouse IgG, highlighting the importance of blocking with this reagent before bovine cell immunophenotyping to avoid erroneous results.

5. Conclusion

Our results highlight the importance of using Fc blocking reagents in flow cytometry experiments and, due to the lack of commercial blocking reagents for bovine cells, demonstrate the effectiveness of purified mouse IgG as a blocking reagent.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetimm.2019.109889.

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