



Application and effects of mouse Foxp3 antibody and fixation/permeabilization buffer on the detection of CD4⁺ regulatory T cells in various mammal species

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ABSTRACT. CD4⁺ regulatory T lymphocytes (Treg cells) play a crucial role in maintaining the normal immune homeostasis. Foxp3, as a key marker for Treg cells, is widely used to identify Treg cells, not only in humans but also in other species, like mouse, porcine, ovine, and bovine. To detect reproducible Treg cells is important for evaluating the state of the immune system, and thus, it is necessary to optimize Foxp3 staining. Here, we present a comparative study of MF23 and FJK-16s clones of anti-mouse Foxp3 antibodies, used in combination with two different fixation/permeabilization buffers. For Foxp3 staining, the fixation/permeabilization buffer and Foxp3 antibody FJK-16s clone from eBioscience were better than those from BD Pharmingen, with the best fluorochrome PE. Moreover, when using the best combination, there was a highly significant positive correlation between CD25⁺ T cells

and CD25⁺Foxp3⁺ T cells. Therefore, the CD25 marker can be used as an alternative to the Foxp3 antibody. As FJK-16s is also applicable for detecting bovine, porcine, canine, ovine, and equine Foxp3 antibodies, these results will be helpful not only in quantifying the frequencies of mouse Treg cells, but also in accurately detecting Treg cells of the other species mentioned above by multicolor flow cytometry.

Key words: Foxp3; FJK-16s; MF23; CD4⁺ regulatory T lymphocytes; CD25

INTRODUCTION

CD4⁺ regulatory T lymphocytes (Treg cells) play a central and nonredundant role in the control of immune responses to self and nonself antigens (Sakaguchi et al., 2008). The best-characterized regulatory T cell populations are different from the other populations in mouse by their expressions of CD4 and high levels of CD25 (IL-2-receptor α -chain), and the forkhead/winged helix transcription factor called Foxp3 (Brunkow et al., 2001), which is a key regulatory protein for their development and function (Hori et al., 2003) and the definitive marker for mouse Treg cells (Fazekas de St Groth et al., 2011). Therefore, mouse Treg cells are often identified by panels of markers CD3⁺CD4⁺CD25⁺Foxp3⁺ (Hodgson et al., 2011).

Several publications noted concerns about the influence of different clones of Foxp3 antibodies coupled to different fluorochromes and paired with different fixation/permeabilization buffers on the staining of human Treg cells (Tran et al., 2007; Pillai and Karandikar, 2008; Grant et al., 2009; Law et al., 2009; Presicce et al., 2010). However, the variables influencing the staining of Treg cells in other mammal species have not been reported. Because there are no commercial specific Foxp3 antibodies for cattle, cat, pig, sheep, dog, and horse Treg cells, the antibodies for their homologous species are usually used in the studies, like mouse Foxp3 antibodies. In current studies, although several anti-mouse Foxp3 clones and corresponding buffer sets can be obtained, the FJK-16s clone from eBioscience (San Diego, CA, USA) is the most frequently used in published studies (Tsang et al., 2006; Izcue et al., 2008; Prochazkova et al., 2009; Janikashvili et al., 2011; Lei et al., 2011). Here, we chose C57BL/6 mice as a model, and compared two kinds of Foxp3 clones: MF23 from BD Pharmingen (San Diego, CA, USA) and FJK-16s. MF23 recognizes an epitope between the 1 and 87 amino acids in the N-terminal domain of the mouse Foxp3 protein, whereas FJK-16s maps the epitope to amino acids 75 and 125 of the mouse, rat, bovine (Gerner et al., 2010), porcine (Bolzer et al., 2009), canine (Pinheiro et al., 2011), ovine (Rocchi et al., 2011), and equine (Robbin et al., 2011) Foxp3 protein. Simultaneously, the Foxp3 buffer sets from the two vendors above were used in our system. Our results showed that the variability of the staining of mouse Treg cells depended not only on the fixation/permeabilization buffer, but also on the clone of anti-mouse Foxp3 antibody and the fluorochrome used.

MATERIAL AND METHODS

Cells

Peripheral blood mononuclear cells (PBMCs) and splenocytes were isolated from

C57BL/6J mice and filtered through 40- μ m nylon cell strainers. For PBMCs, red blood cells were lysed with lysing buffer (BD Pharmingen). Spleen lymphocytes were separated in EZ-Sep™ mouse lymphocyte separation medium (Dakewe, Beijing, China). The cells were maintained in phosphate-buffered saline (PBS) containing 1% fetal calf serum (FCS). The study was approved by the Ethics Committee of the National Center for Clinical Laboratories.

Antibodies

The antibodies to surface markers consisted of the following antibodies: anti-mouse CD3-PerCP-Cy5.5 (clone 145-2C11), anti-mouse CD4-FITC (clone RM4-5), anti-mouse CD25-PE (clone PC61), anti-mouse CD25-APC (clone PC61), and their isotype controls (all from BD Pharmingen). The antibodies to intracellular markers consisted of the following antibodies: anti-mouse Foxp3-Alexa Fluor 647 (clone MF23) and its isotype, anti-mouse Foxp3-PE (clone MF23) and its isotype (all from BD Pharmingen), and anti-mouse Foxp3-PE (clone FJK-16s) and its isotype (both from eBioscience). Each antibody was added at the recommended volume (1 μ g/ 10^6 cells). One million cells were used for each test.

Immunofluorescence staining of cell surface antigens

Cell surface antibodies (anti-mouse CD3, CD4, CD25 coupled to corresponding fluorochromes and their isotype controls) were added to each sample, incubated for 20 min at room temperature, and washed with 1 mL PBS containing 1% FCS.

Fixation/permeabilization and Foxp3 staining

Foxp3 staining buffer set from eBioscience

After surface staining, 1 mL freshly prepared fixation/permeabilization buffer was added to each sample, mixed well, and incubated for 30 min at 4°C in the dark. Cells were washed once with 2 mL permeabilization buffer. Foxp3 antibodies were used to stain intracellular markers, washed twice with 2 mL permeabilization buffer, and then washed once with 1 mL PBS containing 1% FCS.

Mouse Foxp3 buffer set from BD Pharmingen

After surface staining, 2 mL freshly prepared cold mouse Foxp3 fixation buffer was added to each sample, mixed well, and incubated for 30 min at 4°C in the dark. Cells were washed once with 2 mL permeabilization buffer, incubated in 2 mL permeabilization buffer for 30 min at 37°C, and washed once with 2 mL PBS containing 1% FCS. Foxp3 antibodies were then added. A 30-min incubation at room temperature in the dark was followed by two washes with 2 mL PBS containing 1% FCS.

Compensation

Compensation controls were created for each fluorochrome in the Treg detection. Compensation tubes were checked to ensure that each stain was the brightest in its own channel.

Flow cytometry data collection and analysis

Flow cytometry was performed with a FACSCalibur flow cytometer and the data were evaluated by the BD CellQuest analysis software (BD Bioscience, San Diego, CA, USA). The instrument setup was standardized to reduce experiment-to-experiment variation. Before each experiment, the instrument was calibrated by using BD CaliBRITE 3 Beads and BD CaliBRITE APC Beads (BD Biosciences) according to the manufacturer instructions. Lymphocytes were gated based on their side scatter and forward scatter (SSC/FSC) characteristics. At least 30,000 gated lymphocytes were collected for each sample. CD3⁺CD4⁺ cells were gated within the lymphocyte gate. The CD25⁺Foxp3⁺ gate was set based on their isotypes. Treg cells were gated as CD25⁺Foxp3⁺ events as a percentage of CD3⁺CD4⁺ cells.

Statistical analysis

Results were analyzed using the GraphPad Prism 5 software (GraphPad, La Jolla, CA, USA) and statistical analyses were performed using the paired two-tailed Student *t*-test and correlation analysis. Statistical significance was defined as $P < 0.05$, $P < 0.01$, and $P < 0.001$.

RESULTS

This study first compared two clones of anti-mouse Foxp3 antibodies and two fixation/permeabilization buffers from BD Pharmingen and eBioscience, using PBMCs and spleen lymphocytes from five mice. Then, the MF23 clones with two different fluorescences were also compared, when treated with two different fixation/permeabilization buffers. Next, the correlation between CD25⁺ T cells and CD25⁺Foxp3⁺ T cells was analyzed. Finally, we identified the alignments of Foxp3 amino acid residues 1-87 and 75-125 between mouse and eight other species.

Comparison of fixation/permeabilization buffers

In this experiment, the FJK-16s clone was used with two fixation/permeabilization buffers, respectively. Each buffer was used according to the manufacturer instructions. We observed that fixation/permeabilization buffers affected the SSC/FSC characteristics of the cells (Figure 1, row 1), and this result was identical to the staining results of human Treg cell (Hodgson et al., 2011). The CD3 staining was consistent, with 23 to 37% of CD3⁺ events in the lymphocyte gate of PBMCs and 22 to 29% in the spleen lymphocyte gate (Figure 1, row 2). Moreover, the CD4 staining was also consistent, with 48 to 58% of CD4⁺ events in the CD3⁺ lymphocyte gate of PBMCs or spleen (Figure 1, row 2). For CD25 staining, the staining percentages in the CD3⁺CD4⁺ lymphocyte gate of PBMCs and spleen were 4 to 10% and 7 to 13%, respectively (Figure 1, row 3). On the contrary, the staining levels of Foxp3⁺ cells in CD4⁺ T cells with the Foxp3 buffer set from BD Pharmingen were lower than with the Foxp3 staining buffer set from eBioscience, in PBMCs or spleens ($P < 0.01$) (Figure 1, row 3).

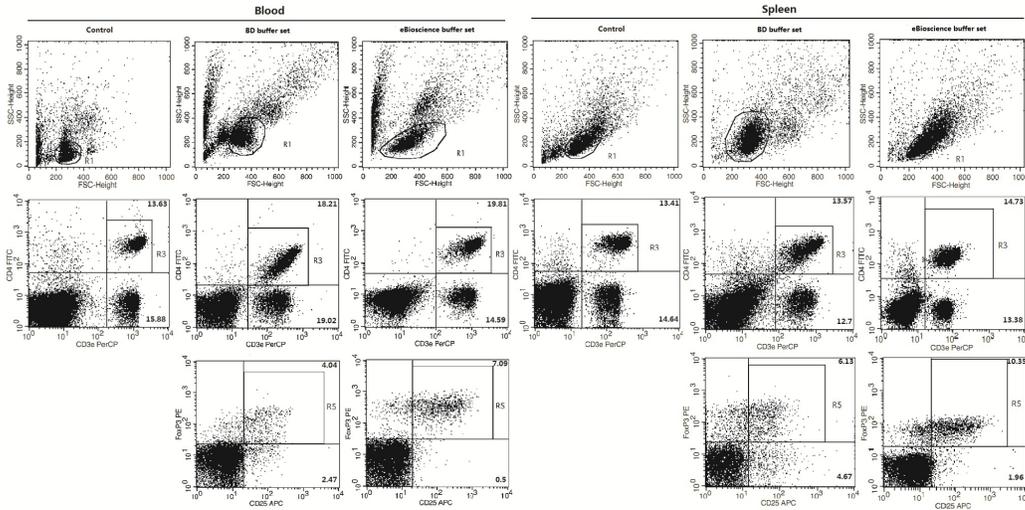


Figure 1. Comparison of fixation/permeabilization buffers. The Foxp3 antibodies used here were FJK-16s from eBioscience. The R1 plots are lymphocyte populations, gated based on SSC/FSC characteristics. The R3 gates are CD3⁺CD4⁺ cells, gated based on their isotypes, and the displayed numbers are CD3⁺CD4⁺ (above) and CD3⁺CD4⁻ events (below) expressed as percentages of lymphocytes (R1). Then CD25 and Foxp3 gates were set based on their isotypes, and the displayed numbers are CD25⁺Foxp3⁺ (above) and CD25⁺Foxp3⁻ events (below) expressed as percentages of CD4⁺ T cells. Columns 1 and 4 = control (no fixation/permeabilization); columns 2 and 5 = mouse Foxp3 buffer set from BD Pharmingen; columns 3 and 6 = Foxp3 staining buffer set from eBioscience. Columns 1-3 = PBMCs; columns 4-6 = spleen lymphocytes.

In PBMCs, compared with the control and with the eBioscience Foxp3 staining buffer set treatment, the signal-to-noise ratio (SNR) for CD3-PerCP-Cy5.5 with the BD Pharmingen Foxp3 buffer set treatment decreased markedly ($P < 0.01$) (Figure 2, column 1). On the contrary, in spleens, the SNR rose significantly, especially compared with the eBioscience Foxp3 staining buffer set treatment (Figure 2, column 1). Furthermore, compared with the control, the SNR also decreased significantly with the eBioscience Foxp3 staining buffer set treatment in the spleen lymphocytes ($P < 0.001$), but not in PBMCs ($P = 0.1096$) (Figure 2, column 1). In PBMCs, the SNR for CD4-FITC with both Foxp3 buffer sets treatments was lower than the control ($P < 0.05$), but was not different between each other (Figure 2, column 2). However, in the spleen lymphocytes, only the SNR for CD4-FITC with the eBioscience Foxp3 staining buffer set treatment was lower than the control ($P = 0.0307$), but it did not decrease significantly compared with the BD Pharmingen Foxp3 buffer set treatment ($P = 0.2478$) (Figure 2, column 2). For Foxp3-PE staining, compared with the BD Pharmingen Foxp3 buffer set treatment, the SNR with the eBioscience Foxp3 staining buffer set treatment was higher whether in spleen lymphocytes ($P < 0.0001$) or in PBMCs ($P = 0.0047$) (Figure 2, column 3).

Comparison of mouse Foxp3 antibodies

In this set of experiments, two clones of anti-Foxp3 antibodies were used in combination with the two Foxp3 buffer sets previously described, respectively (Figure 3). By using a CD25⁺Foxp3⁺ gate based on their isotype controls for CD25 staining and on CD3⁺CD4⁺CD25-

T cells for the Foxp3 staining, the percentages of the Foxp3⁺ cells in CD3⁺CD4⁺ T cells were compared across the different staining conditions. Compared with the eBioscience Foxp3 buffer set treatment, the two clones combined with the BD Pharmingen Foxp3 buffer set treatment all yielded lower staining (Figure 3, row 1). Moreover, in the same fixation/permeabilization conditions, compared with another two Foxp3 antibodies, the best staining was obtained using the FJK-16s clone from eBioscience (Figure 3, column 3). Furthermore, for the MF23 clone, the Foxp3 staining obtained with Foxp3-PE from BD Pharmingen was lower than with Foxp3-Alexa Fluor 647, regardless of the buffer used (Figure 3, columns 1 and 2), but their SNRs were not different from each other (data not shown). Moreover, the SNR for Foxp3-PE from eBioscience was better than that from BD Pharmingen, regardless of the buffer used ($P < 0.05$). Therefore, the best antibody-buffer combination was the FJK-16s clone from eBioscience used with eBioscience Foxp3 staining buffer set (Figure 3, row 2, column 3).

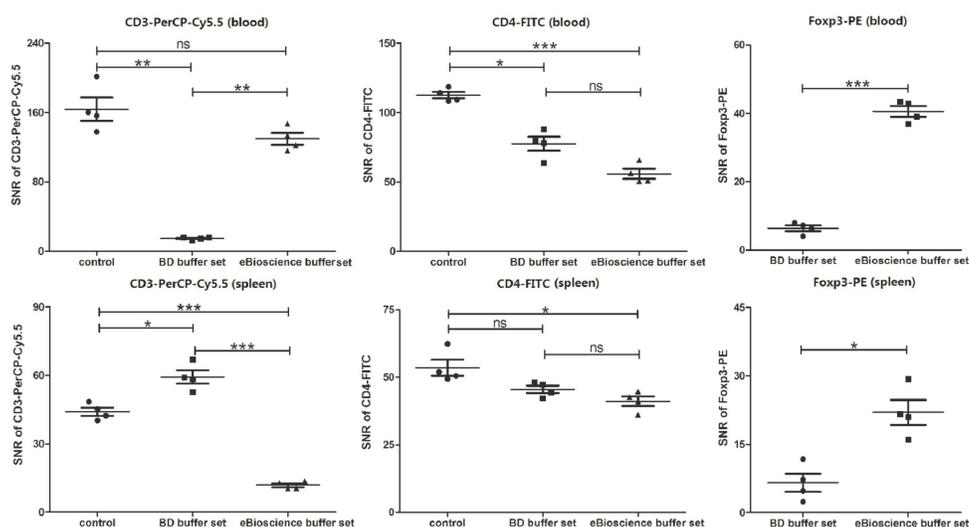


Figure 2. Comparison of the signal-to-noise ratio (SNR) for different fluorochromes after different fixation/permeabilization buffer treatment. Fresh PBMCs and spleen lymphocytes from 4 mice were used in the same fixation/permeabilization conditions and the FJK-16s clone was used here. The gating strategy was the same as described in Figure 1. Row 1 = PBMCs; Row 2 = spleen lymphocytes. Control = no fixation/permeabilization; BD buffer set = mouse Foxp3 buffer set from BD Pharmingen; eBioscience buffer set = Foxp3 staining buffer set from eBioscience. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns, $P > 0.05$.

Correlation analysis between CD25⁺ and Foxp3⁺ T cells

CD25 and Foxp3 are thought to be the key markers of mouse CD4⁺ Treg cells, which are different from the markers of human CD4⁺ Treg cells (Law et al., 2009). To determine whether there was a correlation between CD25⁺ T cells and Foxp3⁺ T cells, we compared their numbers and percentages in CD4⁺ T cells of murine PBMCs, respectively. As demonstrated in Figure 4A, the numbers of CD25⁺ T cells were strongly correlated with the Foxp3⁺ T cells ($r = 0.996$). A practically identical correlation was also found when the percentages of CD25⁺ T cells and Foxp3⁺ T cells were compared ($r = 0.993$) (Figure 4B).

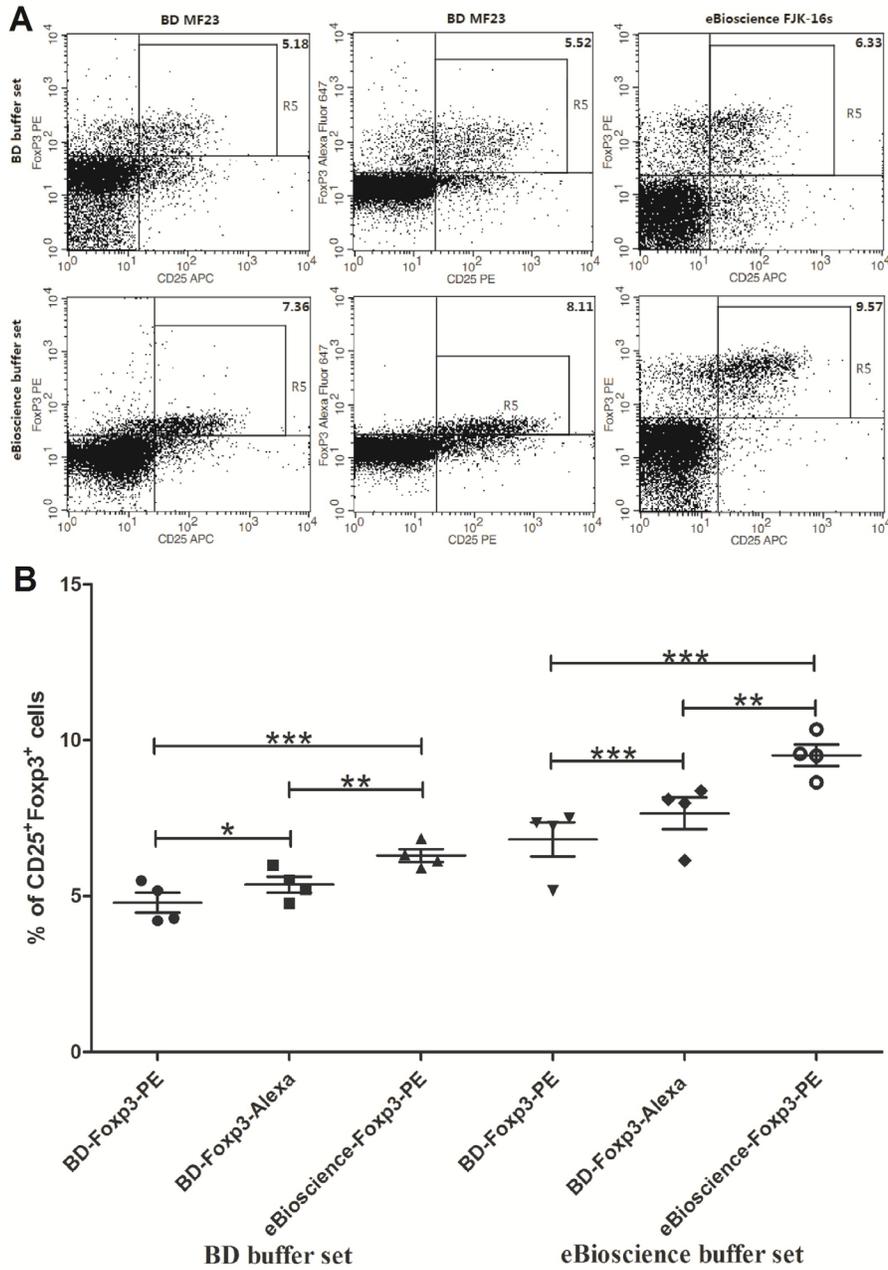


Figure 3. Comparison of mouse Foxp3 antibodies. Fresh spleen lymphocytes from 4 mice were used in the same fixation/permeabilization conditions and mouse Foxp3 antibodies were used here. The gating strategy was the same as described in Figure 1. **A.** Row 1 = treated with Foxp3 buffer set from BD Pharmingen; row 2 = treated with Foxp3 staining buffer set from eBioscience. Column 1 = MF23 clone with PE; column 2 = MF23 clone with Alexa Fluor 647; column 3 = FJK-16s clone with PE. **B.** Percentages of CD25⁺Foxp3⁺ events in CD4⁺ T cells. *P < 0.05; **P < 0.01; ***P < 0.001.

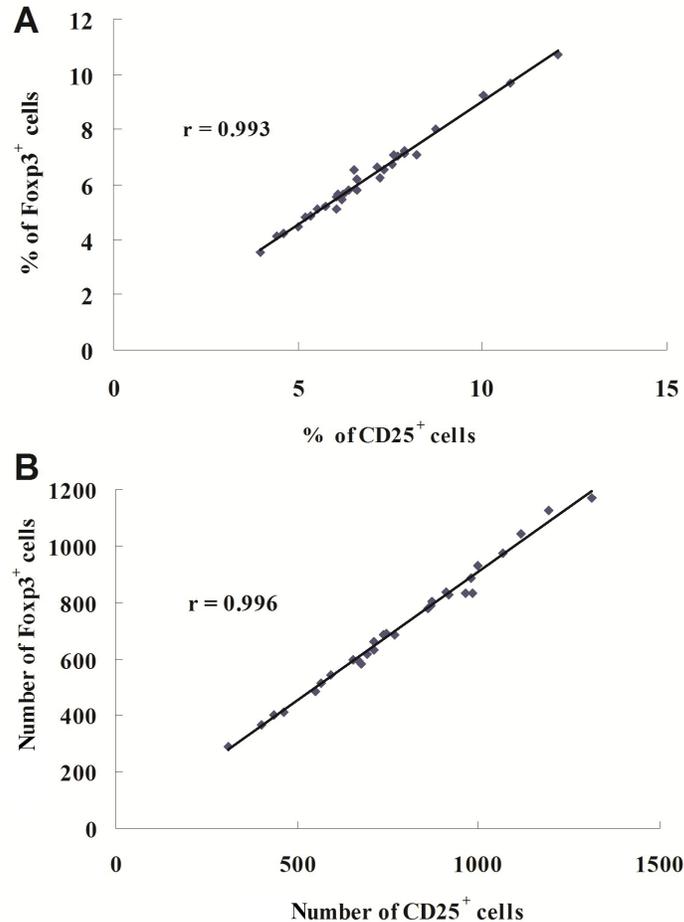


Figure 4. Correlate analysis of CD25⁺ and CD25⁺Foxp3⁺ T cells. Fresh PBMCs were treated with eBioscience Foxp3 buffer set, and the Foxp3-PE antibodies or its isotype from eBioscience were added according to the manufacturer instruction. The gating strategy was the same as described in Figure 1. **A.** Correlation (r) between the numbers of CD25⁺ and Foxp3⁺ T cells. **B.** Correlation (r) between the percentages of CD25⁺ and Foxp3⁺ T cells.

Alignments of amino acid sequences

The FJK-16s clone recognizes the 75-125 amino acids of murine Foxp3, whereas the MF23 clone recognizes the 1-87 amino acids. Here, we used DNAMAN 6.0 to calculate the pairwise p -distances. Except for human Foxp3, the homology of the 75-125 amino acid sequences of murine Foxp3 to the known orthologs from seven other mammal species was higher than that of the 1-87 amino acid sequences, and the greatest homology was found with Norway rat, followed by cattle, horse, pig, and sheep (Table 1 and Figure 5). The results predict that, when used in the detection of Foxp3 in rat, cattle, horse, pig, sheep, pig, and cat, the FJK-16s clone is superior to the MF23 clone.

Table 1. Sequence alignment of Foxp3 amino residues 1-87 and 75-125 between murine and other mammalian species.

Species ^a	1-87 amino acids ^b	75-125 amino acids ^b
Norway rat	95.402	96.078
Cattle	80.460	94.118
Dog	81.609	90.196
Horse	81.609	94.118
Pig	73.563	94.118
Sheep	79.301	94.118
Cat	78.161	92.157
Human	63.218	23.529

^aAccession Nos. for protein sequences are given in parentheses: mouse = *Mus musculus* (NP_473380). Norway rat = *Rattus norvegicus* (NP_001101720); cattle = *Bos taurus* (NP_001039398); dog = *Canis familiaris* (NP_001161933); horse = *Equus caballus* (NP_001156744); pig = *Sus scrofa* (NP_001121910); sheep = *ovis aries* (NP_001138419.1); cat = *Felis catus* (NP_001077421); human = *Homo sapiens* (NP_001107849). ^bFoxp3 amino acid residues 1-87 and 75-125 represent the binding regions of the MF23 and the FJK-16s monoclonal antibodies, respectively. Sequence alignments were obtained from pairwise p-distances computed with DNAMAN 6.0.

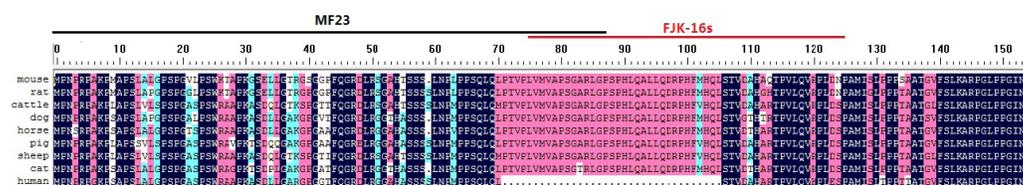


Figure 5. Sequence alignment of Foxp3 amino acid residues 1–125 from various species. For scientific names and accession numbers of mammalian species examined in this study please refer to Table 1.

DISCUSSION

The best flow cytometry staining panels to identify human Treg cells has been published by Law et al. (2009), but no study has determined the best combination of antibody and fixation/permeabilization buffer for the staining of Treg cells in PBMCs and spleen lymphocytes in other mammal species. Therefore, we chose the mouse as a model, and compared two clones of anti-mouse Foxp3 antibodies and two Foxp3 staining buffer sets, which were the most frequently used in published studies in various species (Tsang et al., 2006; Izcue et al., 2008; Bolzer et al., 2009; Prochazkova et al., 2009; Gerner et al., 2010; Janikashvili et al., 2011; Lei et al., 2011; Pinheiro et al., 2011; Robbin et al., 2011; Rocchi et al., 2011).

Although they all identified a population of CD4⁺CD25⁺Foxp3⁺ T cells, the Foxp3 staining varied strikingly depending on the antibody or the fixation/permeabilization buffer used. Our results indicated that the fixation/permeabilization buffers had the most important influence on the quantity of mouse Treg cells. Notably, the clone of anti-mouse Foxp3 antibodies and fluorochromes also influenced the quality of Foxp3 staining. We observed that the FJK-16s clone coupled to PE yielded statistically higher levels of Foxp3 cells and clearer separation of Foxp3⁺ and Foxp3⁻ populations than the other antibodies. Our findings highlighted that it was necessary to use the same staining conditions during the course of a particular study; for example, when measuring intergroup or intragroup variations over time. The alignment results demonstrated that the homology of Foxp3 75-125 amino acids between mouse and other mammal species was greater than that of Foxp3 1-87 amino acids, and therefore,

the FJK-16s clone is also more appropriate for the detection of bovine, porcine, canine, ovine, equine, and feline Foxp3.

However, the CD25 staining did not depend on the staining assay for mouse Treg cells, which was different from the staining of human CD25 (Shevach, 2001; Lühn et al., 2007). Furthermore, regardless of the percentage or the number, there was a highly significant positive correlation between CD25⁺ T cells and CD25⁺Foxp3⁺ T cells using the Foxp3-PE antibodies from eBioscience combined with its own Foxp3 staining buffer set. Therefore, the CD25 marker can be used as an alternative to the Foxp3 antibody in the detection of mouse Treg cells when using the combination mentioned above.

In brief, the present study aids in the optimization of flow cytometry staining panels for the identification of Treg cells in various mammal species, and highlights the importance of the choice of the antibody, fluorochrome, and fixation/permeabilization buffer to achieve optimal results, which should greatly facilitate comparative studies between these species and humans, and is helpful for future studies of human diseases based on these mammal models. Moreover, the results from this study will assist future work to characterize the function of the Treg cells in these animals, which is useful for addressing questions relevant to animal health.

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