

Foxp3 Expression in Bovine CD8⁺ T Cells Is Associated with the Intensity of CD25 Expression

Tomasz MAŚLANKA^{1)*} and Jerzy Jan JAROSZEWSKI¹⁾

¹⁾Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, University of Warmia and Mazury, Oczapowski Street 13, Olsztyn 10-718, Poland

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ABSTRACT. The aim of the undertaken research was to determine whether Foxp3⁺CD25⁺CD8⁺ cells exist in cattle and whether Foxp3 expression in CD8⁺ T cells is correlated with the intensity of CD25 expression. It has been found that 0.66 and 2.36% of CD8⁺ cells on average showed high and low expression of the CD25 molecule, respectively. On average, 11.61% of CD25^{high}CD8⁺ cells expressed Foxp3, while the mean percentage of Foxp3⁺ cells within CD25^{low}CD8⁺ cells was 3.66%. The absolute count of Foxp3⁺CD25^{high}CD8⁺ cells was not significantly different from the absolute count of Foxp3⁺CD25^{low}CD8⁺ cells. The obtained results indicate that CD8⁺ cells with the regulatory phenotype, i.e. Foxp3⁺CD25^{high}CD8⁺ and Foxp3⁺CD25^{low}CD8⁺ cells, occur naturally in bovine peripheral blood, although both of these subpopulations are relatively small. There is a positive correlation between the intensity of CD25 expression and the expression of the transcription Foxp3 factor in bovine CD8⁺ cells.

KEY WORDS: bovine, CD8, CD25, Foxp3, regulatory.

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Regulatory T (Treg) cells (Tregs) are heterogeneous with sub-populations which differ in their phenotype, immune inhibitory mechanisms and function. These cells are responsible for the regulation of immune response and play a leading role in developing immune tolerance through active suppression. Functions of regulatory T cells include: prevention of autoimmune diseases by maintaining self-tolerance, oral tolerance and suppression of allergy and pathogen-induced immunopathology [4, 10, 13, 14]. There are two general types of the regulatory lymphocytes: natural cells, which develop in the thymus, and induced cells, which are derived from naive lymphocytes in the periphery. Regulatory T cells consist of heterogeneous subsets which include CD4⁺ cells, CD8⁺ cells and $\gamma\delta$ ⁺ cells. The first two types of cells can be subdivided on the basis of phenotypic or functional criteria into several subsets (e.g. Foxp3⁺CD25⁺CD4⁺, Tr1, Th3, Foxp3⁺CD25⁺CD8⁺, CD122⁺CD8⁺ and CD28⁺CD8⁺ cells [4, 10, 13, 14]). Currently, naturally-occurring CD25⁺CD4⁺ Treg cells (nTregs) represent a major lymphocyte population maintaining dominant self-tolerance and controlling a variety of pathological immune responses. However, CD25 is not a specific marker for Tregs, because it is also expressed by activated T cells. To address this problem, scientists have been searching for marker molecules to discriminate between activated and regulatory T cells within the population of CD25-expressing CD4⁺ cells. In 2003, three independent groups identified the Forkhead Box P3 protein (Foxp3) as

a unique marker and a “master” regulator of the development and suppressive function of CD25⁺CD4⁺ Treg cells [5, 8, 9]. Foxp3 currently represents the most specific marker used to distinguish regulatory cells (Foxp3⁺CD25⁺CD4⁺) from activated effector cells (Foxp3⁺CD25⁺CD4⁺) within the CD25⁺CD4⁺ cell subpopulation. Interestingly, Hoek *et al.* [7] demonstrated that bovine WC1.1⁺ and WC1.2⁺ $\gamma\delta$ cells rather than Foxp3⁺CD25^{high}CD4⁺ cells act as immune regulatory cells *ex vivo*, however, the results of a study conducted by Seo *et al.* [11] showed that there are certain cells with regulatory properties among bovine CD4⁺ and CD8⁺ cells.

Although CD4⁺ regulatory T cells are the best-studied and characterized regulatory lymphocytes, in recent years, substantial progress has been made in the phenotypic and functional characterization of CD8⁺ T regulatory cells. Foxp3⁺CD25⁺CD8⁺ cells are one of the better-described regulatory subsets in the CD8⁺ T cell population. Although there are much more data regarding inducible Foxp3⁺CD25⁺CD8⁺ Tregs [10, 13], the existence of naturally-occurring regulatory cells with this phenotype has also been demonstrated [1–3]. Cosmi *et al.* [3] revealed the existence of human regulatory thymocytes being CD25⁺CD8⁺ cells sharing the phenotype, functions and mechanisms of action with CD25⁺CD4⁺ human Tregs. CD25⁺CD8⁺ thymocytes exhibited poor, if any, proliferation in response to allogeneic stimulation and suppressed in a dose-dependent fashion the allogeneic response of both CD25⁺CD4⁺ and CD25⁺CD8⁺ autologous T cells. CD25⁺CD8⁺ thymocytes selectively expressed Foxp3 and GITR (Glucocorticoid-induced TNF-Receptor) mRNAs and exhibited significantly higher CCR8, tumor necrosis factor receptor 2 (TNFR2) and CTLA-4 mRNA expression than the CD25⁺CD8⁺ subpopulation [3]. Taking the above into consideration, it can be said that the results of research by Cosmi *et al.* [3] revealed the

*CORRESPONDENCE TO: T. MAŚLANKA, Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, University of Warmia and Mazury, Oczapowski Street 13, Olsztyn 10-718, Poland.

e-mail: tomasz.maslanka@uwm.edu.pl

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presence of naturally-occurring Foxp3⁺CD25⁺CD8⁺ regulatory T cells in the human body which resemble naturally-occurring Foxp3⁺CD25⁺CD4⁺ regulatory T cells in many respects. The results of research by Correale and Villa [2] confirmed the existence of such cells in humans and indicated that defects in their function can cause induction of autoimmune reactions, including multiple sclerosis. The presence of natural regulatory CD25⁺CD8⁺ cells expressing Foxp3 [1] has also been demonstrated in mice. Recently, it has been found [12] that cells with this phenotype occur in swine, although they have not been studied for their regulatory properties. There is a lack of data in the available literature on the occurrence of Foxp3⁺CD25⁺CD8⁺ cells in cattle. The results of research by Gerner *et al.* [6] indicate the possibility that such cells exist because these researchers demonstrated that a small Foxp3⁺ cell population was found within bovine CD8⁺ cells. It should be noted that this research did not investigate whether Foxp3 expression in CD8⁺ cells was connected with CD25 expression by these cells. The percentage of Foxp3⁺ cells within CD8⁺ cells was not studied either, but the percentage of Foxp3⁺CD8⁺ cells within the total lymphocyte population was estimated. For this, the main aims of the undertaken research were to determine: a) whether Foxp3⁺CD25⁺CD8⁺ cells existed in cattle and if yes, how numerous the subpopulation was; b) whether Foxp3 expression by bovine CD8⁺ cells was correlated with the intensity of CD25 expression by these cells.

Studies were carried out on 24 clinically-healthy heifers (Polish Black and White breed), aged 12 months, kept indoors and originating from a dairy farm located in Baldy (Poland). The animals were housed and treated in accordance with the rules approved by the Local Ethics Commission (Ethic Commission Opinion No 82/2010). Blood was drawn by venipuncture from the jugular vein into heparinized sterile vacutainer tubes [Becton Dickinson (BD) Biosciences, San Jose, CA, U.S.A.]. The following staining procedure was applied: 100 μ l of whole blood samples were transferred into individual tubes and kept in ice. Erythrocytes were removed using Red Blood Cell Lysing Buffer (RBCLB) (8.3 g/l ammonium chloride in 0.01 M Tris-HCl buffer). The samples were treated with 2 ml of RBCLB, then incubated for 7 min at 4°C, and washed twice with 2 ml of FACS buffer [FB, 1 \times Dulbecco's PBS (Sigma-Aldrich) devoid of Ca²⁺ and Mg²⁺ with 2% (v/v) heat-inactivated fetal bovine serum] by centrifugation at 300 \times g, for 5 min at 4°C (these parameters were used for all cell-washing procedures). The cells were re-suspended in FB and stained with FITC-conjugated mouse anti-bovine CD8 (1:20, CC63, IgG2a, Serotec, Oxford, U.K.) and PE-conjugated mouse anti-bovine CD25 (1:200, IL-A111, IgG1, Serotec). After a 45 min incubation (on ice and in the dark), the cells were washed in 2 ml FB and fixed by adding 100 μ l Leucoperm-Reagent A (Serotec) to each tube and then incubating them for 15 min at RT (room temperature) in the dark. After this, the cells were washed with 3 ml FB, then permeabilized by adding 100 μ l of Leucoperm-Reagent B (Serotec) and subsequently stained with AF647-conjugated human anti-bovine Foxp3 mAb (1:20, 7627, HuCAL Fab bivalent, Serotec) for

60 min at RT in the dark. Isotype control was performed as above, except that the cells were not stained for surface antigens (CD8 and CD25) and AF647-conjugated anti-Foxp3 mAb was replaced with hucal fab-dhlx-mh isotype control-AF647 (Serotec). After incubation, the cells were washed twice with 2 ml FB and analyzed by flow cytometry. Flow cytometry analysis was performed using a FACSCanto II cytometer (BD Biosciences). The data were acquired by FACSDiva version 6.1.3 software (BD Biosciences) and analyzed by FlowJo software (Tree Star Inc., Stanford, CA, U.S.A.). Cytometry setup and tracking beads (CST, BD Biosciences) were used to initialize PMT (Photomultiplier tubes) settings. Unstained control cells, as well as single stain control for every fluorochrome, were prepared and used to set up flow cytometric compensation. The entire volume of each sample (prepared from 100 μ l of whole blood) was acquired by flow cytometer. Thus, the absolute count of Foxp3⁺CD25^{high}CD8⁺ and Foxp3⁺CD25^{low}CD8⁺ cells represents the number of collected cells per sample obtained from 100 μ l of peripheral blood. FlowJo software directly converted a percentage count of a subpopulation of cells to an absolute count. Background fluorescence for Foxp3 staining (controlled by labeled isotype control) was very low. The gates for the Foxp3 expression within particular CD8⁺ cell subpopulations (CD25^{high}CD8⁺, CD25^{low}CD8⁺ and CD25⁻CD8⁺) were set so that the positive signal for isotype control staining was less than 0.2%. All data are presented as the mean \pm SEM. Student's unpaired *t*-test (GraphPad Prism 3, GraphPad Software, San Diego, CA, U.S.A.) was used to compare differences in the percentage and absolute number of Foxp3⁺CD25^{high}CD8⁺ and Foxp3⁺CD25^{low}CD8⁺ cells.

It was found that 0.66 and 2.36% of CD8⁺ on average showed high and low expression of CD25 molecule, respectively (Fig. 1); the mean percentage of CD25^{high}CD8⁺ and CD25^{low}CD8⁺ cells within the total lymphocyte population was 0.12 and 0.42%, respectively (data not shown). On average, 11.61% of CD25^{high}CD8⁺ cells expressed Foxp3, while the mean percentage of Foxp3⁺ cells within CD25^{low}CD8⁺ cells was 3.66% (Fig. 2A); the mean percentage of Foxp3⁺CD25^{high}CD8⁺ and Foxp3⁺CD25^{low}CD8⁺ cells within the total lymphocyte population was 0.0139 and 0.0154% (data not shown), respectively, and these values did not differ significantly from each other. The absolute count of Foxp3⁺CD25^{high}CD8⁺ cells was also not significantly different from the absolute count of Foxp3⁺CD25^{low}CD8⁺ cells (Fig. 2B). The mean percentage of Foxp3⁺ cells within CD25⁻CD8⁺ cells was only 0.45%, and it should be assumed that a certain part of this value could be the result of background fluorescence.

The obtained results indicate that there is a positive correlation between the expression of the transcription Foxp3 factor and the intensity of CD25 expression in bovine CD8⁺ cells. However, it should be noted that although the percentage of Foxp3⁺ cells within the CD25^{high}CD8⁺ subpopulation was over 3 times higher compared to the CD25^{low}CD8⁺ subpopulation, the percentage of CD25^{high}CD8⁺ cells was 3.5 times lower compared to the percentage of CD25^{low}CD8⁺ cells. Thus, the absolute count of Foxp3⁺CD25^{high}CD8⁺ cells

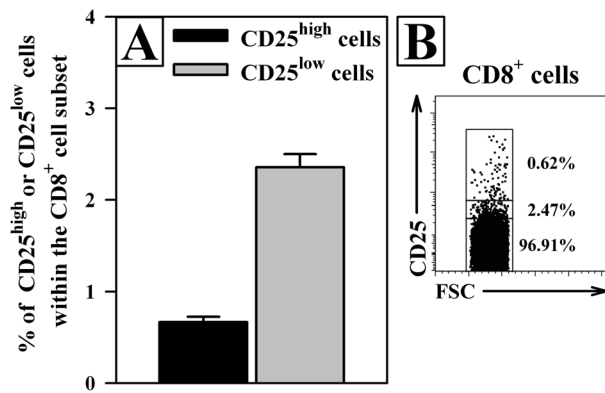


Fig. 1. CD25 expression on CD8⁺ cells. (A) Results are given as a percentage of CD8⁺ cells with high and low expression of CD25. The values represent the mean (\pm SEM) of four independent experiments with six different animals per experiment (overall $n=24$). (B) Representative cytogram showing CD25 expression on CD8⁺ cells.

could not, and was not, significantly different compared to Foxp3⁺CD25^{low}CD8⁺ cells, because the lower Foxp3 expression by CD25^{low}CD8⁺ cells was “compensated” by the higher count of these cells (compared to CD25^{high}CD8⁺ cells). Assuming that Foxp3 confers suppressive properties and is confined to regulatory T cells, the obtained results indicate that CD8⁺ cells with the regulatory phenotype, i.e. Foxp3⁺CD25^{high}CD8⁺ and Foxp3⁺CD25^{low}CD8⁺ cells, occur naturally in bovine peripheral blood, and both of these subpopulations are equal in regard to the absolute count value. It should be noted that these subpopulations are incomparably smaller compared to the naturally-occurring human regulatory Foxp3⁺CD25⁺CD8⁺ lymphocytes detected by Correale and Villa [2]. These investigators demonstrated that all CD25⁺CD8⁺ cells of human peripheral blood expressed Foxp3 and the mean percentage of Foxp3⁺CD25⁺CD8⁺ cells in human peripheral blood was ca. 3.5%. In contrast, our research found that the mean percentage of CD25⁺CD8⁺ (CD25^{high} + CD25^{low}) cells within the total lymphocyte population was just 0.54% and only a small share of these

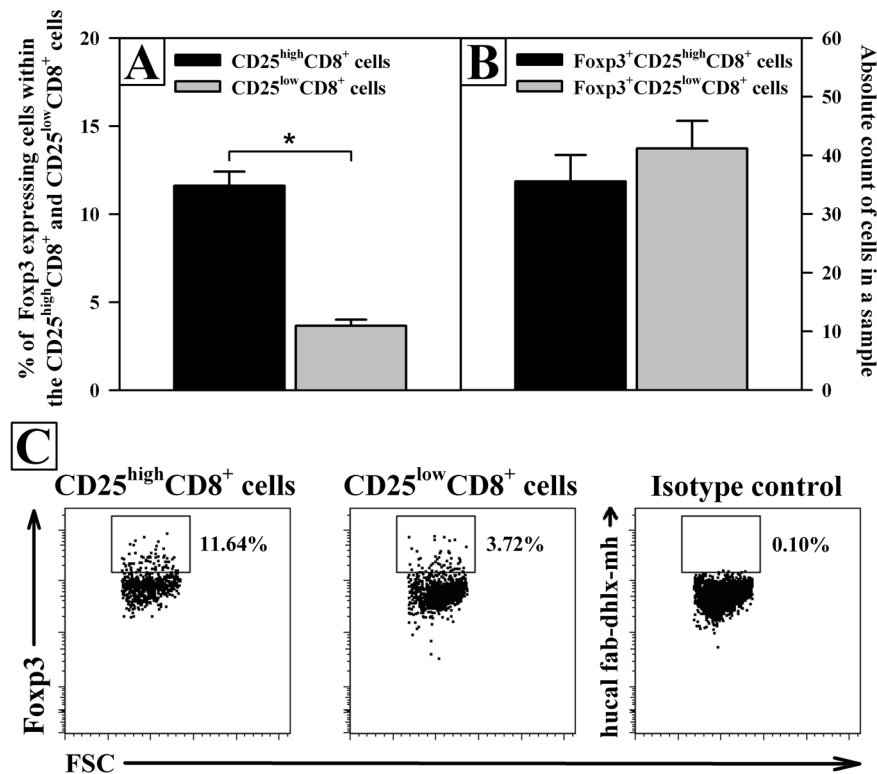


Fig. 2. Relative and absolute counts of Foxp3⁺CD25^{high}CD8⁺ and Foxp3⁺CD25^{low}CD8⁺ cells. (A) The relative count is expressed as a percentage of Foxp3⁺ cells within CD25^{high}CD8⁺ and CD25^{low}CD8⁺ cell subpopulations. (B) The absolute count represents the number of Foxp3⁺CD25^{high}CD8⁺ and Foxp3⁺CD25^{low}CD8⁺ cells per sample obtained from 100 μ l of peripheral blood. Results are the mean (\pm SEM) of four independent experiments with six different animals per experiment (overall $n=24$); * $P<0.001$. (C) Typical cytograms demonstrating Foxp3 expression within the CD25^{high}CD8⁺ and CD25^{low}CD8⁺ cells.

cells expressed Foxp3. Thus, the obtained results indicate that, in terms of quantity, bovine Foxp3⁺CD25⁺CD8⁺ peripheral blood T cells are not the counterpart of human cells with this phenotype. Although other reports on naturally-occurring Foxp3 expressing CD25⁺CD8⁺ regulatory T cells are found in literature [1, 3], there are no other data except for the research by Correale and Villa [2] on whether the whole population of these cells expresses Foxp3 or this concerns a certain percentage of CD25⁺CD8⁺ cells. Cosmi *et al.* [3] found the existence of human regulatory CD25⁺CD8⁺ thymocytes with high levels of Foxp3 expression. Furthermore, Bienvenu *et al.* [1] demonstrated the existence of a small subpopulation of Foxp3 expressing CD25⁺CD8⁺ cells with *in vitro* regulatory capacity in the thymus and peripheral lymphoid organs of mice. However, the results obtained by Cosmi *et al.* [3] and Bienvenu *et al.* [1] cannot be compared quantitatively to our data because those researchers measured the mRNA concentration of Foxp3 in purified CD25⁺CD8⁺ and CD25⁻CD8⁺ cells, while we evaluated the percentage of Foxp3 expressing cells within particular CD8⁺ T cell subsets.

We found that only a trace amount of CD25⁻CD8⁺ cells expressed Foxp3, which is in line with the results obtained by Bienvenu *et al.* [1] and Cosmi *et al.* [3], who also detected only very slight Foxp3 expression in CD25⁻CD8⁺ cells. Moreover, Correale and Villa [2] did not detect Foxp3⁺ cells at all within the CD25⁻CD8⁺ cell subset in human peripheral blood.

It should be stressed that there are no comparable data on cattle in the available literature. Only the research by Gerner *et al.* [6] addressed the issue of Foxp3 expression in bovine CD8⁺ cells, although this was not done in the context of CD25 expression. This research demonstrated that the percentage of Foxp3⁺CD8⁺ cells within the total lymphocyte population was just 0.09%, which generally speaking confirms our observations that Foxp3 expression in bovine CD8⁺ lymphocytes is very poor.

In conclusion, the conducted research revealed the presence of naturally-occurring CD8⁺ cells with the regulatory phenotype, i.e. Foxp3⁺CD25^{high}CD8⁺ and Foxp3⁺CD25^{low}CD8⁺ cells in bovine peripheral blood, although both of these subpopulations are relatively small. The regulatory properties of these cells require confirmation in a further research, particularly in functional studies. The obtained results indicate that there is a positive correlation between the intensity of CD25 expression and the expression of the transcription Foxp3 factor in bovine CD8⁺ cells.

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