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Original article

The presence of CD25 on bovine WC1$^+$ γδ T cells is positively correlated with their production of IL-10 and TGF-β, but not IFN-γ

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Abstract

WC1$^+$ cells in cattle exhibit both regulatory and effector activities. However, it has not been elucidated whether they are so plastic that both activities co-exist in one cell or there are separate subpopulations of effector and regulatory cells. Since the production of IFN-γ and IL-10 seems to be related to WC1$^+$ cells' effector and regulatory function, respectively, the main aim of this study was to determine whether those cytokines are produced by separate subpopulations of WC1$^+$, or are co-produced by the same cells. Due to increasingly frequent emphasised role of consumption of IL-2 in the mechanism of suppressor action of mouse CD25$^+$CD4$^+$ T regulatory cells, expression of the receptor's α chain for interleukin 2 (CD25) on WC1$^+$ lymphocytes has been evaluated. An average of 5.21% of WC1$^+$ cells obtained from PBMCs of 12-month-old heifers show constitutive expression of the CD25 molecule, with CD25^{high}WC1$^+$ and CD25^{low}WC1$^+$ cells accounting for 1.05% and 4.10% of WC1$^+$ lymphocytes, respectively. For detection of intracellular cytokine production, PBMCs were stimulated with concanavalin A. Both IFN-γ- and IL-10-producing cells within the CD25$^-$WC1$^+$ and CD25$^+$WC1$^+$ subpopulations were mainly separate subpopulations. The average percentage of IFN-γ$^+$IL-10$^-$, IFN-γ$^-$IL-10$^+$ and IFN-γ$^+$IL-10$^+$ cells among CD25$^-$WC1$^+$ lymphocytes was 4.03%, 2.67% and 0.51%, respectively. A positive correlation was observed between the presence of the CD25 molecule on WC1$^+$ lymphocytes and production of IL-10 and TGF-β, because the average percentage of IFN-γ$^-$IL-10$^+$ and IFN-γ$^+$IL-10$^+$ among CD25$^+$WC1$^+$ lymphocytes was 3 and 4.5 times higher as compared to the corresponding cells in the CD25$^-$WC1$^+$ subpopulation, whereas the percentage of IFN-γ$^+$IL-10$^-$ cells in both the subpopulations was not significantly different. The percentage of TGF-β$^+$ cells within the CD25$^+$WC1$^+$ subpopulation was 2.72 times as high as that of CD25$^-$WC1$^+$ lymphocytes. Therefore, with respect to the production of IFN-γ, IL-10 and TGF-β, CD25$^+$WC1$^+$ lymphocytes turn out to have a more suppressor profile than CD25$^-$WC1$^+$.

Key words: WC1$^+$ cells, IFN-γ, TGF-β, IL-10, CD25, cattle

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Introduction

There are two major types of T lymphocytes in vertebrates, those that express the αβ TCR and those that express the γδ TCR on their cell surface. Despite a considerable range of reported immunological activities, γδ T cells exist in relatively small numbers in the human and murine immune systems [approximately 2-7% of peripheral blood mononuclear cells (PBMCs)] (Bucy et al. 1989, Itohara et al. 1989) compared with ruminants. Adult cattle have in the region of 10-15% circulating γδ T cells and young calves can have up to 40% of these cells within their PBMCs (Wyatt et al. 1994, Wilson et al. 1996, Pollock and Welsh 2002). The majority of γδ T cells in ruminant blood express WC1 (Workshop Cluster 1), a group of γδ-lineage-specific cell-surface molecules belonging to the SRCR (The scavenger receptor cysteine-rich) family and previously shown to be involved in cellular responses (Handby-Flarida et al. 1996, Takamatsu et al. 1997). Within the $WC1^+$ population of γδ T cells (further in the article they are referred to as $WC1^+$ cells), there are two subpopulations expressing different isoforms of the WC1 molecule, WC1.1 and WC1.2. The $WC1.1^+$ cells appeared to be the main producers of IFN-γ, whereas the $WC1.2^+$ cells have been reported to proliferate in response to stimulation with mitogens (Rogers et al. 2005a, b). Although the two populations can be distinguished, there is too little information to conclude that they differ functionally (Toka et al. 2011). Several investigations have been designed specifically to determine the function of bovine γδ T cells, and particularly cells expressing WC1, in immune responses against various pathogens. Some studies suggested that $WC1^+$ cells function by interacting with or directing the behaviour of other "effector-type" cells (reviewed in Pollock and Welsh 2002). In addition, $WC1^+$ cells have been reported to have an array of immunological functions, including proinflammatory cytokine production (TNF-α, IL-12 and IFN-γ) (Brown et al. 1994, Collins et al. 1996, Fikri et al. 2001) and antigen presentation to CD4 T cells (Collins et al. 1998). A number of studies have reported bovine γδ T-cell natural killer (NK)-like and CTL activity in viral (Amadori et al. 1995) and parasitic studies (Brown et al. 1994, reviewed in Pollock and Welsh 2002). The response of γδ T cells to stimulation with cytokines (White et al 2002, Price et al. 2007) and with bacterial and protozoan antigens (Welsh et al. 2002, Lahmers et al. 2005) is characterized by the release of IFN-γ. Moreover, it has been suggested that the $WC1^+$ cells of cattle represent the inflammatory subpopulation characterized by production of IFN-γ (Hedges et al. 2003, Meissner et al. 2003). However, the proinflammatory cytokine profile of the $WC1^+$ cells is coincident with a regulatory cytokine profile. It has been demonstrated that bovine $WC1^+$ cells simultaneously express transcripts for IFN-γ and for anti-inflammatory/immunosuppressive cytokines such as IL-10 and TGF-β, with TGF-β being constitutively expressed by *ex vivo* cells (Rogers et al. 2005a). It is very well known that TGF-β and IL-10 are regulatory cytokines involved in the development and suppressive function of $Foxp3^+CD25^+CD4^+$ regulatory cells (Tregs) in humans and mice. Moreover, the suppressive activity of human Th3 and Tr1 regulatory cells is related to the cytokines they produce, TGF-β and IL-10, respectively (for review, see Workman et al. 2009, Maślanka 2010). From these data, it could be speculated that bovine $WC1^+$ cells can exert regulatory activity, such as Treg, Tr1 or Th3 cells. Indeed in 2009, Hoek et al. (2009) demonstrated, that bovine $WC1.1^+$ and $WC1.2^+$ cells rather than $Foxp3^+CD25^{high}CD4^+$ cells act as immune regulatory cells *ex vivo*. Moreover, these authors exhibited that IL-10 mRNA was transcribed in all $WC1^+$ cells as well as in the $WC1.1^+$ and $WC1.2^+$ subpopulations ranging from a 7.3-25.9 fold change in IL-10 gene expression compared to that found in total PBMCs. Moreover, they demonstrated IL-10 production in the Con A-stimulated PBMCs. Thus, although data remain limited, information obtained thus far indicate that bovine $WC1^+$ cells possess regulatory and effector activity. A question arises at this point: are bovine $WC1^+$ cells so plastic that regulatory activity and effector function co-exist in the same cell? $WC1^+$ cells are known to produce IFN-γ and IL-10, but it has not been elucidated whether they are separate subpopulations with respect to production of cytokines ($IFN\text{-}\gamma^+IL\text{-}10^-WC1^+$ and $IFN\text{-}\gamma^-IL\text{-}10^+WC1^+$), or they co-produce IFN-γ and IL-10 ($IFN\text{-}\gamma^+IL\text{-}10^+WC1^+$), or whether there are subpopulations with the $IFN\text{-}\gamma^+IL\text{-}10^-WC1^+$, $IFN\text{-}\gamma^-IL\text{-}10^+WC1^+$ and $IFN\text{-}\gamma^+IL\text{-}10^+WC1^+$ phenotype among these lymphocytes. Considering the fact that secretion of IFN-γ indicates the effector function and its pro-inflammatory effect, whereas secretion of IL-10 is indicative of immunosuppressive and anti-inflammatory effect, determination of production/co-production of these cytokines in bovine $WC1^+$ could shed some light on the issue of separation or coexistence of the effector and regulatory function of the cells. There are data (Rogers et al. 2005a) which point to expression of mRNA for TGF-β in $WC1^+$ cells, but the issue of production of the cytokine as such has not been elucidated so far, therefore, research has been conducted to verify this problem. Due to the fact that constitutive expression of the CD25 molecule was observed on a certain percentage of $WC1^+$ cells in the course of research of these cells, it was considered important to

describe the phenomenon quantitatively and to establish the cytokine profile of WC1$^+$ cells depending on whether the molecule is present or not. Moreover, considering the data which suggest that CD25$^+$WC1$^+$ lymphocytes may be an equivalent of mouse Tregs, the cells were examined for the presence of the forkhead family transcription factor Foxp3, which is expressed in Tregs controlling their growth, development and function.

Materials and Methods

Animals

Studies were carried out on eighteen clinically healthy heifers (Polish Black and White breed), aged 12 months, kept indoors and originating from a dairy farm located in Bałdy (Poland). The animals were housed and treated in accordance with the rules approved by the Local Ethics Commission (Ethic Commission Opinion No 82/2010).

Isolation of peripheral blood mononuclear cells and culture conditions

Blood was aseptically drawn by venipuncture from the jugular vein into heparinized sterile vacutainer tubes [Becton Dickinson (BD) Biosciences, San Jose, CA, USA]. PBMCs were isolated by Histopaque 1.077 (Sigma-Aldrich, Munich, Germany) density gradient centrifugation at 400 x g for 30 min at room temperature (RT). PBMCs were recovered from the interface, washed (300 x g for 10 min at 4^{o}C; these parameters were used for all cell-washing procedures) three times and resuspended in complete medium [CM; RPMI 1640, 10% FBS, 10 mM HEPES buffer, 10 mM nonessential amino acids, 10 mM sodium pyruvate and 10 U/ml penicillin/streptomycin (all from Sigma-Aldrich)]. PBMCs were adjusted to 5 x 10^6 cells/mL in CM and seeded in 24-well plates in 1 mL aliquots. For detection of intracellular cytokine production, PBMCs were stimulated with the T-cell mitogen concanavalin A (Con A, 5 μg/mL, Sigma-Aldrich) for 6 h (for evaluation of IL-10 and IFN-γ production) or 12 h (for evaluation TGF-β production) in the presence of brefeldin A (Sigma-Aldrich) (10 μg/mL) during the last 5 h. Moreover, in order to determine whether the stimulation could affect CD25 expression on WC1$^+$ cells PBMCs were cultured simultaneously without Con A. Plates were incubated at 37^{o}C in an atmosphere of humidified incubator with 5% CO_2 and 95% air.

Flow cytometry

Extracellular staining. Cells were removed from the wells by pipetting and rinsing with FACS buffer [FB, 1 x Dulbecco's PBS (Sigma-Aldrich) devoid of Ca^{2+} and Mg^{2+} with 2% (v/v) heat-inactivated fetal bovine serum] and transferred into individual tubes and centrifuged. After additional washing in 2 mL FB, the cells were resuspended in FB and stained with FITC-conjugated mouse anti-bovine WC1 (1:200, CC15, IgG2a, Serotec, Oxford, UK) and PE-conjugated mouse anti-bovine CD25 mAb (1:200, IL-A111, IgG1, Serotec). After 45 min incubation (on ice and in the dark), cells were washed in 2 mL FB.

Intracellular staining for IL-10 and IFN-γ. Cells stained for surface markers (as described above) were fixed with 200 μL 2% paraformaldehyde in Dulbecco's PBS for 15 min on ice. Then, the samples were washed with 2 mL FB and permeabilized by washing with 2 mL 0.2% (w/v) saponin (Sigma-Aldrich) in FB. Subsequently, cells were stained with biotinylated mouse anti-bovine IL-10 mAb (1:1000, CC320, IgG1, Serotec) for 45 min on ice in the dark, followed by washing with 2 mL 0.2% saponin in FB. Then, cells were stained with PerCP-conjugated streptavidin (1:400, BD Biosciences) and Alexa Fluor (AF) 647-conjugated mouse anti-bovine IFN-γ mAb (1:200, CC302, IgG1, Serotec) for 45 min on ice in the dark. Finally cells were washed twice with 2 mL FB and analyzed by flow cytometry. Isotype controls were performed as above except that biotinylated mouse anti-bovine IL-10 and AF647-conjugated anti-IFN-γ mAb were replaced with biotinylated mouse IgG1 isotype control (Serotec) and AF647-conjugated mouse IgG1 isotype control (Serotec), respectively.

Intracellular staining for TGF-β. After extracellular staining (as described above) and fixing (200 μL 2% paraformaldehyde in Dulbecco's PBS per sample for 15 min on ice) cells were permeabilized with 2 mL SAP buffer [0.1% (w/v) saponin, 0.05% (w/v) NaN_3 in Hanks' Balanced Salt Solution (HBSS) (all from Sigma-Aldrich)] and stained with APC-conjugated mouse anti-TGF-β mAb (1:20, 1D11, IgG1, [this antibody reacts with human, mouse and bovine TGF-β1 and TGF-β2], R&D Systems, Minneapolis, MN, USA) for 45 min at RT in the dark. After this step cells were washed twice with 2 mL SAP buffer and analyzed by flow cytometry. Isotype control was performed as above except that APC-conjugated anti-TGF-β mAb was replaced with APC-conjugated mouse IgG1 isotype control (R&D Systems).

Intracellular staining for Foxp3. Cells stained for surface markers (as described above) were fixed by adding 100 μL Leucoperm-Reagent A (Serotec) to each tube and then incubating for 15 min at RT in the

dark. After this, the cells were washed with 3 mL FB and then permeabilized by adding 100 µL 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. After incubation cells were washed twice with 2 mL FB and analyzed by flow cytometry. Isotype control was performed as above except that AF647-conjugated anti-Foxp3 mAb was replaced with hucal fab-dhlx-mh isotype control-AF647 (Serotec).

FACS acquisition and analysis. Flow cytometry analysis was performed using a FACSCanto II cytometer (BD Biosciences). Data were acquired by FACSDiva version 6.1.3 software (BD Biosciences) and analyzed by FlowJo software (Tree Star Inc., Stanford, CA, USA). Cytometry setup and tracking beads (CST, BD Biosciences) were used to initialize PMT settings. Unstained control cells as well as single stain control for every fluorochrome were prepared and used to set up flow cytometric compensation. Figure 1 presents FACS analysis strategy.

Statistical analysis

All data are presented as the mean ± SEM. Student's unpaired t test (GraphPad Prism3, GraphPad Software, San Diego, CA, USA) was used to compare groups. A P value less than 0.05 was considered to be statistically significant. All data were graphed with Sigmaplot software (version 12, Systat Software, Inc, Chicago, IL, USA).

Results

The study has shown that a certain percentage of $WC1^+$ cells, from both Con A-stimulated and non-stimulated PBMC's, showed the presence of CD25 on its surface (Fig. 1, 2). It was established that there were two subpopulations within a population of $CD25^+WC1^+$ cells, i.e. those with high ($CD25^{high}WC1^+$) and those with low ($CD25^{low}WC1^+$) intensity of CD25 expression (Fig. 1, 2A, B). It was found that an average of 5.21% (± 0.21) of $WC1^+$ cells obtained from PBMCs incubated without Con A, showed the presence of CD25 (Fig. 2C), with $CD25^{high}WC1^+$ and $CD25^{low}WC1^+$ cells accounting for 1.05% (± 0.08) and 4.10% (± 0.17) of $WC1^+$ lymphocytes, respectively (Fig. 2A, B). No significant differences were observed with respect to the percentage of $CD25^{high}WC1^+$, $CD25^{low}WC1^+$ and $CD25^{+(total)}WC1^+$ cells between Con A-stimulated and non-stimulated PBMCs (Fig. 2A, B, C). Moreover, the presence of Foxp3 in $WC1^+$ cells was not detected (Fig. 3). The evaluation of cytokine production established that IFN-γ- and IL-10-producing $WC1^+$ cells were mostly separate subpopulations (Fig. 4A, 6), because the percentage of IFN-γ^+IL-10^- and IFN-γ^-IL-10^+ cells among $WC1^+$ lymphocytes was 6.87 and 5.09 times higher, respectively, than the percentage of cells which co-produced IFN-γ and IL-10. The results for $CD25^-WC1^+$ cells (Fig. 4B, 6) were almost the same as those for the entire population of $WC1^+$ lymphocytes, which was a consequence of the fact that $CD25^-WC1^+$ cells accounted for 94.79% of $WC1^+$ lymphocytes, on average. It is noteworthy that the average percentage of IFN-γ^-IL-10^+ and IFN-γ^+IL-10^+ cells within the $CD25^+WC1^+$ subpopulation was 3- and 4.5 times higher, respectively, as compared to the corresponding cells in the $CD25^-WC1^+$ subpopulation (Fig. 4B), and the differences were highly significant ($P < 0.001$). However, no significant differences were found to exist in the percentage of IFN-γ^+IL-10^- cells between $CD25^-WC1^+$ and $CD25^+WC1^+$ subpopulations (Fig. 4B). Moreover, the percentage of TGF-β^+ cells in the $CD25^+WC1^+$ subpopulation was found to be 2.72 times higher ($P < 0.05$) as compared to $WC1^+$ lymphocytes not expressing CD25 (Fig. 5, 6).

Discussion

As was stated in the introduction, $WC1^+$ lymphocytes perform an effector and regulatory function. Therefore, the question was raised whether $WC1^+$ lymphocytes have such plasticity that both functions co-exist in one cell, or are there separate subpopulations of effector and regulatory cells within a population of $WC1^+$ lymphocytes? Hoek et al. (2009) showed that bovine $WC1^+$ cells had a regulatory effect on $CD25^-CD4^+$ lymphocytes. However, considering the fact that $WC1^+$ cells form a large population of T lymphocytes (especially in young ruminants), it does not seem too probable that the entire population were regulatory cells. One should rather assume that there is a subpopulation or subpopulations of regulatory cells, as is the case of human and mouse $CD4^+$ and $CD8^+$ lymphocytes. It is now known that there is a range of specialised kinds/types of regulatory cells among those lymphocytes, such as Tr1 and Th3 cells and lymphocytes with the phenotypes $Foxp3^+CD25^+CD4^+$, $CD8^+CD25^+Foxp3^+$, $CD8^+Foxp3^+$, $CD8^+CD122^+$ and $CD8^+CD28^-$ (for review, see Maślanka 2010, 2011). IFN-γ and IL-10 are cytokines related to the effector and regulatory activity, respectively, of the cells which secrete them. From this point of view, the results obtained seem to indi-

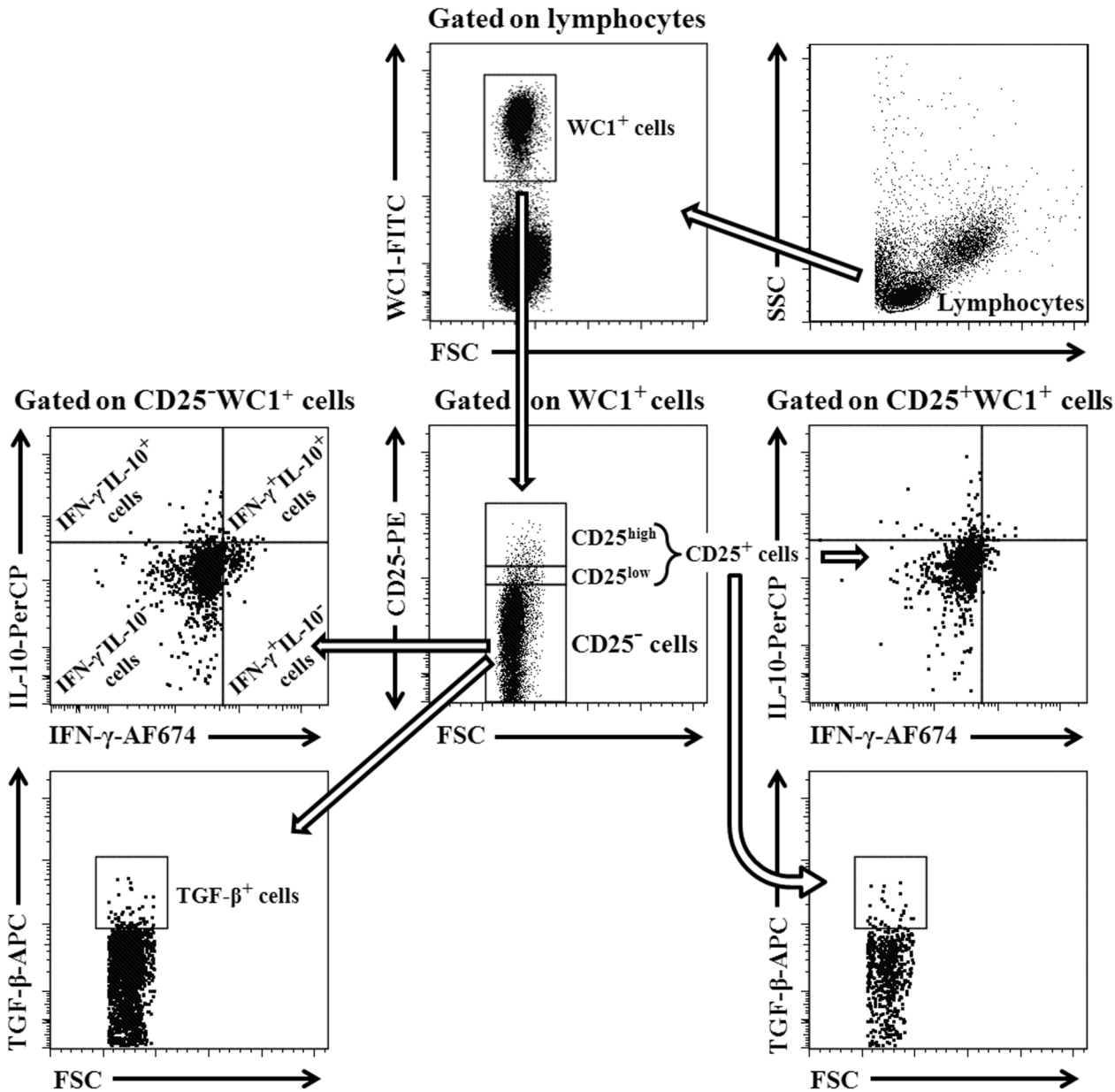

Fig. 1. FACS analysis strategy. Lymphocytes were gated based on forward and side scatter (FSC/SSC) parameters and then gated for WC1⁺ T cells. Based on the expression of the interleukin 2 (IL-2) receptor-α chain (CD25), WC1⁺ cells were divided into 2 major subpopulations $CD25^{-}WC1^{+}$ and $CD25^{+}WC1^{+}$ cells (however, $CD25^{+}WC1^{+}$ cells could be further subdivided into $CD25^{high}WC1^{+}$ and $CD25^{low}WC1^{+}$ subsets). Both subpopulations were analyzed for production/co-production of IFN-γ and IL-10. Moreover, each of these cell populations was evaluated for TGF-β production.

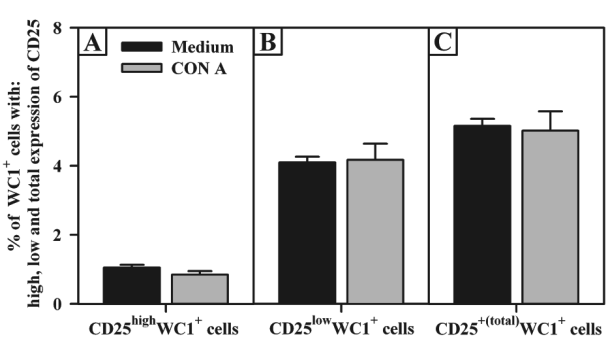

Fig. 2. CD25 expression on WC1⁺ cells after 6 h incubation without (medium) and with Con A stimulation. Results are expressed as a percentage of WC1⁺ cells with high (A), low (B) and total (high + low) (C) expression of CD25. Data reported are the mean (± SEM; n = 18) of three independent experiments (each one using six animals).

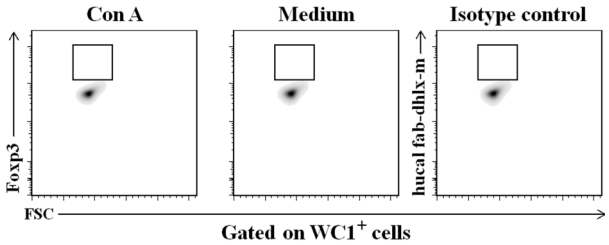

Fig. 3. Representative cytograms showing flow cytometry analysis of Foxp3 expression within $WC1^+$ cells from PBMCs stimulated and not stimulated with Con A.

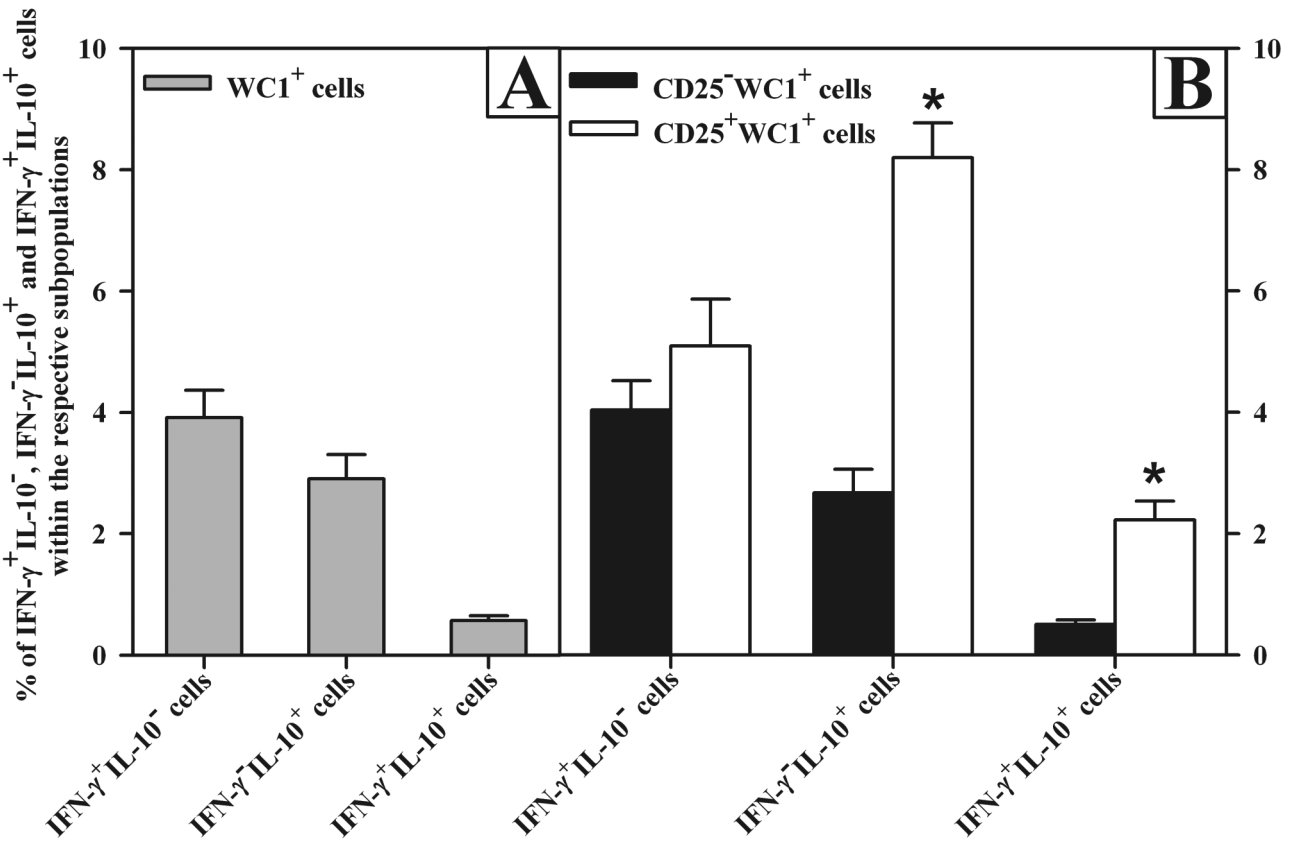

Fig. 4. Production/co-production of IFN-γ and IL-10 by $WC1^+$ (A), $CD25^-WC1^+$ and $CD25^+WC1^+$ (B) cells. Results are expressed as a percentage of IFN-γ^+IL-10^-, IFN-γIL-10^+ and IFN-γ^+IL-10^+ cells among $WC1^+$ (A), $CD25^-WC1^+$ and $CD25^+WC1^+$ (B) subpopulations. Data reported are the mean (± SEM; n = 18) of three independent experiments (each one using six animals). *P < 0.001, $CD25^-WC1^+$ cells versus $CD25^+WC1^+$ cells.

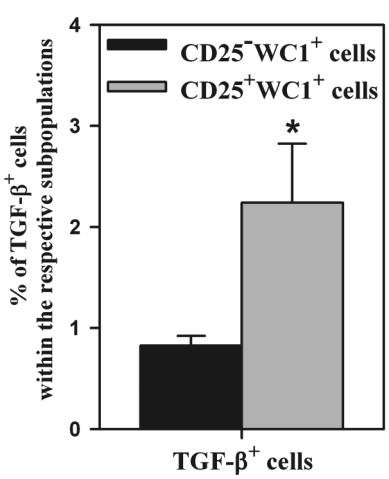

Fig. 5. TGF-β production by $CD25^-WC1^+$ and $CD25^+WC1^+$ cells. The results are expressed as a percentage of TGF-β producing $CD25^-WC1^+$ and $CD25^+WC1^+$ cells. The values represent the mean (± SEM; n = 18) of three independent experiments (each one using six animals). *P < 0.05, $CD25^-WC1^+$ cells versus $CD25^+WC1^+$ cells.

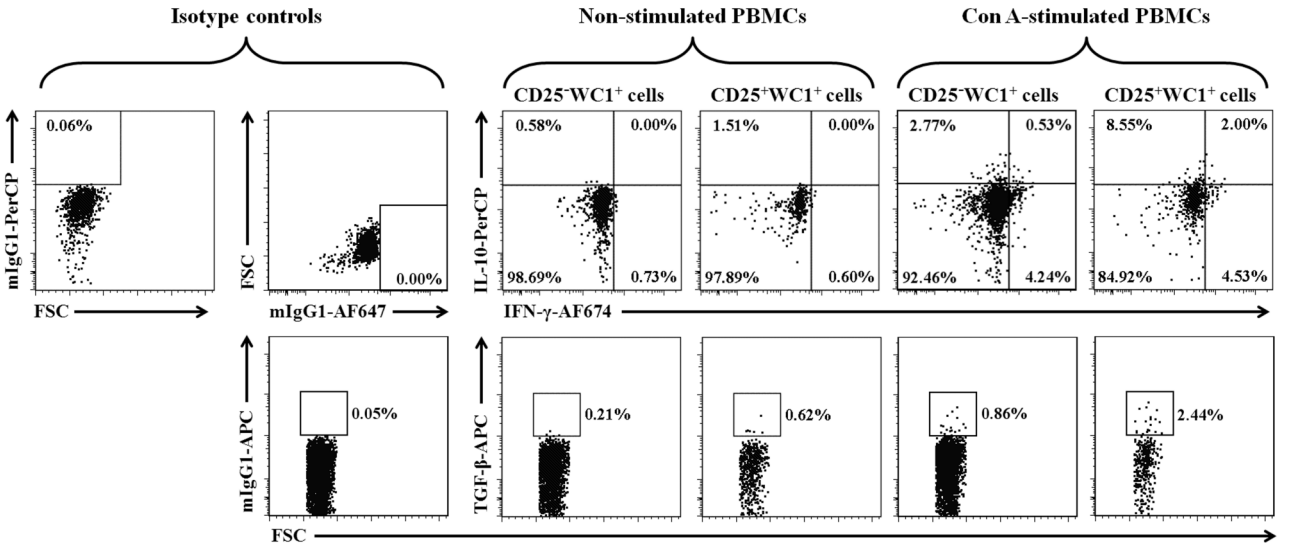

Fig. 6. Representative cytograms illustrating IFN-γ, IL-10 and TGF-β production by $CD25^-WC1^+$ and $CD25^+WC1^+$ cells from non-stimulated and Con A-stimulated PBMCs.

cate that there are separate effector and regulatory cells within $WC1^+$ lymphocytes because it was shown that IFN-γ- and IL-10-producing $WC1^+$ cells were mainly separate subpopulations and they overlapped ($IFN\text{-}\gamma^+IL\text{-}10^+WC1^+$ cells) to a relatively low extent. Therefore, it is possible that $IFN\text{-}\gamma^+WC1^+$ lymphocytes are a pool of effector cells (Teffs), whereas IL-10-producing $WC1^+$ lymphocytes take part in regulating immune response. There are reasons to suppose that $CD25^+WC1^+$ cells may have regulatory potential. The results of this study show that a certain percentage of bovine $WC1^+$ cells constitutively express CD25 molecules. This molecule corresponds to the α chain of interleukin 2 receptor (IL-2rα) and, basically, it is an indicator of lymphocyte activation. However, it should be explained that activated lymphocytes show only temporary presence of CD25, whereas its constitutive expression on mouse $CD4^+$ lymphocytes is correlated with the presence of regulatory properties in these cells; hence, they are referred to as "regulatory T lymphocytes with constitutive expression of CD25", or Tregs or Treg for short. Therefore, Tregs share the $CD25^+CD4^+$ phenotype with activated $CD4^+$ lymphocytes. It has been found in this study that CD25 is expressed on an average of 5.21% of $WC1^+$ cells from non-stimulated PBMCs and 6-hour stimulation of PBMCs with Con A did not significantly affect the percentage of $CD25^+$ cells. It is noteworthy that 12-hour incubation with the stimulant did not significantly affect the parameter (the author's observations, unpublished). This proves that the presence of $CD25^+$ on $WC1^+$ cells was not an effect of the lymphocytes activation by Con A. Therefore, it should be stressed that the results obtained with respect to production of cytokines relate to the $CD25^+WC1^+$ subpopulation with constitutive expression of CD25, and not to *in vitro* Con A-induced cells with that phenotype. It is important to point to the issue because it is known that prolonged (e.g. 24 hours) exposure to Con A upregulates the expression of CD25 on $WC1^+$ lymphocytes (Rogers et al. 2005a). Furthermore, Smyth et al. (2001) showed that 24-hour incubation of PBMCs (collected from clinically healthy calves) with Mycobacterium bovis sonic extract considerably increased the percentage of $CD25^+WC1^+$ cells. Therefore, the pool of bovine $CD25^+WC1^+$ lymphocytes in peripheral blood includes cells with that phenotype with constitutive expression of CD25, but it can also include such cells, but with temporary expression of CD25, which should be regarded as cells in a state of activation. In addition, other reports have shown that a certain percentage of $WC1^+$ cells constitutively express CD25, although there are significant quantitative divergences in this area. Rogers et al. (2005a) demonstrated that a very low level of CD25 was constitutively expressed on cells cultured in medium alone, with identical profiles found for $WC1.1^+$ and $WC1.2^+$ cells. CD25 expression substantially increased on both $WC1.1^+$ and $WC1.2^+$ cells after only 1 day of culture with Con A relative to that on cells cultured with medium. Furthermore, the diagrams presented in Toka et al. (2011) show that about 20% of $WC1^+$ lymphocytes collected from healthy 3-5-month-old calves constitutively expressed CD25, whereas the value was several times higher 2-3 days after infection of the animals with the O1 Manisa strain of FMDV. The study conducted by Sanbulte and Roth (2002) on 6-month-old calves showed that

the percentage of γδ T lymphocytes with CD25 expression varied, because it ranged from 10 to 50% for different calves. It is difficult to provide a reason for the differences, but possible ones may include age and breed of animals and perhaps environmental influence. So far, $CD25^+WC1^+$ cells have been regarded as $WC1^+$ in the state of activation (Smyth et al. 2001, Toka et al. 2011), which may raise doubts in the light of the study results provided here, because a question arises about the nature of $WC1^+$ cells with constitutive expression of CD25. Some light has been shed on the issue by the results of evaluation of production of IFN-γ, IL-10 and TGF-β by the cells and, in particular, by a comparison of the production of cytokines by $CD25^+WC1^+$ and $CD25^-WC1^+$ lymphocytes. It has been shown that the majority of IFN-γ and IL-10 were produced by separate subpopulations of $CD25^-WC1^+$ cells, and the percentage of overlapping IFN-γ^+ and IL-10^+ cells was relatively small. If one takes the total of IFN-γ^+IL-10^-, IFN-γ^-IL-10^+ and IFN-γ^+IL-10^+ lymphocytes in the $CD25^-WC1^+$ subpopulation as 100%, then the percentage of cells which produce only IFN-γ ($CD25^-WC1^+$IFN-γ^+IL-10^-), only IL-10 ($CD25^-WC1^+$ IFN-γ^-IL-10^+) and those which co-produce IFN-γ and IL-10 ($CD25^-WC1^+$IFN-γ^+IL-10^+) was 55.95%, 37.04% and 7.01%, respectively. If one calculates the results for $CD25^+WC1^+$ cells in the same manner, the percentage of cells which produce only IFN-γ, only IL-10 or which co-produce IFN-γ and IL-10 is 32.83%, 52.81% and 14.36%, respectively. Therefore, the cells in the $CD25^-WC1^+$ subpopulation are mainly IFN-γ producers, whereas the lymphocytes in the $CD25^+WC1^+$ subpopulation produce IL-10 to a greater extent. The results show that the former of the populations has rather effector profile, whereas the profile of the latter is more regulatory. The assumption that the constitutive expression of CD25 on $WC1^+$ cells may be related to their regulatory properties is supported by the fact that the presence of the molecule was not correlated with an increase in the production of IFN-γ, whereas the percentage of IFN-γ^-IL-10^+ cells was 3 times higher among the $CD25^+WC1^+$ cells as compared to the $CD25^-WC1^+$ subpopulation. Moreover, the percentage of TGF-β^+ cells was 2.72 times higher within the population of $WC1^+$ lymphocytes which express CD25 as compared to $CD25^-WC1^+$ cells. However, the question arises at this point how to reconcile the regulatory function of $CD25^+WC1^+$ cells with concurrent production of IFN-γ, which is an important cytokine with pro-inflammatory action and one which activates a cellular response? It may be that regulatory effect is exerted only by IFN-γ^-IL-$10^+CD25^+WC1^+$ cells and not by IFN-γ^+IL-$10^+CD25^+WC1^+$ or IFN-γ^+IL-10^-CD25^+ $WC1^+$ ones. It should be emphasised that production of IFN-γ does not have to exclude the regulatory action, because cells producing IFN-γ and IL-10 simultaneously have been described as a potential regulatory subset of $CD4^+$ T cells maintaining a balance between Thl and Th2-type cells (Kemp et al. 1999). Moreover, some mouse regulatory cells also secrete IFN-γ. Although in small amounts, Tr1 lymphocytes also secrete the cytokine (Roncarolo et al. 2001), and production of IFN-γ by Tregs is involved in inducing tolerance to donor's alloantigens (Sawitzki et al. 2005). One should mention that the contribution of immunosuppressor cytokines to the regulatory effect of mouse $CD25^+CD4^+$ lymphocytes is only one of many mechanisms, which mediate the effect that the cells exert on Teffs (for review, see Tang and Bluestone 2008, Maślanka 2010). Such mechanisms include intensified uptake of IL-2 from the environment as an effect of expression of CD25 by Tregs. Because Tregs constitutively express CD25, the high-affinity receptor for IL-2, it has been suspected that Treg cells suppress it by "sopping up" IL-2 produced by Teffs, thereby preventing their proliferation and differentiation (Tang and Bluestone 2008). This hypothesis has been supported by results demonstrating that $CD25^+CD4^+$ cells also induce apoptosis of Teffs *in vitro* through consumption of IL-2 (Pandiyan et al. 2007). Recently, McNally et al. (2011) have provided direct experimental evidence that consumption of IL-2 is a key *in vivo* mechanism by which murine $CD25^+CD4^+$ regulatory cells control $CD8^+$ T-cell effector differentiation. Taking into account the above, one may suppose that by virtue of their constitutive expression of CD25, $CD25^+WC1^+$ could have a suppressor effect on Teffs by depriving them of IL-2 as a result of intensified uptake of this cytokine from the microenvironment. However, it is noteworthy that we have shown the existence of a subpopulation with a low and high intensity of CD25 expression within $CD25^+WC1^+$ cells, as is the case with human and mouse $CD25^+CD4^+$ lymphocytes. A high density of the IL-2 receptor on $CD25^{high}WC1^+$ cells should be naturally correlated with high consumption of IL-2. To sum up, one may claim that not only the cytokine profile but also – maybe in the first place? – constitutive expression of CD25 by bovine $CD25^+WC1^+$ lymphocytes suggest that the cells can be a pool of regulatory lymphocytes. However, in order to elucidate the problem clearly, functional research should be taken up using sorted $CD25^+WC1^+$ cells.

Considering: (a) that with respect to production of IFN-γ, IL-10 and TGF-β, $CD25^+WC1^+$ lymphocytes better "match" the regulatory profile rather than $CD25^-WC1^+$ cells; (b) that the regulatory properties in cattle reside in $WC1^+$ cells rather than in $CD4^+CD25^+$ lymphocytes (Hoek et al. 2009), (c) an

analogy with respect to constitutive expression of CD25 by mouse regulatory CD4$^+$ cells and bovine WC1$^+$ lymphocytes (d) only slight expression of Foxp3 in bovine CD25$^+$CD4$^+$ cells (Maślanka and Jaroszewski 2012), it has been regarded as appropriate to check whether CD25$^+$WC1$^+$ cells show Foxp3 expression, as is the case with mouse and human Tregs. Foxp3 is a critical regulator of CD25$^+$CD4$^+$ regulatory cells development, function, and homeostasis (Workman et al. 2009), and at the same time it is the main marker which enables identification of the right Tregs, i.e. separation of regulatory cells (Foxp3$^+$CD25$^+$CD4$^+$) from effector ones (Foxp3$^-$CD25$^+$CD4$^+$) within the CD25$^+$CD4$^+$ subpopulation. However, the presence of Foxp3 in WC1$^+$ cells has not been detected, which indicates that bovine WC1$^+$ lymphocytes, including CD25$^+$WC1$^+$, are not equivalent to human or mouse Tregs, at least not with respect to expression of Foxp3.

Finally, it should be mentioned that based on the available literature the results with respect to the percentage of WC1$^+$ cells (a) which produce/co-produce IFN-γ and IL-10, (b) which produce TGF-β and (c) which show low and high expression of CD25, presented in this study, are the first on the subject.

Summary

WC1$^+$ cells which produce IFN-γ and IL-10 were mainly separate subpopulations, although the presence of a small subpopulation which co-produced both cytokines was also found. A certain percentage of WC1$^+$ cells was characterised by constitutive expression of CD25, with two subpopulation – with low and high expression of CD25 – present within the population of CD25$^+$WC1$^+$ cells. This suggests that these cells, and especially CD25^{high}WC1$^+$ lymphocytes, could have a suppressor effect on Teffs by depriving them of IL-2 as a result of intensified uptake of the cytokine from the microenvironment. Both CD25$^-$WC1$^+$ and CD25$^+$WC1$^+$ lymphocytes produced IFN-γ and IL-10, and the synthesis took place mainly in separate subpopulations. CD25$^-$WC1$^+$ cells produced mainly IFN-γ, whereas CD25$^+$WC1$^+$ lymphocytes produced IL-10 to a greater extent. The presence of CD25 was not related to the number of IFN-γ producing cells, whereas the percentage of IFN-γIL-10$^+$ and TGF-β$^+$ cells was considerably higher within the population of CD25$^+$WC1$^+$ cells as compared to the CD25$^-$WC1$^+$ subpopulation, which indicates a positive correlation between production of these cytokines and the presence of CD25 on WC1$^+$ lymphocytes. Therefore, with respect to production of IFN-γ, IL-10 and TGF-β, CD25$^+$WC1$^+$ lymphocytes showed a more suppressor profile than CD25$^-$WC1$^+$ cells. The results of the study provide some evidences supporting hypothesis that bovine WC1$^+$ lymphocytes with constitutive expression of CD25 – or maybe only their IFN-γ$^-$CD25$^+$WC1$^+$ subpopulation which produces IL-10 or TGF-β, or one which co-produces both the cytokines – can be a pool of regulatory cells, which does not exclude the possibility that the function may be performed by cells in the CD25$^-$WC1$^+$ subpopulation, for example, those with the phenotype IFN-γ$^-$IL-10$^+$CD25$^-$WC1$^+$.

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