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Original article

Subpopulations of bovine WC1$^+$ γδ T cells rather than CD4$^+$CD25^{high}Foxp3$^+$ T cells act as immune regulatory cells ex vivo

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Abstract – Regulatory T cells (Treg) are regarded essential components for maintenance of immune homeostasis. Especially CD4$^+$CD25^{high} T cells are considered to be important regulators of immune reactivity. In humans and rodents these natural Treg are characterized by their anergic nature, defined as a non-proliferative state, suppressive function and expression of Foxp3. In this study the potential functional role of flowcytometry-sorted bovine white blood cell populations, including CD4$^+$CD25^{high} T cells and γδ T cell subpopulations, as distinct ex vivo regulatory cells was assessed in co-culture suppression assays. Our findings revealed that despite the existence of a distinct bovine CD4$^+$CD25^{high} T cell population, which showed Foxp3 transcription/expression, natural regulatory activity did not reside in this cell population. In bovine co-culture suppression assays these cells were neither anergic nor suppressive. Subsequently, the following cell populations were tested functionally for regulatory activity: CD4$^+$CD25^{low} T cells, WC1$^+$, WC1.1$^+$ and WC1.2$^+$ γδ T cells, NK cells, CD8$^+$ T cells and CD14$^+$ monocytes. Only the WC1.1$^+$ and WC1.2$^+$ γδ T cells and CD14$^+$ monocytes proved to act as regulatory cells in cattle, which was supported by the fact that these regulatory cells showed IL-10 transcription/expression. In conclusion, our data provide first evidence that cattle CD4$^+$CD25^{high}Foxp3$^+$ and CD4$^+$CD25^{low} T cells do not function as Treg ex vivo. The bovine Treg function appears to reside in the γδ T cell population, more precisely in the WC1.1$^+$ and the WC1.2$^+$ subpopulation, major populations present in blood of cattle in contrast to non-ruminant species.

bovine / T cells / regulation / cytokines / suppression

1. INTRODUCTION

Immunoregulation comprises complex mechanisms involving the activities of various immune cell subtypes, among which natural regulatory T cells (Treg). Disbalance between responses that control the infection and counteracting responses that prevent chronic inflammation can lead to insufficient clearance of pathogens i.e. chronic infection and/or chronic inflammation. Regulatory T cells, especially CD4$^+$CD25^{high} T cells, capable of suppressing immune responses in vitro and/or in vivo, were shown to express the transcription factor Foxp3 in the human and rodent systems [13]. Transfer of T cells depleted of CD4$^+$CD25$^+$ T cells, in contrast to transfer of the total T cell population, into athymic

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nude mice caused spontaneous development of T cell-mediated autoimmune responses. Thus the function of $CD4^+CD25^+$ T cells as natural Treg was defined [35]. Subsequently, $CD4^+CD25^+$ natural Treg were shown to function in controlling and regulating the immune system in infectious diseases [19], self-tolerance and autoimmune diseases [2]. Other cell types like suppressor monocytes shown to have a regulatory/suppressor function in the human immune system in tissue injury and during inflammation [6] e.g. allergic inflammation [31]. In the bovine immune system a role for monocytes has been suggested in the control of γδ T cell responses [25], probably mediated by IL-10 secretion [23]. Literature suggests immunomodulation by γδ T cells [7, 32] and in addition potential age related differences in immune regulatory roles of $WC1^+$ γδ T cell subsets in ruminants [16,33]. A functional regulatory/suppressive role of $WC1^+$ γδ T cells, comprising $WC1.1^+$, $WC1.2^+$ [12, 21] and $WC1.3^+$ subsets, and $WC1^-$ γδ T cells has not been shown thus far ex vivo. In humans an immunoregulatory role is suggested for Vδ2 T cells induced in the presence of BCG infected dendritic cells (DC) [22] and tumor-infiltrating Vδ1 T cells suppressing T and DC function [27]. The aim of the present study was to identify potential regulatory cells in cattle ex vivo with special emphasis on $CD4^+CD25^{high}$ T cells, as potential natural Treg in comparison to $WC1^+$ γδ T cells, $CD8^+$ T cells, NK^+ and $CD14^+$ subpopulations. Their regulatory potential was investigated functionally using co-culture assays [40] and by analysis of Foxp3, IL-10 and TGF-β transcription by quantitative RT-PCR and intracellular staining for Foxp3 and IL-10 protein expression as additional regulatory cell characteristics.

2. MATERIALS AND METHODS

2.1. Animal and human cell donors

Seven adult Holstein-Frisian cows (A–G, age > 2 years) were used in the current study. The cows were housed under conventional conditions, and were checked daily for general health. Two healthy human volunteers donated blood to serve as positive controls in an intracellular staining assay to determine cross reactivity of an anti-murine/rat/human Foxp3 mAb with bovine cells. The use of animals was approved by the Ethical Committee of Utrecht University and performed according to their regulations.

2.2. Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) from human ($n = 2$) and bovine donors (A–G) were isolated from aseptically drawn heparinized blood samples by Histopaque 1.077 (Sigma-Aldrich, St. Louis, MO, USA) density gradient centrifugation. From PBMC of cow A a γδ T cell line ($W15B = N24^+/WC1.2^+$) was obtained by culturing these cells for more than 10 weeks in the presence of 100 U recombinant human IL-2/mL according to a published protocol [10].

2.3. Antibodies and flow cytometry

Unlabeled primary mouse mAb against bovine cell surface markers that were used in this study include: anti-CD25 (CACT108A, IgG2a), anti-CD14 (MM61A, IgG1), anti-CD21 (GB25A, IgG1), anti-γδ TCR (N24; GB21A, IgG2b), anti-CD3 (MM1A, IgG1), anti-Workshop cluster 1 (WC1; IL-A29, IgG1), anti-WC1.1 (BAQ159A, IgG1), anti-WC1.2 (CACTB32A, IgG1) from Veterinary Medical Research & Development (VMRD, Pullman, WA, USA); anti-CD335 (AKS1; NKp46, IgG1) from AbD Serotec (Kingston, NH, USA); anti-CD8 (IL-A105, IgG2a) kindly provided by J. Naessens (International Livestock Institute (ILRI) Nairobi, Kenya). As murine isotype controls biotinylated CD107 (IgG1), OX8, (IgG1), UD17 (IgG2a) and OX40 (IgG2b) from Hybridoma Center (Utrecht University) were used. The following conjugated antibodies against cell surface markers, intracellular cytokine and nuclear protein that were used in this study include: anti-bovine CD4 (IL-A11, IgG2a)-FITC, kindly provided by J. Naessens (ILRI); anti-bovine IL-10 (MCA2111B; CC320, IgG1)-biotin from AbD Serotec; anti-mouse/rat/human Foxp3 (150D, IgG1k)-Alexa Fluor 647® and murine IgG1k isotype control (MOPC-21, IgG1k)-Alexa Fluor 647® (Biolegend, San Diego, CA, USA) kindly provided by G. Lay (CEO Biolegend); anti-human CD4 (SK3, IgG1)-Cy-Chrome and anti-human CD25 (2A3, IgG1)-PE from Becton Dickinson (BD

pharmingen, San Diego, CA, USA), kindly provided by Y. Vercoulen en B. Prakken (Wilhelmina Children's Hospital, Utrecht, The Netherlands). The following were secondary antibodies that were used: goat anti-mouse-PE, goat anti-mouse-FITC and streptavidin-PE from BD pharmingen.

For phenotype analysis and FACS sorting, cells were single and double-color surface stained in predetermined optimal concentrations according to methods described previously [18]. Data were acquired on a FACScan™, FACSCalibur™ or Vantage™ SE flowcytometer (BD) and analyzed using CELLQuest™ (BD) and WinMDI 2.8 software.

2.4. FACS sorting of bovine cell subpopulations

All bovine cell subpopulations were purified by FACS on a BD Vantage™ SE flow cytometer based on their characteristic forward and side scatter properties in combination with fluorescence intensity after surface staining. Isotype controls were used as technical controls to set fluorescence thresholds. Percentages of low frequency cell types (0.3–3% of all PBMC) like $CD4^+CD25^{high}$ (trace population), $WC1.1^+$ and $WC1.2^+$ γδ T cells were initially determined for 7 cows (A–G) on multiple independent occasions. Subsequently the cells were isolated from 2–6 × 10^8 PBMC per cow per experiment. Purities of cell subpopulations as determined in 125 separate FACS sort experiments was 93.5 ± 6.0%.

2.5. Quantitative real time Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR) for bovine Foxp3, IL-10 and TGF-β

To determine a possible regulatory profile of the PBMC subpopulations $CD3^+$, $CD4^+$, $CD8^+$ T cells, $N24^+$ γδ T cells, $CD21^+$ B cells, $CD14^+$ monocytes and $NKp46^+$ cells (cow A–C) mean expression of Foxp3, IL-10 and TGF-β was assessed by qRT-PCR. In addition a similar assessment was performed on a panel of subpopulations of $CD4^+$ T cells and γδ T cells, being $CD4^+CD25^{high}$, $CD4^+CD25^{low}$, $CD4^+CD25^-$, $CD4^-CD25^-$ cells (cow A–C) and $N24^+$, $WC1.1^+$ and $WC1.2^+$ γδ T cells (cow A and C). Sorted cells were used to isolate RNA with the RNeasy Mini Kit and the RNase-Free DNase set (Qiagen Benelux BV, Venlo, The Netherlands). For reverse transcription of mRNA the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA) was used. According to the manufacturer's recommendations 5 ng of cDNA was used per PCR reaction. Bovine specific primer sequences were designed using the Primer Express software (Applied Biosystems, Foster City, CA, USA) and published bovine cytokine mRNA sequences (GenBank). As a housekeeping/reference gene the β2-microglobulin gene was chosen. Primers (Invitrogen, Breda, The Netherlands) used in this study include β2-microglobulin forward (50 nM): TTACCTGAACTGCTATGTGTA-TGG; β2-microglobulin reverse (300 nM): GCTGTACTGATCCTTGCTGTTG (GenBankno: X69084), IL-10 forward (300 nM): TGACATCAAGGAGCACGT-GAA; IL-10 reverse (300 nM): TCTCCACCGCCTTGCTCTT (GenBankno: U00799), TGF-β forward (300 nM): TTCTTCAACACGTCCGAGCTC; TGF-β reverse (300 nM): AGCGCCAGGAATTGTTGCTAT (GenBankno: M36271), Foxp3 forward (300 nM): CACAACCTGAGCCTGCACAA; Foxp3 reverse (300 nM): TCTTGCGGAACTCAAACTCATC (GenBankno: DQ322170).

QRT-PCR was performed with the ABI Prism 7000 Real-Time PCR Cycler (Applied Biosystems) using iTaq SYBR Green Supermix with ROX (Bio-Rad). All PCR reactions were set up in 96-well microAmp plates (Applied Biosystems) using predetermined forward and reverse primer concentrations in a reaction volume of 25 μL. After 10 min dissociation at 95 °C, the reactions were cycled 40 times at 95 °C for 15 s and 60 °C for 1 min. Melting point analysis was done after the last cycle to verify the amplification specificity. A 20 min temperature gradient was performed after the last cycle by cooling samples to 60 °C and increasing the temperature to 95 °C at 0.5 °C/min. A single product at a specific melting temperature was found for each target gene. Specificity of the PCR products, based on the predicted sizes according to the designed primer sets, was also confirmed by gel electrophoresis (2% agarose).

All samples were tested in triplicate and the mean cycle threshold (Ct) was used for further calculations. Each run included a non-template control to test for contamination of assay reagents. Real-time PCR efficiencies (E) were calculated, using different cDNA concentrations of total PBMC to produce a standard curve. The relative expression ratio (R) of a target gene was calculated based on the PCR efficiency and the Ct deviation of a tested sample versus the control (total PBMC) [28], and

expressed in comparison to β2-microglobulin using the ABI PRISM 7000 sds software version 1.1 and quantification software REST© MCS(Relative Expression Software Tool) [29]. Relative expression ratio calculated as 2-log ratio were converted in fold change in gene expression.

2.6. Intracellular staining (ICS) for Foxp3 and IL-10

Since no bovine Foxp3 specific antibodies were available, cross specificity of the anti-murine/rat/human Alexa Fluor 647®-Foxp3 mAb (clone 150D – IgG1k – Biolegend) [34] with bovine Foxp3 was investigated. As an isotype control the mouse mAb Alexa Fluor 647® (clone MOPC-21 – IgG1k – Biolegend) was used. Intracellular Foxp3 staining of human $CD4^+CD25^{high}$, $CD4^+CD25^{low}$, $CD4^+CD25^-$ and $CD4^-CD25^-$ cells gated from PBMC prelabeled with CD4Cy-Chrome/CD25PE and bovine sorted bovine $CD4^+CD25^{high}$, $CD4^+CD25^{low}$, $CD4^+CD25^-$ and $CD4^-CD25^-$ cells sorted from PBMC prelabeled with CD4FITC/CD25PE was performed according to the protocol of the manufacturer (Biolegend) with minor modifications. Cells, 1×10^6, were aliquoted into 5 mL tubes (BD/Falcon™, NJ, USA) and pelleted to be fixed and permeabilized. Incubation with Foxp3 Fix/Perm solution (Biolegend) was extended for 120 min at 4 °C after the standard 20 min at room temperature, furthermore incubation with Foxp3 Perm solution (Biolegend) was extended for 30 min at 4 °C after the standard 15 min at room temperature. Finally cells were resuspended in staining buffer and analyzed for Foxp3 expression using a FACSCalibur™ with CELLQuest™ software (BD).

Expression of intracellular IL-10 was investigated in bovine isolated PBMC stimulated with Con A (Sigma-Aldrich) (5 μg/mL) for 6 h and in the presence of brefeldin A (Sigma-Aldrich) (10 μg/mL) during the last 5 h. PBMC were single stained by unlabeled WC1, CD21, CD3, CD14, or NKp46 followed by goat anti–mouse-FITC (BD Pharmingen). Fixation and permeabilisation of PBMC were performed in Cytofix/Cytoperm and Perm/Wash solution according to the manufacturer's protocol (BD). Cells were incubated in Perm/Wash containing a predetermined optimal concentration (1/1000, IgG1, 1 mg/mL stock) of biotinylated anti-bovine IL-10 mAb MCA2111B (CC320, AbD Serotec). As an isotype IgG1 control mAb mouse anti-chicken biotinylated CD107 (1/750, IgG1, 1 mg/mL stock, Hybridoma Center, Utrecht University) was used. As a second step streptavidin-PE (1/1000, BD) for PBMC was used. Finally, cells were washed twice and analyzed for IL-10 expression using a FACSCalibur™ with CELLQuest™ software (BD).

2.7. Bovine co-culture suppression assay

In a co-culture suppression assay [40], described for human use and adjusted for bovine purposes, the suppressive function of the following sorted potential regulatory cell subpopulations was tested; $CD4^+CD25^{high}$, $CD4^+CD25^{low}$ and $CD8^+$ T cells, $CD14^+$ monocytes, $NKp46^+$cells, $WC1^+$, $WC1.1^+$ and $WC1.2^+$ γδ T cells, (cow A–G) and the $WC1^+$ γδ T cell line W15B (cow A).

Their impact on proliferation of sorted $CD4^+CD25^-$ responder T cells (Tresp) activated by plate-bound mouse anti-bovine CD3 mAb (MM1A, 3 μg/mL, inducing approximately 40–50% of the potential maximal Tresp response) in the presence of irradiated sorted $CD4^-CD25^-$ APC was studied. In assay 3×10^4, 6×10^4 and 9×10^4 potential regulatory cells in combination with 3.5×10^4 $CD4^+CD25^-$ Tresp (respectively 0.9:1, 1.7:1 and 2.6:1) and 7×10^4 irradiated (3000 cGy) $CD4^-CD25^-$ APC were co-cultured in a 96 well roundbottom microtiter plate (Corning Costar Corp., Acton, MA, USA). As controls all subpopulations of cells used were cultured as individual populations (Treg, Tresp or APC) and in all possible combinations of double/triple co-cultures. All tests were performed in triplicate in RPMI 1640 tissue culture medium (Gibco®-Invitrogen, Breda, The Netherlands) supplemented with 10% FCS (Bodinco BV, Alkmaar, The Netherlands), 50 IU/mL penicillin, 50 μg/mL streptomycin, 2 mM L-glutamine, 5×10^{-5} M β-mercapto-ethanol (Flow Laboratories, Irvine, UK) and 0.5 μg/mL amphotericin B (Sigma-Aldrich) (complete medium (CM)) at 37 °C and 5% CO_2 in a humidified incubator for 5 days. Finally 0.4 μCi ^{3}H-thymidine (Amersham, Buckinghamshire, UK) was added to each well and cells were cultured for an additional 18 h. Subsequently, cells were harvested onto glass fiber filters and incorporation of ^{3}H-thymidine was measured by micro-Betaplate liquid scintillation counting (EG & G® Wallac, Turku, Finland) and expressed as average cpm + 1SD.

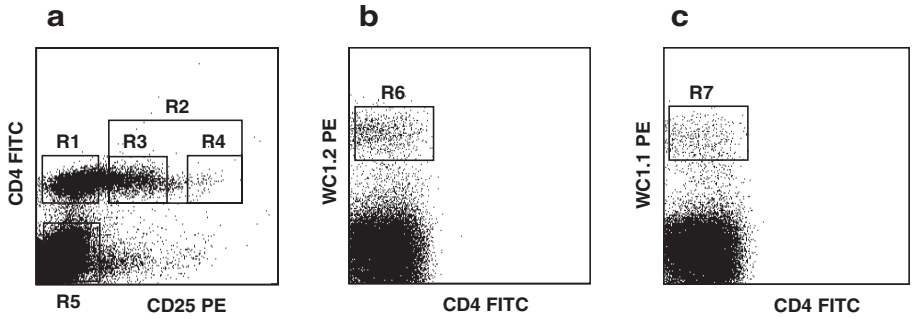

Figure 1. CD4CD25, WC1.2$^+$ and WC1.1$^+$expression on stained bovine PBMC. (a) Representative dotplot of CD4FITC/CD25PE double-color surface stained PBMC (cow A) and gate regions for sorting and/or frequency analysis of CD4$^+$CD25$^-$ (gate R1), total CD25$^+$ (gate R2), CD4$^+$CD25^{low} (gate R3), CD4$^+$CD25^{high} (gate R4) and CD4$^-$CD25$^-$ (gate R5) cells. (b) Representative dotplots of WC1.2$^+$, (gate R6) respectively (c) WC1.1$^+$ (gate R7) γδ T cell subsets stained PBMC and gate regions for sorting and frequency analysis.

2.8. Statistical analysis

All experiments were performed in duplicate or triplicate and results shown are representative of two or three independent experiments. Differences between overlay histograms of Foxp3 stainings were calculated using Kolmogorov Smirnov (two sample K-S test, testing goodness of fit between distributions, p-values < 0.05 were considered statistically significant) statistics as part of CellQuest software (Becton Dickinson). Power calculations for determining animal sample size via transcriptional analysis of fold changes in gene expressions were performed by Episcope 2.0 software. For all other comparisons, a paired two-tailed t test was used to determine significance differences, p-values < 0.05 were considered statistically significant.

3. RESULTS

3.1. Quantification of bovine CD4$^+$CD25^{high}, CD4$^+$CD25^{low} T cells and WC1.1$^+$, WC1.2$^+$γδ T cell populations by flowcytometry

Independent samplings in 7 animals (A–G) showed that in average $27.8 \pm 9.3\%$ of all bovine CD4$^+$ T cells were CD25$^+$ (Fig. 1a, gate R2) (i.e. $6.0 \pm 2.0\%$ of all PBMC), $23.1 \pm 3.7\%$ of all CD4$^+$ T cells were CD4$^+$CD25^{low} T cells (Fig. 1a, gate R3) (i.e. $5.0 \pm 0.8\%$ of all bovine PBMC) and $1.9 \pm 0.6\%$ of all CD4$^+$ T cells were CD4$^+$CD25^{high} T cells (Fig. 1a, gate R4) (i.e. $0.4 \pm 0.1\%$ of all bovine PBMC). Of bovine PBMC $7.3 \pm 2.0\%$ were γδ T cells and $5.5 \pm 1.4\%$ were WC1$^+$ T cells (data not shown). Mean WC1.2$^+$ percentages (Fig. 1b, gate R6) of $48.8 \pm 18.2\%$ of all WC1$^+$ γδ T cells ($2.7 \pm 1.0\%$ of all PBMC) and mean WC1.1$^+$ percentages (Fig. 1c, gate R7) of $36.7 \pm 6.9\%$ of all WC1$^+$ γδ T cells ($2.0 \pm 0.4\%$ of all PBMC) were measured.

3.2. Differential transcription of IL-10, TGF-β and Foxp3 in sorted bovine leukocyte subpopulations

In total PBMC and sorted subpopulations: CD3$^+$, CD4$^+$, CD8$^+$, N24$^+$, CD21$^+$, CD14$^+$ and NK$^+$ cells, isolated from cows A, B, and C (Fig. 2a), mean transcription levels of Foxp3, IL-10 and TGF-β were determined by qRT-PCR, at 3 or more independent occasions, and representative results were depicted as fold change in gene expression. Specificity of the PCR products, based on the predicted sizes according to the designed primer sets, confirmed by gel electrophoresis, showed expected products of 171 bp for β2-microglobulin, 141 bp for TGF-β, 112 bp for IL-10 and 88 bp for Foxp3 (Fig. 2b).

The transcription in the specific cell populations compared to that in total PBMC, (mean fold change expression in 3 animals), CD3$^+$ T cells showed a fold change in Foxp3 gene

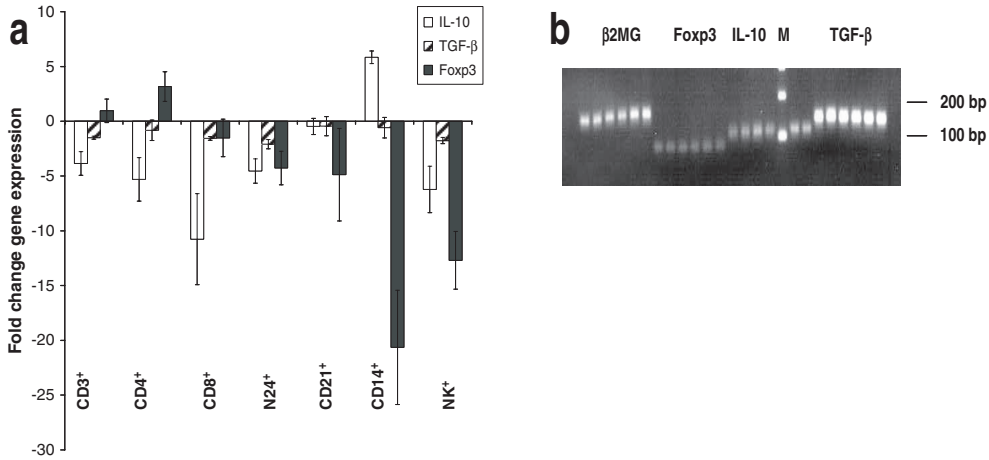

Figure 2. Transcription of IL-10, TGF-β and Foxp3 in 7 sorted bovine cell subpopulations as determined by qRT-PCR. (**a**) PBMC were isolated from cows A-C, respectively PE stained and sorted $CD3^+$, $CD4^+$, $CD8^+$, $N24^+$, $CD21^+$, $CD14^+$, $NKp46^+$ cells were tested. Results show mean fold changes in gene expression (±1SEM) of triplicate samples from three representative experiments of specific cell populations compared to total PBMC, calculations based on REST© (Relative Expression Software Tool) software [29] relative to the gene expression of β2-microglobulin. (**b**) MW sizes of products of qRT-PCR (6 independent PCR), for β2-microglobulin (171 bp), Foxp3 (88 bp), IL-10 (112 bp) and TGF-β (141 bp) (cow A) performed on bovine PBMC derived samples on a 2% agarose gel and compared to a MassRuler™ low range DNA ladder.

expression of 1.0 and $CD4^+$ T cells showed a fold change in Foxp3 gene expression of 3.2. In $CD14^+$ cells a 5.8 fold change in IL-10 gene expression was observed. In all other cell subpopulations IL-10, TGF-β and Foxp3 transcription showed a –0.5 to –20.6 fold change in gene expression compared to that found in total PBMC.

In sorted $CD4^+CD25^{high}$ T cells from all three animals, a 51.2 fold change in Foxp3 gene expression than in total PBMC was observed, in $CD4^+CD25^{low}$ cells a 12.5 fold change, in $CD4^+CD25^-$ a 1.5 fold change and in $CD4^-CD25^-$ a –1.7 fold change in Foxp3 gene expression was measured (Fig. 3a). In all CD4CD25 subsets mean changes in IL-10 and TGF-β gene expression were measured ranging from a 1.2 to –5.4 fold change in gene expression compared to total PBMC (Fig. 3a). Finally, in the γδ T cell subpopulations of cow A and cow C (Fig. 3b, cow B had to be culled during the experiments due to an infectious disease), 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 PBMC. A mean change in Foxp3 and TGF-β expression was only detected in low amounts (ranging from 1.2 to –5.0) compared to that found in total PBMC (Fig. 3b).

3.3. Intracellular expression of Foxp3, but not IL-10, in bovine $CD4^+CD25^{high}$ T cells

Flowcytometric analysis of human PBMC surface double stained for CD4 and CD25 revealed that only $CD4^+CD25^{high}$ and not $CD4^+CD25^{low}$ (Fig. 4a) or $CD4^+CD25^-$ and $CD4^-CD25^-$ (data not shown) showed intracellular staining by the crossreactive anti-Foxp3 mAb compared to an isotype control.

Bovine CD4 and CD25 surface double stained PBMC sorted for $CD4^+CD25^{high}$, $CD4^+CD25^{low}$, $CD4^+CD25^-$ T cells and $CD4^-CD25^-$ cells after ICS with anti-human/mouse/rat Foxp3-647 mAb showed staining in $CD4^+CD25^{high}$ T cells and not

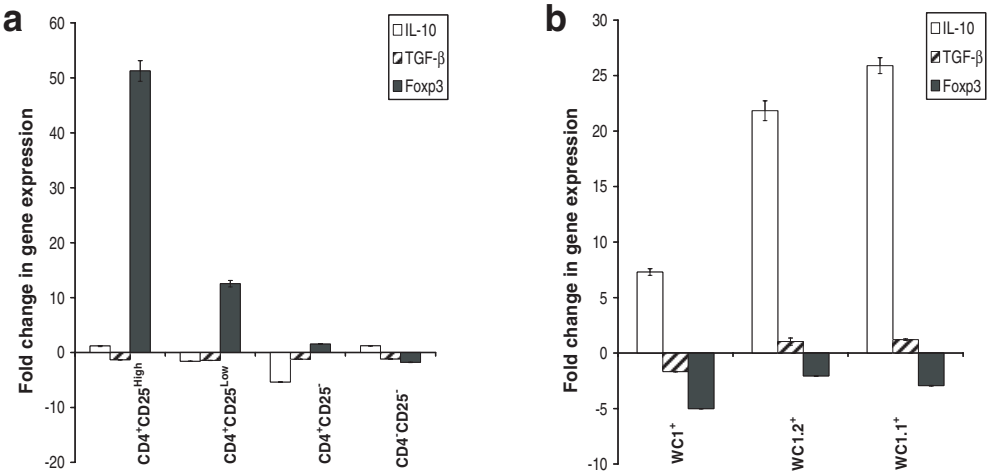

Figure 3. Transcription of IL-10, TGF-β and Foxp3 in CD4CD25 and γδ T cell sorted bovine cell subpopulations as determined by qRT-PCR. (a) $CD4^+CD25^{high}$, $CD4^+CD25^{low}$, $CD4^+CD25^-$, and $CD4^-CD25^-$ cells were FACS sorted (CD4FITC/ CD25PE) from cow A–C and (b) $WC1^+$, $WC1.2^+$ and $WC1.1^+$ PE stained γδ T cells were sorted from cow A and C. Results show mean fold changes in gene expression (±1SEM) of triplicate samples from three representative experiments of specific cell populations compared to total PBMC, calculations based on REST© (Relative Expression Software Tool) software [29] relative to the gene expression of β2-microglobulin.

in $CD4^+CD25^{low}$ (Fig. 4a) or $CD4^+CD25^-$ and $CD4^-CD25^-$ (data not shown) cells after intracellular staining by the cross reactive anti-Foxp3 mAb compared to an isotype control.

Bovine PBMC, Con A stimulated and brefeldin A treated, stained by mouse anti-chicken CD107 as an isotype control mAb irrelevant for the bovine system showed low staining compared to anti-bovine IL-10 mAb in a representative experiment (Fig. 4b). Furthermore intracellular IL-10 staining was shown in $CD14^+$ monocytes, low intracellular IL-10 staining in $CD3^+$ T cells and $WC1^+$ γδ T cells and no IL-10 staining in $CD21^+$ or $NKp46^+$ cells (Fig. 4c).

3.4. Bovine $CD4^+CD25^{high}$ and $CD4^+CD25^{low}$ T cells are non-anergic and lack suppressive properties; $WC1.1^+$, $WC1.2^+$ γδ T cells and $CD14^+$ monocytes show suppressive properties

Inhibition of proliferation of a fixed number of anti-CD3 stimulated bovine $CD4^+CD25^-$ responder T cells (Tresp) + irradiated APC ($CD4^-CD25^-$), by increasing numbers of the potential regulatory cell (sub)populations: $CD4^+CD25^{high}$, $CD4^+CD25^{low}$, $CD8^+$ T cells, the γδ T cell subsets $WC1^+$, $WC1.1^+$, $WC1.2^+$ and $WC1.2^+$ T cell line W15B, NK^+ cells, and $CD14^+$ cells (cow A–G) was determined in a bovine co-culture assay (Figs. 5a–5i).

Proliferation ranging from 77 000–247 000 cpm was observed when $CD4^+CD25^-$ Tresp cells were cultured in the presence of plate-bound aCD3 and irradiated APC confirming the potency of the bovine readout system. When increasing numbers of $CD4^+CD25^{high}$ T cells were cultured in the presence of plate-bound aCD3 and irradiated APC (without Tresp) a dose dependent proliferation ranging from 87 000–211 000 cpm was shown (Fig. 5a) indicating that these cells are non-anergic. Addition of increasing numbers of these $CD4^+CD25^{high}$ T cells to bovine $CD4^+CD25^-$ Tresp cells in a co-culture assay showed a dose dependent significant increase of proliferation ranging from 49–72% ($p < 0.0005$) compared to the proliferation of Tresp + APC only (Fig. 5a).

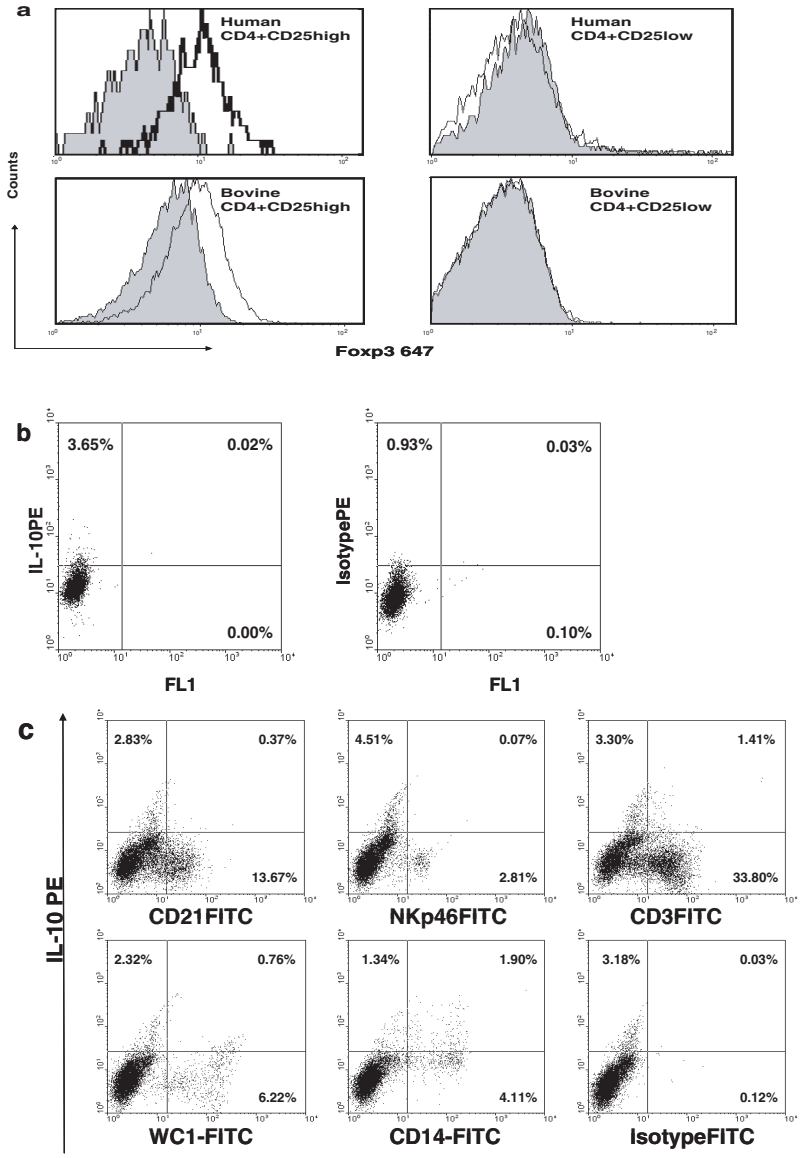

Figure 4. Intracellular staining of bovine Foxp3, IL-10, and human Foxp3. (**a**) Histograms showing intracellular Foxp3 fluorescence intensity of gated human $CD4^+CD25^{high}$, $CD4^+CD25^{low}$ and FACS sorted bovine $CD4^+CD25^{high}$, $CD4^+CD25^{low}$ T cells after ICS with anti-human/mouse/rat Foxp3 mAb (150DAlexa 647, unfilled histogram) compared to a murine IgG1k isotype control mAb (MOPC-21Alexa 647, filled histogram). (**b**) Dotplots representing intracellular staining of IL-10 in bovine PBMC gated for live cells after Con A stimulation + Brefeldin A, stained with intracellular biotinylated anti-bovine IL-10 (IgG1) and StreptavidinPE as a second step and the relevant isotype control biotinylated anti-chicken CD107 (IgG1). (**c**) Dotplots representing intracellular staining of IL-10 in bovine PBMC gated for live cells after Con A stimulation + Brefeldin A, surface stained with anti-bovine CD21, NKp46, CD3, WC1, CD14 and goat anti-mouseFITC as a second step in combination with intracellular biotinylated anti-bovine IL-10 and StreptavidinPE as a second step.

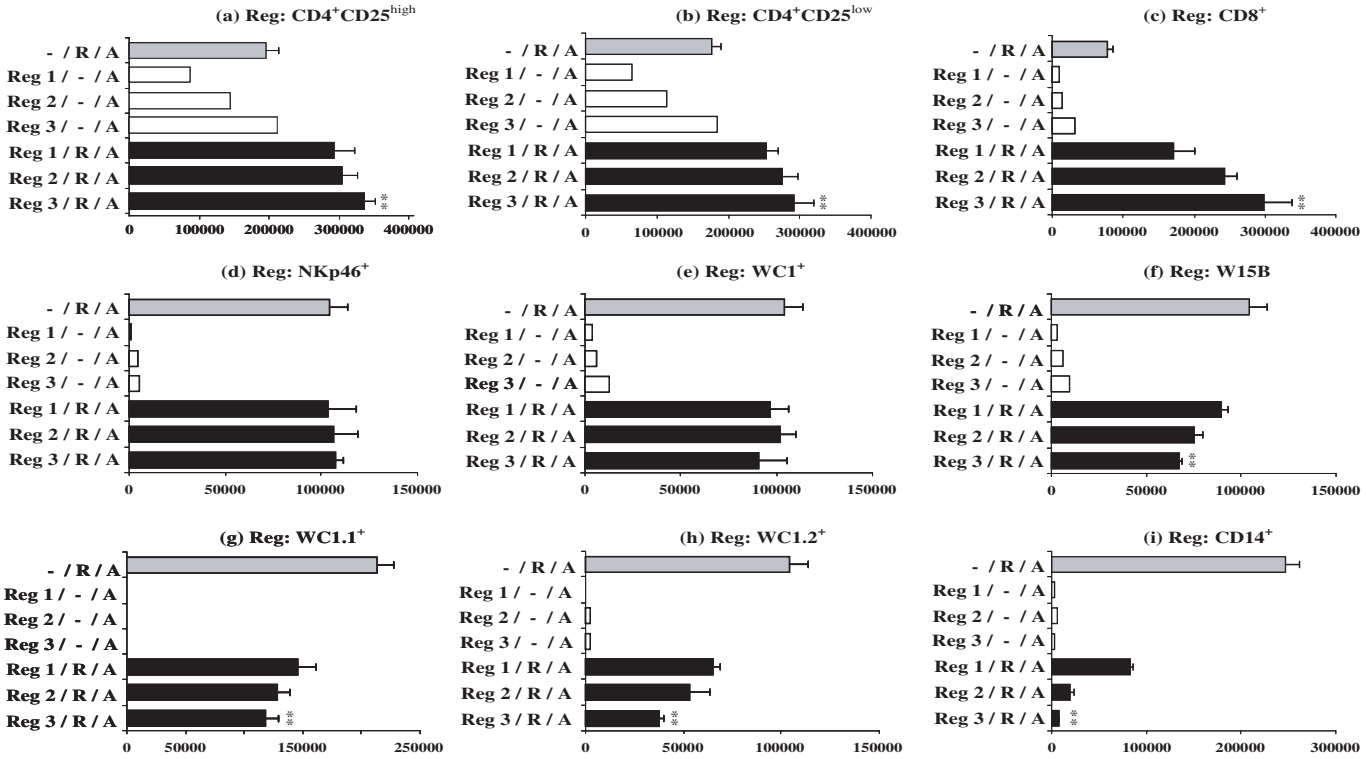

Figure 5. Ex vivo regulatory functions of 9 bovine cell populations in a co-culture assay. As a read out system (grey bars) proliferation of 35 000 $CD4^+CD25^-$ Tresp cells (population R1 in Fig. 1, indicated as R along the Y-axis), isolated from peripheral blood (cow A as a representative example) combined with 70 000 irradiated $CD4^-CD25^-$ APC (population R5 in Fig. 1, indicated as A along the Y-axis) is shown. In white bars control results of co-culturing 30 000 (indicated as Reg 1 along the Y-axis), 60 000 (indicated as Reg 2 along the Y-axis) or 90 000 (indicated as Reg 3 along the Y-axis) potential regulatory cells (a) $CD4^+CD25^{high}$, (b) $CD4^+CD25^{low}$, (c) $CD8^+$, (d) $NKp46^+$, (e) $WC1^+$, (f) W15B ($WC1^+$ T cell line), (g) $WC1.1^+$, (h) $WC1.2^+$ and (i) $CD14^+$ monocytes combined with 70 000 irradiated $CD4^-CD25^-$ APC are shown. In black bars results are shown of co-cultures with the 9 potential regulatory cells (30 000–90 000 cells indicated as Reg 1-3 along the Y-axis) in combination with $CD4^+CD25^-$ Tresp (R) and irradiated $CD4^-CD25^-$ APC (A). The data are representative experiments and are presented as dose dependent (30 000, 60 000 and 90 000) potential Treg proliferation or ratio dependent (potential Treg: Tresp = 0.9:1, 1.7:1 and 2.6:1, R1-R3:R) as the mean of proliferation on day 5 (+ 1 SD) in cpm. The stimulus used was plate-bound anti-bovine CD3 at a sub maximal concentration of 3 μg/mL. (p values reflect comparison of R1 + R (2.6:1) + A versus R + A, ** $p \leq 0.01$).

Significantly increased proliferation was also measured after addition of $CD4^+CD25^{low}$ T cells (43–65%, $p < 0.003$) (Fig. 5b) and $CD8^+$ T cells (122–287%, $p < 0.0007$) (Fig. 5c). The $NKp46^+$ cell population (Fig. 5d) and $WC1^+$ γδ T cell subpopulation (Fig. 5e) showed no significant effect ($p > 0.05$) on proliferation of Tresp + APC.

Dose dependent inhibition of proliferation of Tresp was caused by the $WC1^+$γδ T cell line W15B (14–36%, $p < 0.003$), $WC1.2^+$(WC1-$N3^+$) γδ T cells (38–64%, $p < 0.0003$) and $WC1.1^+$ (WC1-$N4^+$) γδ T cells (32–44%, $p < 0.0009$) (Figs. 5f–5h). Furthermore, the $CD14^+$ cell population caused dose dependent inhibition (67–97%, $p < 0.000007$) of proliferation of Tresp + APC (Fig. 5i) (p values reflect comparison of proliferation of potentially Treg + Tresp (2.6:1) + APC vs. Tresp + APC).

4. DISCUSSION

To our knowledge this is the first report describing the identification and functional characterization of sorted ex vivo bovine $WC1^+$ γδ T cell subsets, WC1.1 and WC 1.2, and $CD14^+$cells as leukocytes with regulatory function. In addition it was shown that in cattle in contrast to humans and rodents the $CD4^+CD25^{high}$ and $CD4^+CD25^{low}$ T cell subpopulations do not have regulatory capacity ex vivo.

Regulatory cells are regarded essential components in maintenance of homeostasis of the immune system capable of suppressing other immune responses in vitro and/or in vivo. In rodents approximately 5–15% of the $CD4^+$ cells identified in the spleen are $CD25^+$ cells and 6–10% of all $CD4^+$ T cells demonstrate regulatory function [36]. The percentage of circulating $CD25^+$ cells identified in humans is approximately 10–15% of all $CD4^+$ T cells and approximately 1–3% of all human $CD4^+$ T cells demonstrates regulatory function [34] as tested in a co-culture assay. The percentage of circulating $CD25^+$ cells identified in cattle was approximately 19–37% of all $CD4^+$ T cells, and the percentage of $CD4^+CD25^{high}$ T cells found in cattle (1.3–2.5% of all $CD4^+$ T cells) was similar to that in humans. The two main characteristics of $CD4^+CD25^{high}$ natural Treg are their functional regulatory (suppressive) activity and their anergic state (non-proliferative) upon stimulation e.g. with plate-bound anti-CD3. In the mouse both the $CD4^+CD25^{high}$ and $CD4^+CD25^{low}$ T cells seem to be regulatory while in humans this is confined to the $CD4^+CD25^{high}$ T cell population [3]. The anergic nature of $CD4^+CD25^{high}$ Treg was proven by stimulation via cross linking of the TCR with an anti-CD3 mAb. Addition of low concentrations of IL-2 resulted in minor proliferation of natural Treg in these species. In contrast our functional studies using bovine cells showed that the $CD4^+CD25^{high}$ and $CD4^+CD25^{low}$ populations are non-anergic (proliferative) and has no regulatory/suppressive effect.

The expression of Foxp3 is regarded as a hallmark of natural Treg ($CD4^+CD25^{high}Foxp3^+$) and is considered as critically important for the development and function of natural Treg in humans and rodents [43]. Deficiency of Foxp3, a disorder called scurfy in mice [8] and IPEX in humans [5] leads to a fatal autoimmune lympho-proliferative disease as a consequence of chronic T cell activation while overexpression of Foxp3 in the mouse leads to a reduction in the mature T cell population and decreased T cell function, all proving a regulatory disbalance. Foxp3 was also identified in murine $B220^+$ B cells [8] however these cells displayed no suppressor function in vitro in contrast to certain human $CD8^+$LAG-$3^+CD25^+Foxp3^+CCL4^+$ T cells that did display suppressor function [14] indicating that the links between Foxp3 and suppression and between suppression and CD4/CD25 expression may not be absolute. The present study showed that the bovine Foxp3 gene is highly transcribed in $CD4^+CD25^{high}$ and $CD4^+CD25^{low}$ T cells, while transcription was low or almost absent in $CD4^+CD25^-$ or $CD4^-CD25^-$ T cells, consistent with the situation in humans and rodents. Low expression of intracellular bovine and moderate expression of human Foxp3 protein

was detected in $CD4^+CD25^{high}$ T cells, but none could be detected in $CD4^+CD25^{low}$, $CD4^+CD25^-$ T cells or $CD4^-CD25^-$ human or bovine cells. Foxp3 staining in bovine $CD4^+CD25^{high}$ T cells proved to be weaker, probably due to less specificity of the cross-reactive anti-human/mouse/rat Foxp3 mAb for bovine Foxp3 as compared to human cells and/or lower amounts of intracellular Foxp3 present in bovine versus human cells.

In humans two different isoforms of Foxp3 are known and the ectopic expression of the Foxp3Δ2 (lacking exon 2) failes to induce the development of suppressor T cells but leads to Foxp3 expression without regulatory T cell development [1]. Human IPEX-like-3 patients show moderate to low, full length, Foxp3 expression, so the affected $CD4^+CD25^{high}$ T cell regulation may result from mutations in other genes, questioning the role of Foxp3 as the "master regulator" of human natural Treg development and function [11]. Seo et al. identified the bovine Foxp3 gene and sequenced the bovine Foxp3 protein, and found a high similarity with other homologs of Foxp3 [38]. Since the mAb 150D (Biolegend) used in the current study, recognizes a Foxp3 epitope in the exon 2 region, a positive ICS shows the expression of exon 2 and no expression of Foxp3Δ2. The exclusive high presence of Foxp3 transcription in cattle $CD4^+CD25^{high}$ T cells which lack regulatory function argues against a prominent role for Foxp3 as driving force in development and function of 'natural Treg' in cattle. Foxp3 may be an important but not unique marker [17] that defines all cells with regulatory activity in most species however plays a functional role following activation of conventional T cells [34].

In conclusion, the presence of bovine $CD4^+CD25^{high}$ and $CD4^+CD25^{low}$ T cells was demonstrated; Foxp3 mRNA was detected in both cell types and protein expression in $CD4^+CD25^{high}$ T cells only. In functional assays these cells failed to confirm being regulatory T cells in cattle. Our data therefore indicate that co-expression on T cells of $CD4^+CD25^{high}$ and endogenous Foxp3, at least in cattle, is not exclusive for functional bovine regulatory T-cells, as has recently also been described in humans [41]. This indicates that the bovine immune system may be governed by different regulatory mechanisms as compared to rodents and humans.

Apart from $CD4^+CD25^{high}$ and $CD4^+CD25^{low}$ T cells, different cell types have been suggested as potential regulatory cells (i.e. $CD4^+$, $CD8^+$ T cells, $CD14^+$ cells and $WC1.1^+$, $WC1.2^+$ γδ T cells) in humans, rodents [6, 20, 26, 31, 39], and cattle [7, 16, 25, 33, 38] but has until now not been proven functionally ex vivo.

In the present study ex vivo functional evidence of the regulatory potential of the bovine $WC1.1^+$, $WC1.2^+$ γδ T cell subsets and $CD14^+$ monocytes has been generated in the co-culture suppression assay. Furthermore it was shown that NK cells and CD8 T cells do not show regulatory functions ex vivo.

Activation of γδ T cells studied by Sathiyaseelan et al. [37] and Baldwin et al. [4] showed that bovine γδ T cells could be activated to a low degree in response to CD3 crosslinking. We observed a similar response when, $WC1^+$, $WC1.1^+$ and $WC1.2^+$ γδ T cells + irradiated APC were tested in a co-culture assay (Fig. 5), proliferation ranging from 2 000–13 000 cpm by 90 000 cells which is low compared to the proliferation of $CD4^+CD25^-$ T responder cells + irradiated APC (ranging from 77 000–247 000 cpm by 35 000 cells). Crosslinking of CD3 may not be the optimal stimulation for γδ T cell proliferation but it does stimulate suppressive activity as shown in our co-culture experiments. Finally it is possible that γδ T cells are already activated in vivo or require less stringent (or none at all) activation requirements for suppressive function.

Suppression as a biproduct of sequestration of IL-2 by natural Treg has been suggested in the past, and although IL-2 is a vital cytokine maintaining and activating natural Treg, it is not sequestration but rather inhibition of IL-2 production in Tresp cells [36]. Sathiyaseelan et al. [37] showed that the sequestration of IL-2 by γδ T cells does not seem a likely explanation for the observed suppressive effect as addition of IL-2 in combination with CD3 crosslinking did not show a substantial

increase in γδ T cell replication in bovine PBMC cultures. Sequestration of growth factors can not formally be excluded, although in the presented experiments we found dose dependent decreased proliferation which is not in accordance with sequestration of IL-2 by γδ T cells. First because these cells are not likely to proliferate to a large extent as argued above (in contrast to the responder cells, that even though in comparable or lower numbers were shown to be readily activated, thus to produce IL-2). Second because it is likely that activation of the γδ T cells would have contributed to the total proliferation, not in accordance with the observed decreased proliferation.

In view of the apparent discrepancy in suppressive activity between sorted $WC1.1^+$/$WC1.2^+$ and $WC1^+$ γδ T cells other cell populations like $WC1.3^+$ γδ T cells or other $WC1^+$ subpopulations, as yet unidentified within the $WC1^+$ γδ T cell population, may influence the potential regulatory or stimulatory cells within the $WC1^+$ γδ T cell population. Furthermore potentially regulatory T cells present in the $WC1^+$ populations may influence each others function.

$WC1.1^+$ cells described in this study as immune suppressors showed to be IFN-γ secretors [33], after specific defined stimulation, that can act as innate immune cells potentially by directing adaptive immune responsiveness. These functions seem contradictory, however 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 [15]. Besides IFN-γ has been reported to act in a suppressive fashion [24]. Different ways of stimulation may result in differential activities of relevance in adaptive versus innate immune functioning by $WC1.1^+$ cells. Also further subdivision of $WC1.1^+$ and $WC1.2^+$cell populations can not be excluded.

Since a role for cytokines like IL-10 and TGF-β [42] has been implicated in the regulatory mechanisms of murine and human natural Treg, T_R1 and T_H3 regulatory T cells, we assessed the production of these cytokines by the different bovine celltypes after stimulation [9, 30]. In our study bovine $CD4^+CD25^{high}$ T cells produced low amounts of IL-10 and TGF-β specific mRNA in comparison to the levels in the total bovine PBMC population. Similar low amounts of IL-10 and TGF-β transcription were found in $CD4^+CD25^{low}$, $CD4^+CD25^-$ and $CD4^-CD25^-$ cells. Intracellular staining of bovine PBMC confirmed that no or very little IL-10 was present in $CD4^+CD25^{high}Foxp3^+$ T cells, in agreement to what was found before in the murine system where expression of Foxp3 was proven not to be correlated to IL-10 expression [17]. However, IL-10 transcription was clearly increased in sorted $WC1.1^+$, $WC1.2^+$ γδ T cells and in CD14 monocytes, in addition we found actual expression of this cytokine in both $CD14^+$ and $WC1^+$ γδ T cells which is in accordance with earlier publications [10, 33].

In contrast Foxp3 transcription/expression were very low or absent in these cell types.

In conclusion, we have functionally characterized $WC1.1^+(WC1\text{-}N4^+)$ and $WC1.2^+(WC1\text{-}N3^+)$ γδ T cell subpopulations and $CD14^+$ monocytes, in which IL-10 transcription and expression could be detected but no Foxp3 transcription, as suppressive/regulatory bovine cells. The exact mechanism of the suppression observed was not addressed in the present study. In contrast, $CD4^+CD25^{high}Foxp3^+$ and $CD4^+CD25^{low}$ T cells did not perform the regulatory function shown in other species. The fact that γδ cells are a major T cell population in cattle, varying in numbers in age with as yet undefined function, calls for further analysis of the mechanism of their regulatory function in health and disease in cattle.

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