

Original Article

***In vitro* effects of meloxicam on the number, Foxp3 expression, production of selected cytokines, and apoptosis of bovine CD25⁺CD4⁺ and CD25⁻CD4⁺ cells**

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The purpose of this study was to evaluate the effect of meloxicam (MEL) on selected immune parameters of bovine CD25^{high}CD4⁺, CD25^{low}CD4⁺, and CD25⁻CD4⁺ cells. Peripheral blood mononuclear cells (PBMCs) collected from 12-month-old heifers were treated with MEL at a concentration corresponding to the serum level of this medication following administration at the recommended dose (MEL 5×10^{-6} M) and at a concentration 10 times lower (MEL 5×10^{-7} M). After 12 and 24 h of incubation with the drug, the percentage of CD25^{high}CD4⁺ cells decreased; however, this disturbance was quickly reversed. Furthermore, the absolute number of CD25^{high}CD4⁺ cells in the PBMC populations treated with MEL 5×10^{-6} M for 48 and 168 h was increased. Prolonged (168 h) exposure to the drug increased the percentage of Foxp3⁺ cells in the CD25^{high}CD4⁺ cell subpopulation. The higher dose of MEL was found to significantly increase the percentage of IFN- γ cells among the CD25⁻CD4⁺ cells. These results indicated that MEL does not exert an immunosuppressive effect by depleting CD4⁺ cells and suppression of IFN- γ production by these cells. Furthermore, IL-10 and TGF- β production was not changed following exposure to MEL.

Keywords: cattle, CD4⁺ cells, cytokines, Foxp3, meloxicam

Introduction

Meloxicam (MEL) is an anti-inflammatory drug commonly used to treat cattle [6]. This drug belongs to a general class of compounds known as non-steroidal anti-inflammatory drugs (NSAIDs). NSAIDs inhibit the activity of cyclooxygenase (COX), an enzyme that

initiates prostanoid formation [25]. Analysis of the available literature has shown that current knowledge about the effect of MEL on bovine CD4⁺ lymphocytes is based on two publications. A single dose of the drug given to calves was not found to have any significant effect on the percentage of CD4⁺ cells in peripheral blood in our earlier study [18] nor in the one conducted by Bednarek *et al.* [2]. Likewise, we did not find MEL to exert any significant effect on the percentage of cells that were in an early stage of apoptosis. This is essentially the information that makes up the entire body of knowledge about the effects of MEL on bovine CD4⁺ lymphocytes.

Considering the number of reports showing that COX-2 helps regulate the production of interferon- γ (IFN- γ) [23], interleukin-10 (IL-10) [7], and transforming growth factor- β (TGF- β) [4,12], it would be extremely interesting and useful to understand the consequence of COX-2 inhibition on the production of these cytokines which are very important for regulating the immune response. The only available data showing the possible effect of MEL on cytokine production in cattle were published by Maeda *et al.* [14]. This study found that the drug did not significantly change the mRNA expression of IFN- γ , IL-2, or IL-4 in bovine peripheral blood mononuclear cells (PBMCs). There have been a few publications demonstrating the effects of MEL on the production of IFN- γ [20,21] and IL-10 [19,20]; however, these investigations were conducted with animals other than ruminants. Our preliminary research (unpublished data) showed that MEL does not equally affect the production of cytokines by different subpopulations of T cells. Therefore, we initiated comprehensive studies of this issue in CD4⁺ lymphocytes and have presented our findings in this paper.

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It was also of interest to assess the effects of MEL on CD25+CD4+ cells and Foxp3 expression in these cells. In mice, T cells with a CD25+CD4+ phenotype are regulatory cells (T_{regs}) that play an important role in maintaining immunologic balance in the host by suppressing a wide variety of immune responses [24]. However, CD25 (the IL-2 receptor α chain) is not a specific T_{regs} marker because this factor is also expressed by activated T cells. Currently, the forkhead family transcription factor Foxp3 is the most specific marker used to distinguish regulatory cells (Foxp3+CD25+CD4+) from activated effector cells (Foxp3-CD25+CD4+) within CD25+CD4+ cell subpopulations [26]. However, it seems that the regulatory properties of bovine CD25+CD4+ cells are not equivalent to those of mouse lymphocytes with the same phenotype. Hoek *et al.* [8] were unable to demonstrate that bovine CD25^{high}CD4+ and CD25^{low}CD4+ lymphocytes have suppressive properties using a functional assay, and concluded that these subpopulations in cattle are not composed of regulatory cells. Findings from our earlier research [17] are consistent with this conclusion since only a small percentage of bovine CD25^{high}CD4+ and CD25^{low}CD4+ cells were found to express Foxp3, a factor which helps determine the regulatory properties of lymphocyte subpopulations.

There have been many recently published papers [3,22,27] on generating Foxp3+CD25+CD4+ regulatory cells using steroidal anti-inflammatory drugs (SAIDs). There have also been reports [1,9] indicating a possibility of inducing such cells with NSAIDs. We assumed that an absence of regulatory properties of bovine CD25^{high}CD4+ and CD25^{low}CD4+ lymphocytes is a result of insufficient Foxp3 expression. Based on reports indicating the possibility of increasing Foxp3 expression in human or mouse CD25+CD4+ lymphocytes with SAIDs or NSAIDs, research was conducted to determine the effect of dexamethasone (a SAID) and MEL on the percentage of Foxp3+ cells among bovine CD25^{high}CD4+ and CD25^{low}CD4+ lymphocytes, and on the number of cells in these subpopulations. We considered the possibility that inducing Foxp3 expression in CD25^{high}CD4+ and CD25^{low}CD4+ cells may be one of the mechanisms underlying SAID and NSAID action that is responsible for some pharmacological effects of these drugs in cattle. We have shown [17] that dexamethasone increases the number of bovine CD25^{high}CD4+ and CD25^{low}CD4+ lymphocytes but unlike in mice and humans, this drug reduces Foxp3 expression in these cells. This current study produced results demonstrating the effect of MEL on the numbers of bovine CD25^{high}CD4+ and CD25^{low}CD4+ cells, and on the expression of Foxp3 in these cells.

Considering the potential effect that MEL may have on CD4+ cells and scarce knowledge on this issue with respect to cattle, the present study was conducted to

determine whether MEL could affect the number of these cells. The effects of MEL on apoptosis, production of selected cytokines, and Foxp3 expression were also evaluated. Three subpopulations of CD4+ lymphocytes (CD25^{high}CD4+, CD25^{low}CD4+, and CD25- CD4+) were used for our investigation.

Materials and Methods

Animals

This study was carried out on 36 clinically healthy heifers (Polish black and white) 12 months old. The animals were kept indoors and originated from a dairy farm located in Bałdy (Poland). The heifers were housed and treated in accordance with the rules approved by the Local Ethics Commission of the University of Warmia and Mazury in Olsztyn (Ethic Commission Opinion No. 82/2010).

Isolation of PBMCs and cell culture conditions

Blood was aseptically drawn by venipuncture from the jugular vein into sterile heparinized vacutainer tubes (BD Biosciences, USA). PBMCs were isolated with a Histopaque 1.077 (Sigma-Aldrich, USA) density gradient by centrifugation at $400 \times g$ for 30 min at room temperature (RT). PBMCs were recovered from the interface, washed ($300 \times g$ for 10 min at $4^{\circ}C$; these parameters were used for all cell washing procedures) three times with complete medium [CM; RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES buffer, 10 mM nonessential amino acids, 10 mM sodium pyruvate, and 10 U/mL penicillin/streptomycin (all reagents were purchased from Sigma-Aldrich, USA)], and resuspended in CM.

For all experiments, the cells were treated with MEL (Sigma-Aldrich, USA) at a concentration reflecting plasma levels achieved *in vivo* with therapeutic doses of the drug (MEL 5×10^6 M) and a concentration 10 times lower (MEL 5×10^7 M). For phenotypic analysis and detection of apoptosis, PBMCs were diluted to a final concentration of 10^6 cells/mL in CM, seeded in 12-well plates (BD Falcon, USA) in 2-mL aliquots, and incubated for 12, 24, 48, and 168 h in the absence (control) or presence of MEL. Apoptosis was monitored after 12 and 24 h of incubation. To analyze the intracellular expression of IL-10, IFN- γ , and TGF- β , the PBMCs were diluted to 5×10^6 cells/mL in CM and seeded in 24-well plates (BD Falcon, USA) in 1-mL aliquots. The cells were pre-incubated for 1 h without (control) or with the two different concentrations MEL followed by stimulation for 6 h (to evaluate IL-10 and IFN- γ production) or 12 h (to evaluate TGF- β production) with 5 μ g/mL concanavalin A (Con A; Sigma-Aldrich, USA) in the presence of 10 μ g/mL brefeldin A (Sigma-Aldrich, USA) during the last 5 h. The plates were incubated at $37^{\circ}C$ in an atmosphere containing

5% CO₂ and 95% air.

Flow cytometry

Extracellular staining: The cells were removed from the wells by pipetting and rinsing with FACS buffer [FB, 1 × Dulbecco's phosphate-buffered saline (DPBS) devoid of Ca²⁺ and Mg²⁺ with 2% (v/v) heat-inactivated FBS; both reagents were purchased from Sigma-Aldrich, USA], transferred into individual tubes (12 × 75 mm, BD Falcon, USA), and centrifuged. After washing in 2 mL FB, the cells were resuspended in 200 µL of FB and stained with FITC (fluorescein isothiocyanate) -conjugated mouse anti-bovine CD4 (1 : 20, CC8, IgG2a; AbD Serotec, UK) and PE(phycoerythrin)-conjugated mouse anti-bovine CD25 (1 : 200, IL-A111, IgG1; AbD Serotec, UK). After 45 min incubation on ice and in the dark, the cells were washed in 2 mL FB.

Intracellular staining for Foxp3: Cells stained for surface markers (as described above) were fixed by adding 100 µL leucoperm reagent A (AbD Serotec, UK) to each tube and incubating for 15 min at RT in the dark. Next, the cells were washed with 3 mL FB, permeabilized by adding 100 µL of leucoperm reagent B (AbD Serotec, UK), and subsequently stained with AF (alexa fluor) 647-conjugated human anti-bovine Foxp3 mAb (1 : 20, 7627, HuCAL Fab bivalent; AbD Serotec, UK) for 60 min at RT in the dark. The cells were then washed twice with 2 mL FB and analyzed by flow cytometry. An isotype control was made using the protocol described above except that the AF647-conjugated anti-Foxp3 mAb was replaced with hucal fab-dhlx-mh isotype control-AF647 (AbD Serotec, UK).

Staining for intracellular IL-10 and IFN-γ: Cells stained for surface markers (as described above) were fixed with 200 µL 2% paraformaldehyde in DPBS for 15 min on ice. After this, the samples were washed with 2 mL FB and then permeabilized with 2 mL 0.2% (w/v) saponin (Sigma-Aldrich, USA) in FB. The cells were subsequently stained with biotinylated mouse anti-bovine IL-10 mAb (1 : 1000, CC320, IgG1; AbD Serotec) for 45 min on ice in the dark and were then washed with 2 mL 0.2% saponin in FB. Next, the cells were stained with PerCP (peridinin-chlorophyll-protein complex) -conjugated streptavidin (1 : 400; BD Biosciences, USA) and AF647-conjugated mouse anti-bovine IFN-γ mAb (1 : 200, CC302, IgG1; AbD Serotec, UK) for 45 min on ice in the dark. Finally, the cells were washed twice with 2 mL FB and analyzed by flow cytometry. Isotype controls were made as described above except that the biotinylated mouse anti-bovine IL-10 and AF647-conjugated anti-IFN-γ mAb were replaced with biotinylated mouse IgG1 isotype control (AbD Serotec, UK) and AF647-conjugated mouse IgG1 isotype control (AbD Serotec, UK), respectively.

Staining for intracellular TGF-β: After extracellular staining (as described above) and fixation (200 µL 2%

paraformaldehyde in Dulbecco's PBS per sample for 15 min on ice; both reagents were purchased from Sigma-Aldrich, USA), the cells were permeabilized with 2 mL SAP buffer [0.1% (w/v) saponin and 0.05% (w/v) NaN₃ in Hanks' balanced salt solution (HBSS; all from Sigma-Aldrich, USA)] and stained with APC (allophycocyanin) -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, USA) for 45 min at RT in the dark. The cells were then washed twice with 2 mL SAP buffer and analyzed by flow cytometry. An isotype control was made as described above except that the APC-conjugated anti-TGF-β mAb was replaced with APC-conjugated mouse IgG1 isotype control (R&D Systems, USA).

Apoptosis analysis: Cells stained for surface markers were washed once in 1 mL of 1 × annexin V binding buffer (BD Biosciences, USA). The supernatant was removed by centrifugation, and the cells were suspended in 100 µL of 1 × annexin V binding buffer. Next, 5 µL of APC-conjugated annexin V (BD Biosciences, USA) and 5 µL of 7-AAD (7-aminoactinomycin D; BD Biosciences, USA) were added to the cells. The cells were gently mixed, incubated for 15 min at RT in the dark, diluted with 400 µL of 1 × annexin V binding buffer, and analyzed by flow cytometry within 1 h.

FACS data acquisition and analysis

Flow cytometry was performed using a FACSCanto II cytometer (BD Biosciences, USA). Data were acquired with FACSDiva version 6.1.3 software (BD Biosciences, USA) and analyzed by FlowJo software (Tree Star, USA). Cytometry setup and tracking beads (CST; BD Biosciences, USA) were used to initialize the photomultiplier tube (PMT) settings. Unstained control cells as well as a single stain control for every fluorochrome were prepared and used to establish flow cytometric compensation. The absolute count represented the number of collected cells of each subset per sample taking into account that a fixed volume of sample (100 µL) was always analyzed. Values obtained in this manner would reflect changes in the absolute numbers of lymphocytes because (a) the same number of cells was seeded in each well, (b) total contents of each well were transferred to individual tubes after culturing, (c) the same volume of each sample (100 µL) was always analyzed by flow cytometry, and (d) all cytometry assays was performed using the same forward scatter (FSC) threshold. FlowJo software (Tree Star, USA) directly converted the percentage count of a cell subpopulation into an absolute count.

Statistical analysis

All data are presented as the mean ± standard error (SE). Student's unpaired *t*-test was used to compare groups. A *p*-value less than 0.05 was considered to be statistically significant. All data were analyzed with GraphPad Prism3

(GraphPad Software, USA) and graphed with SigmaPlot software (ver. 12; Systat Software, USA).

Results

The effect of MEL on the percentage and absolute number

of CD25^{high}CD4⁺, CD25^{low}CD4⁺, and CD25CD4⁺ lymphocytes was evaluated after 12, 24, 48, and 168 h of exposure to the drug. It was found that MEL significantly [12 h: $p < 0.01$ (MEL 5×10^{-6}), $p < 0.05$ (MEL 5×10^{-7}); 24 h: $p < 0.01$] reduced the percentage of CD25^{high}CD4⁺ cells (Fig. 1A and E) at 12 and 24 h; however, the absolute

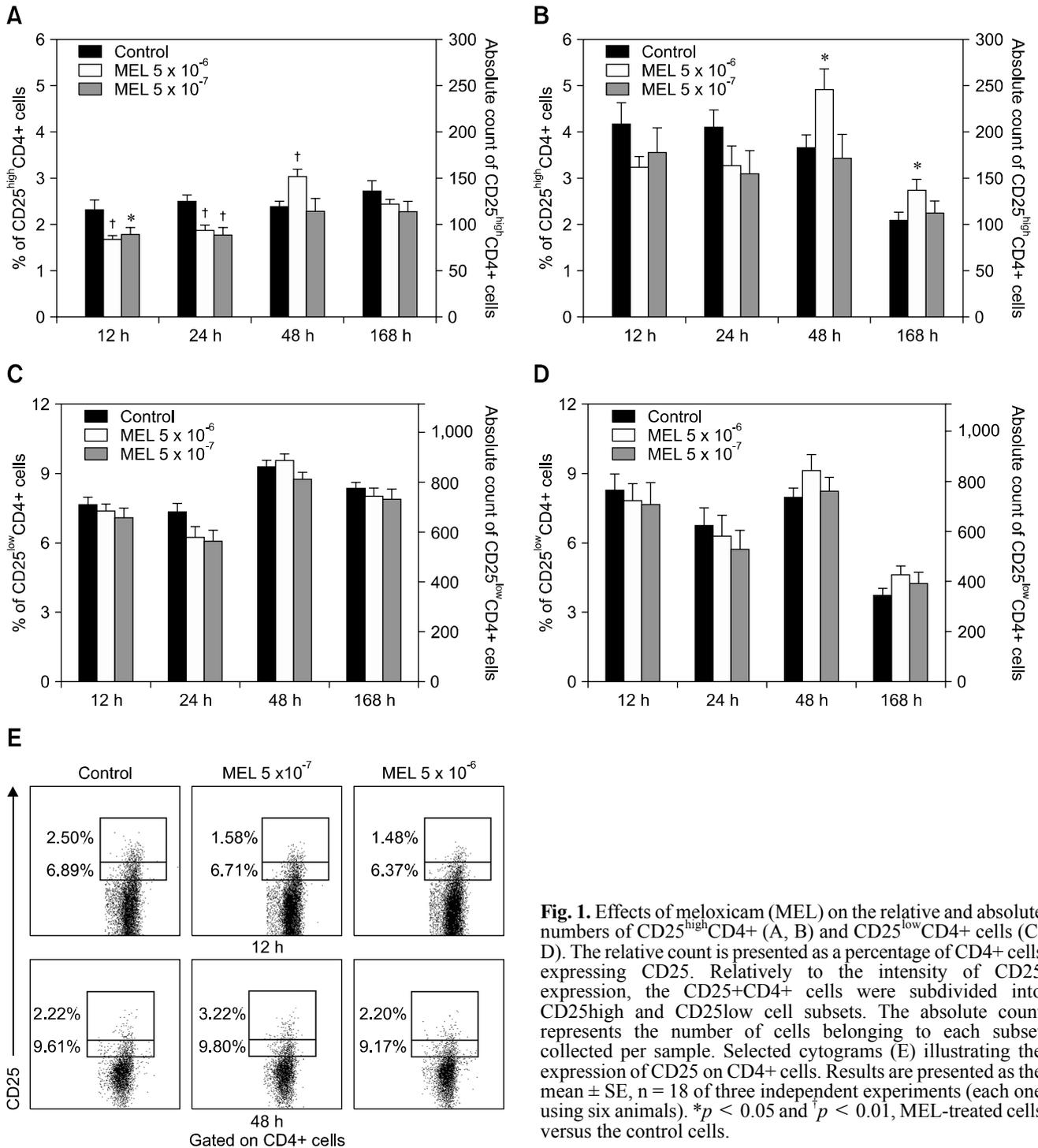


Fig. 1. Effects of meloxicam (MEL) on the relative and absolute numbers of CD25^{high}CD4⁺ (A, B) and CD25^{low}CD4⁺ cells (C, D). The relative count is presented as a percentage of CD4⁺ cells expressing CD25. Relatively to the intensity of CD25 expression, the CD25⁺CD4⁺ cells were subdivided into CD25^{high} and CD25^{low} cell subsets. The absolute count represents the number of cells belonging to each subset collected per sample. Selected cytograms (E) illustrating the expression of CD25 on CD4⁺ cells. Results are presented as the mean \pm SE, $n = 18$ of three independent experiments (each one using six animals). * $p < 0.05$ and [†] $p < 0.01$, MEL-treated cells versus the control cells.

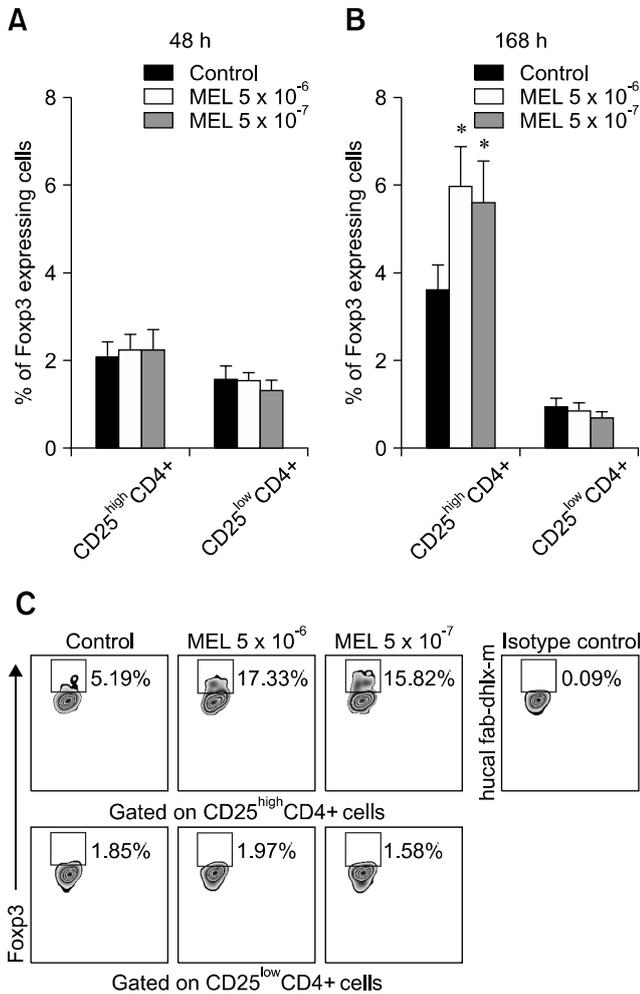


Fig. 2. Effect of MEL on the proportion of Foxp3+CD25^{high}CD4+ and Foxp3+CD25^{low}CD4+ cells. The results are expressed as a percentage of CD25^{high}CD4+ and CD25^{low}CD4+ cells expressing Foxp3. PBMCs were cultured alone or with MEL for 48 (A) and 168 h (B). Selected cytograms (C) illustrating Foxp3 expression within the CD25^{high}CD4+ and CD25^{low}CD4+ cells derived from Peripheral blood mononuclear cells (PBMCs) cultured long-term in the presence or absence of MEL. Data are expressed as the mean ± SE, n = 18 of three independent experiments (each one using six animals). **p* < 0.01, MEL-treated cells versus the control cells.

number of these cells was not significantly different from the control values. Exposure to MEL 5 × 10⁻⁶ for 48 and 168 h led to a considerable (*p* < 0.05) increase in the absolute number of CD25^{high}CD4+ cells (Fig. 1B). However, a significant (*p* < 0.05) increase in the relative cell number was recorded only at 48 h after the treatment (Figs. 1A and E). MEL did not affect the absolute number of CD25^{low}CD4+ lymphocytes (data not shown).

The influence of MEL on Foxp3 expression in CD25^{high}CD4+, CD25^{low}CD4+, and CD4+CD25^{low} cells was evaluated after 48 and 168 h of culturing in the presence or absence of the drug. Exposure to the drug for

48 h was not found to significantly affect the expression of Foxp3+ in the cell subpopulations (Fig. 2A). In contrast, prolonged (168 h) exposure of the PBMCs to MEL significantly (*p* < 0.05) increased the percentage of Foxp3+ cells among the CD25^{high}CD4+ lymphocytes (Figs. 2B and C). No Foxp3+ cells were present among the CD4+CD25^{low} cells in either the control or MEL-treated cultures (data not shown). The effect of MEL on apoptosis of CD25^{high}CD4+, CD25^{low}CD4+, and CD25^{low}CD4+ cells was evaluated after 12 and 24 h of incubation in the presence or absence of MEL. The drug was not found to have any significant effect on the percentage of cells undergoing early apoptosis among the CD4+ cell population (Fig. 3).

The effect of MEL on IL-10, TGF-β, and IFN-γ production was evaluated in PBMCs incubated with or without the drug and concurrent Con A stimulation in the presence of brefeldin A. MEL was not found to affect the percentage of IL-10+ or TGF-β+ cells in any of the lymphocyte subpopulations (Figs. 4A and B). MEL administered at the higher dose was shown to significantly increase (*p* < 0.05) the percentage of IFN-γ+ cells among CD4+CD25^{low} lymphocytes. On the other hand, the production of this cytokine by CD25^{high}CD4+ or CD25^{low}CD4+ cells was not significantly affected (Figs. 4C and D).

Discussion

Results of the present study showed that MEL did not deplete CD4+ lymphocyte populations, and could even increase the number of these cells relative to CD25^{high}CD4+ cell populations. Despite temporary fluctuations in the relative number of CD25^{high}CD4+ cells, MEL was not found to significantly affect the percentage of CD4+ lymphocytes (data not shown). This is consistent with our earlier findings [18] and those of other researchers [2] who observed that MEL administered at a single dose does not impact the percentage of CD4+ lymphocytes in calves. The absence of any negative effects of MEL on the absolute number of CD4+ lymphocytes concurs with the results of our evaluation showing the impact of MEL on apoptosis. We did not find MEL to have a significant effect on the percentage of cells in the early apoptotic stage in any of the subpopulations we assessed. Our previous investigation conducted on calves [18] also demonstrated that MEL does not alter the percentage of early apoptotic cells among CD4+ lymphocytes. An increase in the percentage of late apoptotic/necrotic cells among the CD4+ lymphocytes was observed after 24 h of exposure to the drug, but the increase was relatively small (the average difference between the drug-treated animals and the control group was about 1.5%) and short-term. It was thus doubtful that this effect was clinically relevant. Apart from

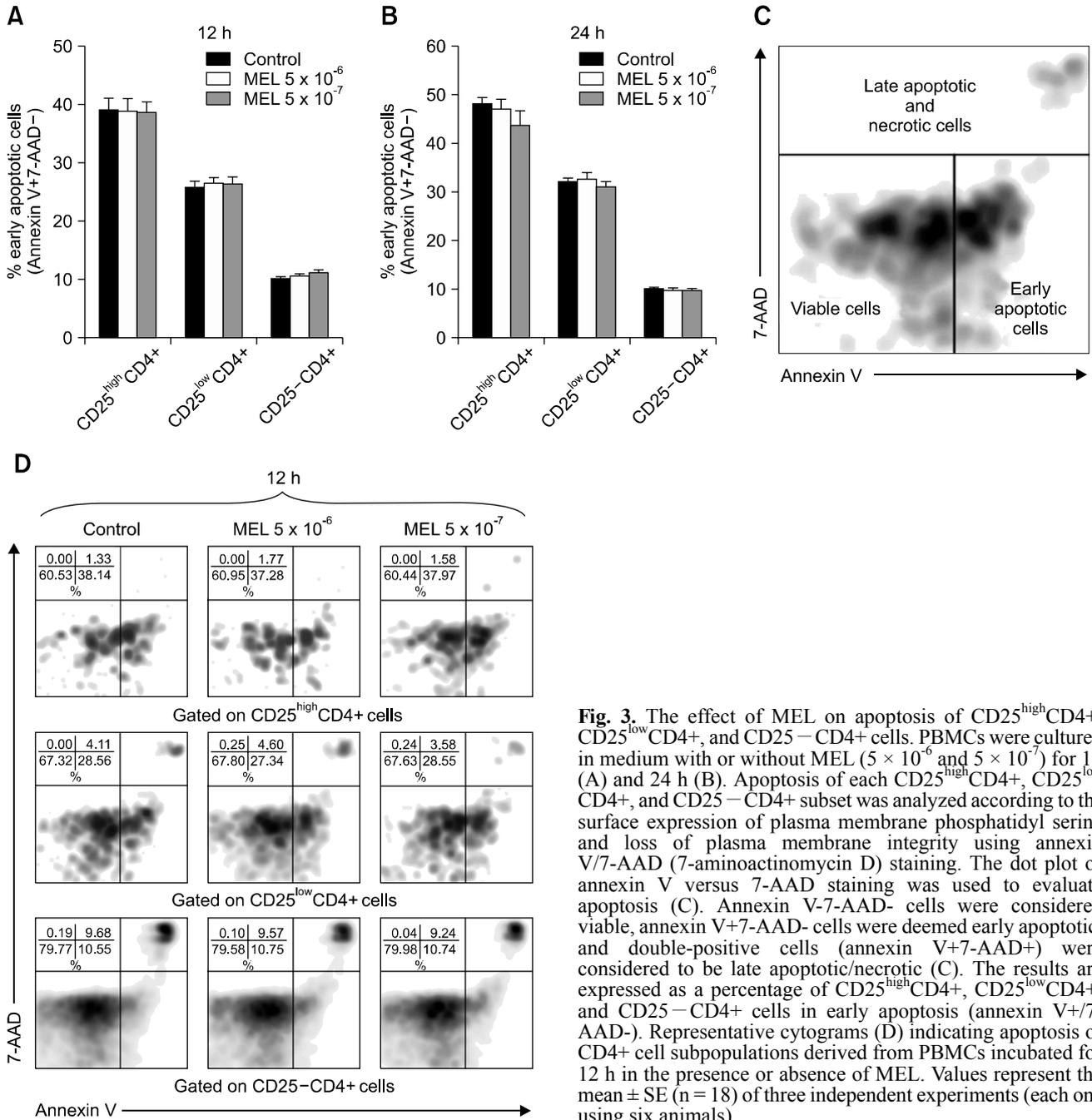


Fig. 3. The effect of MEL on apoptosis of CD25^{high}CD4⁺, CD25^{low}CD4⁺, and CD25⁻CD4⁺ cells. PBMCs were cultured in medium with or without MEL (5×10^{-6} and 5×10^{-7}) for 12 (A) and 24 h (B). Apoptosis of each CD25^{high}CD4⁺, CD25^{low}CD4⁺, and CD25⁻CD4⁺ subset was analyzed according to the surface expression of plasma membrane phosphatidyl serine and loss of plasma membrane integrity using annexin V/7-AAD (7-aminoactinomycin D) staining. The dot plot of annexin V versus 7-AAD staining was used to evaluate apoptosis (C). Annexin V-7-AAD⁻ cells were considered viable, annexin V+7-AAD⁻ cells were deemed early apoptotic, and double-positive cells (annexin V+7-AAD⁺) were considered to be late apoptotic/necrotic (C). The results are expressed as a percentage of CD25^{high}CD4⁺, CD25^{low}CD4⁺, and CD25⁻CD4⁺ cells in early apoptosis (annexin V+7-AAD⁻). Representative cytochrome plots (D) indicating apoptosis of CD4⁺ cell subpopulations derived from PBMCs incubated for 12 h in the presence or absence of MEL. Values represent the mean \pm SE (n = 18) of three independent experiments (each one using six animals).

our research, there has only been one report on the effect of MEL on lymphocyte mortality although the study focused on B cells. In this investigation, Kobayashi *et al.* [11] did not observe a decrease in the viability of normal B lymphocytes treated with etodolac or MEL.

After 12 and 24 h of incubation with MEL, a relatively small but statistically significant reduction of CD25^{high}CD4⁺ lymphocyte percentage was found. However, this was not reflected by the absolute numbers.

This disturbance was temporary and quickly resolved in that the percentage of CD25^{high}CD4⁺ after 48 h of drug exposure was the same as the control values (MEL 5×10^{-7}) or even higher (MEL 5×10^{-6}). Moreover, cultures treated with MEL 5×10^{-6} for 48 and 168 h were observed to contain more CD25^{high}CD4⁺ lymphocytes in terms of absolute numbers. These findings indicate that this phenomenon was not due to the pro- or anti-apoptotic effect of MEL on CD25^{high}CD4⁺, CD25^{low}CD4⁺, or

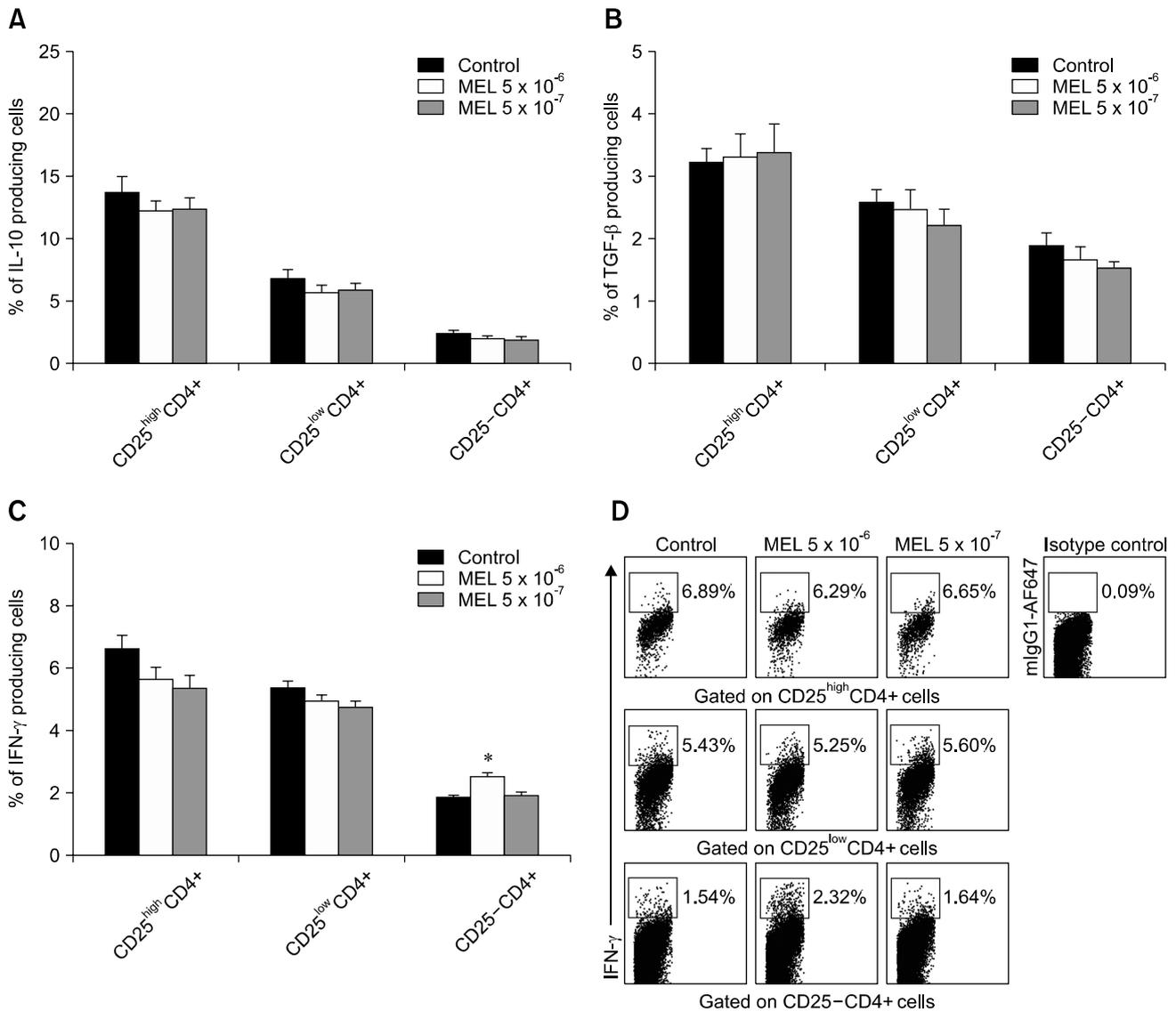


Fig. 4. The effect of MEL on IL-10 (A), TGF- β (B), and IFN- γ (C) production. The results are expressed as a percentage of CD25^{high}CD4⁺, CD25^{low}CD4⁺, and CD25-CD4⁺ cells expressing IL-10, TGF- β , or IFN- γ . Typical cytograms (D) illustrating IFN- γ expression in particular lymphocyte subpopulations are shown. Results are presented as the mean \pm SE (n = 18) of three independent experiments (each one using six animals). **p* < 0.01, MEL-treated cells versus the control cells.

CD25-CD4⁺ cells. Moreover, our latest study [16] provides evidence suggesting that MEL does not affect the proliferation of cells belonging to these subsets. Thus, alterations in the percentage and absolute number of CD25^{high}CD4⁺ cells observed in the present study could be caused by MEL-induced changes in the expression of CD25 molecules. It could be also speculated that MEL initially down-regulates and subsequently up-regulates CD25 expression on bovine CD4⁺ lymphocytes. Moreover, results obtained in the present study suggest that this later effect is dose-dependent and requires a MEL concentration achieved *in vivo* after therapeutic doses of the drug (0.5 mg/kg, MEL 5×10^{-6} M) are administered.

This hypothesis requires further study.

Next, the nature of MEL-induced CD25^{high}CD4⁺ lymphocytes was addressed. Our previous research [17] demonstrated that dexamethasone increases the absolute number of CD25^{high}CD4⁺ cells although this increase was several times higher than that induced by MEL. This has been interpreted as an increase in the number of activated rather than regulatory lymphocytes due to the fact that dexamethasone significantly reduces the percentage of Foxp3⁺ cells in the CD25^{high}CD4⁺ lymphocyte subpopulation. The situation was different with MEL. One cannot rule out the possibility that CD25^{high}CD4⁺ lymphocytes generated in response to MEL 5×10^{-6} have

regulatory properties since our current research revealed that prolonged exposure of PBMCs to MEL increased the percentage of Foxp3+ cells in the CD25^{high}CD4+ subpopulation. Considering the fact that treatment with MEL 5×10^{-6} increased the absolute number of CD25^{high}CD4+ lymphocytes, it should be assumed that the drug also increased the absolute number of Foxp3+CD25^{high}CD4+ cells. Theoretically, one could conclude that MEL can promote the generation of inducible T_{regs} (iT_{regs}) in cattle, but it seems that such a conclusion would be premature and far-fetched. It must be pointed out that the number of Foxp3+CD25+CD4+ cells in cattle is relatively small and increases induced by MEL were rather modest despite being statistically significant. Therefore, it is not certain whether such an effect is clinically relevant.

Considering the data presented above, it is obvious that caution should be exercised when interpreting the results indicating the effect of MEL on Foxp3+CD25^{high}CD4+ cells. It should be emphasised that the current literature is not helpful for interpreting results from the present study because the effects of MEL on CD25+CD4+ cells or Foxp3 expression remain unclear. These issues have been examined with regard to other NSAIDs [1,9,13,28] or other substances acting as COX-2 inhibitors [29], but many of the results are contradictory. In line with our findings, Atarashi *et al.* [1] reported that ketoprofen completely inhibits prostaglandin E₂ (PGE₂) production and simultaneously induces Foxp3 expression in lymph node cells, indicating an inverse correlation between PGE₂ production and Foxp3 expression. Other investigators [9] have demonstrated that acetyl-salicylic acid (ASA), another NSAID, significantly increases the relative levels of CD4+CD25+Foxp3+ T_{regs} cells among CD4+ cells in the periphery and thymus of mice. However, these studies did not prove that the drug-induced cells with a T_{reg} phenotype have, in fact, a regulatory effect. Nevertheless, it should be emphasized that there are reasons to believe that the cells generated via NSAID action may have regulatory potential. It has been demonstrated [1] in mice that skin application of ketoprofen systemically suppresses contact hypersensitivity to picryl chloride by increasing CD25+CD4+ regulatory cell numbers. Moreover, Yokoyama *et al.* [29] found that the COX-2 inhibitor NS-398 promotes the generation of CD4+ regulatory cells and has an immunomodulatory effect that induces the indefinite survival of cardiac allografts. Completely opposite results and conclusions were reported by other groups [13,15,28]. Lönnroth *et al.* [13] demonstrated a decrease in the levels of molecules associated with immunosuppressive T_{reg} cells, such as Foxp3 and IL-10, in tumor tissue from indomethacin-treated patients. Another study by Mahic *et al.* [15] found that the mechanism of CD25+CD4+ iT_{regs} cell production depends on COX-2

expression and PGE₂ secretion. This group concluded that PGE₂ acts in a paracrine manner to inhibit effector T cell responses, and exerts autocrine effects on iT_{regs} cells that lead to increased levels of intracellular cAMP and up-regulated Foxp3 expression. Moreover, other results of this team [28] suggest that iT_{regs} cells contribute to the formation of an immunosuppressive microenvironment in colorectal cancer (CRC) and inhibit effector T cells through a COX-2/PGE₂-dependent mechanism, thereby facilitating tumor growth. It was shown in this previous study that the COX inhibitor indomethacin significantly improves anti-tumor immune activity. Thus, it was concluded that therapeutic strategies targeting T_{reg} cells and the PGE₂-cAMP pathway may be useful for enhancing anti-tumor immune activity in CRC patients. However, there are contradictory data on the consequences of blocking COX-2 activity.

In the present study, the duration of exposure to MEL was highly correlated to *in vivo* conditions. The half-life of MEL in cattle is extremely long as previously shown by Coetzee *et al.* [5]. Researchers have found that the average half-life of MEL after a single oral administration is 27.54 h with a range (for different animals) from 19.97 to 44.29 h. The drug concentration decreases 10 times relative to the maximum concentration after about 100 h following drug administration. One should also take into account the fact that in cattle the drug is administered a second time several days after the first dose if needed.

The current study showed that MEL used at a higher dose increased IFN- γ production by CD25-CD4+ lymphocytes while the drug did not affect the percentage of IFN- γ + cells within the CD25^{high}CD4+ and CD25^{low}CD4+ cell subpopulations. Thus, MEL-induced increases of IFN- γ production in bovine CD4+ lymphocytes clearly involves only resting cells (*i.e.*, CD25-CD4+ cells) while CD25^{high}CD4+ and CD25^{low}CD4+ cells are “resistant” to this action of the drug. Determining whether this “resistance” is conditioned by the presence CD25 or whether the observed correlation is accidental requires further research. The available literature does not contain any precisely comparable data, but there have been reports about the effect of MEL on the levels of some cytokines in rat serum [21] or culturing medium of mouse splenocytes [20], or the mRNA expression of these cytokines [14]. Maeda *et al.* [14] did not observe any effect of MEL on IFN- γ mRNA expression in cultured PBMCs from calves. On the other hand, Oliveira *et al.* [21] found that administration of MEL in rats infected with *Trypanosoma cruzi* significantly increases the serum level of IFN- γ whereas splenocytes from mice infected with the same parasite produce significantly less of the cytokine when cultured with the drug [20]. These findings show that data demonstrating the effect of MEL on IFN- γ production are contradictory while our observations indicating that MEL

may increase production of the cytokine are not unique. The most probable explanation for our result is that IFN- γ production depends on COX-2 activity and PGE₂ production. Sharma *et al.* [23] demonstrated that COX-2 inhibition strongly induces IFN- γ production. Splenocytes from COX-2 inhibitor-treated mice have an enhanced capacity to secrete IFN- γ . Moreover, it has been demonstrated [10] that PGE₂ inhibits the production of IFN- γ by CD4+ cells from human cord blood. It was not observed in the present study that MEL significantly changed the percentage of IL-10+ or TGF- β + cells in the subpopulations being evaluated. We hypothesized that MEL might reduce IL-10 production because it has been previously demonstrated that COX-2-mediated PGE₂ enhances the production of IL-10, and that COX-2-produced PGE₂ might be essential for the reciprocal regulation of IL-10 and IL-12 production because it enhances IL-10 levels and spontaneously inhibits IL-12 production in LPS-treated bone marrow-derived dendritic cells (BM-DC) [7]. Moreover, there have been reports indicating that PGE₂ can also regulate IL-10 synthesis in lymphocytes. MEL has been shown to reduce production of PGE₂ by mouse splenocytes obtained from animals infected with *Paracoccidioides brasiliensis* [19] or *Trypanosoma cruzi* [20]. COX-2 is also associated with TGF- β production because its inhibition decreases TGF- β expression in some types of cultured cells [4]. However, these results clearly indicate that the production of IL-10 and TGF- β in bovine CD4+ lymphocytes does not depend on PGE₂, or that the doses of MEL used do not inhibit COX-2 activity to an extent sufficient for reducing cytokine production.

In summary, our results demonstrated that MEL did not induce immunosuppression via the depletion of CD4+ cells or decreased production of IFN- γ and increased IL-10 or TGF- β synthesis by these cells. The ability to generate Foxp3+CD25^{high}CD4+ cells indicates that the potential for such an effect exists. However, due to the small scale observed one should be cautious in attributing any clinical importance to this finding until appropriate studies have been conducted. The results obtained showed that with respect to the parameters being evaluated, meloxicam seems a relatively safe anti-inflammatory drug to be used in infectious diseases in cattle. Moreover, the drug seems to be able to induce IFN- γ production by CD4+ lymphocytes, which can be a positive attribute in patients who suffer from infections caused by intracellular pathogens.

References

1. **Atarashi K, Mori T, Yoshiki R, Kabashima K, Kuma H, Tokura Y.** Skin application of ketoprofen systemically suppresses contact hypersensitivity by inducing CD4⁺CD25⁺ regulatory T cells. *J Dermatol Sci* 2009, **53**, 216-221.
2. **Bednarek D, Zdzisińska B, Kondracki M, Kandeferszerzeń M.** Effect of steroidal and non-steroidal anti-inflammatory drugs in combination with long-acting oxytetracycline on non-specific immunity of calves suffering from enzootic bronchopneumonia. *Vet Microbiol* 2003, **96**, 53-67.
3. **Braitch M, Harikrishnan S, Robins RA, Nichols C, Fahey AJ, Showe L, Constantinescu CS.** Glucocorticoids increase CD4⁺CD25^{high} cell percentage and Foxp3 expression in patients with multiple sclerosis. *Acta Neurol Scand* 2009, **119**, 239-245.
4. **Chávez E, Segovia J, Shibayama M, Tsutsumi V, Vergara P, Castro-Sánchez L, Salazar EP, Moreno MG, Muriel P.** Antifibrotic and fibrolytic properties of celecoxib in liver damage induced by carbon tetrachloride in the rat. *Liver Int* 2010, **30**, 969-978.
5. **Coetzee J, Kukanich B, Mosher R, Allen P.** Pharmacokinetics of intravenous and oral meloxicam in ruminant calves. *Vet Ther* 2009, **10**, E1-8.
6. **Friton GM, Cajal C, Ramirez-Romero R.** Long-term effects of meloxicam in the treatment of respiratory disease in fattening cattle. *Vet Rec* 2005, **156**, 809-811.
7. **Harizi H, Juzan M, Pitard V, Moreau JF, Gualde N.** Cyclooxygenase-2-inhibited prostaglandin E₂ enhances the production of endogenous IL-10, which down-regulates dendritic cell functions. *J Immunol* 2002, **168**, 2255-2263.
8. **Hoek A, Rutten VPMG, Kool J, Arkesteijn GJA, Bouwstra RJ, Van Rhijn I, Koets AP.** Subpopulations of bovine WC1⁺ $\gamma\delta$ T cells rather than CD4⁺CD25^{high}Foxp3⁺ T cells act as immune regulatory cells ex vivo. *Vet Res* 2009, **40**, 6.
9. **Javed A, Zhang B, Qu Y, Zhang A, Sun C, Zhang L, Liu J, Zeng C, Zhao Y.** The significantly enhanced frequency of functional CD4⁺CD25⁺Foxp3⁺ T regulatory cells in therapeutic dose aspirin-treated mice. *Transpl Immunol* 2009, **20**, 253-260.
10. **Katamura K, Shintaku N, Yamauchi Y, Fukui T, Ohshima Y, Mayumi M, Furusho K.** Prostaglandin E₂ at priming of naive CD4⁺ T cells inhibits acquisition of ability to produce IFN- γ and IL-2, but not IL-4 and IL-5. *J Immunol* 1995, **155**, 4604-4612.
11. **Kobayashi M, Nakamura S, Shibata K, Sahara N, Shigeno K, Shinjo K, Naito K, Ohnishi K.** Etodolac inhibits EBER expression and induces Bcl-2-regulated apoptosis in Burkitt's lymphoma cells. *Eur J Haematol* 2005, **75**, 212-220.
12. **Liu H, Peng Y, Liu F, Li J, Chen X, Liu Y, Zhang H.** A selective cyclooxygenase-2 inhibitor decreases transforming growth factor- β 1 synthesis and matrix production in human peritoneal mesothelial cells. *Cell Biol Int* 2007, **31**, 508-515.
13. **Lönnroth C, Andersson M, Arvidsson A, Nordgren S, Brevinge H, Lagerstedt K, Lundholm K.** Preoperative treatment with a non-steroidal anti-inflammatory drug (NSAID) increases tumor tissue infiltration of seemingly activated immune cells in colorectal cancer. *Cancer Immunol* 2008, **8**, 5.
14. **Maeda Y, Tanaka R, Ohtsuka H, Matsuda K, Tanabe T, Oikawa M.** Comparison of the immunosuppressive effects

- of dexamethasone, flunixin meglumine, and meloxicam on the *in vitro* response of calf peripheral blood mononuclear cells. *J Vet Med Sci* 2011, **73**, 957-960.
15. **Mahic M, Yaqub S, Johansson CC, Taskén K, Aandahl EM.** FOXP3⁺CD4⁺CD25⁺ adaptive regulatory T cells express cyclooxygenase-2 and suppress effector T cells by a prostaglandin E₂-dependent mechanism. *J Immunol* 2006, **177**, 246-254.
 16. **Maślanka T, Jaroszewski JJ.** Dexamethasone, but not meloxicam, suppresses proliferation of bovine CD25⁺CD4⁺ and CD25⁻CD4⁺ cells. *Pol J Vet Sci* 2013, **16**, 219-222.
 17. **Maślanka T, Jaroszewski JJ.** *In vitro* effects of dexamethasone on bovine CD25⁺CD4⁺ and CD25⁻CD4⁺ cells. *Res Vet Sci* 2012, **93**, 1367-1379.
 18. **Maślanka T, Jaroszewski JJ, Markiewicz W, Jakubowski P.** Evaluation of the influence of meloxicam and flunixin meglumine on the apoptosis of peripheral blood CD4⁺ and CD8⁺ T cells in calves. *Pol J Vet Sci* 2010, **13**, 3-12.
 19. **Michelin MA, Figueiredo F, Cunha FQ.** Involvement of prostaglandins in the immunosuppression occurring during experimental infection by *Paracoccidioides brasiliensis*. *Exp Parasitol* 2002, **102**, 170-177.
 20. **Michelin MA, Silva JS, Cunha FQ.** Inducible cyclooxygenase released prostaglandin mediates immunosuppression in acute phase of experimental *Trypanosoma cruzi* infection. *Exp Parasitol* 2005, **111**, 71-79.
 21. **Oliveira LGR, Kuehn CC, Santos CD, Toldo MPA, do Prado JC Jr.** Enhanced protection by melatonin and meloxicam combination in experimental infection by *Trypanosoma cruzi*. *Parasite Immunol* 2010, **32**, 245-251.
 22. **Provoost S, Maes T, van Durme YM, Gevaert P, Bachert C, Schmidt-Weber CB, Brusselle GG, Joos GF, Tournoy KG.** Decreased FOXP3 protein expression in patients with asthma. *Allergy* 2009, **64**, 1539-1546.
 23. **Sharma S, Zhu L, Yang SC, Zhang L, Lin J, Hillinger S, Gardner B, Reckamp K, Strieter RM, Huang M, Batra RK, Dubinett SM.** Cyclooxygenase 2 inhibition promotes IFN- γ -dependent enhancement of antitumor responses. *J Immunol* 2005, **175**, 813-819.
 24. **Sakaguchi S.** Naturally arising CD4⁺ regulatory T cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol* 2004, **22**, 531-562.
 25. **Vane JR, Botting RM.** The mechanism of action of aspirin. *Thromb Res* 2003, **110**, 255-258.
 26. **Workman CJ, Szymczak-Workman AL, Collison LW, Pillai MR, Vignali DAA.** The development and function of regulatory T cells. *Cell Mol Life Sci* 2009, **66**, 2603-2622.
 27. **Xie Y, Wu M, Song R, Ma J, Shi Y, Qin W, Jin Y.** A glucocorticoid amplifies IL-2-induced selective expansion of CD4⁺CD25⁺FOXP3⁺ regulatory T cells *in vivo* and suppresses graft-versus-host disease after allogeneic lymphocyte transplantation. *Acta Biochim Biophys Sin (Shanghai)* 2009, **41**, 781-791.
 28. **Yaqub S, Henjum K, Mahic M, Jahnsen FL, Aandahl EM, Bjørnbeth BA, Taskén K.** Regulatory T cells in colorectal cancer patients suppress anti-tumor immune activity in a COX-2 dependent manner. *Cancer Immunol Immunother* 2008, **57**, 813-821.
 29. **Yokoyama T, Aramaki O, Takayama T, Takano S, Zhang Q, Shimazu M, Kitajima M, Ikeda Y, Shirasugi N, Niimi M.** Selective cyclooxygenase 2 inhibitor induces indefinite survival of fully allogeneic cardiac grafts and generates CD4⁺ regulatory cells. *J Thorac Cardiovasc Surg* 2005, **130**, 1167-1174.