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Dexamethasone affects day/night development and function of thymus-derived T regulatory cells

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ABSTRACT

Thymus-derived T regulatory (tTregs) cells play a crucial role in the maintenance of tolerance and immune homeostasis. Mechanisms and factors regulating tTreg development and function are widely investigated, but to a large degree still remain unclear. Our previous findings demonstrated that, in physiological conditions, the development and suppressive function of tTregs demonstrated day/night rhythmicity, which correlated with the concentration of plasma corticosterone and the expression of glucocorticoid receptors. In this study we ask whether synthetic glucocorticoids commonly used to inhibit excessive activity of the immune system, can modulate the development and suppressive function of tTregs *in vivo* depending on the time of administration. Young C57BL/6 male and female mice were injected intraperitoneally with a single dose of dexamethasone at two time points of the day: 7.00–8.00 a.m. and 7.00–8.00 p.m. The experimental can be used to indicate on the potentially expected positive or adverse side effects and can constitute also a good model for the assessment of the effects of long-term therapy. The results of our studies demonstrated the increase of the percentage of tTregs at both time points in male mice, but only in the evening in females. The suppressive activity of tTregs increased independently on the day time of in female mice, but in the morning only in males. We concluded that in the condition of dexamethasone supplementation, the elevated suppressive potential of tTregs is balanced by the induction apoptosis in order to prevent excessive suppression.

1. Introduction

Regulatory T cells (Tregs) characterized by the $CD4^+CD25^+Foxp3^+$ phenotype develop naturally in the thymus or in peripheral lymphoid organs upon activation (Engel et al., 2013; Bin Dhuban et al., 2014). Currently, they are classified according to their origin as thymus-derived regulatory T cells Tregs (tTregs) and peripherally-derived Tregs (pTregs) (Elkord, 2014). Tregs, regardless of their origin, suppress various physiological and pathological immune responses and the activity of these cells is dependent on a variety of agents constituting the local microenvironment. Thymus-derived Tregs migrate to the peripheral lymphoid organs as mature T cells with suppressive activity. They play a crucial role in the maintenance of self-tolerance, immune homeostasis (Sakaguchi, 2005; Wing and Sakaguchi, 2010), and have a therapeutic potential to suppress autoreactive and overactivated T cells leading to the protection from autoimmune diseases and chronic inflammation. Mechanisms and factors regulating tTreg development and function *in vivo* and *in vitro* are widely investigated but to a large degree still remain unclear (Thornton et al., 2010; Sojka et al., 2008; Sakaguchi et al., 2009). Thymus-derived regulatory T cells are not easily distinguished by the expression of a characteristic surface marker from Tregs developed in peripheral lymphoid organs thus, the thymus remains the best organ to follow changes in their development and relative distribution. Commonly accepted molecular marker and cell lineage specification factor for Treg, which is also required for their suppressive function, is still the transcription factor Foxp3 (Fontenot et al., 2003; Rudensky, 2011). It is also considered that the majority of Tregs are generated in the thymus and exported to the periphery as

Abbreviations: CFSE, carboxyfluorescein diacetate succinimidyl ester; Dex, dexamethasone; DN, double negative thymocytes; DP, double positive thymocytes; Foxp3, forkhead box P3; GC, glucocorticoids; GCR, glucocorticoid receptor; GITR, glucocorticoid induced TNF-family receptor; HPA, hypothalamic-pituitary-adrenal axis; MFI, mean fluorescence intensity; Nrp1, neuropilin-1; pTregs, peripherally-derived regulatory T cells; SP, single positive thymocytes; Tregs, T regulatory cells; tTregs, thymus-derived T regulatory cells

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Foxp3-expressing cells, where they cannot be unambiguously distinguished from pTregs by the expression of Neuropilin-1 or Helios (Szurek et al., 2015).

Different factors are involved in the induction of Foxp3, which is mandatory for the generation and activity of tTregs: interaction with dendritic cells, anti-inflammatory cytokines, vitamins, pathogen-derived molecules and hormones (Bettelli et al., 2006). Special attention is focused on the role of glucocorticoids, which are indispensable and widely used anti-inflammatory drugs in a variety of diseases related to the overactive immune system. The bad side of GC therapy is related to systemic side effects, mainly in chronic and high dose application. The results of Bordag et al. demonstrated a severe metabolic deregulation induced by single-dose, short-term application of synthetic glucocorticoid in humans (Bordag et al., 2015). The good side of GC activity showed by Chen et al. is to increase the proportion of $CD4^+CD25^+$ of T cells and the ratio of $CD4^+CD25^+/CD4^+CD25^-$ cells especially in the thymus (Chen et al., 2004). This correlated with higher level of GCR, Bcl-2 and resistance to Dex-induced apoptosis of $CD4^+CD25^+$ thymocytes compared to $CD4^+$CD25- cells (Chen et al., 2004; Huang and Cidlowski, 2002). This effect was induced by i.p. injection of single-dose in BALB/c mice. Glucocorticoids may act indirectly and modulate the tolerogenic activity of antigen-presenting cells or directly by inducing Foxp3 and GITR expression (Karagiannidis, 2004; Ronchetti et al., 2015; van Kooten et al., 2009). GCs are produced mainly in the adrenal gland, but they are also produced in the thymus where they are involved in thymocyte development (Ashwell et al., 2000). The recent paper of Berki's group confirm the role of these hormones as selecting factor for $CD4^+CD25^+Foxp3^+$ generation (Ugor et al., 2018).

The activity of the immune system shows day/night variation, which is strongly coordinated with sleep regulation and circadian system (Irwin, 2002; Besedovsky et al., 2012). The activity of the immune system is higher at night in humans, and during the day in mice, which are nocturnal animals. During sleep the activity of the immune system is potentiated by hormones showing immunostimulatory properties (melatonin, prolactin). In contrast, during the active phase, the immune system is suppressed. In our previous paper we demonstrated that day/night rhythmicity of the activity of the immune system correlates with the plasma concentration of corticosterone in C57BL/6 mice, and can be regulated by thymus-derived T regulatory cells, which change their development and suppressive activity in day/night dependent manner (Kiernozek et al., 2014). In addition, changes in the suppressive activity of tTregs correlated with the expression of glucocorticoids receptors.

In this study we ask the question whether dexamethasone, a synthetic glucocorticoid used in therapy of a variety of diseases can be considered a factor modulating the rhythmic activity and distribution of thymus-derived T regulatory cells. The experimental design of our study is based on the assumption that a short-term single-dose glucocorticoid application may indicate on the potential expected positive or adverse side effects and constitute a good model of the assessment of the effects of long-term therapy. We compared the role of dexamethasone in male and female mice based on the sexual dimorphism of the immune system and contribution of HPA hormones to gender differences in health risk (Uhart et al., 2006).

2. Materials and methods

2.1. Mice

8-10 weeks old female and male mice of C57BL/6 strain were used. Animals were maintained in standardized conditions for temperature (22–24 °C) and light (12/12 h light/dark cycles). All experiments were performed according to the permission of the Local Ethical Commission.

2.2. Dexamethasone treatment

The mice were injected intraperitoneally with a single dose of dexamethasone at two time points in which the concentrations of endogenous GC are the lowest and the highest: in the morning at 7.00–8.00 a.m. and in the evening at 7.00–8.00 p.m. corresponding to ZT1–ZT2 ZT13–ZT14, respectively. The dose of dexamethasone used in the experiments was chosen based on the results of preliminary study and on the literature data (Chen et al., 2004; Karssen et al., 2009; Gomez et al., 1998). In our preliminary experiments, we checked a panel of doses from low (1.25 μg/kg of body weight) to high (5 mg/kg of body weight) doses of Dex. However, we have taken into consideration that injected, infused, or orally administered systemic glucocorticoids have been associated with immediate hypersensitivity reactions, including life-threatening anaphylaxis. The dose 5 mg/kg resulted in severe adverse life-threatening effects immediately post-injection. For these study we have used single-dose 1.25 mg/kg, the highest which did not give noticeable adverse affects. Dexamethasone was dissolved in DMSO and the final concentration of the solvent did not exceeded 2.5%. Control mice were injected with the same volume of 2.5% DMSO in PBS. Thymus and blood samples were taken for further analysis 24 h after every single dose injection.

2.3. Isolation of thymocytes

Isolated thymus were homogenized in cold PBS and filtered through 100 μm cell restrainer.

2.4. Flow cytometric analysis of the main thymocyte subsets and tTregs

The main thymocyte subsets were distinguished on the basis of the expression of CD4 and CD8 molecules: DN ($CD4^-CD8^-$), DP ($CD4^+CD8^+$), SP $CD4^+$ ($CD4^+CD8^-$) and SP $CD8^+$ ($CD8^+CD4^-$). Thymus-derived Tregs are recognized as $CD4^+CD25^+Foxp3^+$ within SP $CD4^+CD8^-$ thymocytes.`

Thymocytes were stained with monoclonal antibodies anti-CD4/FITC, CD8/PercP and CD25/APC (BD PharMingen). The selection of concentrations of antibodies were made based on titration. PE anti-mouse/rat/human Foxp3 Flow Kit (BioLegend) was used to detect the transcription factor Foxp3 and to determine the level of its expression by MFI value (mean fluorescence intensity). Four-colour analysis was performed using CellQuest or FACSDiva software.

2.5. Measurement of the suppressive activity of tTregs

Thymic tTregs were isolated basing on the expression of CD25. The suppressive activity of tTregs was examined by the inhibition of proliferation of activated $CD4^+CD25^-$ T from lymph nodes. $CD4^+CD25^+$ T cells and $CD4^+CD25^-$, respectively, from thymus and axillary lymph nodes were sorted at two time points at 7.00–8.00 a.m. and 7.00–8.00 p.m. using FACSAria (Becton Dickinson). To check the level of viability, sorted cells were examined by trypan blue exclusion. To evaluate the proliferation of responding activated T cells, the CFSE staining was performed (Lyons and Parish, 1994).

In brief: 25×10^3 CFSE stained $CD4^+CD25^-$ T cells were activated by 0.5 μg/ml anti-CD3 and 0.05 μg/ml anti-CD28 mAb (BD Biosciences) and cultured with or without 5×10^3 tTreg cells in 96-well plates ($CD4^+CD25^-$ / tTreg cell ratio 5:1). Cells were incubated 72 h in RPMI 1640 + GlutaMAX medium (GIBCO) containing 20 mM HEPES, 1 mM sodium pyruvate, 5×10^{-5} M 2-ME and 10% heat-inactivated FCS in standard conditions of 37 °C and 5% of CO_2.

Non-activated T $CD4+CD25^-$ cells were used as negative control. The proliferation of responding T cells was measured by flow cytometry using FACSCalibur (Becton Dickinson) and the percentage of proliferating cells was calculated using FACSDiva software. Suppressive activity of tTregs was evaluated by the percentage of inhibition,

according to the following formula (Bollinger et al., 2009):

$$\%\text{inhibition} = 100 - \frac{\%\text{of proliferated cells in the tTreg assay} \times 100}{\%\text{ of proliferated cells in the T cell assay}}$$

2.6. Measurement of glucocorticoids plasma level

Glucocorticoids concentration were determined in plasma samples stored at −80 °C.

Corticosterone plasma level: total plasma corticosterone was extracted with ethyl ether for the measurement of corticosterone using a commercial kit (Corticosterone ELISA Kit, Neogen Corporation) according to the procedure recommended by the company.

Dexamethasone plasma level: plasma dexamethasone concentration was determined using a commercial kit (Dexamethasone ELISA Kit, Neogen Corporation) according to the recommended procedure.

Quantitative results were obtained by measuring the absorbance at 450 nm using the microplate reader (VICTOR 3, Multilabel Plate Readers).

2.7. Glucocorticoid receptor expression

Flow cytometry was used for the identification of GCR based on the binding of fluorescein-dexamethasone complex (Kowalik et al., 2013).

2.8. Apoptosis assay

Apoptotic thymocytes were detected by Annexin V/PE conjugated staining and analyzed by flow cytometry. The percentage of apoptotic cells was examined for all thymocyte subsets and tTregs.

2.9. Statistical analysis

Analysis of variance (ANOVA) was used to evaluate the significance of differences in the percentage of DN, DP, SP thymocytes and thymic Tregs between control and experimental groups ($P < 0.05$). The Kolmogorov-Smirnov statistic was used to evaluate changes in the expression of Foxp3 and GC receptors. Statistically significant differences are marked on the figures or representative histograms and density plots.

3. Results

3.1. Corticosterone concentration in the plasma

In vertebrates, endogenous glucocorticoids concentration, usually peaks shortly before the onset of activity. In mice, the lowest concentration of corticosterone is observed in the morning and the highest in the evening. After the administration of dexamethasone, the plasma concentration of corticosterone didn't change significantly 24 h post injection both in male and female mice (Fig. 1). Detectable level of dexamethasone in the plasma of injected mice was found only 24 h after evening injection (7.00–8.00 p.m.) regardless of the sex of animals (data not shown).

3.2. Day/night changes of thymocyte number and thymocyte subset distribution in dexamethasone-injected mice

Dexamethasone decreased the number of thymocytes at ZT1–ZT2 (7.00–8.00 a.m.) and ZT13–ZT14 (7.00–8.00 p.m.) regardless of the sex of animals, however the number of thymocytes remained still higher in females (Fig. 2A). The average percentage difference between control and Dex-treated mice was greater in females in the morning, and in males in the evening. We didn't observe changes in the distribution of thymocyte subsets (DN $CD4^-CD8^-$, DP $CD4^+CD8^+$, SP $CD4^+CD8^-$ and SP $CD8^+CD4^-$) in Dex-injected mice at 7.00–8.00 a.m. (Fig. 2B, C). However, we observed statistically significant differences in Dex-injected mice in DP $CD4^+CD8^+$, SP $CD4^+$ and SP $CD8^+$ thymocyte subsets at 7.00–8.00 p.m.: the percentage of DP $CD4^+CD8^+$ was decreased and of SP $CD8^+$ was increased in males, in contrast to increased percentage of DP and decreased of SP $CD4^+$ in females compared to the physiological condition (Ctrl). These changes resulted in the statistically significant diminution of CD4:CD8 ratio at 7.00–8.00 p.m. which is greater in males (Fig. 2D).

3.3. Changes of thymus-derived T regulatory cell development in Dex-injected mice

The sequential stages of tTreg development were analyzed based on the expression of CD25 and Foxp3: $CD4^+CD25^+Foxp3^-$ represent the less mature stage, and the fully mature tTregs are characterized by the $CD4^+CD25^+Foxp3^+$ phenotype. The results of our studies have shown that in Dex-injected male mice the percentage of $CD4^+CD25^+$ thymocytes in SP $CD4^+CD8^-$ subset significantly increased independently of the day time. Dex-injection of females resulted in the increase of the percentage of $CD4^+CD25^+$ thymocytes in the evening only (Fig. 3A). Dexamethasone induced the expression of $Foxp3^+$ cells within $CD4^+CD25^+$ thymocytes independently of the day time in males, and at 7.00–8.00 p.m. in females (Fig. 3B). In addition, the percentage of mature tTregs after Dex injection was higher in the thymus of male than female mice independently of the day time. The average percentage difference between control and Dex-treated mice was greater in males in the morning at 7.00–8.00 a.m.

Thymus-derived Treg development can be followed based on the increase of CD25 expression and percentage of $Foxp3^+$ thymocytes within $CD4^+CD25^{low}$ and $CD4^+CD25^{high}$ populations pointing on the kinetics of development. Dexamethasone treatment resulted in the increase of the percentage of Foxp3+ cells in both $CD4^+CD25^{low}$ and $CD25^{high}$ population at 7.00–8.00 a.m. and $CD25^{high}$ population at 7.00–8.00 p.m. in the thymus of male mice, and in $CD4^+CD25^{high}$ population of the female thymus in the evening only (Fig. 4A) In addition, dexamethasone caused a higher increase in the percentage of $CD4^+CD25^{high}$ population in female thymus. The level of expression of Foxp3 in tTregs increased significantly after Dex-injection in $CD4^+CD25^{low}$ and $CD4^+CD25^{high}$ populations at 7.00–8.00 p.m., both in males and females (Fig. 4B). Furthermore, the greater difference in Foxp3 expression was demonstrated in female thymus at 7.00–8.00 p.m.

3.4. Dexamethasone-induced apoptosis

The immune cells, especially thymocytes and T cells are very sensitive to glucocorticoids-induced apoptosis [30]. Therefore, we examined if the changes in the contribution of tTregs in the thymus of Dex-injected mice might be due to changes in their development or different sensitivity to apoptosis induction compared to other thymocyte subsets. The percentage of apoptotic thymocytes in Dex-injected male mice increased significantly in DP, $CD4^+CD25^+$ thymocytes at 7.00–8.00 a.m. and in SP $CD4^+$, SP $CD8^+$, DP thymocytes at 7.00–8.00 p.m. (Fig. 5). The percentage of apoptotic thymocytes in female thymus significantly increased in DN, $CD4^+CD25^+$ at 7.00–8.00 a.m. and in all thymocyte subsets at 7.00–8.00 p.m. The higher difference in the percentage of apoptotic thymocytes resulting from Dex treatment was observed in DP at 7.00–8.00 p.m. in males and in $CD4^+CD25^+$ at ZT1–ZT2 and in SP $CD4^+$ at ZT13–ZT14 in females.

3.5. Dexamethasone influence on the expression of glucocorticoids receptors

Dex-injection resulted in the decrease of GCR expression in DP, SP $CD4^+$, SP CD8+ thymocytes at 7.00–8.00 a.m. and SP $CD4^+$, SP $CD8^+$, $CD4^+CD25^+$ thymocytes at.700–8.00 p.m. in males. In females, in contrary, dexamethasone increased GCR expression in DN, SP $CD4^+$,

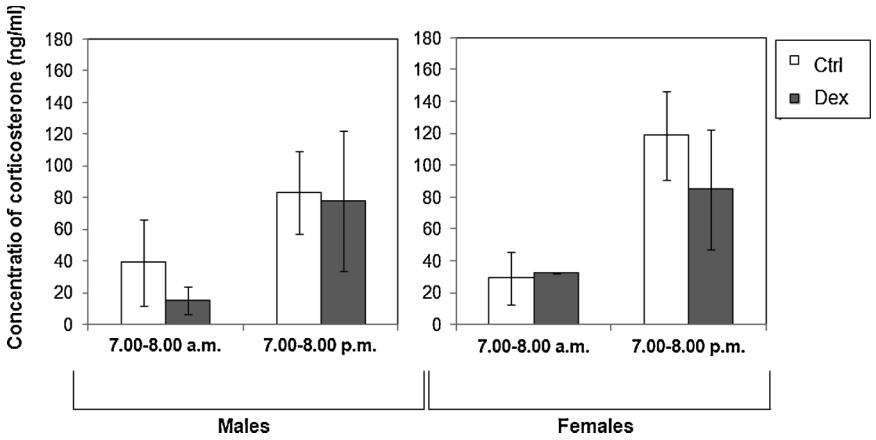


Fig. 1. Concentration of corticosterone in the plasma.
Three independent experiments were performed. 2–3 mice were used for each time point in each experiment. $p < 0.05$.

$CD4^+CD25^+$ thymocytes at 7.00–8.00 p.m. The opposite effect of Dex on the expression of glucocorticoid receptors of $CD4^+CD25^+$ thymocytes in male and female mice, may at least in part, explain the sensitivity of these cells to Dex-induced apoptosis. However, the same effect did not affect changes in apoptosis of thymocytes of other subsets with one exception of DN thymocytes in females in the evening (Fig. 6).

3.6. Suppressive activity of tTregs of dexamethasone-injected mice

The results of our study showed that the suppressive activity of tTregs was potentiated by Dex treatment (Fig. 7). The percentage of proliferating responder T cells (T $CD4^+CD25^-$ T cells) was decreased in males at 7.00–8.00 a.m. and in females in both time points after administration of dexamethasone (Fig. 7A and B). The percentage of

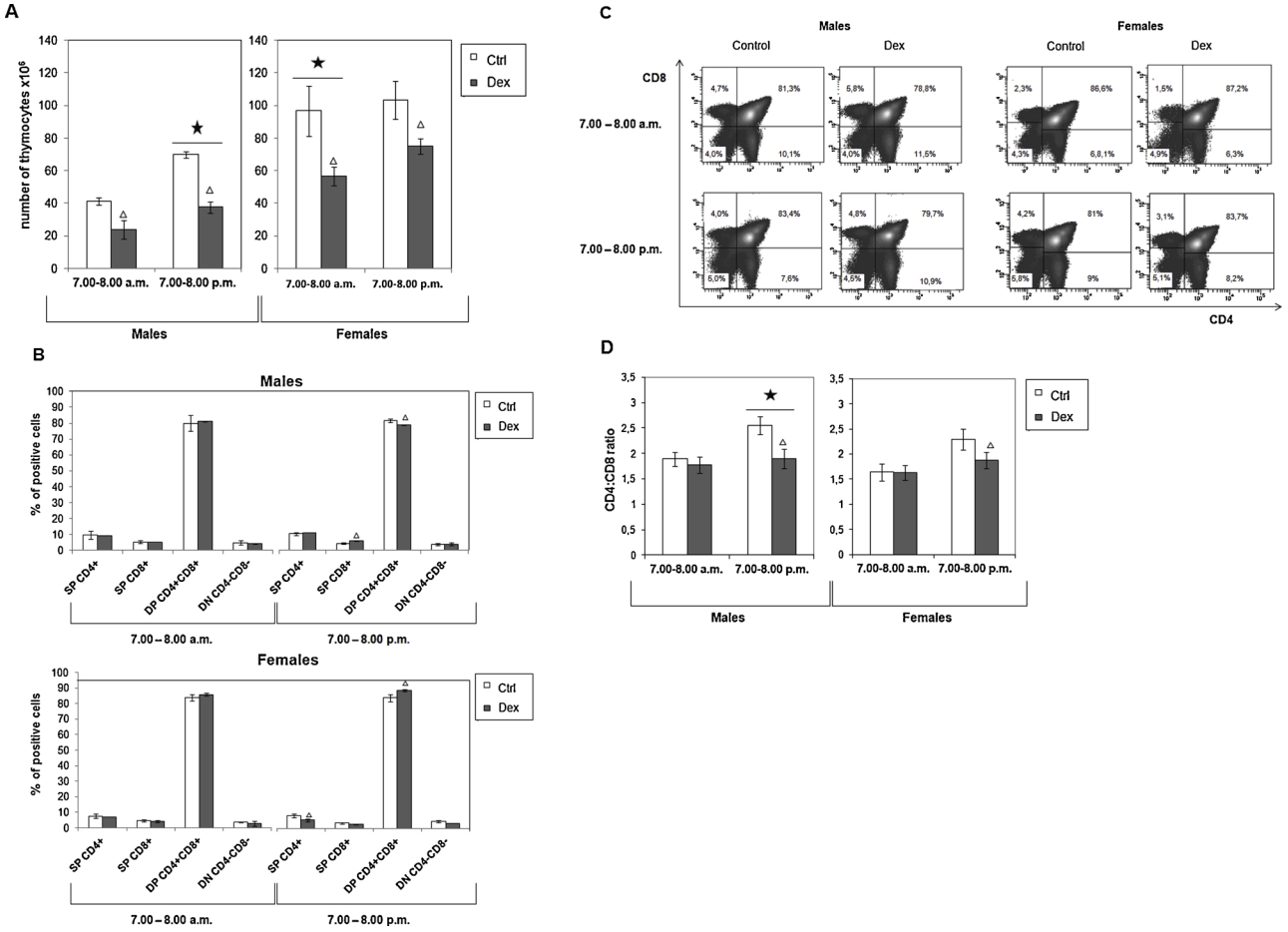


Fig. 2. Thymocyte number and thymocyte subset distribution in Dex-injected mice.
(A) Thymocyte number; (B) Thymocyte subset distribution; (C) Thymocyte subsets on representative cytograms; (D) CD4:CD8 ratio.
Data represent the results of three independent experiments performed on 2–3 mice for each day time. $p < 0.05$.
△ Statistically significant differences after Dex treatment.
★ The average percentage difference between control and Dex-treated mice depending on animal's sex.

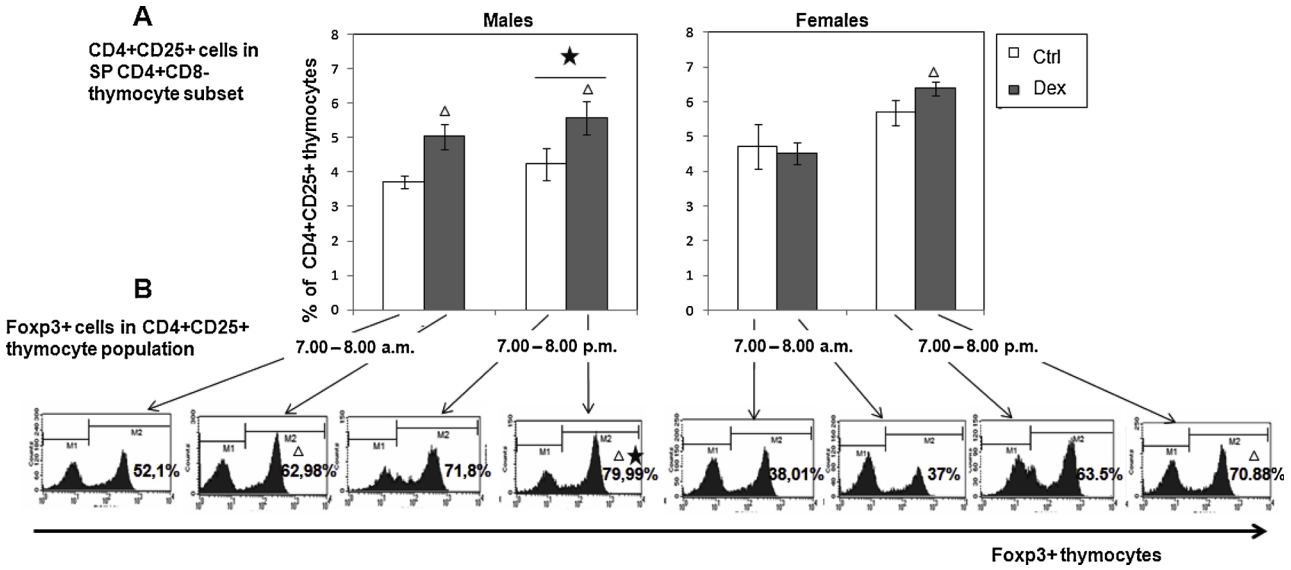


Fig. 3. tTreg development in dexamethasone-injected mice.
(A) CD4 + CD25 + thymocytes in SP CD4 + CD8- subset; (B) Foxp3 + thymocytes in CD4 + CD25 + population.
Data represent the results of three independent experiments performed on 2–3 mice for each day time. $p < 0.05$.
△ Statistically significant differences after Dex treatment.
★ The average percentage difference between control and Dex-treated mice depending on animal's sex.

inhibition increased from 58% to 97% at 7.00–8.00 a.m. in males, and from 18% to 28% and 67% to 89% at 7.00–8.00 a.m. and 7.00–8.00 p.m. respectively in female mice (Fig. 7C). The greater effect of Dex on the tTreg suppressive activity was observed in males at 7.00–8.00 a.m.

4. Discussion

The results of our previous study demonstrated day/night rhythmic variation of thymus-derived regulatory T cell development and suppressor activity (Kiernozek et al., 2014). The main features of this variation are the increase of the percentage of $CD4^+CD25^+$ in SP $CD4^+$ thymocyte subset, the increase of the percentage of fully mature tTregs $CD4^+CD25^+Foxp3^+$ in $CD4^+CD25^+$ thymocyte population and expression of Foxp3, and finally, the potentiation of the suppressive activity of tTregs at 7.00–8.00 p.m. These changes correlated with the level of corticosterone in the plasma and the expression of glucocorticoid receptors in $CD4^+CD25^+$ thymocytes. Gender-dependent differences were also pointed: lower content of fully mature tTregs accompanied by less suppressive activity in females in the morning. Considering all mentioned above, in this study we investigated the effect of single dose of dexamethasone on the development and suppressive activity of tTregs dependently on the day time, and additionally, to predict long-term application in therapy, and also took into account the contribution of HPA-axis to gender differences in health risk (Uhart et al., 2006). The total number of thymocytes decreased after Dex-injection, which is observed in almost all experiments *in vivo*, and results from the induction of apoptosis mainly of DP thymocytes (Ugor et al., 2018; Berki et al., 2002; Pálinkás et al., 2008). However, the greater number of thymocytes was still observed in female thymus similarly to steady-state condition of control mice. The results presented in this paper show that dexamethasone did not substantially changed the distribution of the main thymocyte subsets, however small changes, occasionally significant in SP $CD8^+$ or SP $CD4^+$ resulted in the decrease of CD4:CD8 ratio (Fig. 2). This could result from the dose of dexamethasone, which in this study was chosen based on the lack of visible adverse effects and distribution of thymocyte subsets. Usually, doses of dexamethasone used to study its impact on the development of Tregs change the distribution of the thymocyte subsets by decreasing the precentage of DP thymocytes (Ugor et al., 2018). Dexamethasone, support the survival of thymus-derived and peripheral regulatory $CD4^+CD25^+$ T cells, while inducing apoptosis of their $CD4^+CD25^-$ counterparts (Chen et al., 2004). There are reports on the upregulation of Foxp3 expression by glucocorticoids resulting in an increase of regulatory $CD4^+CD25^+$ T cell number in asthmatic patients (Karagiannidis, 2004). Development of thymus-derived regulatory T cells is a two step process related to the transition of SP $CD4^+$ CD25-Foxp3- thymocytes to $CD25^+$Foxp3-, and next to $CD25^+Foxp3^+$ in response to IL-2 (Lio and Hsieh, 2008). We showed the increase of the percentage of $CD4^+CD25^+$ and $CD4^+CD25^+Foxp3^+$ thymocytes in Dex-injected male mice at both time points of the experiment and at 7.00–8.00 p.m. in females (Fig. 3A and B). These results indicate that dexamethasone at the dose of 1.25 mg/kg, similarly to endogenous glucocorticoids, affects the development of tTregs. Foxp3 expression is required for the function of Tregs and is considered a marker for fully differentiated Tregs in the thymus (Fontenot et al., 2003; Rudensky, 2011; Hori et al., 2003). The majority of Foxp3 T cells express CD25 at a high or low level, even if it was shown that Foxp3-positive T cells in peripheral tissues lacking CD25 exhibited suppressor function in mouse model (Fontenot et al., 2005). Glucocorticoids treatment was shown to increase the expression of mRNA for Foxp3 and to restore the number of $CD4^+CD25^+Foxp3^+$ cells (Karagiannidis, 2004). Here we suggest a direct role of exogenous glucocorticoids on the development of tTregs based on their potential to induce Foxp3. However the results of Kooten et al. proved that dexamethasone induced tolerogenic dendritic cells what can result in the development of T regulatory cells at least in the periphery (van Kooten et al., 2009). We showed that dexamethasone increased the percentage of Foxp3 + thymocytes in $CD4^+CD25^{low}$ and $CD25^{high}$ populations at both time points in males, but only in $CD4^+CD25^{high}$ in female mice (Fig. 4A). This point on the higher sensitivity of male thymocytes to Dex-induced expression of Foxp3, and the modulatory role of the hormone in the evening restricted to $CD25^{high}$ thymocytes. These results suggest a different modulatory mechanism of Foxp3 induction by Dex in females, which is highly dependent on the level of CD25 expression. In addition, we showed that dexamethasone increased the expression of Foxp3 both in $CD25^{low}$ and $CD25^{high}$ thymocytes at 7.00–8.00 p.m., in a gender-independent fashion (Fig. 4B). This may be an important observation if it will correlate with the suppressive potential of tTregs. The results presented on Fig. 7C show

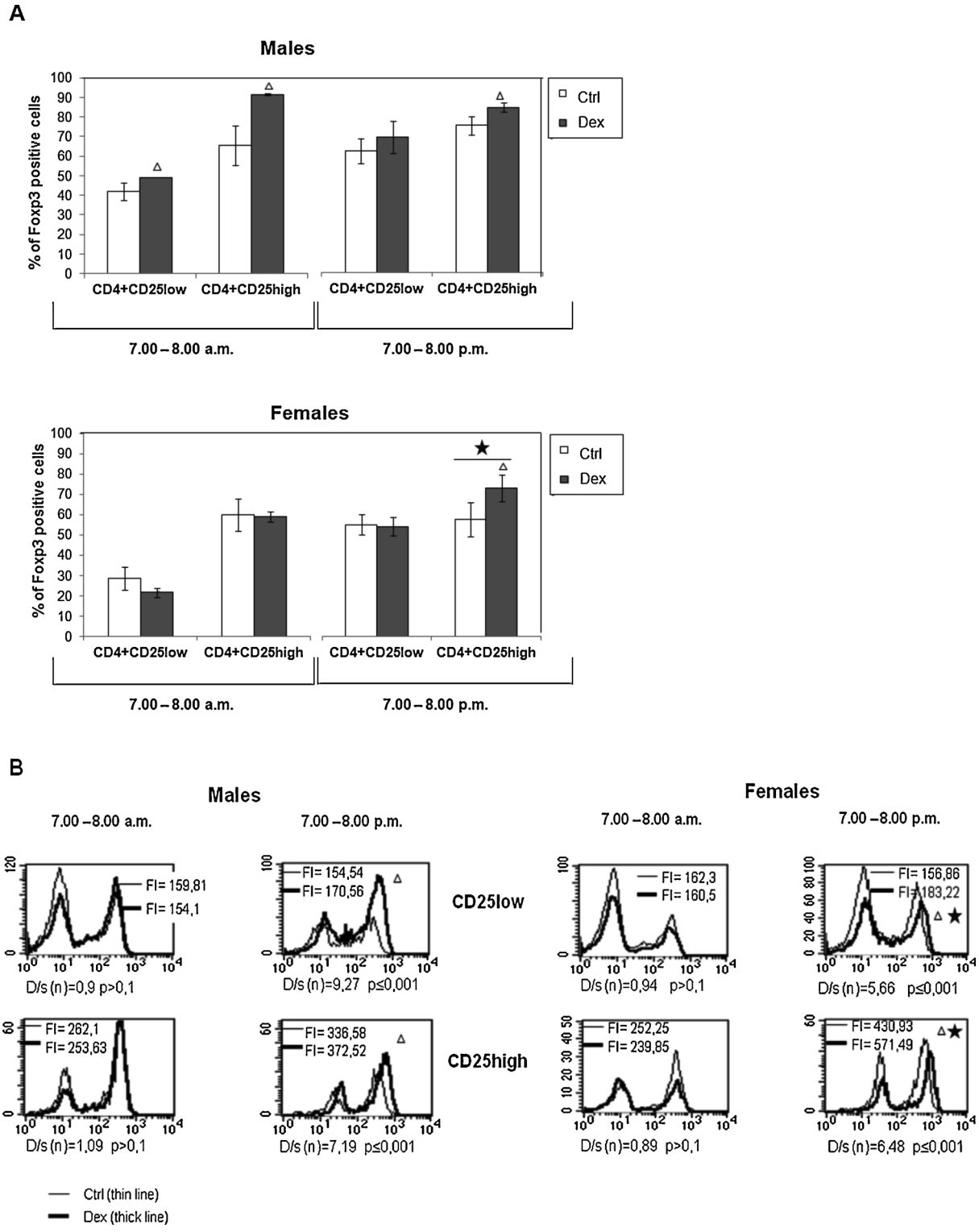


Fig. 4. CD25 and Foxp3 expression in dexamethasone-injected mice.
(A) Foxp3+ thymocyte content in CD4+CD25low and CD4+CD25high thymocytes; (B) Level of Foxp3 expression in CD4+CD25low and CD4+CD25high thymocytes. Foxp3 expression was analyzed based on the fluorescence intensity value (FI), representative histograms of Foxp3 expression in control (thin line), and in Dex-treated mice (thick line).
Data represent the results of three independent experiments performed on 2–3 mice for each day time. $p < 0.05$.
△ Statistically significant differences after Dex treatment.
★ The average percentage difference between control and Dex-treated mice depending on animal's sex.

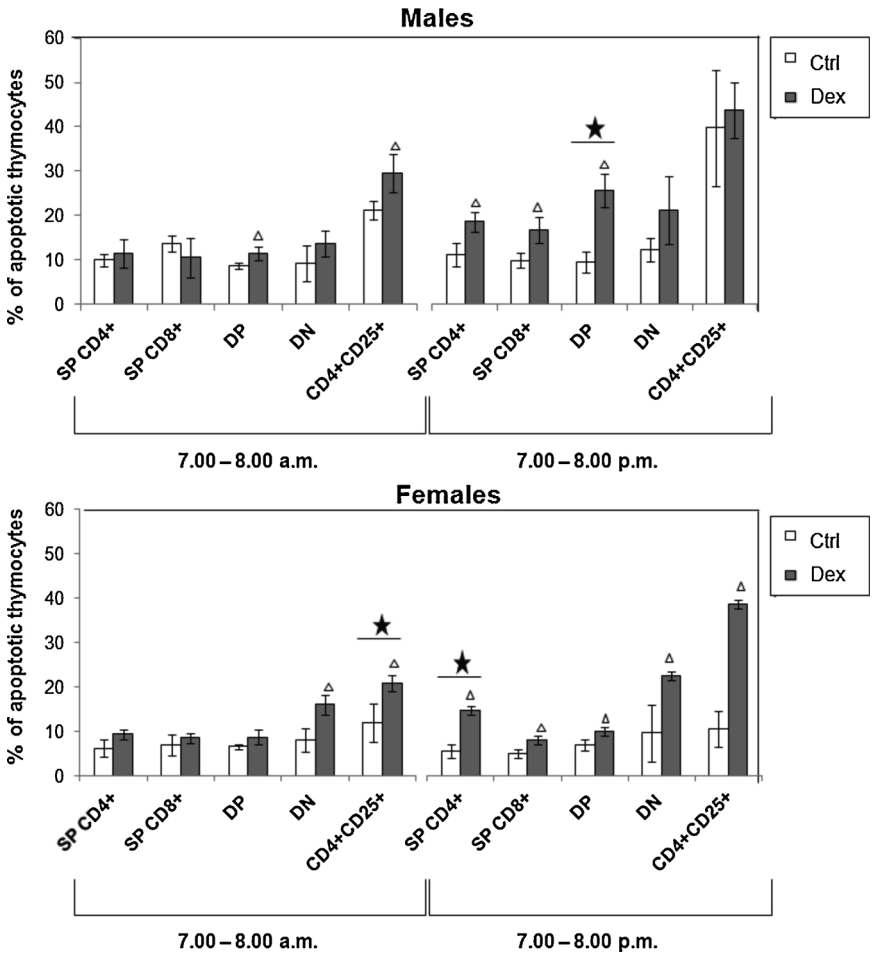


Fig. 5. Dexamethasone-induced apoptosis in thymocyte subsets.
The percentage of apoptotic cells was determined in separate thymocyte subsets: SP CD4+, SP CD8+, DP CD4+CD8+, DN CD4-CD8- and CD4+CD25+ thymocytes.
Data represent the results of three independent experiments performed on 2–3 mice for each day time. $p < 0.05$.
△ Statistically significant differences after Dex treatment.
★ The average percentage difference between control and Dex-treated mice depending on animal's sex.

that the increase of the expression of Foxp3 can be sufficient to increase the suppressive activity of tTregs, which was evidenced in females treated with Dex in the morning. In contrast, the increase of Foxp3 expression over some level can not correlate with the suppressive activity of tTregs despite the increase of their percentage.

One of the most often investigated effects of glucocorticoids is their activity to induce thymocyte apoptosis (Delfino et al., 2004; Bruscoli et al., 2006). To answer the question whether the increase of tTregs resulted from the induction of their development by dexamethasone, or may be due to preferential apoptosis of other thymocyte subsets we followed the level of apoptosis in different thymocyte subsets (Fig. 5). According to Berki et al. the level of Dex-induced thymocyte apoptosis increase with the dose of dexamethasone (Berki et al., 2002). The percentage of apoptotic thymocytes increased after Dex-treatment among all thymocyte subsets in females and DP, SP $CD4^+$ and SP $CD8^+$ in male mice at 7.00–8.00 p.m. The results of the study of Wiegers et al. (2001) have shown that the most sensitive to corticosterone-induced apoptosis *in vitro* are DP thymocytes. Paradoxically, at 7.00–8.00 p.m., we have demonstrated the greatest sensitivity to Dex-induced apoptosis of DP thymocytes in males only, in contrast to female thymocytes revealing the greatest sensitivity of SP $CD4^+$ and $CD4^+CD25^+$ thymocytes. These contrasting results requires further investigations on the role of other agents influencing the effect of glucocorticoids on thymocytes *in vivo*. It has been demonstrated that signals delivered by GC and TCR ligands antagonize each other's activity to induce apoptosis in immature DP thymocytes (Ashwell et al., 2000). We can hypothesize that the final effect of exogenous glucocorticoids administration in mice on the distribution of thymocyte subsets and apoptosis induction may depend on simultaneous interactions of TCRs and their ligands presented by thymic APC, which can change with the time of the day and can differ in a gender-dependent manner. This hypothesis should be the subject of separate research. The proapoptotic activity of Dex at 7.00–8.00 a.m. was directed mainly on $CD4^+CD25^+$ thymocytes both in males and females. Independently of day time, we observed the highest percentage of apoptotic thymocytes among $CD4^+CD25^+$ thymocyte population, which increased after Dex treatment with one exception of males injected in the evening. This is opposite to the results of Chen et al. demonstrating the low sensitivity of $CD4^+CD25^+$ thymocytes to Dex-induced apoptosis (Chen et al., 2004). Our results led to the conclusion that the increase of the percentage of tTregs in the thymus of Dex-injected mice did not result directly from differential level of apoptosis, but from the effect of dexamethasone on tTreg development documented by the increase of the percentage of $CD4^+CD25^+Foxp3^+$ thymocytes and Foxp3 expression (Figs. 3 and 4). We demonstrated that the level of apoptosis did not correlate with the expression of GCR, which may suggest that Dex-induced apoptosis and sensitivity of thymocytes to Dex is not directly dependent on the expression of glucocorticoids receptors, what was also demonstrated by other authors (Wiegers et al., 2001; Gruver-Yates et al., 2014).

Finally, we asked a question whether dexamethasone, in addition to increasing the percentage of tTregs and expression of Foxp3, increased the suppressive activity of tTregs derived from male as well as female thymus. Application of dexamethasone at 7.00–8.00 a.m. resulted in the increase of the suppressive activity of tTregs. The sensitivity of tTregs to Dex in this aspect was higher in male mice. Application of the same dose of Dex at 7.00–8.00 p.m. resulted in the increase of the potential of female tTregs to suppress the proliferation of activated $CD4^+$T cells while it did not affect the activity of male mice tTregs. Regulatory T cells can act through a diversity of suppressive mechanisms, which are still incompletely understood (Sojka et al., 2008; Sakaguchi et al.,

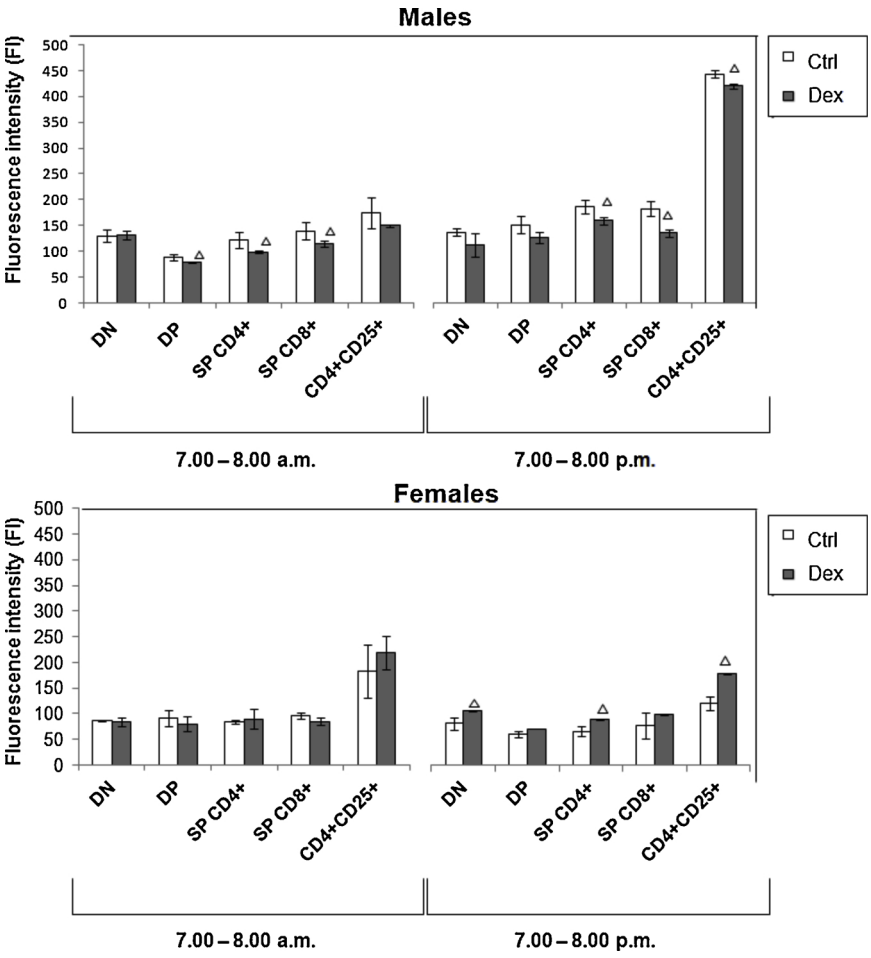


Fig. 6. Glucocorticoids receptor expression in thymocyte subsets.
GCR expression was determined in separate thymocyte subsets: DN CD4-CD8-, DP CD4+CD8+, SP CD4+, SP CD8+ and CD4+CD25+ thymocytes. Receptor expression was calculated by mean fluorescence intensity value (FI).
Data represent the results of three independent experiments performed on 2–3 mice for each day time. $p < 0.05$.
△ Statistically significant differences after Dex treatment.
★ The average percentage difference between control and Dex-treated mice depending on animal's sex.

2009). Their suppressive activity in the *in vitro* co-culture with responding conventional $CD4^+CD25^+$ T cells can involve soluble factors or cell-to-cell contact involving generation of adenosine or transfer of cAMP to conventional T cells (Schmidt et al., 2012). Here we can consider the mechanism of IL-2 dependent metabolic disruption based on immediate binding of this cytokine by tTregs having constitutive high expression of CD25. This mechanism remains controversial, however still considered in terms of IL-2 deprivation-mediated inhibition of survival and proliferation. Recently, it was documented that the effect of exogenous IL-2 is rather dose-dependent (Moon et al., 2015). We can hypothesize that differences in the suppressive potential of tTregs may result from the level of IL-2 produced *in vitro* by responding activated CD4+ lymph node T cells depending on the gender of mice and sorting time.

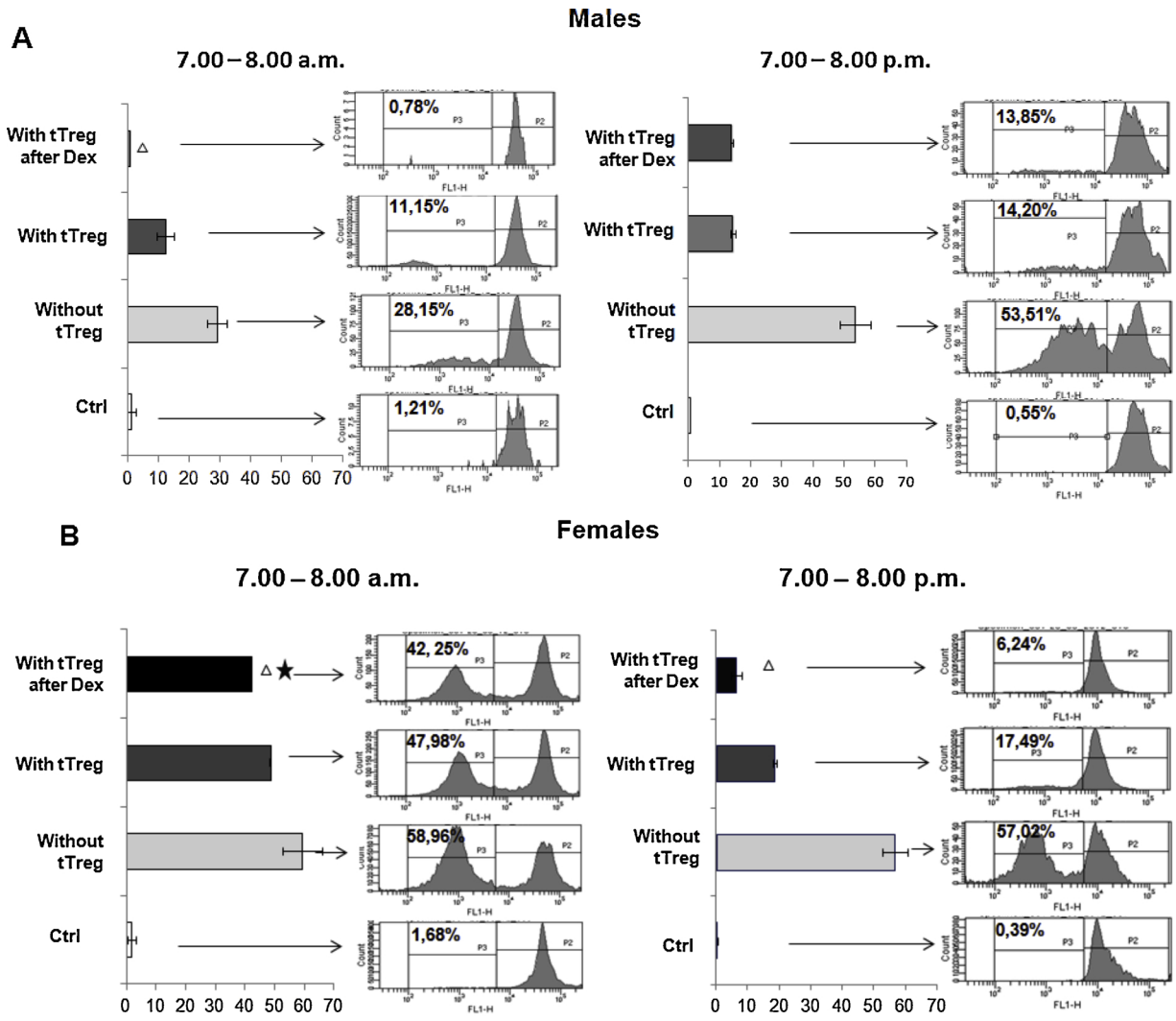
Table 1 contains summarized results of our studies, the analysis of which led to state that dexamethasone can modulate the development and suppressive activity of tTregs depending on the time of application by the contribution of GCR expression.

We have demonstrated previously that the increase of the percentage and suppressive activity of tTregs at 7.00–8.00 p.m. in physiological condition correlates with the increase of the percentage of tTregs and GCR expression (Kiernozek et al., 2014). The application of dexamethasone resulted in the following changes:

a increase of the percentage of $CD4^+CD25^+Foxp3^+$ thymocytes accompanied by elevated suppressive activity and GCR expression in females at 7.00–8.00 p.m. This is in accordance with the physiological condition, but at the same time is related to higher sensitivity of $CD4^+CD25^+$ thymocytes to Dex-induced apoptosis;
b no change of the percentage of tTregs nor GCR expression, moderate increase of their suppressive activity in females at 7.00–8.00 a.m., and additionally, moderate increase of the sensitivity of $CD4^+CD25^+$ thymocytes to Dex-induced apoptosis;
c increase of the percentage of $CD4^+CD25^+Foxp3^+$ thymocytes, no changes of their suppressive activity, decrease of the expression of GCR, and unchanged sensitivity to Dex-induced apoptosis in male mice at 7.00–8.00 p.m.;
d increase of the percentage of $CD4^+CD25^+Foxp3^+$ thymocytes accompanied by the increase of their suppressive activity, no change of the expression of GCR, and in addition, elevated level of apoptotic $CD4^+CD25^+$ thymocytes in males at 7.00–8.00 a.m.

Based on these observations we can conclude that in the condition of dexamethasone supplementation, the elevated suppressive potential of tTregs is balanced by the induction of apoptosis in order to prevent excessive suppression. We can hypothesize that apoptotic thymocytes are phagocytozed by dendritic cells, which results in tolerance induction. When the level of apoptosis is to high, the clearance of apoptotic bodies is not effective, which in turn, may result in the release of autoantigen, their presentation by DCs, and break of tolerance.

Summarizing gender-related results of our study, we can conclude that single-dose of dexamethasone resulted in: a/ the increase of the percentage of tTregs at both time points in male mice, while increasing their percentage only in the evening in females, b/ elevation of the percentage of Foxp3 thymocytes both in $CD4^+CD25^{low}$ and $CD25^{high}$ populations in male mice, while increasing their percentage only in $CD4^+CD25^{high}$ population in females, c/ increase of the suppressive activity of tTregs at both time points in female mice, but at 7.00–8.00 a.m. only in males. This contrasting effect should be taken into account in the therapeutical application of dexamethasone, or other synthetic



C

Thymus-deriving tTregs from:	% inhibition of responder cell proliferation	
	7.00 – 8.00 a.m.	7.00 – 8.00 p.m.
Males	58%	73%
Males after Dex	97%△ ★	74%
Females	18%	67%
Females after Dex	28%△	89%△

Fig. 7. Proliferation suppressive activity of thymus-derived regulatory T cells.
(A) Proliferation suppression by male tTregs; (B) Proliferation suppression by female tTregs. Suppressive activity of tTregs was determined by the inhibition of proliferation of responder $CD4^+CD25^-$ lymph node T cells. The percentage of proliferating activated responder T cells cultured alone, with tTregs or with tTregs after Dex, was compared. As the control (Ctrl) of activation responder T cells were cultured without anti-CD3 and anti-CD28 antibodies; (C) Percentage of inhibition of responder T cell proliferation.
Data represent the results of three independent experiments performed on 2–3 mice for each day time. $p < 0.05$.
△ Statistically significant differences after Dex treatment.
★ The average percentage difference between control and Dex-treated mice depending on animal's sex.

glucocorticoids, implying a different timing of dexamethasone application in male and female mice, which can result also from the simultaneous effect of estrogens having a stimulatory activity toward immune cells in physiological concentration (Olsen and Kovacs, 1996; McMurray, 2000). Recent results of the study of Bordag et al. demonstrated the impact of dexamethasone on the metabolome induced by single dose or short-term glucocorticoid application, which may result in the interruption of the rhythmicity of processes controlled by endogenous hormones (Bordag et al., 2015), which additionally confirm the usefulness of single-dose Dex application to broadly predict or identify potential changes in long-term use.

Table 1
Summarized changes induced by single-dose Dex injection.

Males		Parameter analyzed	Females	
7.00–8.00 a.m.	7.00–8.00 p.m.		7.00–8.00 a.m.	7.00–8.00 p.m.
no change	no change	Corticosterone conc.	no change	no change
↓	↓	Thymocyte number	↓	↓
no change	no change	% DN	no change	no change
no change	↓	% DP	no change	↑
no change	no change	% SP $CD4^+$	no change	↓
no change	↑	% SP $CD8^+$	no change	no change
no change	↓	CD4:CD8 ratio	no change	↓
↑	↑	% $CD4^+CD25^+$ within SP $CD4^+$	no change	↑
↑	↑	% Foxp3 within SP $CD4^+CD25^+$	no change	↑
↑	no change	% Foxp3 within SP $CD4^+CD25^{low}$	no change	no change
↑	↑	% Foxp3 within SP $CD4^+CD25^{high}$	no change	↑
no change	↑	Foxp3 expression	no change	↑
no change	no change	DN apoptosis	↑	↑
↑	↑	DP apoptosis	no change	↑
no change	↑	SP $CD4^+$ apoptosis	no change	↑
no change	↑	SP $CD8^+$ apoptosis	no change	↑
↑	no change	SP$CD4^+CD25^+$ apoptosis	↑	↑
no change	no change	GCR expression DN	no change	↑
↓	no change	GCR expression DP	no change	no change
↓	↓	GCR expression SP $CD4^+$	no change	↑
↓	↓	GCR expression SP $CD8^+$	no change	no change
no change	↓	GCR expression SP $CD4^+CD25^+$	no change	↑
↑	no change	Suppressive activity of tTregs	↑	↑

Declaration of Competing Interest

The authors declare no financial or commercial conflict of interest.

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