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# Role of Endogenous Melatonin in the Regulation of Th17/Treg Balance during Pregnancy

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We studied the role of endogenous melatonin in the development and functioning of T cells that produce IL-17 (Th17) and regulatory T cells (Treg) during pregnancy. The study was performed *ex vivo* and *in vitro* with auto-serum as the source of endogenous melatonin under conditions of blockade of melatonin-dependent signaling. Participation of the hormone in the regulation of differentiation of both $CD4^+ROR\gamma t^+$ and $CD4^+FoxP3^+$T cells and their key products IL-17A and TGF-β was demonstrated. It is known that the normal gestational process is accompanied by a decrease in Th17/Treg ratio due to hormonal changes. The sensitivity of the studied subpopulations to melatonin during pregnancy can affect its outcome.

**Key Words:** *differentiation; Th17; Treg; melatonin; pregnancy*

Epiphyseal hormone melatonin regulates a wide range of biological reactions and plays an important role during pregnancy: being an effective antioxidant, it protects the fetal and placental tissues from oxidative damage [7,10,13]. At the same time, the hormone produces an immunomodulatory effect; changes in its level during gestation should affect activity of the immune processes [3]. Of special interest is the role of melatonin in the regulation of the proportion of two T-cell subpopulations, Th17 and Treg. On the one hand, this balance is important for successful development of pregnancy [5,8,11]. On the other, $CD4^+$T cells are under the direct control of melatonin, because they express specific membrane receptors for this hormone, MT1 and MT2 [12]. Transcription factor RORα effectively regulated by melatonin and considered until recently as the third, intracellular, receptor for this hormone [6] acts as one of the two main differentiation factors for Th17 [14] and at the same time serves as a negative regulator of Treg differentiation [4].

We have previously shown [1] that melatonin, in both physiological and pharmacological concentrations, can induce *in vitro* differentiation of intact $CD4^+$T cells in Th17. Therefore, pharmacological use of this hormone as the antioxidant in pregnancy can shift the Th17/Treg balance towards Th17, which is fraught with spontaneous abortion [8].

Here we evaluated the role of endogenous melatonin in the regulation of Th17/Treg differentiation at different stages of pregnancy.

## MATERIALS AND METHODS

The study involved pregnant women of the trimester I (20 women, mean age 28.05±4.70 years) and trimester III (10 women, mean age 33.10±6.61 years). These periods were chosen because they are most critical for induction of spontaneous abortion [2] and development of preeclampsia [9], respectively. In addition, these periods differ from each other in the level of melatonin in the blood [13]. The control group consisted of 10 non-pregnant women of reproductive age (mean age 31.80±6.43 years). All women gave informed consent for participation in the study. In view of daily fluctuations in the blood melatonin level, the blood was taken at the same time (from 08.00 to 09.00 h).

Leukocytes were isolated from heparinized venous blood by density gradient centrifugation in ficoll—urografin ($\rho$=1.077 g/cm$^3$). A suspension of

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mononuclear cells, as well as $CD4^+$T cells fractionated on immunomagnetic particles with appropriate separation system (R&D Systems) was used. $CD4^+$T cells were cultured for 48 h with autologous serum (as source of an endogenous melatonin) in RPMI-1640 medium (Gibco) with 1 mM HEPES (Sigma-Aldrich), 2 mM L-glutamine (Serva), and 40 U/ml gentamicin (Pharmacia) at 37°C and 5% $CO_2$ under conditions of polyclonal activation (system for activation based on monoclonal antibodies to CD3/CD28; Invitrogen). One hour prior to activation, a blocker of membrane receptors of melatonin (MT1/MT2) lusindol (R&D Systems) was added to the culture. At this stage, we did not use blockade of RORα that is effectively regulated by melatonin, because, first, RORα is simultaneously a differentiation factor for Th17, and, as shown by us earlier [1], its inhibition can block differentiation of these cells, and, second, affinity of RORα binding with melatonin is by 2.5 orders of magnitude lower than affinity of membrane melatonin receptors [12], and theoretically, it should not be sensitive to physiological concentrations of melatonin.

The serum level of melatonin was assessed by ELISA (IBL International). The expression of transcription factors RORγt (Th17 differentiation marker) and FoxP3 (Treg marker) by cells was determined *ex vivo* and after 48-h culturing (flow cytometry with monoclonal antibodies: anti-CD4*FITC, anti-RORγt*PE, anti-FoxP3*PE; Novus Biologicals, R&D Systems). Synthesis of the key cytokines of the studied subpopulations IL-17 (by the main IL-17A isoform) and TGF-β was evaluated by ELISA by their level in supernatants (BioLegend); *ex vivo* expression of the key membrane receptor MT1 and factor RORα was also measured (flow cytometry with the appropriate monoclonal antibodies: anti-CD3*PerCP, anti-Melatonin Receptor 1B*Alexa Fluor, anti-CD4*FITC, anti-RORα*PE, Novus Biologicals, R&D Systems). Nonspecific binding and release of fluorescence-negative lymphocytic gate were evaluated using the corresponding isotypic controls. Lymphocyte viability measured in the test with eosin after 48 h in culture was 95-98%.

Statistical analysis of the results was conducted using non-parametric Wilcoxon's *T* test and Mann—Whitney *U* test, because data distribution did not fit the normal law. The descriptive characteristics of the quantitative parameters are represented in the form of median and lower and upper quartiles: Me (LQ; UQ). The degree of correlation between the parameters was estimated by Spearman rank correlation coefficient (*r*).

## RESULTS

*Ex vivo* study showed an increase in serum melatonin concentration in the trimester III in comparison with non-pregnant women, significant differences also were found between pregnant women in the trimesters I and III (Table 1). Evaluation of MT1 melatonin receptor expression did not reveal differences between the groups of pregnant and non-pregnant women, but there was a tendency to increase in this parameter during gestation. A significant decrease was found in the fraction of $CD4^+$T cells that express transcription factor RORα (Table 1). This effect may be due to the increase in melatonin concentration during pregnancy: we previously showed a decrease in RORα expression by $CD4^+$T cells *in vitro* under the influence of exogenous hormone [1].

The groups of pregnant and non-pregnant women did not differ by the count of $CD4^+RORγt^+$T cells (Table 2) and serum concentration of their main cytokine IL-17A, but a tendency was shown towards decrease in this parameter during the trimester III (Table 2). However, analysis of correlation showed a direct dependence of $CD4^+RORγt^+$T cells content on serum melatonin level ($r$=0.810, $p$<0.05). In contrast, the count of $CD4^+FoxP3^+$T cells and serum concentration of their main cytokine TGF-β (Table 2) increased during pregnancy. Thus, during gestation, the ratio of the level and activity of Th17 and Treg changed, and these changes were associated with melatonin content in the peripheral blood.

Analysis of the role of endogenous melatonin in the regulation of Th17 differentiation *in vitro* in re-

**TABLE 1.** Serum Level of Melatonin and Expression of Melatonin Receptors and RORα *Ex Vivo* (Me (LQ; UQ)

| Parameter | Non-pregnant women | Trimester I | Trimester III |
|---|---|---|---|
| Serum concentration of melatonin, pg/ml | 21.37 (18.69; 38.47) | 19.55 (17.70; 34.90) | 48.59*+ (42.41; 135.85) |
| $CD3^+MT1^+$ cells, % | 1.48 (0.96; 5.22) | 1.57 (1.03; 4.22) | 3.45 (2.38; 5.49) |
| $CD4^+RORa^+$ cells, % | 1.03 (0.50; 1.58) | 0.94 (0.76; 1.35) | 0.50 (0.44; 0.78)+ |

**Note.** *$p$<0.05 in comparison with *non-pregnant women, +trimester I.

**TABLE 2.** Content of Th17, Treg, and Their Products *Ex Vivo* (Me (LQ; UQ)

| Parameter | Non-pregnant women | Trimester I | Trimester III |
|---|---|---|---|
| $CD4^+ROR\gamma t^+$T cells, % | 1.46 (1.25; 1.64) | 1.26 (1.13; 1.52) | 1.01 (0.78; 1.75) |
| Serum concentration of IL-17A, pg/ml | 8.48 (8.12; 10.07) | 4.92** (4.71; 5.36) | 5.14 (4.92; 6.60) |
| $CD4^+FoxP3^+$T cells, % | 0.16 (0.05; 0.24) | 0.21 (0.15; 0.27) | 0.46*+ (0.34; 0.62) |
| Serum concentration of TGF-β, pg/ml | 5309.89 (4135.67; 8832.55) | 12,355.21* (10,277.75; 15,697.22) | 11,948.75* (10,277.75; 12,626.19) |

**Note.*** $p<0.05$, ****$p=0.05$ in comparison with non-pregnant women, $^+p<0.05$ in comparison with trimester I.

**TABLE 3.** Role of Endogenous Melatonin in the Regulation of Th17 and Treg Differentiation *In Vitro* in Response to Polyclonal Activation (Me (LQ; UQ)

| Parameter | Experimental condition | Non-pregnant women | Trimester I | Trimester III |
|---|---|---|---|---|
| $CD4^+ROR\gamma t^+$T cells in 48-h culture, % | without blockade of MT1/MT2 | 2.48 (2.10; 2.95) | 1.68 (1.36; 3.66) | 2.66 (2.20; 3.10) |
| | with blockade of MT1/MT2 | 1.47° (1.16; 1.85) | 1.32° (0.92; 1.86) | 1.79° (1.31; 2.20) |
| Concentration of IL-17A in cultural supernatants, pg/ml | without blockade of MT1/MT2 | 128.63 (46.47; 263.84) | 95.84 (47.64; 235.45) | 211.62 (152.04; 817.41) |
| | with blockade of MT1/MT2 | 77.97 (23.27; 198.68)° | 65.76 (31.53; 203.07) | 247.97 (92.41; 310.85) |
| $CD4^+FoxP3^+$T cells in 48-h culture, % | without blockade of MT1/MT2 | 0.68 (0.56; 1.03) | 0.24* (0.21; 0.34) | 0.45+ (0.35; 0.57) |
| | with blockade of MT1/MT2 | 0.68 (0.53; 0.97) | 0.18*° (0.12; 0.25) | 0.34*+° (0.23; 0.42) |
| Concentration of TGF-β in cultural supernatants, pg/ml | without blockade of MT1/MT2 | 88,589.17 (43,697.85; 135,016.01) | 63,569.26 (49,749.59; 90,124.69) | 33,671.82* (25,813.58; 70,253.28) |
| | with blockade of MT1/MT2 | 69 891.98° (41,620.38; 127, 519.07) | 71,337.17 (50,381.87; 93,557.02) | 27,800.72*+° (23,103.84; 58,962.70) |

**Note.** MT1/MT2: membrane receptors of melatonin, *$p<0.05$ in comparison with *non-pregnant women, +trimester I, °without blockade of MT1/MT2.

sponse to polyclonal activation with auto-serum revealed the effects of the hormone mediated by signals from membrane melatonin receptors. A significant decrease in $CD4^+ROR\gamma t^+$T cells contents was detected in both non-pregnant and pregnant women in a 48-h activated culture under conditions of blockade of MT1/MT2-dependent signals (Table 3). Evaluation of IL-17A synthesis in the presence of membrane melatonin receptor blocker revealed a decrease in cytokine concentration in culture supernatants, but these changes were statistically significant only in non-pregnant women (Table 3). A positive correlation was shown between the level of serum melatonin and IL-17A concentration in culture supernatants ($r=0.738$, $p<0.05$) in the group of pregnant women of the trimester III.

Evaluation of $CD4^+FoxP3^+$T cell levels under condition of polyclonal activation in the presence of autoserum revealed no significant differences after addition of inhibitors of membrane melatonin receptor to cultures from non-pregnant women, but showed significant changes in this parameter in pregnant women (Table 3). Similar patterns were found for non-pregnant and pregnant in the trimester III during studies of the concentration of TGF-β in supernatants of activated $CD4^+$T cell cultures. In addition, in non-pregnant women a negative correlation was found between the

percentage of RORα receptor and concentration of TGF-β in culture supernatants ($r$=0.774, $p$<0.05). This effect is apparently associated with previously shown RORα-dependent inhibition of FoxP3 expression [15], the key transcription factor in the induction of TGF-β synthesis.

In general, the data obtained both *ex vivo* and *in vitro* indicate the involvement of endogenous melatonin in the development and functioning of the two studied T-cell subpopulations, Th17 and Treg; for Th17 this regulation was positive, whereas for Treg cells it was negative. As a consequence, the increase in the level of melatonin during pregnancy affects the balance of Th17/Treg. It is known that these T-cell subpopulations play an important role in gestational processes [8,11]. Normally, pregnancy is accompanied by a decrease in Th17/Treg ratio [4,8], which is provided by hormones and proteins of pregnancy [2,4]. The effect of melatonin was contrary to this trend and was balanced under physiological conditions, apparently by the effects of other factors.

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