VILNIUS UNIVERSITY

VITALIJ ČERNYŠIOV

THE EFFECT OF MELATONIN ON THE ANTIBODY PRODUCTION AND LEUKOCYTE MIGRATION IN BALB/c LINE MOUSE

Summary of doctoral dissertation

Biomedical Science, Biology (01B)

Vilnius, 2014 years

This dissertation was prepared during the period of 2010-2014 at the Department of Microbiology and Biotechnology, Faculty of Natural Sciences of Vilnius University and at the State Research Institute Centre for Innovative Medicine.

Scientific Supervisor:

Doc. dr. Irutė Girkontaitė (State Research Institute Centre for Innovative Medicine, biomedical sciences, biology – 01B).

Consultant:

Prof. dr. Lilija Kalėdienė (Vilnius University, biomedical sciences, biology – 01B).

The dissertation is defended at the Vilnius University Council of Biological Science:

Chairman*:*

Prof. dr. Dainius Characiejus (Vilnius University, biomedical sciences, medicine – 06B).

Members:

Prof. dr. Nomeda Kuisienė (Vilnius University, biomedical sciences, biology – 01B).

Prof. dr. Genė Bizulevičienė (State Research Institute Centre for Innovative Medicine, biomedical sciences, biology – 01B).

Dr. Vita Pašukonienė (National Cancer Institute, biomedical sciences, biology – 01B).

Dr. Vilma Urbonavičiutė (Karolinska Institute (Sweden), biomedical sciences, biology – 01B).

The dissertation will be defended at the public session of the Council of Biological Science at 14 p.m. on 14 November, 2014 in the Grand auditorium (214 cabinet) at the Faculty of Natural Sciences, Vilnius University.

Address: M.K. Čiurlionio 21/27, LT-03101, Vilnius, Lithuania

Date of posting: 10 October, 2014.

This dissertation is available at the Library of Vilnius University.

VILNIAUS UNIVERSITETAS

VITALIJ ČERNYŠIOV

MELATONINO POVEIKIS BALB/c LINIJOS PELIŲ ANTIKŪNŲ GAMYBAI IR LEUKOCITŲ MIGRAVIMUI

Daktaro disertacija,

Biomedicinos mokslai, Biologija (01 B)

Vilnius, 2014 metai

Disertacija rengta 2010-2014 metais Vilniaus universiteto Gamtos mokslų fakultete, Mikrobiologijos ir biotechnologijos katedroje ir Valstybiniame mokslinių tyrimų institute Inovatyvios medicinos centre.

Mokslinis vadovas:

Doc. dr. Irutė Girkontaitė (Valstybinis mokslinių tyrimų institutas Inovatyvios medicinos centras, biomedicinos mokslai, biologija - 01B).

Konsultantas:

Prof. dr. Lilija Kalėdienė (Vilniaus universitetas, biomedicinos mokslai, biologija – 01B).

Disertacija ginama Vilniaus universiteto Biologijos mokslų krypties taryboje:

Pirmininkas:

Prof. dr. Dainius Characiejus (Vilniaus universitetas, biomedicinos mokslai, medicina – 06B).

Nariai:

Prof. dr. Nomeda Kuisienė (Vilniaus universitetas, biomedicinos mokslai, biologija – 01B).

Prof. dr. Genė Bizulevičienė (Valstybinis mokslinių tyrimų institutas Inovatyvios medicinos centras, biomedicinos mokslai, biologija – 01B).

Dr. Vita Pašukonienė (Nacionalinis vėžio institutas, biomedicinos mokslai, biologija – 01B).

Dr. Vilma Urbonavičiutė (Karolinsko institutas (Švedija), biomedicinos mokslai, biologija – 01B).

Disertacija bus ginama viešame Biologijos mokslo krypties tarybos posėdyje 2014 m. lapkričio mėn. 14 d. 14 val. Vilniaus universiteto Gamtos mokslų fakulteto Didžiojoje fakulteto auditorijoje (214 kabinetas).

Adresas: M.K. Čiurlionio 21/27, LT-03101, Vilnius, Lietuva

Disertacijos santrauka išsiuntinėta 2014 m. spalio mėn. 10 d.

Disertaciją galima peržiūrėti Vilniaus universiteto bibliotekoje.

INTRODUCTION

Melatonin – is a hormone produced by the pineal gland during the dark time (Lerner et al., 1958). Light during the night suppresses melatonin production (Wurthman et al., 1963). Melatonin is an important biological body regulator: it controls daily and seasonal biorhythms, glucose metabolism, gonadal activity, cardiovascular system, gastrointestinal tract and the activity of the immune system (Jaworek et al., 2007, Masana et al., 2002, Lang et al., 1983). The night shift work disrupts the production of melatonin (Carrillo-Vico et al., 2013). There is a lot of information on night shift work being harmful to the body: it causes health problems and disrupts body`s homeostasis. In 2007, World Health Organization's International Agency for Research on Cancer classified the night shift work to a possible group of carcinogens (Group A2) (Straif et al., 2007). It is well known that working night shifts also increases the risk of developing diabetes, cardiovascular and metabolic syndrome diseases (Wang et al., 2011).

There is a lot of scientific information about the immunoregulative properties of melatonin. Melatonin modulates the development of some organs of the immune system, cell differentiation, immune response and cytokine production (Tian et al., 2003). The imunomodulatory activity of melatonin is usually determined by the following experimental models: surgical pinealectomy, *in vivo* treatment with melatonin or *in vitro* treatment of the immune cells with melatonin. However, during the experiments while keeping animals under constant light conditions, melatonin production was rarely naturally inhibited. So far, the effect of suppressed melatonin synthesis in BALB/c line mouse immune system has not been evaluated. In the literature, there is no information about the effect of suppressed melatonin synthesis on the antibody production, immune cell numbers in organs and in the site of inflammation as well as granulocyte migration. Also, in the literature there is no data on the dependence of antibody production on the immunization time of the day. So far it is not known through which cell receptors could melatonin regulate T-cell-dependent antibody production in mouse. Though it is known that melatonin inhibits granulocyte migration acting through endothelial cells, however, it is not clear whether melatonin can regulate granulocyte migration while acting through the same granulocytes.

The results of this study contribute to the current knowledge on the effects of melatonin on the immune system and help to understand its functional mechanisms.

The aim of the dissertation work is to investigate the effect of suppressed melatonin synthesis on the immune system of BALB/c line mouse.

The following **tasks** have been formulated to achieve this aim:

1. To identify the role of melatonin in T-cell-independent antibody production.
2. To evaluate the role of melatonin in T-cell-dependent antibody production.
3. To produce monoclonal antibodies against murine MT1 and MT2 receptors and to examine the expression of melatonin membrane receptors in BALB/c line mouse lymphocytes.
4. To explore the role of melatonin on the leukocyte migration into sites of inflammation.
5. To study the role of melatonin on fMLP-induced granulocyte migration.

Scientific novelty

- For the first time, it was shown that melatonin modulates the T-cell-dependent antibody production through effects on splenic B lymphocyte MT2 receptors.
- For the first time, it was established that melatonin directly (in the absence of T cells) can affect B cell activation and antibody secretion.
- Monoclonal antibodies against mouse MT1 and MT2 receptors were produced. Using these monoclonal antibodies, MT2 melatonin receptors were detected in splenic B lymphocytes.
- For the first time, it was shown that the production of antibodies depends on the time of the day of immunization.
- For the first time, it was proved that melatonin inhibits granulocyte adhesive properties through granulocyte *MT3*/CR2 receptor**.**

MATERIAL AND METHODS

Experimental animals. BALB/c, C57BL/6 RAG2$^{-/-}$ line mouse and Lewis line rats were bred and housed in our animal facility in accordance with the institutional guidelines for Ethical Conduct in the Care and Use of Animals. The rodents were given *ad libitum* access to food and water and maintained under a 12/12-h light/dark cycle. In order to study the influence of melatonin on antibody production, one group of mouse (LD) was kept under normal light/dark conditions; another group (LL) was kept under constant artificial lighting (around 50 luxes) for about one week before the experiments.

Determination of melatonin concentration in sera. The concentration of melatonin was determined by ELISA using IBL melatonin ELISA kit. The samples were extracted and ELISA performed according to the company's guidelines.

Granulocytes, B220^{+} B cell and CD4^{+} T cell preparation. The granulocytes were purified using negative selection with anti-R-Phycoerythrin (PE) Magnetic Particles - DM (BD Biosciences).

Splenocytes were stained with PE labelled antibodies to mouse B220 (BD Biosciences). Lymph node cells were stained with PE labelled antibodies to mouse CD4 (BD Biosciences). The stained cells were separated using positive selection with anti-R-Phycoerythrin (PE) Magnetic Particles - DM (BD Biosciences). The cell isolation was performed according to the manufacturer's recommendations. Cell purity was estimated by flow cytometry.

Isolation of leukocytes from the blood. The erythrocytes were lysed using lysing buffer (168 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM tetrasodium EDTA, pH to 7.3). 1 ml of blood was diluted with 14 ml of lysing buffer and incubated at room temperature for 3-5 minutes. The cells were centrifuged at 300 g for 5 min at 4^{o} C and the pellet was resuspended in cold PBS buffer. The cells were centrifuged again and resuspended in the medium required for future analysis.

T cell depend and T cell independ antigen immunization. Mouse were immunized intraperitoneally with T cells independent antigens: 10 μg TNP-Ficoll or 50 μg TNP–LPS in PBS and T cells dependent antigen: 100 μg of alum-precipitated TNP–ovalbumin. All antigens were obtained from Biosearch Technologies. TNP–ovalbumin-immunized mouse were boosted on 14 and 21 days of immunization. Serum samples were collected before immunizations and on day 7 after the immunization with T cell independent antigens or on days 7, 14, 21 and 28 after the immunization with T cell dependent antigen.

Antibody secretion *in vitro*. Splenic cells were isolated from T cell dependent antigen immunized mouse 1 or 3 weeks after the last immunization.

Splenic cells were purified using Lympholyte-M gradient centrifugation. The cells were plated into 96-well round-bottom plates at the concentration 2.2*10^{5} cells per well in RPMI medium containing 10 % FCS. The cells were unstimulated or stimulated with TNP–ovalbumin 10 μg/ml, melatonin 10 μM (Sigma), luzindole 10 μM (Sigma) or 1–10000 nM 4P-PDOT (Tocris) and cultured at 37ºC with 5 % CO_2 for 9–10 days. The antibodies in the cell culture supernatant were detected by ELISA.

ELISA (Enzyme-Linked Immunosorbent Assay) assay. ELISA maxisorb plates were coated with TNP-ovalbumin. Antigen-specific antibodies were detected with biotin conjugated antibodies to mouse IgM, IgG1, IgG2a, IgG2b and IgG3 (all from Pharmigen). The coupled biotinilated antibodies were detected using streptavidin-conjugated horseradish peroxidase (Pierce). Ortho-phenylenediamine was used as a substrate for horseradish peroxidase. The amount of each antigen-specific isotype was determined by comparing test samples with a standard serum (pooled sera from immunized mice and set at 100 AU).

Flow cytometry assay. The expression of surface markers of on cells from the peritoneal cavity, thymus, spleen, lymph nodes, blood and bone marrow was analyzed by using a four-color flow cytometer (FACScalibur, Becton Dickinson, New Jersey, USA) and CellQuest software. The following antibodies were used: fluorescein isothiocyanate (FITC)-Gr1, -IgM, -B220, -CD8, -CD21, -CD3 –CD18; phycoerythrin (PE)-CD5, -CD8, -CD23, -B220, -DX5, -Mac1; peridinin chlorophylla protein (PerCP)-B220, allophycocyanin (APC) -Mac1and-CD8. Macrophages, monocytes and granulocytes were identified staining the cells with Gr1 FITC/Mac1-APC; peritoneal B cells were stained using antibodies IgM-FITC/CD5-PE or IgM-FITC/ CD5-PE/ B220-PerCP/ Mac1-APC.

RNA isolation, cDNA synthesis. RNA was extracted using the GenElute™ Mammalian Total RNA Kit (Sigma) or GeneJET ™ RNA Purification Kit (Thermo Fisher Scientific). $0.5x10^7$ - $1x10^7$ cells were used for one RNA preparation. RNA was prepared according to the manufacture instructions. To remove the DNA contaminations, RNA was incubated 30 minutes at 37°C with DNase (final concentration 0.1 u/ml). Prepared RNA was immediately used for cDNA synthesis or frozen at -80^{o}C. cDNA was synthesized using Maxima Reverse Transcriptase and random hexamer primers (Thermo Fisher Scientific).

PCR. PCR was performed in a real-time PCR cycler Rotor-Gene RG 6000-time PCR instrument. Primers (Integrated DNA Technologies) and their sequences, also annealing temperatures are listed in Table 1. All primers (except MT1(C) and RORα) were designed using Lasergene software. The sequences of MT1(C) and RORα primers were as described (Carrillo-Vico et al., 2003). The primers for nuclear receptors could be used to amplify all isoforms. The cDNA was amplified in a reaction containing 2 μl of cDNA, 2 μl of 10×PCR buffer, 2 μl of 2 mM dNTP/dUTP mix, 2 μl of 25 mM MgCl, 4 μl of betain, 1 μl of 10 pM of each 5′- and 3′-primer, 26.6 μM of a fluorescence dye SYTO 9 (Invitrogen), 4.5 μl of nuclease-free water, 0.4 units of Uracl-DNA glycosidase (Thermo Fisher Scientific) and 0.5-0.75 units of Hot start Taq polymerase (Thermo Fisher Scientific). Uracil-DNA Glycosylase and dNTP/dUTP mixture was used to avoid PCR contaminations. The possible DNA contaminations are digested by Uracil-DNA Glycosylase during initial 2 min PCR step at 50°C. The PCR reaction started by a 5 min activation of hot start DNA polymerase at 95°C followed by 40-45 cycles of target cDNA amplification (45 s at 95°C, 45 s depending on the primers from 54°C to 61°C and 45 s at 72°C). The last elongation was 2 min for 72°C. All primers (except MT2 and MT1 (B)) are localised on different exons. To exclude the contaminations of genomic DNA, control PCR's with primers that are localised on the same exon were performed using isolated RNA (without cDNA synthesis).

According to the literature (Hardeland et al., 2011) all melatonin receptors are expressed in the brain. Therefore, brain cDNA served as a calibrator. A five-fold dilution of brain cDNA

was prepared and the PCR was performed with each dilutions of calibrator cDNA and tested samples. We accepted that brain cDNA has certain amount of relative units of target gene. The relative standard curve was constructed adding relative units on Y axis and threshold values (Ct) on X axis. Target quantity was determined from the standard curve using Rotor-Gene RG 6000 Ver. 1.7 software. The ubiquitously expressed HPRT (hypoxanthine guanine phosphoribosyl transferase) mRNA was used to monitor the quality of RNA and the efficiency of the RT and the PCR processes. The relative quantities of each target gene were normalized to HPRT as a reference gene and were carried out according to the ABI PRISM 7700 Sequence Detection System bulletin #2. Additionally, calculation of the relative mRNA concentrations of target genes was carried out according to the $2^{-\Delta\Delta c}$T method of Livak and Schmittgen (Livak et al., 2001). The results obtained according relative calibration curve and $2^{-\Delta\Delta c}$T methods were similar.

Table1. PCR primers.

Receptors name	Ta (°C)	Amplicon size (bp)	Sequences of primers
MT1 (A)	58	352	F: GGGCCCCACTCAACCTCATAG R: AGCAGTAAGACCCCAACCAGTGTG
MT1 (B)	59	374	F: ATCGCCATCATGCCCAACCT R: TAACTAGCCACGAACAGCCACTCT
MT1 (C)	59	248	F: CCGCAACAAGAAGCTCAGGAACTC R: TCGTACTTGAGGCTGTGGCAAATG
MT2	58	341	F: AACCGCTACTGCTGCATCTGTCAT R: AAACTGCGCAAATCACTCGGTCTC
MT3	59	396	F: CAATGGGTCCCTGAAGAAAGTGG R: CCCGTGGTTAAGGAAAGGAGAGC
RORα	59	417	F: GGAAGAGCTCCAGCAGATAACG R: GCTGACATCAGTACGAATGCAG
RORβ	58	373	F: TGGGGATATCAGAACAGCAGAGGA R: AATCAAGGCGTATCAAAGCAAGTC
RORγ	59	423	F: GGCCCCCTGGACCTCTGTTTTG R: CGCCGCAGCCAGCAGTGTAATG
HPRT	54	249	F: GCT GGT GAA AAG GAC CTC T R: CAC AGG ACT AGA ACA CCT GC

Ta(°C) – PCR hybridization stage temperature; F– forward primer; R – reverse primer; HPRT (hypoxanthine guanine phosphoribosyl transferase).

Production of monoclonal antibodies to mouse MT1 and MT2 melatonin receptors. Hybridomas producing rat monoclonal antibodies to murine MT1 and MT2 melatonin antibodies were established in our lab. The antigens were N-terminal extracellular domains of MT1 and MT2 receptors expressed as glutathione-S-transferase (GST) fusion proteins. Synthetic genes (Eurofins MWG Operon) that correspond to the positions 1-109 bp of MT1

(NCBI NM_008639) and 1-136 bp of MT2 (NCBI NM_145712) were cloned into pGEX-4T-3 vector (GE Healthcare). The GST-MT1 and GST-MT2 fusion proteins were expressed in *E.coli* BL21 strain, purified using glutathione sepharose (Pierce). Lewis rats were immunized with GST-MT1 or GST-MT2 fusion protein. SP2/0 myeloma cells were fussed with splenocytes from the immunized rat according to the method described by G.Kohler and C.Milschten (Kohler et al., 1975). The production of monoclonal antibodies to MT1 and MT2 was tested by ELISA and Western blotting using GST, GST-MT1 and GST-MT2 proteins. The antibodies to MT1 bounded GST-MT1 but did not bound GST-MT2 or GST. The antibodies to MT2 bounded GST-MT2 but did not bound GST-MT1 and GST.

The analysis of MT1 and MT2 melatonin receptor expression by flow cytometry. The expression of MT1 and MT2 melatonin receptors on the surface of the cells from spleen, thymus, bone marrow and lymph nodes was analyzed. The cells were incubated with monoclonal antibodies against MT1 and MT2 in the presence of Fc block (antibodies to CD16/CD32, BD Biosciences). Hybridoma cell culture supernatants were used as sources of antibodies. Bound antibodies were visualized using biotin-labelled antibodies to rat immunoglobulin (GE Healthcare) and Streptavidin labelled with PerCP (BD Biosciences). The cells were identified staining with antibodies to cell specific surface markers: CD8-FITC, CD4-PE, B220-APC, B220-PerCP, CD21-FITC, CD23-PE, IgM-FITC, CD5-PE (BD Biosciences). The cells were measured by BD FACScan cytometer and analyzed by CellQuest software.

Granulocyte adhesion to plate bound ICAM-1Fc. Nunc Maxisorb ELISA plates were coated with rmICAM-1Fc (R&D Systems). rmICAM-1Fc was diluted in PBS at concentration 4 µg/ml and distributed 50 µl per each well. The plates were coated overnight, blocked for 1 h with 150 µl of 1 % BSA in PBS, washed 3 times with RPMI medium without serum. The cells were resuspended in RMPI medium with 0.25 % BSA, 10 mM HEPES at concentration $0.5x10^6$ cells/ml and treated 15 min with *MT3*/QR2 antogonist prazosin chidrochloride (Tocris), RORα agonist CGP52608 (Sigma-Aldrich), *MT3*/QR2 agonist GR135531 (Tocris), MT2 antogonist 4P-PDOT (Tocris), MT1/MT2 antogonist luzindole (Sigma-Aldrich), melatonin (Sigma-Aldrich). In case receptor antagonists and melatonin were used together, the cells were preincubated for 15 minutes with antagonist and then the melatonin was added.

The cells were transferred to the coated wells (100 µl/well) and incubated for 30-40 min at 37°C with 5 % CO_2. The non-adherent cells were washed out, the plates gently washed 4-5 times with RPMI medium. The antibody master mix for cell staining was prepared in staining medium (RPMI with 5 mM EDTA and 0.1 % Na aside). 50 µl/well of ice cold master mix was added to each well and incubated for 30 minutes. The cells were released by pipetting. Additional 150 µl of ice cold staining medium was added to every well, then mixed and all the medium with detached cells was transferred to the tubes for analysis. The cells were analyzed by flow cytometry. The adhesion % was calculated according to the formula: adhesion % = (number of adherent cells)/(number of added cells to the well) x 100%.

Data analysis. The paired Student's *t*-test was used to investigate possible differences among the groups in ELISA. The unpaired Student's *t*-test was used to investigate possible

differences among the groups in granulocyte adhesion. The Mann-Whitney *U* test was used to investigate possible differences among the groups in flow cytometry. The post hoc Tukey's Multiple Comparison test was used to investigate possible differences among the groups in PCR. Differences were considered statistically significant when the *P*-value was < 0.05.

RESULTS

The influence of melatonin on T-cell-independent (TI) antibody production.

The effect of melatonin on antibody production was investigated using a mouse model with suppressed melatonin synthesis. As the melatonin secretion is inhibited by light, the BALB/c line mouse in our study were exposed to constant lighting conditions (Fig. 1).

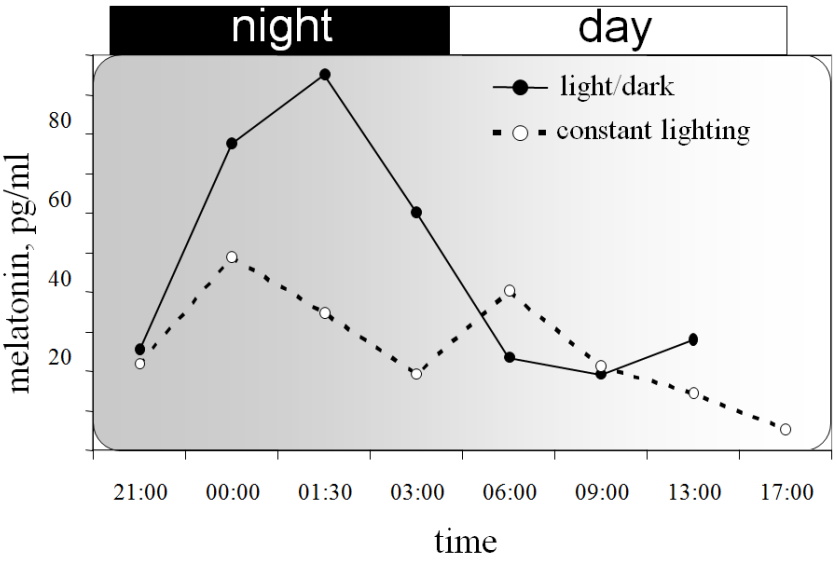

Fig. 1. Melatonin concentration in the sera of mouse kept at different lighting conditions.
BALB/c mouse (females, 8–9 weeks old) were divided into two groups and kept at normal light/dark conditions and at constant lighting for 2 weeks. The blood was collected at different time during one day and one night. The blood was pooled from two to three mouse. The melatonin concentration in the sera was determined by ELISA. The dark bar represents the duration of the night.

The control mouse were kept under normal light/dark conditions. To find out if the altered antibody secretion was due to the suppressed melatonin synthesis or only due to dramatically altered circadian rhythms, we included the second control: mouse were kept under constant light but got daily melatonin injections. Experimental mouse were kept at certain lighting conditions for 1 week and immunized with TI (TNP-Ficoll and TNP-LPS) antigens as described in Methods. The animals throughout the experiment were kept at the

same lighting conditions. The TNP-specific antibodies were measured in the pre-immune sera and sera of the immunized animals. TNP-specific antibodies of IgM, IgG1, IgG2b and IgG3 isotypes were detected in the sera after the immunization with TI type II antigen TNP-Ficoll (Fig. 2). The amount of TNP-specific antibodies was higher in the sera of mouse kept under constant lighting (inhibited melatonin synthesis) as compared with the control mouse maintained under normal light/dark conditions. The amount of antibodies in the mouse exposed to constant lighting but provided with daily melatonin treatment was similar to the level of antibodies in mouse kept at normal light/dark conditions. Thus, the production of specific antibodies increased when melatonin synthesis was inhibited (constant light).

To prove the influence of melatonin on TI antibody production, we immunized the mouse with TI type I antigen TNP-LPS. The increased amount of IgG1- and IgG3-specific antibodies was obtained in the mouse group that was kept under the constant lighting as compared with the control mouse group (living at the normal light/dark conditions). The amount of IgM antibodies in that group varied from experiment to experiment. The IgM anti-TNP antibodies were increased under the constant lighting or similarly as in the control group. It means that melatonin inhibited the secretion of TI antibodies of IgM, IgG1, IgG2b and IgG3 classes.

Direct influence of melatonin on B cells is not clear. Some data indicate that melatonin failed to influence the activity of B cells in a TI manner. Melatonin had no influence on LPS stimulated B cell proliferation or secretion of immunoglobulins (IgA, IgM, IgG1 and IgG2a) (Raghavendra et al., 2001). Our data demonstrate that melatonin can modulate TI antibody secretion by B cells. The amount of antibodies increased when melatonin synthesis was suppressed (mouse kept at constant artificial lighting). Mouse maintained at constant lighting and treated daily with melatonin produced a similar amount of antibodies as the control group. This means that melatonin negatively regulates the TI antibody production.

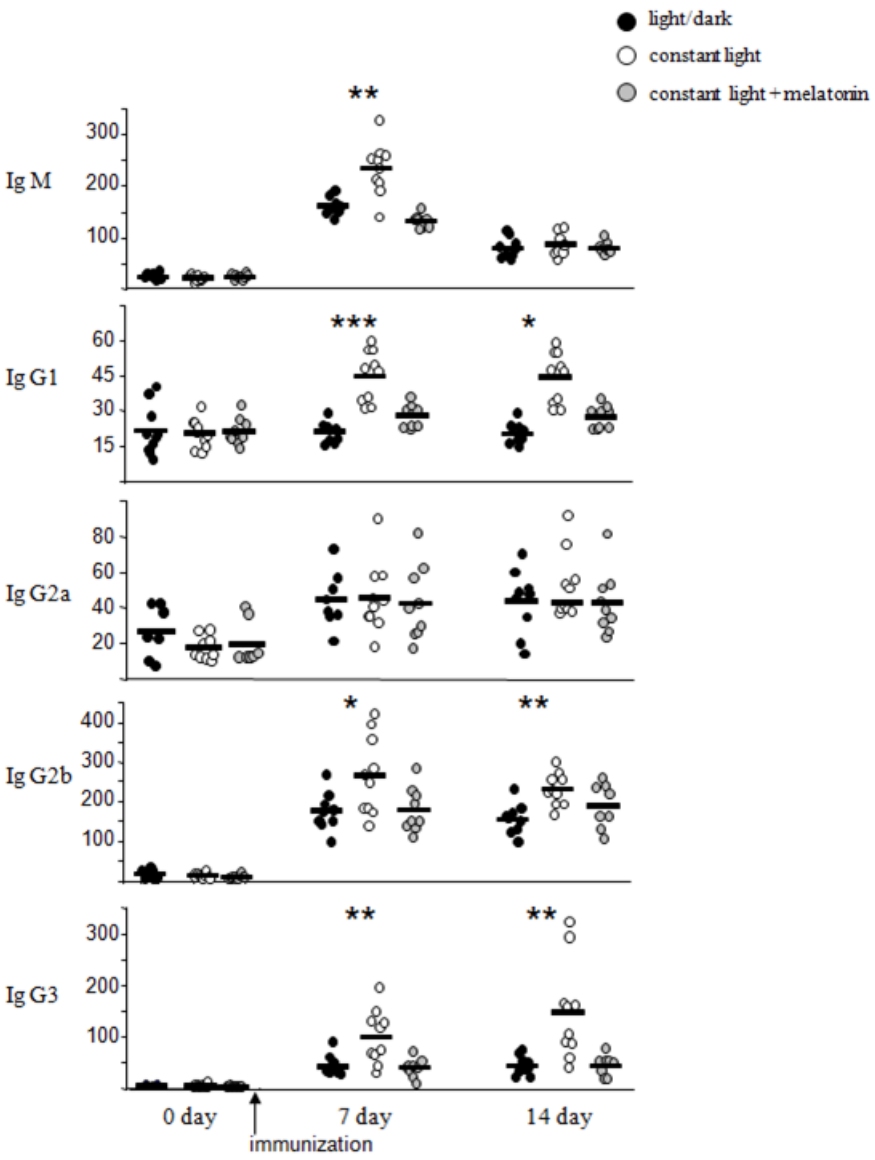

Fig. 2. Production of TI antibodies at different lighting conditions.
BALB/c mouse were divided into three groups and kept at different lighting conditions: at normal light/dark (black circles), constant light (white circles) and constant light with daily administration of melatonin (gray circles). One week later, the mouse were immunized with TI type antigen TNP-Ficoll. The immunizations and daily melatonin injections were performed in the evening. The blood was collected before immunization (day 0) and at days 7, 14 after the immunization. TNP-specific antibodies were detected by ELISA. One circle represents the antibody concentration (arbitrary units) in the serum of one mouse. Eight to ten mouse were per group. Representative graphs from 3 independent experiments are shown. The significant differences (calculated by Student's t-test) between the mouse groups kept in normal dark/light conditions and constant light are marked: *P < 0.05, **P < 0.005, ***P < 0.0005.

The influence of melatonin on T-cell-dependent (TD) antibody production.

In order to reveal the differences and similarities between the action of TI and TD antigens, we performed the immunizations with TD antigen TNP-ovalbumin (Fig. 3). Similar to the immunization with TI antigen, the increased amounts of IgM, IgG2a, IgG2b and IgG3 TNP-specific antibodies in the sera of mouse kept at constant lighting conditions versus control groups were detected. The amount of IgG1 class TNP-specific antibodies, on the contrary, was significantly lower in the sera of this mouse group where melatonin synthesis was inhibited by the light. The influence of melatonin on regulation of TD antibody production was different: it stimulated IgG1 and inhibited IgM, IgG2a, IgG2b and IgG3 class antibody production. Similar data of the melatonin effect on antibody production were obtained by immunizing BALB/c females and males.

The effect of melatonin on B cells could be indirect due to T helper cells. According to the literature, a daily injection of melatonin specifically enhances the production of IgG1 but decreases IgG2a antigen-specific antibody secretion and has no effect on the production of IgG2b- and IgG3-specific antibodies in BALB/c line mouse (Shaji et al., 1998). The primary and secondary antibody production in response to sheep red blood cell (SRBC) was markedly decreased in mouse treated with propranolol (inhibition of the night-time rise of melatonin) in the evening, while the responses of the morning-treated mouse were similar to those of the controls. The immunoenhancing effect of melatonin was evident only when it was administered in the afternoon or in the presence of TD antigenic stimulation (Guerrero et al., 2002). According to our results, inhibition of melatonin synthesis caused the increased amount of IgG2a, IgG3 and IgM antibodies after the immunization with TD antigen. On the other hand, the amount of IgG1 antibodies significantly decreased. The same tendency was observed with the primary and secondary immune responses.

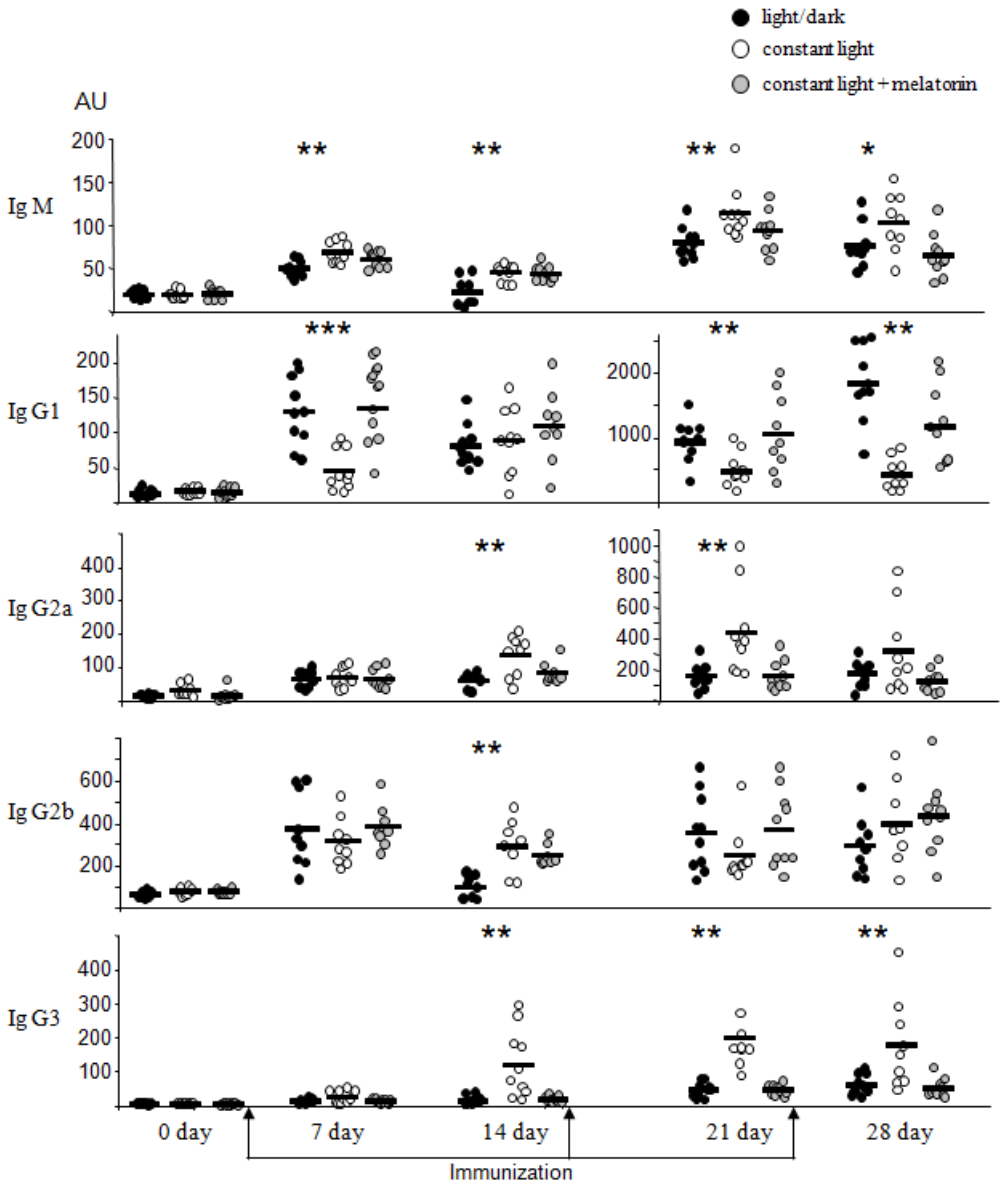

Fig. 3. Production of TD antibodies at different lighting conditions.
BALB/c line mouse were divided into three groups and kept at different lighting conditions: at normal light/dark (black circles), constant light (white circles) conditions and constant light conditions with daily administration of melatonin (gray circles). The mouse were immunized with TD antigen TNP-ovalbumin. The immunizations (indicated by arrows) and daily melatonin injections were performed in the evening. The blood was collected before immunization (day 0) and after the immunizations (day 7, 14, 21 and 28). One circle represents the antibody concentration (arbitrary units) in the serum of one mouse. Nine to ten mouse were per group. One representative experiment of three is shown. The significant differences (calculated by Student's t-test) between the mouse groups kept in normal dark/light conditions and constant light are marked: *P < 0.05, **P < 0.005, ***P < 0.0005.

After *in vivo* and *in vitro* experiments using melatonin membrane receptor inhibitors it has been shown that melatonin modulates T cell-dependent immune response by acting through the MT2 receptor.

To find out, through which cell type MT2 receptors (MT2R) does melatonin modulate the T-cell-dependent immune response, we investigated the expression of membrane melatonin receptors in purified splenic B cells, and lymph node T_H0 cells using PGR methods. The transcripts of MT1R and MT2R in the cDNA from lymph node T_H0 cells almost failed to detect. The presence of MT1 and MT2 transcripts in splenic B cells depends on lighting conditions. We detected mRNA of MT1R and MT2R in splenic B cells only at night, which corresponds to the increased melatonin concentration in the blood. The transcripts of MT1R and MT2R in B cells were not detected in the samples from "LD day" and "LL night" mouse, but melatonin injection into the "LL night" mouse (kept at constant light) induced the expression of MT1 and MT2 receptors.

To sum up the results, melatonin, acting through the splenic B lymphocyte MT2 receptors, modulates the T-cell-dependent humoral immune response. After constant lighting, the disruption of melatonin production affected the expression of MT2 receptor in B lymphocytes. For this reason, the decreased antibody levels of IgG1 isotype and increased IgG2a antibody titers were observed. Additional injections of melatonin restored the expression of MT2 receptor in splenic B lymphocytes, induced the increased antibody levels of IgG1 isotype and reduced the IgG2a antibody titer.

The expression of MT1 and MT2 melatonin receptors in murine lymphocytes at protein level.

The expression of MT1 and MT2 receptors was investigated at protein level using our produced rat monoclonal antibodies to MT1 and MT2 receptors. Monoclonal antibody production was carried out as described in Methods. We investigated the expression of melatonin receptors in the murine lymphocytes (thymocytes, bone marrow cells, purified splenic B cells and lymph node T cells) isolated from the mouse kept at different lighting conditions. The expression of MT1 receptor was not found in all cells studied (bone marrow

cells, thymocytes, splenocytes). The expression of MT2 receptor on B cells was near the detection level (Fig 4). The MT2 receptor expression on B cells was very weak and did not depend on the circadian time and lighting conditions. The MT2 receptor was not detected in other investigated cells.

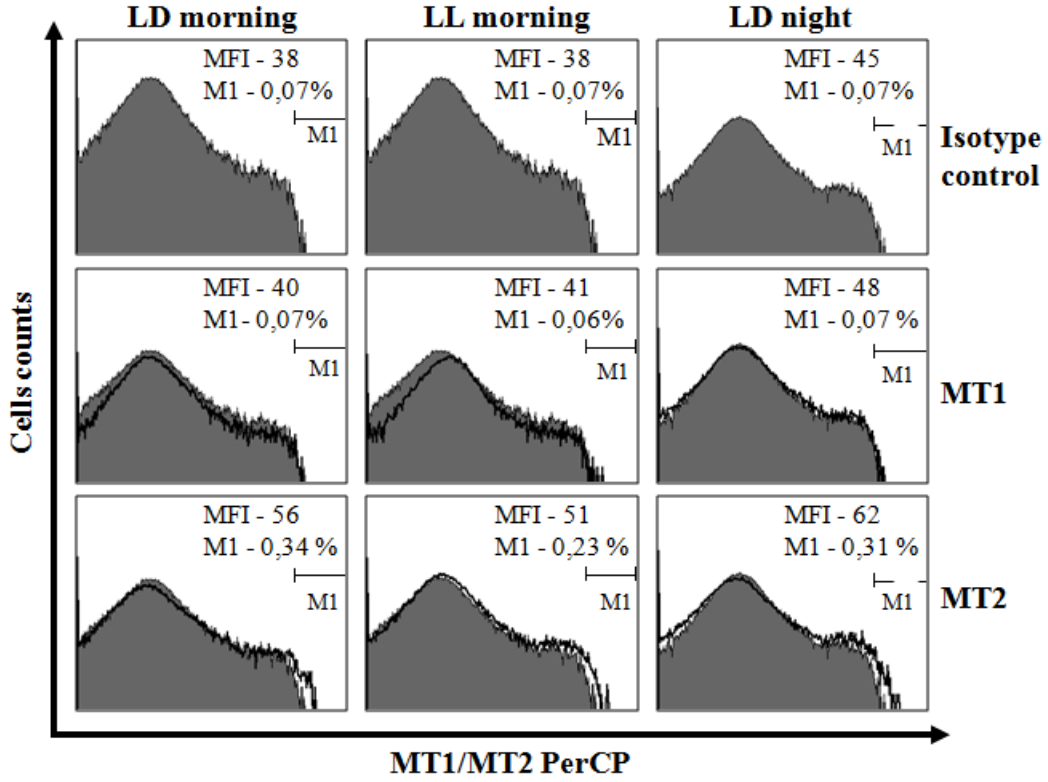

Fig. 4. The expression of melatonin membrane receptors in splenic B cells
MT1 and MT2 receptors expression was measured on 8 weeks BALB/c line mouse splenocytes by flow cytometry. The mouse were kept at constant light (LL) and 12/12 light/dark (LD) conditions and the cells were isolated in the morning and at night. Splenocytes were incubated with unspecific rat antibodies (izotype control), anti-MT1 and anti-MT2 rat monoclonal antibodies. The binding of primary antibodies was detected using anti-rat-biotin and streptavidin-PerCP. The B cells were separated using anti-B220-PE antibodies. Representative histograms from three independent experiments are shown.

The cell analysis using real-time PCR showed that the expression of MT2 melatonin receptor on splenic B lymphocytes depends on time of day and the disruption of melatonin production. Using flow cytometry, this dependence was not detected. The difference could be due to the sensitivity of the methods used.

The influence of melatonin on the amount of leukocytes in organs of the immune system and in the inflammatory site.

The effect of melatonin on the leukocyte numbers in organs of the immune system was investigated using the mouse model with suppressed synthesis of melatonin. As we have shown previously, melatonin secretion is inhibited by light; therefore the BALB/c line mouse were exposed to constant light during the experiment and about a week before. The control mouse were kept under normal light/dark conditions. All mouse were immunized with TI or TD antigens in the evening. Different cell populations were analyzed in lymphoid organs. No significant differences in numbers of separate leukocyte subpopulations (T cells, B cells, monocytes, granulocytes) in thymus, bone marrow, spleen, lymph nodes and Peyer's patches were observed following one week after the last immunization. However, the differences in cell numbers were identified in the peritoneal cavity (inflammatory site). The mouse with inhibited melatonin synthesis and immunized with TD as well as TI antigen had increased numbers of T and B2 B cells, monocytes/macrophages and granulocytes (Fig. 5) versus the control group - mouse kept at normal lighting. To prove the melatonin effect on cell amount in the peritoneal cavity, an additional control was included: the mouse were maintained under constant light conditions and melatonin was injected every evening. The amount of cells in those mouse was similar to the level of peritoneal cavity cells in mouse kept at normal light/dark conditions. Thus, the number of cells increased when melatonin synthesis was inhibited (constant light). Similar results were obtained with BALB/c line males and females. It means that melatonin is involved in the regulation of leukocyte migration into sites of inflammation.

The effect of melatonin on the size of immune organs and the number of cells was mostly investigated using pinealectomized animals or daily treating with melatonin. Pinealectomy promotes weight loss of the main immune organs (thymocytes, spleens, LN) (Vivien-Roels et al.,1998), thymic disorganization, absence of lymphocytes in thymus (Sasaki et al., 2002) and inhibition of IL-2 production by T cells (Park et al., 2007). Pinealectomized animals have defects in B cell activation and antibody formation: the lack of germinal centers in the spleen, which is probably caused by the lack of appropriate T

cells (Vivien-Roels et al.,1998), reduction of lymphocytes in the lymph nodes and loss of secondary follicles in the B cell area (Luo et al., 2004). Daily afternoon administration of melatonin induced the increase of thymus weight in the gerbil (Vivien-Roels et al.,1998), prevented thymic involution in very old mice, increased the number of leukocytes and lymphocytes (Sasaki et al., 2002).

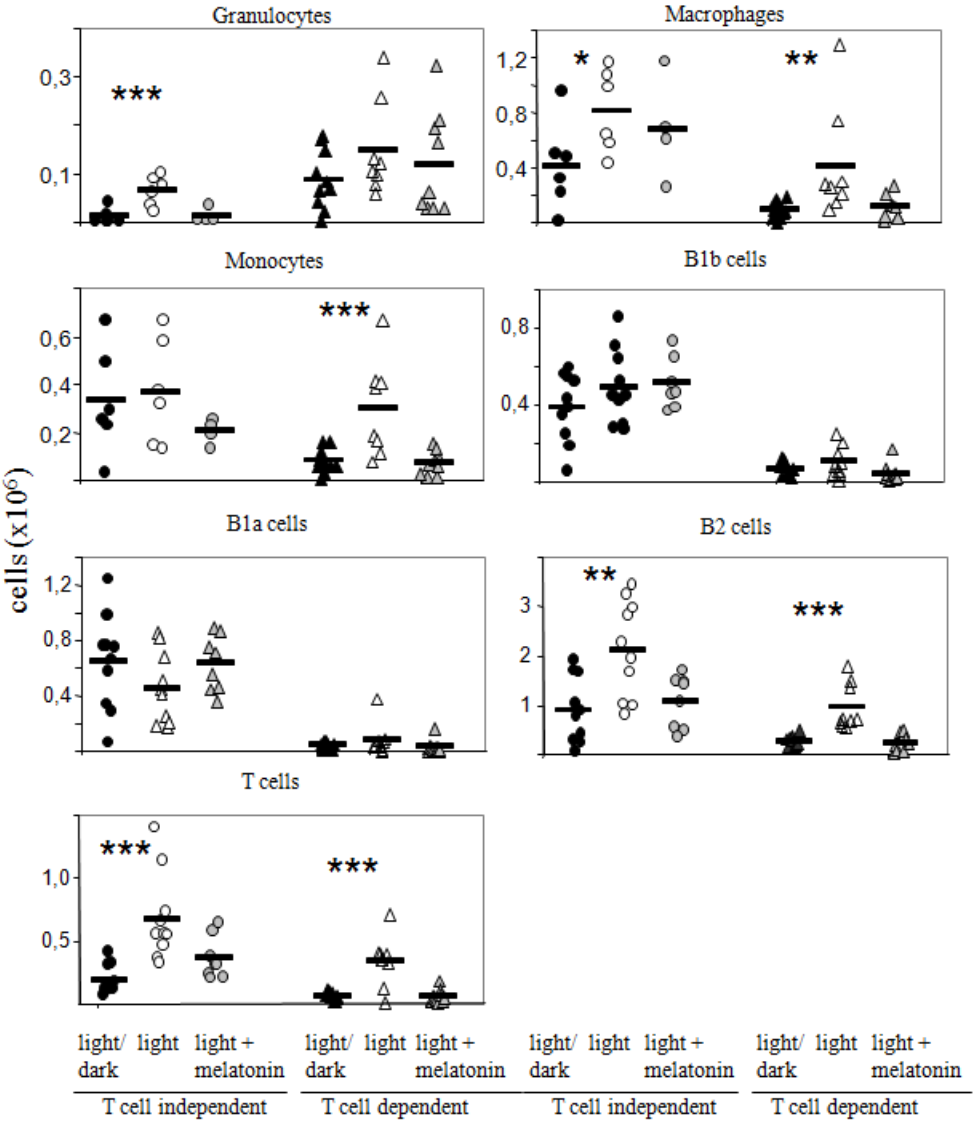

Fig. 5. Total number of cells (x10^6) in the peritoneal cavity of BALB/c line mouse kept at different lighting and immunized at the beginning of dark time.
BALB/c mouse were kept in 12/12h light/dark (closed black circles or triangles), constant light (open circles or triangles) conditions and constant light conditions with daily melatonin injections (closed grey circles or triangles). Peritoneal cavity cells were analyzed by FACS seven days after the last immunization. A representative experiment (out of 3) is shown. Statistical significant differences between the two mouse groups (light/dark vs constant light and constant light vs. constant light + melatonin) were calculated according to the Mann–Whitney U test. Significant differences are as follows: * p= 0.01 - 0.05, ** p=0.001- 0.01, *** p< 0.001.

How can melatonin be involved in regulating the number of peritoneal cells in the immunized mouse? Our hypotheses are in agreement with those suggested by Markus (Markus et al., 2007). Melatonin (produced by the pineal gland during the dark time) suppresses leukocyte rolling and adhesion (Lotufo et al., 2001). Leukocytes are driven to the inflammatory site (peritoneal cavity) by chemotactic substances. The absence of the nocturnal melatonin source allows full migration of leukocytes. Melatonin, produced by the activated cells at the site of injury (Markus et al., 2007), together with the cytokine gradient could "help" to keep the cells in the peritoneal cavity.

The migration of granulocytes into the peritoneum depends on the lighting conditions.

To investigate the role of melatonin in the regulation of granulocyte migration, mouse were injected with fMLP (N-Formyl-Met-Leu-Phe) into the abdominal cavity. The fMLP induced granulocyte migration into the peritoneal cavity was investigated in the mouse kept at different lighting conditions. One group of BALB/c line mouse was kept at normal (12 hours dark and 12 hours light) lighting conditions (LD). The second mouse group was under constant lighting conditions (LL). The amount of granulocytes in the peritoneum of LL mice was significantly higher as compared to the LD mouse (Fig.6), when the cells were counted during the night.

However, the number of granulocytes did not differ when the cells in the peritoneal cavity were counted during the day. Those experiments indicate that the number of fMLP induced granulocytes in the peritoneum depends on lighting conditions. Based on our results, the reduction in melatonin concentration increased the granulocyte migration. Our study results confirm the previous literature data (Serin et al., 2007) which suggest that melatonin negatively regulates granulocyte migration.

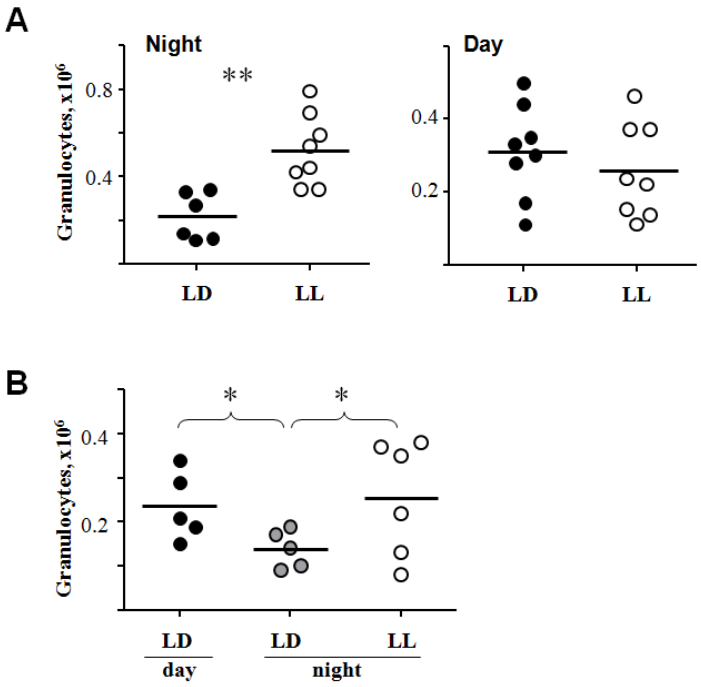

Fig.6. The migration of granulocytes into the peritoneal cavity depends on the lighting conditions.
The BALB/c line mouse were kept at constant light (LL) or 12/12 light/dark (LD) conditions. fMLP was injected into the peritoneum to induce the migration of granulocytes and the cells from the peritoneal cavity were isolated 4h later. A) The influence of lighting conditions on the amount of granulocytes in the peritoneum around the midnight (left) and around the midday (right). B) The influence of circadian time on the migration of granulocytes into the peritoneum. One circle means the number of granulocytes in the peritoneum cavity of one mouse. Significant differences between the two mice groups were calculated by means of the Mann–Whitney U test (* $p \leq 0.05$, ** $p \leq 0.005$). Representative graphs from 3 independent experiments are shown.

Melatonin inhibits granulocyte adhesion to ICAM-1 molecule.

Next we checked if melatonin could influence the granulocyte adhesion to ICAM. The granulocyte adhesion was investigated *in vitro* on the rmICAM-1Fc coated plates. Melatonin significantly reduced granulocyte adhesion (Fig.7). Melatonin receptor *MT3*/CR2 agonist Gr135531 acted the same as melatonin. It has also significantly decreased granulocyte adhesion to rmICAM-1Fc. However, the combination of melatonin and Gr135531 did not show a synergistic effect (Fig.7). *MT3*/CR2 antagonist prazocin hydrocholoride alone did not influence the adhesion; however it blocked the effect of melatonin (Fig.7). The MT1/MT2 antagonist luzindole and MT2 antagonist 4P-PODT did not affect the adhesion, neither each of them alone nor in the combination with melatonin. These data indicate that melatonin reduces granulocyte adhesion acting via the *MT3*/CR2 receptor.

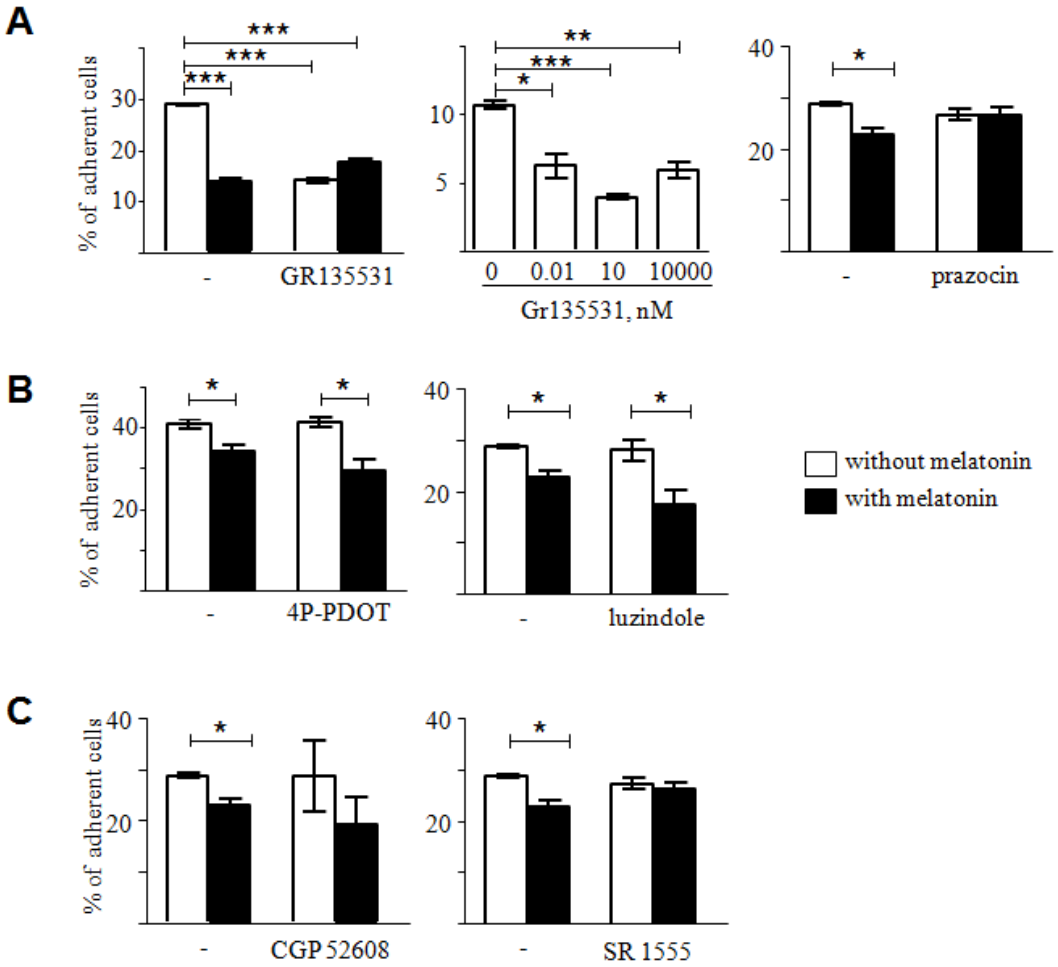

Fig.7. Melatonin inhibits granulocyte adhesion to ICAM-1 molecule.
The mouse were kept at 12/12 light/dark conditions. The granulocytes from the blood were isolated during the day. The granulocyte adhesion to the plates coated with rmICAM-1Fc (R&D Systems) was performed in the absence (white bars) or presence (black bars) of melatonin. **A**. The influence of *MT3*/CR2 receptor agonist GR135531 and *MT3*/CR2 receptor antagonist prazocin hydrocholoride. **B**. The influence of MT2 receptor antagonist 4P-PDOT and MT1/MT2 receptor antagonist luzindole. **C.** The influence of RORa receptor agonist CGP52608 and RORγ receptor inverse agonist SR1555. Significant differences between the two mice groups were calculated by means of the unpaired T test (* $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.0005$).

It is known that melatonin, acting through the endothelial cells, inhibits the expression of adhesion molecules in endothelial cells as well as their adhesion properties (Marcola et al., 2013). Also melatonin, acting through the *MT3*/CR2 receptor, inhibits leukocyte adhesion (Lotufo et al., 2001). However, the evidence that melatonin inhibits granulocyte adhesion has not yet been submitted to scientific journals. Summarizing the results, it was found that melatonin modulating granulocyte migration, acts through the same granulocytes. Melatonin, acting through *MT3*/CR2 granulocyte receptor, inhibits granulocyte adhesion and migration.

The aim of the dissertation was to restore the shift work conditions and to investigate the possible effect of melatonin deficiency on the immune system in people who work night shifts. The results show that keeping mouse at constant lighting conditions causes the disruption of the immune homeostasis: production of antibodies, leukocyte and granulocyte migration. Therefore, shift work and its influence on the immune system could be considered as a factor for possible health disorders.

CONCLUSIONS

1. Melatonin modulated T cell-independent antibody production. Suppressed melatonin synthesis increased the amount of IgM, IgG1, IgG2a, IgG2b and IgG3 antibodies after immunization with TI antigen. Daily melatonin treatment brought the antibody level back to normal. Melatonin directly (in the absence of T cells) inhibited B cell activation and antibody secretion.
2. Melatonin modulated T cell-dependent antibody production. Suppressed melatonin synthesis increased the amount of IgM, IgG2a and IgG3; however, the concentration of antigen-specific antibodies of IgG1 isotype was significantly decreased. Daily melatonin treatment brought the antibody level back to normal. Melatonin modulates the production of antibodies acting via splenic B lymphocyte MT2 receptors.
3. The monoclonal antibodies against mouse melatonin MT1 and MT2 receptors were produced. Using these monoclonal antibodies, we obtained the expression of MT2 receptors on B cells.
4. Melatonin decreased the leukocyte migration into the inflammatory site. The mouse with inhibited melatonin synthesis and immunized with T cell-dependent, as well as T cell-independent antigen, had an increased leukocytes in the injured area as compared to the control group (immunized mouse kept in customary lighting). Melatonin treatment normalized the cell number.
5. Melatonin inhibited the fMLP-induced granulocyte migration. The mouse with inhibited melatonin synthesis had an increased number of granulocytes in the peritoneal cavity as compared to the control group (mouse kept in customary lighting). Melatonin acting through granulocyte *MT3*/CR2 receptor inhibits granulocyte adhesion to endothelial cell ICAM-1 molecule.

REZIUMĖ

Tiriant melatonino trūkumo poveikį BALB/c linijos pelių imuninei sistemai, pirmiausiai buvo nustatytas pastovaus apšvietimo poveikis melatonino sintezei. Naktį, kai įprastai prasideda melatonino gamyba, esant šviesai pasireiškė melatonino sintezės slopinimas. Pelių, gyvenančių pastovaus apšvietimo sąlygomis, hormono koncentracija kraujo serume buvo 2-3 kartus mažesnė nei pelių, gyvenančių natūralaus apšvietimo sąlygomis. Sekančiuose tyrimuose, taikant pastovaus apšvietimo metodą ir, tokiu būdu slopinant melatonino gamybą, buvo ištirtas melatonino poveikis BALB/c linijos pelių imuninei sistemai.

Kadangi antikūnų produkcija gali būti nuo T limfocitų priklausoma ir nepriklausoma, siekiant nustatyti melatonino poveikį antikūnų gamybai, imunizacijai naudoti skirtingi antigenai. Nuo T ląstelių nepriklausomai antikūnų produkcijai tirti BALB/c pelės buvo imunizuotos nuo T ląstelių nepriklausomais antigenais – TNP-fikoliu ir TNP-LPS. Šie antigenai stimuliuoja tik B ląsteles ir skatina antikūnų sintezę, tačiau TNP-fikolis ir TNP-LPS nestimuliuoja T ląstelių, todėl šios ląstelės neturi įtakos B ląstelių funkcijoms. Remiantis literatūros duomenimis, melatoninas neturi poveikio BALB/c linijos pelių B limfocitų proliferacijai ir antikūnų gamybai nesant ir esant LPS stimuliacijai (Raghavendra et al., 2001). Atlikus eksperimentus, nustatyti priešingi rezultatai - melatonino trūkumas stimuliuoja nuo T ląstelių nepriklausomų antikūnų susidarymą. Kita vertus, laikant peles pastovaus apšvietimo sąlygomis, bet kas vakarą leidžiant melatoniną, antikūnų kiekis buvo panašus į kontrolinės grupės (pelės, gyvenančios 12 val. šviesos ir 12 val. tamsos sąlygomis). Remiantis šiais rezultatais, galime teigti, kad melatoninas slopina nuo T ląstelių nepriklausomą antikūnų produkciją. Mūsų tyrimų metu pirmą kartą įrodyta, kad melatoninas tiesiogiai (nedalyvaujant T ląstelėms) gali veikti B ląstelių aktyvinimą ir antikūnų sekreciją.

Remiantis literatūros duomenimis, melatoninas moduliuoja nuo T ląstelių priklausomą pelių antikūnų gamybą, reguliuodamas citokinų gamybą T_H0 ląstelėse. Stimuliuodamas IL-4 ir IL-10 produkciją ir slopindamas IFNγ, šis hormonas skatina B limfocitus sekretuoti IgG1

ir slopina IgG2a gamybą (Raghavendra et al., 2001, Shaji et al., 1998). Šiame darbe atliktų eksperimentų rezultatai taip pat rodo, kad, trūkstant melatonino, padidėja IgM, IgG2a ir IgG3 antikūnų kiekiai bei sumažėja IgG1 izotipo antikūnų kiekis.

Vis dėlto iki šiol literatūroje nebuvo skelbiama duomenų, per kurias ląsteles ir jų receptorius melatoninas reguliuoja nuo T ląstelių priklausomą pelių antikūnų gamybą. Šiame darbe atlikti *in vivo* ir *in vitro* eksperimentai, kuriuose naudotas MT2 receptoriaus selektyvus slopiklis, parodė, kad melatoninas reguliuoja nuo T ląstelių priklausomą antikūnų gamybą veikdamas per MT2 receptorių. Atlikus tikrojo laiko PGR, blužnies B limfocituose nustatyta žymiai intensyvesnė melatonino membraninių receptorių transkriptų raiška, lyginant su vykusia limfinių mazgų T_H0 limfocituose. Taip pat nustatyta, kad blužnies B limfocituose membraninių receptorių transkriptų raiška priklauso nuo paros laiko – vyksta tik nakties metu ir priklauso nuo melatonino koncentracijos. Dėl pastovaus apšvietimo sutrikus melatonino gamybai, MT1 ir MT2 receptorių raiška nakties metu nevyksta, tačiau papildomos melatonino injekcijos atstato blužnies B limfocituose melatonino membraninių receptorių raišką. Apibendrinant visus rezultatus, galime teigti, kad šio darbo metu pirmą kartą buvo įrodyta, kad melatoninas, veikdamas per blužnies B limfocitų MT2 receptorių, moduliuoja nuo T ląstelių priklausomą antikūnų gamybą. Dėl pastovaus apšvietimo sutrikus melatonino gamybai, sutrinka ir MT2 receptoriaus ekspresija B limfocituose, sumažėja IgG1 ir padidėja IgG2a antikūnų kiekis. Kita vertus, papildomos melatonino injekcijos atstato MT2 receptoriaus ekspresiją blužnies B limfocituose, padidina IgG1 ir sumažina IgG2a antikūnų kiekį.

Literatūros duomenis, pašalinus pagrindinį melatonino gamybos organą - kankorėžinę liauką, gyvūnams sumažėja imuninės sistemų organų masė (Vaughan et al., 1971, Cunnane et al., 1979, Brainard et al., 1988). Tyrinėjant melatonino poveikį BALB/c linijos pelių leukocitų migravimui mes nustatėm, kad melatonino sintezės slopinimas nesukelia statistiškai patikimo ląstelių kiekio sumažėjimo imuninės sistemos organuose: užkrūčio liaukoje, limfmazgiuose, blužnyje. Kita vertus, melatonino sintezės slopinimas skatina leukocitų migraciją į uždegimo vietą (pilvo ertmę), bet laikant peles pastovaus apšvietimo sąlygomis ir kas vakarą leidžiant melatoniną, limfocitų skaičius buvo panašus į kontrolinės

grupės (pelės, gyvenančios 12 val. šviesos ir 12 val. tamsos sąlygomis). Remiantis šiais rezultatais, galime teigti, kad melatoninas slopina leukocitų migraciją į uždegimo vietą. Vykdant tolimesnius tyrimus, buvo nustatyta, kad melatoninas slopina leukocitų migraciją veikdamas per MT2 receptorių.

Tiriant melatonino poveikį granuliocitų migravimui pirmą kartą buvo nustatyta, kad melatoninas slopina granuliocitų migraciją, veikdamas per granuliocitus. Šis hormonas skatina *MT3*/CR2 receptoriaus raišką granuliocituose ir, veikdamas per *MT3*/CR2 receptorių, slopina granuliocitų sukibimo savybes, tokiu būdu mažindamas jų migraciją.

Disertacijos darbe buvo taikytas pastovaus apšvietimo metodas modelinėje pelių sistemoje. Taip siekta atkurti pamaininio darbo sąlygas ir ištirti galimą melatonino trūkumo poveikį žmonių, dirbančių pamaininį darbą, imuninei sistemai. Gauti rezultatai rodo, kad, laikant peles pastovaus apšvietimo sąlygomis, sutrinka jų imuninės sistemos homeostazė: antikūnų gamyba, leukocitų, granuliocitų migravimas. Dirbant pamaininį darbą reikėtų atkreipti dėmesį į jo poveikį imuninei sistemai ir įvertinti galimus sveikatos sutrikimus.

LIST OF PUBLICATION

1. V. Černyšiov, N. Gerasimcik, M. Mauricas, I. Girkontaitė. Regulation of T-cell-independent and T-cell-dependent antibody production by circadian rhythm and melatonin, *International Immunology*, 2010, 22(1): 25-34.
2. V. Černyšiov, M. Mauricas, I. Girkontaitė. Leukocyte infiltration in lymphoid organs and peritoneal cavity upon immunization: dependence on circadian rhythmicity and melatonin 24-h profile, *European Journal of Inflammation*, 2011, 9(3): 219-229.
3. V. Černyšiov, R. Bozaitė, M. Mauricas, I. Girkontaitė. The expression of MTNR3 and nuclear receptors in murine leucocytes. *In Vivo,* 2014, 09-10;28(5): 827-830.
4. V. Černyšiov, R. Bozaitė, M. Mauricas, I. Girkontaitė. Influence of circadian time and lighting conditions on expression of melatonin receptors MT1 and MT2 in murine lymphocytes. *In Vivo,* 2014, 09-10;28(5): 831-835.
5. V. Černyšiov, M. Mauricas, I. Girkontaitė. The physiological effectiveness on melatonin. *Health Sciences*, 2014, 24(3): 5-10.
6. V. Černyšiov, M. Mauricas, I. Girkontaitė. The effect of melatonin on the immune system. Submitted *Lithuanian Endocrinology* journal is peer-reviewed and accepted for publication.

ISI Web of Science and ISI proceedings:

1. V. Černyšiov, M. Mauricas, I. Girkontaitė. Regulation of T-cell-independent and T-cell-dependent antibody production by circadian rhythm and melatonin. *European journal of immunology*. 2009, vol. 39, issue S1: abstracts from the 2nd European Congress of Immunology.
2. V. Černyšiov, R. Bozaitė, M. Mauricas, I. Girkontaitė. Expression of melatonin receptors in the cells of immune system. *Immunology*, 2012, 137 Special Issue: Abstracts of the European Congress of Immunology, 5-8 September 2012, Glasgow, Scotland): 296.

SCIENTIFIC CONFERENCES:

Dissertation theme was presented in 15 conferences (6 of them – international). International Conferences:

1. The Second European Congress of Immunology, Berlin, Germany, 13-16 September, 2009, the poster report: „Regulation of T-cell-independent and T-cell-dependent antibody production by circadian rhythm and melatonin".
2. The Third European Congress of Immunology, Glasgow, Scotland, 5-8 Semtember, 2012, the poster report: **„**Expression of melatonin receptors in the cells of immune system**"**.
3. The Tenth International Conference of Lithuanian Chemists, Vilnius, 14-15 October, 2011, the poster report: **„**The role of melatonin and circadian time on the migration of leukocytes into peritoneal cavity upon immunization**"**.
4. The Fifteenth International Congress of Immunology, Milan, Italy, 22-27 August, 2013, the poster report: **„**Melatonin regulates the migration of granulocytes into mouse peritoneal cavity via MT2 receptor**"**.
5. The Fifth Conference of Lithuanian Neuroscience Association, Vilnius, Lithuania, 6-7 December, 2013, the poster report: **„**The role of melatonin in the inflammatory process**"**.
6. The Ninth International Conference of Natural and Life sciences **„**COINS 2014**",** Vilnius, Lithuania, 3-8 March, 2014, the poster report: **„**The immunoreguliatory action of melatonin**"**.

Scientific conferences and competitions, in which the results of the thesis work, were presented and awards received:

1. The winner of the competition for students' scientific works in 2008, the Faculty of Natural Sciences in Vilnius University.
2. The winner of the competition for the best student's scientific work (biology, chemistry, medicine), the Lithuanian Academy of Sciences (2009-2010).
3. In 2011, at the National PhD students' conference "Science for health" the oral presentation "The role of melatonin in mice humoral immunity" was awarded a special Thermo Fisher Scientific Prize.

4. In 2012, at the National PhD students' conference "Science for health" the oral presentation "Melatonin receptor expression in lymphocytes" was awarded a special company Thermo Fisher Scientific Prize, and also was awarded the first place in the work section "Pulmonology and Allergology".

5. In 2013, Lithuanian Academy of Sciences organized the Young Scientists Conference "Biofuture: natural and life sciences perspective", the oral presentation "Melatonin role in regulating neutrophils migration" was awarded the first place.

6. In 2014, at the National PhD students' conference "Science for health" the oral presentation "Melatonin receptor mRNA transcripts expression in BALB/c mice macrophages and dentritic cells in presence and absence of LPS (lipopolysaccharide) stimulation" was awarded a special Thermo Fisher Scientific prize, and also awarded the second place in the work section "Bio- and nanotechnology".

CURRICULUM VITAE

Name Vitalij Černyšiov

Date of birth 18 January 1981

Education

2010 - 2014 PhD studies at the Department of Biotechnology and Microbiology (the Faculty of Natural Sciences at Vilnius University), Lithuania

2008 - 2010 Msc in Microbiology (the Faculty of Natural Sciences at Vilnius University), Lithuania. Msc "Magna Cum Laude" - diploma for the best and clever students of Vilnius University

2004 - 2008 Bsc in Biology (the Faculty of Natural Sciences at Vilnius University), Lithuania

Employment activity

2013 - present Medical biologist in the Vilnius University Hospital, Santariškiu Clinics centre of Hematology, Oncology and Transfusion, Vilnius, Lithuania

2012 - present Junior research associate, SRI Centre for Innovative Medicine, the Division of Immunology, Vilnius, Lithuania

2011 - 2012 Junior research associate, SRI Centre for Innovative Medicine, the Division of Creation for technologies of Individualized Immunotherapy, Vilnius Lithuania

2010 - 2011 Engineer-microbiologist, SRI Centre for Innovative Medicine, the Division of Creation for technologies of Individualized Immunotherapy, Vilnius Lithuania

2008 - 2009 Engineer-microbiologist, the laboratory of Immunotechnology at the Institute of Immunology of Vilnius University, Vilnius, Lithuania

Professional training

2012.05.01 – 2012.07.31 Professional training at Virgen del Rocio University Hospital, Seville, Spain

ACKNOWLEDGEMENTS

I would like to express my greatest gratitude to my supervisor **doc. dr. Irutė Girkontaitė** for giving me the opportunity to be a scientist. Thanks for her great guidance and assistance, stimulating suggestions and extraordinary patence throughhout this long journey.

Special thanks to people who helped me in the preparation of the doctoral dissertation: **dr. Mykolas Mauricas, dr. Natalija German, dr. Raimonda Kvietkauskaitė**, **prof. habil. dr. Lilija Kalėdienė**, **Eugenijus Šimoliunas, Milda Naciūtė, Rūta Bozaitė and Danutė Davidonienė.**

And the biggest gratitude to my family - my **parents Galina and Yurij, and sister Arina** for their constant support, without them this thesis would not have been possible.