Final report on the OTKA-project "Daily rhythm of neutrophil functions under physiological and pathological conditions" (K- 115 953)

The main goal of this project was to examine how the circadian control affects immune processes with a special focus on the role of the circadian regulation of neutrophils. The experimental work was divided into 6 subprojects which are detailed below:

1. Time-keeping mechanisms in human neutrophils

Our preliminary data indicated that neutrophils express core clock components at lower level than other leukocyte types do. As rhythmic changes in clock RNA levels were already well characterized in the mononuclear fraction of blood leukocytes, our first aim was to compare the expression level and pattern of canonical clock genes in neutrophils and mononuclear cells in blood samples of the same subjects. Blood samples were taken every three hours during a day. In mononuclear cells expression of *Per1, Per2, Per3* and *Dbp* showed significant daily rhythm, and also *Bmal1* displayed tendentious oscillation, whereas no rhythm in the expression of *Rev-erbα* was observed. In neutrophils, however only *Per1*, *Dbp* and *Rev-erbα* levels showed low-amplitude but significant oscillation, whereas expression of *Per2, Per3* and *Bmal1* was not rhythmic. As expression levels of housekeeping genes (used as reference genes for the normalization in the real-time PCR) are not evidently equal in different cell types, in a further analysis we used the core clock gene *Bmal1* as a clock-internal control to compare the relative expression of clock genes in both cell types. Although relative *Per1* levels were similar, expression of *Per2*, *Per3*, *Dbp* and *Rev-erbα* was different in neutrophils and mononuclear cells. These findings suggest that operation of the molecular oscillator is different in mononuclear cells and neutrophils.

On the other hand, *Per1* expression followed diurnal fluctuations also in neutrophils. In the blood samples we also determined cortisol concentrations as physiological markers of circadian rhythm and found that *Per1* oscillated in synchrony with cortisol levels. To examine whether cortisol as systemic cue may directly influence *Per1* expression, we treated isolated neutrophils with physiologically relevant amounts of cortisol. Expression of *Per1* became markedly elevated, whereas no significant change in the expression of other clock genes was observed. This result suggests that the *Per1* gene of human neutrophils is under the control of the glucocorticoid receptor and the observed oscillation of *Per1* expression could be a consequence of the daily rhythm of blood cortisol levels.

In our preliminary experiments we observed reduced BMAL1 and PER2 protein expression in human neutrophils. In our further investigations we found that phosphorylation of BMAL1 was also different in neutrophils and mononuclear cells. While in the mononuclear fraction BMAL1 forms with different electrophoretic mobility were present, in neutrophils only a hypophosphorylated single protein band could be detected on Western blots. Nuclear accumulation of BMAL1 is a basic requirement of normal clock function and it correlates with phosphorylation of the protein. Using immunostaining we showed that compared to monocytes and lymphocytes, BMAL1 levels were reduced and the nuclear accumulation of

the protein was impaired in neutrophils. This suggested again that the classical molecular clock does not operate robustly in these cells.

Our preliminary data supported the hypothesis that age distribution of the peripheral neutrophil pool changes in a circadian manner. We addressed whether these changes might be connected to fluctuations in the secretion of bone marrow-derived cytokines regulating neutrophil egress or elimination. We found that levels of the CXCR4 ligand CXCL12 oscillated with a phase similar to that of *Cxcr4* expression detected in neutrophils. These results raise the possibility that time-dependent clearance of neutrophils is supported by daily changes of human bone marrow functions.

In summary, our results suggest that the molecular clock is downregulated in human peripheral neutrophils and diurnal changes in the age composition of the cell pool may form the basis of circadian fluctuations in the intensity of neutrophil responses.

2. Characterization of rhythmic outputs and responsiveness of neutrophil granulocytes

Our preliminary data showed that expression of *Gp91^{phox}*, one of the core components of the superoxide-generating NADPH oxidase, displayed diurnal variations parallel with changes in the intensity of superoxide production. Superoxide production is functionally coupled to phagocytosis. In line with this, we found that human neutrophils isolated at 1 am (when superoxide production was high) engulfed significantly more bacteria (opsonized, GFP-labelled *Staphylococcus aureus*) than cells collected at 1 pm (when superoxide production was relatively low).

To address the background of the temporal regulation of neutrophil responses, we investigated the cell surface expression of complement receptor components at different time points of the day. In human neutrophils complement receptor 3 is one of the main components mediating the phagocytosis of opsonized particles. We found that levels of both subunits (CD18 and CD11b) of the receptor showed diurnal changes, indicating that circadian variations in receptor availability may contribute to the observed differences in phagocytosis.

To study the role of circadian clock in additional neutrophil reactions, we switched to the mouse model *(*C57BL/6J mice) and established the method of neutrophil isolation from mouse bone marrow in the lab. In order to compare the migratory activity of *wt* and *Bmal1$^{-/-}$* neutrophils, we stimulated the cells with TNFα/ fMLP (chemotactic peptide) /ATP / CXCL12 (cytokine) or co-stimulated with TNFα and fMLP and transwell migration assays were performed. Neutrophil migration was quantified, but no statistically significant differences were found between *wt* and *Bmal1$^{-/-}$* cells.

Together, our preliminary data and the new experimental results suggest that phagocytosis and superoxide production of neutrophils are under circadian control. On the other hand, we found no direct evidence that migration capacity of the cells depends on clock function.

3. Examination of changes in clock function during myeloid maturation *in vitro*

Based on our observations in human neutrophils we hypothesized that clock function may change during the myeloid differentiation process. To investigate this possibility, first we used the human acute myeloid leukemia cell line PLB-985 as a model and followed clock protein expression during differentiation of the cells towards the granulocytic pathway. Differentiation of PLB cells can be induced by treating the cells with only dimethylsulfoxide or both dimethylformamide and insulin. However, under all conditions only about 10 % of the cells differentiated to morphologically mature hypersegment neutrophils and majority of the cultured cells remained in the metamyelocyte stage. Nevertheless, when we compared the levels of PER2 at the beginning and on the last day of differentiation, a significant reduction in protein expression was observed. In addition, a similar change in *Per2* RNA expression relative to *Bmal1* was detected. In addition, phosphorylation (reflected by changes in the electrophoretic mobility) of BMAL1 was also affected; between the first and the 4^{th} day of cultivation a significant shift towards the phosphorylated forms was found. These data suggest that the molecular oscillator undergoes a modification along with differentiation to neutrophil-like cells.

Compared to the PLB-985 cells, the newly available ER-HOXB8 cell line can be more efficiently differentiated towards neutrophil granulocytes. Therefore, in our further experiments we used these cells to follow how cell differentiation is associated with changes in clock gene expression. In these mouse myeloid progenitor cells β-estradiol arrests the myeloid differentiation. Following removal of β-estradiol from the culture media, in the presence of G-CSF the cells begin to differentiate. We optimized the culturing conditions for cell differentiation, so that about 25% of the cells were in metamyelocyte stage, more than 10% had banded phenotype and 45% of the cells showed nuclear segmentation. Parallel with the phenotype changes, expression of molecular clock genes also showed marked differences. We analyzed the gene expression every four hours during the day. When compared to the expression on the first day of differentiation, on the 5^{th} day the *Per2*: *Bmal1* mRNA ratio was declined to 15 %. Our results in this new system confirmed our observations on PLB-985 cells indicating that the molecular clock function undergoes characteristic changes during the differentiation towards neutrophils.

4. Studies on the effect of the circadian clock on myelopoesis and the peripheral leukocyte composition

Using the mouse model, we generated bone marrow chimeras by transplanting either *Bmal1*$^{-/-}$ or *wt* bone marrow cells (carrying the CD45.2 leukocyte marker) to irradiated *wt* recipients (carrying the CD45.1 marker). Repopulation of the blood by donor cells was controlled with flow cytometry by using anti-CD45.2 and anti-Ly6G (neutrophil marker) antibodies. In the 4th week after transplantation, more than 98 % of the peripheral leukocyte pool was replaced by donor cells in both types of animals and no significant difference in the leukocyte number was obtained. Also leukocyte composition of the blood assessed by both flow cytometry and microscopy (blood smears) was similar in the two groups. Next, we compared the daily changes of leukocyte number in blood samples of *wt* and *Bmal1*$^{-/-}$ mice,

and in *wt*- and *Bmal1*$^{-/-}$ bone marrow chimeras. Animals were kept in 12h/12h light-dark cycles and total leukocyte number and leukocyte composition were determined in blood samples taken in every 4 hours. In *wt* mice and *wt*- and *Bmal1*$^{-/-}$ bone marrow chimeras both the leukocyte number and composition showed marked oscillation. In the *Bmal1*$^{-/-}$ animals, however, the cell number did not oscillate. Our data suggest, that (1) systemic circadian clock function is required for rhythmic trafficking of leukocytes and the environmental rhythm (light/dark cycles) cannot rescue the lack of *Bmal1*, (2) a functional clock in the hematopoietic cells is not needed for the characteristic daily changes of the peripheral leukocyte number.

Several data from the literature suggest that bone marrow macrophages have a modulatory effect on leukocyte trafficking. To analyze whether the rhythmic release of leukocytes from the bone marrow needs an intact clock function of bone marrow macrophages, we examined the effect of irradiation on the replacement of macrophages during chimera generation. Four weeks after the transplantation, bone marrow samples from *Bmal1*$^{-/-}$ bone marrow chimeras were prepared and labelled with antibodies against the macrophage marker F4/80 and the linage marker CD45.2. All F4/80 positive cells were CD45.2 positive showing that for this time point the whole macrophage population was already replaced by *Bmal1*$^{-/-}$ cells. These data suggest that the cellular clock of macrophages is not required for the rhythmic egress and homing of leukocytes.

5. Examination of the role of the circadian clock in the pathology of autoimmune arthritis

To seek links between circadian regulation and the pathogenesis of autoimmune arthritis, we established the K/BxN serum-transfer arthritis model in our lab. This disease model is primarily characteristic for the acute phase of RA in which neutrophil reactions dominate the pathology. In the first series of experiments we compared arthritis development in *wt* and *Bmal1*$^{-/-}$ mice. Severity of the disease was assessed by clinical scoring of the limbs and measurement of ankle thickness. However, correct quantification and comparison of the pathological changes was difficult because in *Bmal1*$^{-/-}$ mice a non-inflammatory arthropathy developed, usually from the age of 10-12 weeks, independently of arthritis induction. Therefore, in the next experiments we generated bone marrow chimeras by transplanting *Bmal1*$^{-/-}$ or *wt* bone marrow cells (carrying the CD45.2 leukocyte marker) to irradiated *wt* recipients (carrying the CD45.1 marker). Repopulation of the blood by donor cells was controlled with flow cytometry by using anti-CD45.2 and anti-Ly6G (neutrophil marker) antibodies. Four weeks after the bone marrow transplantation arthritis was induced and both morphological and functional signs of inflammation were followed. In animals with *wt* hematopoietic cells a more severe form of arthritis developed than in mice carrying *Bmal1*$^{-/-}$ blood cells, however, the difference did not reach the level of significance.

To further examine the role of *Bmal1*$^{-/-}$ leukocytes in the pathomechanism of arthritis, we generated mixed bone marrow chimeras by transplanting both *wt* (carrying the CD45.1 marker) and *Bmal1*$^{-/-}$ (carrying the CD45.2 marker) bone marrow cells in a ratio of 50%-50% into irradiated recipient (carrying the CD45.1 marker) mice. Four weeks after the transplantation this ratio was similar in the neutrophil subset of the peripheral blood. Arthritis

was induced and the neutrophils in the synovial fluid were analyzed on the 6th day, when the inflammation usually reaches its maximal intensity. The ratio of *Bmal1*$^{-/-}$ and *wt* cells in the synovial space was similar to that in the circulating pool suggesting that *wt* and *Bmal1*$^{-/-}$ neutrophils have similar ability to migrate to the site of inflammation. Taken together, we suggest that arthritis development in this disease model is not significantly affected by the loss of clock function in bone marrow cells.

6. Interconnections between feeding rhythm, metabolism and neutrophil functions

Metabolic processes impact immune responses at both the cellular and the organismal level. The differentiation of hematopoietic cells into various types of leukocytes, the responsiveness of mature leukocytes and the activity of other cell types that interact with the immune cells can all be affected by metabolic activities. Clinical data also support the importance of these relationships; i.e. pathological changes of the metabolism such as obesity, metabolic syndrome and diabetes mellitus, are associated with pathological alterations in the immune functions and a higher incidence of infectious conditions. Our group has been examining interconnections between metabolism and circadian timing of physiology in multiple studies by using the model organism *Neurospora crassa*. Based on this experience and literature data, in the last period of this project we turned our interest to the examination of interconnections between metabolism and the circadian clock also in higher organisms.

Irregular timing of food intake is a common phenomenon in the modern society. An increasing body of data indicates that abnormal eating patterns, and particularly eating during the rest period are related with obesity and other metabolic disturbances. Aim of our investigations was to explore whether metabolic rhythms driven by rhythmic feeding affect neutrophil-dependent immune responses, and particularly the development of autoimmune arthritis. These investigations also provided preliminary data to the application for a next OTKA support.

wt mice housed under a 12 hours light : 12 hours dark schedule were subjected to normal or high-fat chow under either *ad libitum* or time restricted regimen for four weeks. During the period of time-restricted feeding (TRF, 4 weeks) mice had access to food only for the first 10 hours of the dark phase of the day which corresponds to the natural feeding period of the animals. We followed body weight changes and food intake during the entire feeding program, and at the end of the feeding program we performed intraperitoneal glucose tolerance tests. In other sets of experiments K/B×N serum-transfer arthritis or peritonitis were induced and severity of the inflammation was assessed. In further investigations extravasation of leukocytes was examined.

6.1. Metabolic consequences of time-restricted feeding

In animals fed with normal chow, we observed no difference in weekly caloric intake between the ALF (*ad libitum* feeding) and the TRF groups, suggesting a fast adaptation of the animals to the rhythmic availability of food. Mice either in the TRF or the ALF group showed only minimal body weight changes during the four weeks. In animals subjected to high-fat

chow caloric intake was higher, but again no difference was found between the ALF and TRF groups. All mice with high-fat diet showed an increase of the body weight during the four weeks, but there was no significant difference between the ALF and TRF groups directly before and during arthritis development. This was particularly important for the next stage of experiments, because body weight may affect severity of joint inflammation.

While in mice fed with normal chow both basal glucose levels and those measured after the glucose injection were similar in the ALF and TRF groups, significant differences between the experimental groups were observed when animals were subjected to the high-fat diet. The basal glucose level was 2 mmol/l higher in the ALF than in the TRF group and both the 1-hour and 2-hour blood glucose concentrations were significantly elevated in the ALF group. Thus, in contrast to normal chow conditions, when mice are subjected to a high-fat diet, timing of feeding affects basic metabolic regulation and TRF seems to have a beneficial effect.

6.2. Interconnection between feeding rhythm and arthritis development

Following the four weeks of the feeding program, K/B×N serum-transfer arthritis was induced and severity of the arthritis was assessed by clinical scoring of the limbs and ankle thickness measurement. Under both normal and high-fat conditions, disease indicators revealed a less severe form of the inflammation in the TRF group as compared to the ALF group. Repeated measures of ANOVA revealed a group x day effect on both parameters. In addition, the concentration of synovial IL1β, another arthritis indicator was also higher in the ALF group compared to TRF mice. In summary, our results suggest that TRF significantly modified the autoimmune process. To our knowledge, these are the first data indicating that feeding rhythm affects arthritis development.

6.3. Examining the interaction between feeding rhythm and peritonitis development

To further investigate whether changing of the feeding rhythm influences neutrophil-based reactions, we applied a formerly established peritonitis model as well. Mice were subjected to either normal or high-fat diet as described in the previous section. Following the feeding program, peritonitis was induced by the intraperitoneal injection of zymosan. Four hours after treatment, mice were sacrificed, and the peritoneal cavity was washed with equal volumes of buffer. Ly-6G-positive cells (neutrophils) were counted in the samples by flow cytometry. However, no significant difference in the amount of neutrophils between the ALF and TRF group was detected under any diet conditions.

It is important to note that reproducibility of these experiments was rather low and therefore we concluded that this system may not be suitable to detect fine differences between the experimental groups.

6.4. Investigation of the role of feeding rhythm in the control of the extravasation of leukocytes

Mice were subjected to normal chow according to the feeding program described above and extravasation of neutrophils into muscle tissue was analyzed. Briefly, three hours

after intrascrotal injection of TNF-α or sterile PBS cremaster muscles were exteriorized, mounted on adhesive slides, and fixed. After staining (with Giemsa's azure eosin methylene blue) whole mounts were analyzed with microscope and perivasal leukocytes were counted. However, in the experiments performed until now extravasation of leukocytes did not display differences between the ALF and TRF groups.

Based on above detailed but also additional results on the interaction between feeding rhythm and immune responses, we started to prepare a manuscript which we plan to submit in March 2021.