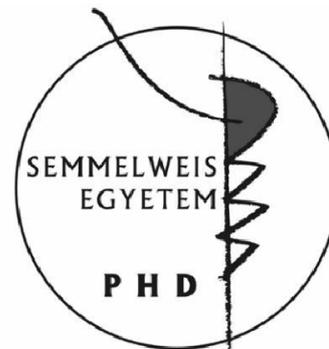


The investigation of short-term lymphocyte activation using flow cytometry

PhD thesis

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1. INTRODUCTION

Short-term activation of T lymphocytes is of utmost importance in the regulation of the immune response. These cells also play an important role in the pathomechanism of autoimmune disorders. Therefore, the investigation of short-term lymphocyte activation kinetics is essential to describe the immune status both under physiological conditions and in autoimmune diseases. Flow cytometry is one of the most common techniques in immunological investigations. However, this high-throughput, widespread method has not been reliably used for kinetic measurements and for exact description of processes that involve change in time. This has been due to the lack of objectivity regarding the evaluation of data, making statistical analysis of the results difficult.

Our team has been developing a method for years that enables objective and reliable evaluation of kinetic flow cytometry recordings based on mathematical analysis. The core of our method is the monitoring of a kinetic process using flow cytometry in the investigated cell population for at least ten minutes. This is followed by the fitting of a series of specified mathematical functions on fluorescent signals describing the process by our software, FacsKin (www.facskin.com). The software also calculates certain parameters of the functions, thus enabling the comparison of these parameters between separate measurements.

We have been using this approach successfully over the past years for investigations in T lymphocyte cell physiology as well as for studying T lymphocyte activation in the neonate, during pregnancy, in preeclampsia, multiple sclerosis and type 1 diabetes. Furthermore, we have thoroughly investigated the role of lymphocyte potassium channels in the course of lymphocyte activation. In my thesis, I would like to summarize our corresponding results.

2. AIMS

In our investigations, we aimed to characterize the differences of the regulation of calcium homeostasis between Th1 and Th2 cells using our kinetic flow cytometry approach. Our aim was to investigate the role of endoplasmic reticulum (ER) calcium release, calcium release activated calcium (CRAC) channels, the mitochondrial calcium uniporter (MCU), the sarco/endoplasmic reticulum calcium ATPase (SERCA) as well as the plasma membrane calcium ATPase (PMCA) in the regulation of cytoplasmic free calcium concentration ($[Ca^{2+}]_{cyt}$) in the early period (first 10 minutes) of lymphocyte activation in Th1 and Th2 cells.

Furthermore, we aimed to characterize the kinetics of activation-elicited calcium influx in major lymphocyte subsets (CD4, Th1, Th2 and CD8 cells) in the neonate, during pregnancy, in preeclampsia, multiple sclerosis and type 1 diabetes compared to healthy, non-pregnant adults. We also aimed to describe the alterations induced by selective inhibition of Kv1.3 and IKCa1 lymphocyte potassium channels in the kinetics of lymphocyte activation.

3. SUBJECTS AND METHODS

3.1. SUBJECTS OF THE INVESTIGATIONS

To describe the differences of the regulation of calcium homeostasis between Th1 and Th2 cells, we took peripheral blood samples from 10 healthy volunteers (5 women and 5 men, age: 24 [23–25] years (median [range])).

To describe the characteristics of lymphocyte activation in the neonate, we collected peripheral blood samples from 9 healthy adults (5 women and 4 men, age: 27 [24–52] years (median [range])) and cord blood samples from 9 healthy, term newborns (4 girls and 5 boys, gestational age: 40 [38–41] weeks (median [range])) following vaginal delivery.

To describe the characteristics of lymphocyte activation in healthy pregnancy and preeclampsia (PE), we obtained peripheral blood samples from 9 healthy non-pregnant women (age: 30 [25–33] years (median [range])), 9 healthy pregnant women (age: 35,5 [34–37] years (median [range])), and 9 PE patients (age: 32 [27–35] years (median [range])). In case of fertile, non-pregnant women, samples were collected in the luteal phase of the menstrual cycle. PE patients were characterized by the mild form of the disease. PE was diagnosed according to standard international criteria.

For our study in multiple sclerosis (MS) we collected peripheral blood samples from 10 healthy volunteers (6 women and 4 men, age: 35 [23–44] years (median [range])), as well as 11 relapsing-remitting MS patients (7 women and 4 men, age: 44 [24–51] years (median [range])) receiving no immunomodulatory therapy, only supportive treatment. Another 6 relapsing-remitting MS patients (4 women and 2 men, age: 39 [32–56] years (median [range])) were involved, who received regular dose interferon (IFN) beta treatment (either IFN beta-1a, 30 ug im. weekly injection (Avonex®); or IFN beta-1a, 44 ug sc. thrice-a-week injections (Rebif®); or IFN beta-1b, 250 ug sc. alternate day injections (Betaferon®)). MS patients were diagnosed according to international standard criteria, and were all in a remission phase of the disease at the time of blood collection. The last IFN beta treatment was administered to patients on the day before (over 24 h to) blood withdrawal. Healthy controls had a negative history of neurological

symptoms and negative status upon detailed neurological and physical examination. No co-morbidities were detected in MS patients based on routine laboratory investigations and physical examination.

For our investigations in type 1 diabetes (T1DM) we took peripheral blood samples from 9 healthy volunteers (4 women and 5 men, age: 27 [24–52] years (median [range])) and 9 T1DM patients (4 women and 5 men, age: 35 [27–55] years (median [range])). T1DM patients were diagnosed according to international standard criteria. Healthy controls had a history of normal glucose homeostasis and T1DM specific autoantibodies were not detected in any of them. No co-morbidities were detected in participants based on routine laboratory investigations and physical examination. BMI and blood pressure values of all participants fell in the normal range.

Informed consent was obtained from all subjects, or in case of neonates, from the parents of subjects, and our study was reviewed and approved by an independent ethical committee of the institution. The studies were adhered to the tenets of the most recent revision of the Declaration of Helsinki.

3.2. ISOLATION OF MONONUCLEAR CELLS

Peripheral blood mononuclear cells (PBMCs) were separated by a standard density gradient centrifugation (Ficoll Paque, Amersham Biosciences AB, Uppsala, Sweden, 27 minutes, 400 g, 22 °C) from 9 ml of freshly drawn peripheral venous blood collected in lithium heparin treated tubes (BD Vacutainer, BD Biosciences, San Jose, CA, USA). This cell suspension was washed twice in phosphate buffer saline (PBS). From then on, cells were kept throughout staining with fluorescent markers, treatment with inhibitors and measurement on flow cytometer in a modified RPMI medium (Sigma-Aldrich, St. Louis, MO, USA). The calcium concentration of this medium was set to 2 mM by the addition of crystalline CaCl₂. In our investigation describing the differences of the regulation of calcium homeostasis between Th1 and Th2 cells, a portion of the cells were kept in PBS instead of the modified RPMI medium.

3.3. THE DETERMINATION OF CELL SURFACE MARKERS AND INTRACELLULAR CALCIUM LEVELS

The population of lymphocytes was gated from PBMCs according to Forward Scatter Characteristics and Side Scatter Characteristics. For surface marker staining, PBMCs were suspended in 500 μ l modified RPMI or PBS. Cells were incubated for 30 minutes at room temperature, in dark with the following conjugated anti-human monoclonal antibodies: anti-CD4 PE-Cy7, anti-CD8 APC-Cy7, anti-CXCR3 APC (for the Th1 subset) and anti-CCR4 PE (for the Th2 subset) (all from PharMingen, San Diego, CA, USA), as well as anti-Kv1.3 channel FITC (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturers' instructions. In our investigation describing the differences of the regulation of calcium homeostasis between Th1 and Th2 cells, the following cell surface monoclonal antibodies were used: anti-CD4 allophycocyanin-Cy7 (BioLegend, San Diego, CA, USA), anti-CXCR3 allophycocyanin (PharMingen), and anti-CCR4 PerCP (BioLegend). Cells were then washed.

For monitoring $[Ca^{2+}]_{cyt}$, PBMCs were loaded with calcium sensitive Fluo-3 and Fura-Red dyes supplemented with Pluronic F-127 according to the manufacturer's recommendations (Invitrogen, Carlsbad, CA, USA). In case of the study describing the differences of the regulation of calcium homeostasis between Th1 and Th2 cells, only Fluo-3 was used. Cells were incubated for 20 min in dark at 30 °C, and then washed. After washing, cells were kept at room temperature in dark. $[Ca^{2+}]_{cyt}$ was calculated based on a fix ratio of Fluo-3 and Fura-Red.

3.4. INHIBITOR TREATMENT OF CELLS

PBMCs were divided into three vials with equal cell numbers. One vial was treated with MGTX (60 nM), a selective blocker of the Kv1.3 channel (Sigma-Aldrich, St. Louis, MO, USA) for 15 minutes before measurement. Another vial was treated with a triarylmethane compound, TRAM-34 (60 nM), a specific inhibitor of the IKCa1 channel (Sigma-Aldrich, St. Louis, MO, USA) for 10 minutes before measurement. The third vial was used as control. In case of the study describing the differences of the regulation of calcium homeostasis between Th1 and Th2 cells, MCU was inhibited by the addition of

ruthenium red (RR), SERCA was inhibited with thapsigargin (TG), while PMCA was blocked with caloxin 2A1 (CLX). RR (Sigma-Aldrich, 1,25 mM) was applied for at least 10 minutes before measurement, TG was added directly before measurement (Sigma-Aldrich, 750 nM), while CLX (AnaSpec, Fremont, CA, USA, 750 μ M) was added at least 5 minutes before measurement. Measurements were initiated directly after the addition of 20 μ g of PHA as an unspecific activating stimulus (Sigma-Aldrich). Basal fluorescence of intracellular calcium binding dyes was calculated at the beginning (zero second) of each measurement. Flow cytometry measurements were conducted on a BD FACSAria flow cytometer (BD Biosciences, San Jose, CA, USA). Cell fluorescence data were measured and recorded for 10 minutes in a kinetic manner.

3.5. FLOW CYTOMETRY FOR THE INVESTIGATION OF SHORT-TERM LYMPHOCYTE ACTIVATION

Data acquired from the measurements were evaluated with our specific software called FacsKin. The core of this software is an improved version of an algorithm based on the calculation of a double-logistic function for each recording. This function is used to describe measurements that have an increasing and a decreasing intensity as time passes. The software also calculates parameter values describing each function such as the Maximum value (Max), the Time to reach maximum value (tmax), the slope of the ascending phase of the curve calculated at 50% of the Maximum value (Slope), and the Area Under the Curve (AUC). These parameters represent different characteristics of lymphocyte calcium influx kinetics. The Maximum value represents the peak value of the calcium influx curve upon lymphocyte activation, thus it reflects the maximal amount of $[Ca^{2+}]_{cyt}$ in the course of activation. The Time to reach maximum value describes how soon the peak value of the calcium influx curve is reached. The Slope value reflects how rapidly the peak of calcium influx is reached. The Area Under the Curve describes the full amount of $[Ca^{2+}]_{cyt}$ during the whole period of lymphocyte activation recorded and thus the magnitude of the elicited calcium response in general. AUC values correspond to the sum $[Ca^{2+}]_{cyt}$ increase, which further corresponds to the level of lymphocyte activation.

3.6. STATISTICAL ANALYSIS

Comparisons were made with Mann-Whitney, Wilcoxon and Kruskal-Wallis tests, since Kolmogorov-Smirnoff tests indicated non-normal distribution of the data. p values less than 0.05 were considered significant. Statistics were calculated using the R software (R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria).

4. RESULTS

4.1. THE REGULATION OF CALCIUM HOMEOSTASIS IN HEALTHY INDIVIDUALS DURING SHORT-TERM LYMPHOCYTE ACTIVATION

In our study, CD4⁺ CXCR3⁺ cells were regarded as the Th1 subset, while CD4⁺ CCR4⁺ cells were regarded as the Th2 subset. First, we compared the kinetics of calcium response in the Th1 and Th2 lymphocyte subsets following PHA stimulation. AUC, Slope and Max values were lower in the Th2 lymphocyte subset upon activation. However, the t_{\max} value did not significantly differ in the two subsets.

To determine the individual contribution of ER calcium release and calcium entry through CRAC channels to the elevation of $[Ca^{2+}]_{\text{cyt}}$ during lymphocyte activation, we measured calcium flux kinetics in an extracellular milieu containing no calcium (thus preventing CRAC channel functionality). We then calculated the contribution of CRAC by subtracting the parameter values of this set of measurements from the values of measurements performed in an extracellular milieu with 2 mM calcium concentration. Subsequently, we determined the ratio of contribution of the two sources for $[Ca^{2+}]_{\text{cyt}}$ elevation (CRAC/ER ratio) in case of each investigated parameter.

RR decreased the AUC values in both investigated lymphocyte subsets. Furthermore, the Slope and Max values were lowered in the Th1 subset, while the t_{\max} value was elevated in the Th2 subset upon RR treatment. This finding suggests that RR plays a role in shaping both the magnitude and kinetics of calcium influx. Our results indicate that mitochondrial calcium uptake is present in both the initial and the peak phase of early lymphocyte activation in Th1 cells (represented by the alteration of both the Slope and Max values upon MCU inhibition), while it plays a role in $[Ca^{2+}]_{\text{cyt}}$ regulation only once the peak of calcium influx is reached in Th2 cells (represented by the alteration of solely the t_{\max} value upon MCU inhibition).

TG elevated AUC, Slope and Max values in both Th1 and Th2 cells compared with the corresponding populations without inhibitor treatment. These results demonstrate that TG shapes both the magnitude and kinetics of calcium clearance in Th1 and Th2 cells. The increase of the Slope value upon TG treatment was two-fold higher in Th2 than in Th1 cells. This indicates that the initiation of ER calcium uptake is more rapid in Th2 cells. Further calcium uptake, however, in the following phase of calcium influx

(around the peak) is similar in Th1 and Th2 cells, since the application of TG elevates the Max value to a similar extent in the two subsets.

We demonstrated alterations in calcium flux properties induced by CLX in the Th1 lymphocyte subset. The Max value was elevated by 24% upon incubation of the samples with CLX compared with samples without inhibitor treatment. These results indicate that CLX induces alterations in the peak phase of the calcium response instead of the ascending slope. Interestingly, Th2 cells are unaffected by CLX treatment.

4.2. T LYMPHOCYTE ACTIVATION AND LYMPHOCYTE POTASSIUM CHANNELS IN HEALTHY INDIVIDUALS

The application of specific inhibitors of the Kv1.3 and IKCa1 channels (MGTX and TRAM, respectively) had distinctive impact on AUC, Max and t_{\max} values of calcium influx of lymphocytes of healthy individuals. TRAM treatment decreased AUC and Max values of all investigated lymphocyte subsets. MGTX also decreased AUC and Max values, except for the Th1 subset. No significant alterations in the t_{\max} values were detected. The extent of the decrease in parameter values was characteristic for the investigated lymphocyte subset and the inhibited potassium channel.

4.3. T LYMPHOCYTE ACTIVATION AND LYMPHOCYTE POTASSIUM CHANNELS IN THE NEONATE

AUC values were lower in the overall lymphocyte population, the Th1 subset and the CD8 subset in newborns compared with adults. Max values were lower in newborns compared with adults in the overall lymphocyte population, the Th1 subset, the Th2 subset and the CD8 subset. t_{\max} values were lower in neonates compared with adults in the Th1 subset and the Th2 subset. AUC and Max values of the CD8 subset were lowered by both MGTX and TRAM. Furthermore, TRAM decreased the t_{\max} value of the overall lymphocyte subset. Calcium influx was not decreased by MGTX or TRAM in any other subsets in neonates.

We evaluated the median fluorescence of the antibody against the voltage-gated Kv1.3 channels in lymphocytes of adults and neonates. Median fluorescence was elevated in newborns in the Th2, CD4 and CD8 subsets.

4.4. T LYMPHOCYTE ACTIVATION AND LYMPHOCYTE POTASSIUM CHANNELS IN HEALTHY PREGNANCY AND PREECLAMPSIA

First, we compared the AUC, Max and t_{\max} parameter values of calcium influx in non-pregnant, healthy pregnant and PE lymphocytes activated with PHA. AUC values were lower in lymphocytes of healthy pregnant compared to non-pregnant women in the Th1 subset, the CD4 subset and the CD8 subset. Comparing healthy pregnancy with PE, we found that the t_{\max} value was lower in PE in the Th2 subset.

AUC values of the overall lymphocyte population, the Th1, CD4 and CD8 subsets were lowered by both MGTX and TRAM in healthy pregnancy. However, in contrast to non-pregnant women, the inhibitors had no effect on calcium influx of the Th2 subset in healthy pregnancy. Max values were decreased by MGTX but not TRAM in the overall lymphocyte population, the CD4 and the CD8 subsets in healthy pregnancy.

In PE, both inhibitors decreased the AUC values in the overall lymphocyte population, the Th2 and CD4 subsets. AUC values of the Th1 and CD8 subsets were not affected by the inhibitors in PE. Max values were decreased by MGTX in the Th2 and CD8 subsets and by both inhibitors in the CD4 subset in PE.

We evaluated the median fluorescence of the antibody against the voltage-gated Kv1.3 channels in non-pregnant, healthy pregnant and PE lymphocytes. No significant alteration was detected in any of the investigated subsets.

4.5. T LYMPHOCYTE ACTIVATION AND LYMPHOCYTE POTASSIUM CHANNELS IN MULTIPLE SCLEROSIS

First, the calculated parameter values (AUC, Max, t_{\max} and Slope) were compared without inhibitor treatment following lymphocyte activation with PHA between healthy subjects and the two investigated groups of MS patients (treated without and with IFN beta). t_{\max} values were lower in MS patients without IFN beta compared with healthy

individuals in the CD4, Th1 and Th2 subsets. Thus the peak of calcium influx is reached more rapidly in these subsets in MS. The t_{\max} value was increased again in MS patients with IFN beta when compared with MS patients without IFN beta in the Th1 subset. The Slope value was higher in MS patients without IFN beta compared with healthy individuals in the CD4, Th1 and Th2 lymphocyte subset supporting a more rapid increase of $[Ca^{2+}]_{\text{cyt}}$. This parameter decreased in the CD4 and the Th1 subsets in MS patients with IFN beta compared with MS patients without IFN beta.

The application of specific inhibitors of the Kv1.3 and IKCa1 channels had distinctive impact on AUC, Max and t_{\max} values of calcium influx of lymphocytes in all three study groups; however the Slope value was unaffected. MGTX and TRAM treatment decreased AUC values of all investigated lymphocyte subsets in samples of all three study groups. The Max value was also decreased upon inhibitor treatment, except for the CD4, Th1 and CD8 subsets in healthy individuals, and the Th2 subset in case of MS patients without IFN beta. t_{\max} values were decreased solely by MGTX in the CD8 subset in MS patients with and without IFN beta. The extent of the decrease in parameter values was characteristic for the investigated lymphocyte subset and the inhibited potassium channel.

We evaluated the median fluorescence of the antibody against the voltage-gated Kv1.3 channels in lymphocytes of the three study groups. Median fluorescence was decreased in MS patients without IFN beta compared with healthy individuals in the CD4 and the Th2 subsets. No significant alterations were found in the median fluorescence in the Th1 and CD8 subsets.

4.6. T LYMPHOCYTE ACTIVATION AND LYMPHOCYTE POTASSIUM CHANNELS IN TYPE 1 DIABETES

The t_{\max} value was lower in T1DM compared to healthy lymphocytes in the overall lymphocyte population and in the Th1 subset. Thus, similarly to MS, the peak of calcium influx is reached more rapidly due to the ongoing autoimmune reaction.

In Th1 cells of healthy individuals, AUC and Max values were decreased by TRAM only, whereas those in the Th2 subset were decreased by both MGTX and TRAM. Calcium influx in the overall lymphocyte population, and CD4 and CD8 lymphocytes

was also inhibited by both MGTX and TRAM (reflected by the decrease of the AUC and Max values). Similarly to healthy lymphocytes, the calcium influx of the overall lymphocyte population was decreased by both MGTX and TRAM in T1DM. However, AUC and Max values of the Th1 subset were lowered by MGTX instead of TRAM. As a further difference from healthy samples, calcium influx in Th2 and CD8 lymphocytes in T1DM was inhibited solely by MGTX. Our findings suggest that lymphocyte subsets are more sensitive to the inhibition of Kv1.3 channels in T1DM compared to healthy individuals.

We evaluated the median fluorescence of the antibody against the voltage-gated Kv1.3 channels in lymphocytes of healthy individuals and T1DM patients. Median fluorescence was elevated in T1DM in the overall lymphocyte population and the Th1 subset compared to the corresponding healthy lymphocyte subsets.

5. CONCLUSIONS

1. During T lymphocyte activation, calcium entering the cell from the extracellular space is of major importance in the increase of cytoplasmic calcium level than calcium released from the endoplasmic reticulum. The amount of calcium released from the endoplasmic reticulum is similar in Th1 and Th2 cells. The lower activity of MCU, and therefore of CRAC channels, along with the higher activity of the SERCA pump account for the notion that Th2 cells go through a lower level of lymphocyte activation compared with Th1 cells upon identical activating stimuli. The function of SERCA pump contributes to the decrease of cytoplasmic calcium levels already from the beginning of calcium influx, and regulates the function of the PMCA pump. The PMCA pump contributes to the clearance of elevated cytoplasmic calcium from the peak phase of calcium flux, and functions more actively in Th1 cells.
2. In healthy individuals, TRAM, the specific inhibitor of the IKCa1 channel decreased calcium influx in Th2 cells to a lower extent than in Th1 cells. This is in part explained by the fact that the cytoplasmic calcium level, which needs to reach a threshold to activate the IKCa1 channels, increases more rapidly in Th1 than in Th2 cells. In contrast with IKCa1, the inhibition of Kv1.3 channels results in a larger decrease of calcium influx in Th2 than in Th1 cells. Calcium influx in Th1 cells was less sensitive to the inhibition of Kv1.3 channels.
3. The kinetics of calcium influx is lower in T lymphocytes of newborns compared to adults. Lower activity of lymphocyte potassium channels may play an important role in this finding. Neonatal T lymphocytes are less sensitive to the specific inhibition of Kv1.3 and IKCa1 channels. The expression of Kv1.3 channels is higher in neonatal T lymphocytes.
4. Calcium influx is lower in Th1 and CD8 cells in healthy pregnancy, but not in preeclampsia. Calcium influx of Th2 cells was insensitive to potassium channel inhibition in lymphocytes isolated from healthy pregnant women, while it decreased

significantly in case of preeclamptic patients. There is a characteristic pattern of calcium influx in T lymphocytes and its sensitivity to potassium channel inhibition in healthy pregnancy that is missing in preeclampsia, where these characteristics are more comparable to the non-pregnant state. This raises the notion that T lymphocyte calcium handling may have a role in the development of immune tolerance during healthy pregnancy.

5. The peak of calcium influx is reached more rapidly in the investigated autoimmune disorders and preeclampsia compared to healthy individuals. This finding is related to the increased reactivity of lymphocytes in the above diseases.
6. Specific immunomodulation of CD8 cells can be reached through the inhibition of the Kv1.3 channel in MS. However, on contrary to previous suggestions, this effect does not seem to be specific enough concerning all lymphocyte subsets playing a role in the development of the autoimmune response, since it also affects anti-inflammatory cytokine producing Th2 cells. This would probably result in a setback of current therapeutic efforts in MS. IFN beta therapy induces compensatory changes in calcium influx kinetics and lymphocyte potassium channel function in MS primarily in the Th1 subset, shaping these properties more similar to those of healthy individuals. However, the elevated functionality of Th2 lymphocytes and the production of anti-inflammatory cytokines are less affected.
7. The increased reactivity of lymphocytes is correlated to the higher expression of Kv1.3 channels in type 1 diabetes compared to controls. Lymphocytes isolated from type 1 diabetes patients are more sensitive to the inhibition of Kv1.3 channels. Lymphocyte activation can be modulated via the inhibition of Kv1.3 channels in this disorder. However, the increased significance of these channels is not exclusive for a specific lymphocyte subset.

6. PUBLICATIONS

6.1. PUBLICATIONS RELATED TO THE THESIS

Cumulative impact factor: 17,978, as a first author: 15,278

International publications:

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6.2. PUBLICATIONS NOT RELATED TO THE THESIS

Cumulative impact factor: 22,808, as a first author: 11,236

International publications:

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