T lymphocyte potassium channel function in pediatric Crohn's disease

PhD thesis

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1 Introduction

In the past 10-15 years the prevalence of pediatric Crohn's disease (CD) has doubled in developed countries. The cause of the disease or the early manifestation is still unknown, however its multifactorial pathogenesis has become evident. The central role of the immune system is indisputable, therefore a large number of studies have aimed to characterize the immunological background and potential therapeutic targets in CD. The majority of these studies are based on either animal models (i.e. knockout mice) or human tissue samples from biopsies. As a result of the development and spreading of genetical study methods we now have a better understanding of the immunogenetic background of CD. However, only few studies have focused on describing the phenotypical and functional characteristics of immune cells.

The MTA-SE Pediatric and Nephrological Research Group, in the work of which I have had the opportunity to participate in the last 4 years, has developed a methodology that allows the characterization of the kinetics of multiple immune cell populations simultaneously in a real time manner using flow cytometry. The research group has also developed an algorithm, that enables the objective analysis and comparison of kinetic data by fitting a function to each dataset. Applying this method we have been able to study the physiological activation processes of immune cells in a uniqe way.

Utilizing these methods we have described the biphasic calcium signal specific for the early activation of T lymphocytes that is indispensable for adaptive immune response in several autoimmune diseases (such as type 1 diabetes or rheumatoid arthritis. An altered calcium signal can lead to pathological immunophenotype and function.

The other major field on which our research group focuses is the role of the potassium channels on the suface of T lymphocytes in different diseases. We have been able to demonstrate that the altered function of these channels contribute to the pathological immune response in multiple auimmune diseases. Our results pointed out that the pharmacological selective blocking of the altered channels is possible without affecting the normal subpopulations. Previous studies have also raised awareness on the potential therapeutic role of potassium channels in CD.

The matter is further complicated, as with the recent developments in the field of immunology several smaller T cell subpopulations have been discovered of which we have little information. The most prominent of these new subtypes are the Th17 and the regulatory T (Treg) lymphocytes which, along with the previously described Th1 and Th2 subtypes play an important role in regulating the balance of inflammation. These subtypes are usually studied isolated from other cell types. This is an important issue, because if the different subtypes are studied individually at different times and often with different methods, then the comparative analysis of data can lead even in the best case only to partially correct conclusions and the cell-cell interactions are completely disregarded.

Building on the research group's many years of experience in the field of immunology I have had the opportunity to participate in the study that is the base of the current thesis.

In these studies, we aimed to characterize the calcium influx kinetics in early T cell activation and assess the role of potassium channels in pediatric Crohn's disease. Later, while this study was ongoing we worked out a methodology enabling the simultaneous characterization of four different T lymphocyte subpopulations, which is an important direction for our future research.

2 Aims

Throughout our study our aims were:

1. To describe the calcium influx kinetics in early T cell activation in pediatric Crohn's disease and compare it with age-matched healthy controls in order to indentify functional alterations in CD.
2. We also aimed to asses the effect of infliximab treatment on calcium influx kinetics in comparison to classic treatment.
3. To measure the calcium signal not only from T lymphocytes, but to assess the activation process at the level of Th1, Th2 and cytotoxic (Tc) subpopulations
4. To evaluate the effect of potassium channel inhibitors TRAM-34 and margatoxin that showed promising results in our previous studies.
5. To characterize the cell surface expression of Kv1.3 potassium channel in each T lymphocyte subpopulation.
6. In the second part of our study we were interested if the simultaneously detection of 4 subsets is possible without losing track of the more subtle kinetic alterations. Therefore we aimed to detect the kinetic alterations of the calcium signal from Th1, Th2, Th17 and Treg cells paralelly following cell suface staining, loading the cells with calcium binding dyes and activating them with phytohemagglutinin (PHA). We aimed to describe the characteristics of the calcium signal from each individual lymphocyte subset isolated from the peripheral blood samples of healthy subjects. Throughout these measurements we not only wanted to evaluate whether 4 subsets can be measured simultaneously without significant loss of signal, but also if the different subsets show different calcium influx characteristics in healthy individuals.
7. Whether the calcium influx kinetics of the above subsets can be altered with potassium channel inhibitors in healthy individuals.

8. We aimed to describe the Kv1.3 expression of the above subpopulations in helathy individuals.

Methods

2.1 Patients enrolled in the study investigating Crohn's disease

The children enrolled in the study were seen at the Department of Gastroenterology in the First Department of Pediatrics, Semmelweis University. The diagnosis of Crohn's disease was established with conventional methods (endoscopy, histopathology, blood count). Patients with colitis ulcerosa or other inflammatory/autoimmune bowel disease were excluded from the study. Conventionally treated patients received azathioprine and 5-acetylsalicylic acid (5-ASA) or mesalazine without systemic steroids. Patients with a sustained PCDAI score > 30, by whom disease control could not be established by conventional treatment were switched to IFX (Remicade®) and enrolled in our severe study group. Measurements were performed before the first and after the 4th IFX-treatment. Age and gender matched children were selected as a control group, who presented with aspecific symptoms at the clinic and following examination showed no sign of inflammatory disease origin. All patients were treated and enrolled by pediatric gastroenterologists and sample collection was supervised by the same specialist.

2.2 Subjects of the Th17 study

Peripheral blood samples were taken from 11 healthy adults free from immune-mediated or any other disease (6 men age, 8 women; average age: 23.9 years). Since the subpopulations in question can be investigated in adult subjects and considering that blood sampling is a much smaller "trauma" for adults, we saw no reason to enroll healthy children for this part of the study. Subjects volunteered for the study.

2.3 Method of peripheral blood sampling

2 x 9 ml of peripheral venous blood was drawn in natrium-heparin treated tubes (BD Vacutainer, BD Biosciences, San Diego, CA, USA) upon each sampling from the cubital vein of each subject, according to rutine protocol. All samples were processed within 8 hours of sampling, until which time the samples were stored at room temperature. Informed consent was obtained from parents of all subjects, and our study was reviewed and approved by an independent ethical committee of the institution. The study was adhered to the tenets of the most recent revision of the Declaration of Helsinki.

2.4 Protocol of calcium influx kinetic measurements in Crohn's disease

Peripheral blood mononuclear cells (PBMCs) were isolated by a standard density gradient centrifugation (Ficoll Paque, Amersham Biosciences AB, Uppsala, Sweden, 27 min, 400 g, room temperature). The cell suspension was washed twice in phosphate buffered saline (2 mM KH2PO4, 9.5 mM Na2HPO4.2H2O, 136.7 mM NaCl). Cells were suspended in 2mL of modified RPMI medium (Sigma-Aldrich, St. Louis, MO, USA) throughout the following steps of the procedure. The Ca^{2+} concentration of this medium was set to the physiological concentration of 2 mM by the addition of crystalline $CaCl_2$.

The cell suspension was divided to four equal aliquots:

- 650µl-Control tube, no inhibitor added
- 650µl-TR tube, 240 nM TRAM-34 (Sigma-Aldrich, St. Louis, MO, USA) added at a later time point
- 650µl-MG tube, 4 nM margatoxint (Sigma-Aldrich, St. Louis, MO, USA) added at a later time point
- 100µl-VD tube, anti-Kv1.3 FITC conjugated antibody added at a later time point.

PBMCs were incubated with the following antihuman mAbs: anti-CD4 PE-Cy7 (clone SK3, BioLegend San Diego, CA, USA), anti-

CD8 APC-Cy7 (BioLegend, San Diego, CA, USA), anti-CXCR3 APC (clone 1C6/ CXCR3, BD Biosciences, San Jose, CA, USA), anti-CCR4 PE (clone 1G1, BD Biosciences) and the VD vial with anti-Kv1.3 FITC polyclonal antibody (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturers' instructions. The samples were incubated at 24 °C in the dark for 30 minutes. Cells were then washed, centrifuged for 7 minutes and resuspended in modified RPMI medium. Cytoplasmic free calcium levels were detected by loading the cells with the 1:2 ratio of Fluo-3-AM and Fura Red fluorescent dyes (Biotium, Hayward, CA, USA) and 0.02 % Pluronic F-127 (Molecular Probes, Carlsbad, CA, USA) for 20 minutes at 30 ^{o}C. Cells were washed once and kept at 30 °C in dark until measurement. The application of the two calcium sensors is favorable, because while the fluorescence intensity of Fluo-3 increases upon calcium-binding, that of FuraRed decreases, therefore calculating the ratio of the two dyes is ideal. If the ratio of the two dyes is calculated, small differences in measurement coming from the different base calcium levels and base fluorescence intensity of different cell types can be minimized.

Cells were suspended in modified RPMI medium for measurement and inhibitors were added to the appropriate vials 15 (MgTX) and 10 (TRAM-34) minutes prior to measurement.

At the beginning of the kinetic measurements, a 2 min baseline of calcium level was recorded. T cell activation was initiated by phytohemagglutinin (PHA, 15 μg/ml final concentration), a lectin leading to T cell activation via TCR binding. Fluorescence emission of sequentially measured cells was monitored for 10 minutes. Average cell acquisition rate was 1000 cells/s. In case of the Kv1.3 expression measurements, 500.000 cells were recorded regardless of time, and the mean fluorescence intensity (MFI) values were calculated.

2.5 Protocol for the measurement of T cell subpopulations

The isolation of PBMC-s, suspending and distribution of samples was performed as described in the previously.

PBMCs were incubated with the following anti-human mAbs: anti-CD4 PE-Cy7 (BioLegend, San Diego, CA), anti-CD25 APC-Cy7 (BD Biosciences, San Jose, CA), anti-CXCR3 APC (BD Biosciences), anti-CCR4 PE (BD Biosciences), and anti-CCR6 PerCP (BD Biosciences), as well as anti-Kv1.3 channel FITC (Sigma-Aldrich, St. Louis, MO) according to the manufactures' instructions. Cells were incubated for 30 mins at 24 °C in the dark, then were washed and centrifuged for 7 minutes to remove al unbound fluorochromes. Cells were then suspended in modified RPMI medium. Cytoplasmic free calcium level was detected by loading the cells with 2.6 lM Fluo-3-AM (Biotium, Hayward, CA) and 0.02 % Pluronic F-127 (Molecular Probes, Karlsbad, CA) for 20 min at 30°C. In this study, due to the high number of fluorochromes added we were able to use a single calcium-binding dye for detection only. Cells were washed once kept at 30 °C in dark until measurement. Inhibitors were added as described previously 15 and 10 minutes prior to measurement.

2.6 Data analysis

The population of lymphocytes was gated according to forward and side scatter characteristics. CD4+ CXCR3+ cells were regarded as Th1 cells, CD4+ CCR4+ cells were regarded as Th2 cells population.

Data acquired from the measurements were evaluated with specific software developed at our laboratory (FacsKin, available at: www.facskin.com) which we have previously successfully applied for the evaluation of several studies. The core of this software is an algorithm, that considers time as well as parameter of interest (i.e. IC calcium fluorescence) and calculates functions for each recording. From the several different functions, using the F test, we are able to choose the function best fitting to a given measurement. In the case of

the Crohn's disease study we used the of double-logistic function, while in the case of the T cell subpopulation study, we found the logistic function to fit best. This difference is the result of the different calcium indicators used in each study. The software also calculates parameter values describing each function, such as the start value, representing the initial cytoplasmic calcium concentration of resting cells, the maximum value, the slope parameter, describing the magnitude of increase during calcium influx, and the AUC value, corresponding to the sum cytoplasmic calcium increase. These values enable the objective description and statistical comparison of our kinetic data

2.7 Statistical analysis

As Kolmogorov-Smirnoff analysis indicated non-normal distribution of data, non-parametric tests were applied. In the study investigating Crohn's disease comparisons of the calculated parameters between T cell subpopulations in the same patient group as well as between patient groups within the same T cell subset were made with the Kruskall-Wallis test, while the effect of the applied channel blockers was calculated using the Wilcoxon-test. Data from Kv1.3 expression was compared using Mann-Whitney U-test. Correlations were calculated using Spearmann test. Comparisons between two T cell subpopulations were made with Mann-Whitney tests, while comparisons between the paired values (with or without treatment of specific channel blockers) in the same population were made with Wilcoxon tests. Statistics were calculated at 5% significance level ($p = 0.05$) using the GraphPad Prism 5 software (La Jolla, CA, USA).

3 Results

3.1 Results from our study on pediatric Crohn's disease

3.1.1 Distribution of investigated cell populations

Our results show, that the percentage of Tc, Th1 and Th2 cells within the lymphocytes does not differ between the different study groups or compared to each other within each group. However, while the difference between healthy controls and conventionally treated CD patients as well as between conventionally treated CD patients and the group before the first IFX dose is minimal, there is a significant difference in the percentage of Th1 and Th2 cells between the first and the fourth IFX treatment. The percentage of both cell populaions is higher following the fourth IFX dose. Upon calculation of Th1/Th2 ratio, it is clear, that the ratio is higher in all CD groups compared to controls. This is different is mainly the result of an increased number of Th1 cells. Interestingly the ratio shows no further elevation in the severe (IFX) group and IFX appears to have no moderating effect on this alteration.

3.1.2 Calcium influx kinetic results

We found no significant difference between the the T cell subsets in any of the parmeters in healthy controls, meaning that the basal calcium level of Th1, Th2 and Tc lymphocytes is similar, expressed in the similar Starting values.

The effect of MgTX and TRAM-34 treatment was minimal in this group. Tc cells showed no significant difference in any of the parameters, while Th2 cells showed a small increase in the Slope parameter upon MgTX treatment. TRAM-34 treatment decreased Start and AUC value in Th1 cells, and Slope in Th2 cells.

These effects can be considered insignificant in light of the alterations observed in the patient populations.

Our results on calcium influx kinetics clearly indicated a distinct profile of classically treated CD from age-matched healthy individuals. This is similar to previous findings of our team in autoimmune disorders like rheumatoid arthritis and type 1 diabetes, which were also characterized by a significant alteration of T cell calcium influx kinetics. Th2 cells in the conventionally treated group and in moderate-severe patients before IFX treatment showed higher initial and peak cytoplasmic calcium concentrations as well as AUC values, hence this may indicate that this subpopulation is more active during early phase signaling compared to Th1 cells. At the first glance, this is in contrast with findings describing that CD is a Th1 type disorder. The difference may come from the fact that most of the studies were performed on adult patients and describe the local cytokine and cellular milieu in the gut. Instead, studies that investigated pediatric subjects also found a shift toward Th2 populations in the peripheral blood. This shift observed in the bloodstream might partially be due to the extravasation of Th1 cells in the gut, however, it is not confirmed by prevalence values of the current study. Upon inhibition with MgTX and TRAM-34 the increased calcium influx was normalized in Th2 cells, reaching levels comparable to healthy controls values. This inhibition appears to bel selective, because the populations less affected by CD (Th1 and Tc) were also inhibited to a lesser extent by potassium channel inhibitors.

One of our most interesting observation comes from the measurements performed on the group where blood samples were obtained before the first and after the 4th IFX therapy on patients with an initial PCDAI score > 30. In samples from before the first IFX dose, Th2 cells showed higher values in nearly all parameters compared to Th1 and Tc cells, which showed values comparable to that of healthy controls. Although the elevation the activation level (AUC) of Th2 cells from severe (IFX treated) group was not significant compared to the moderate group, the tendency was obvious. Supported by the difference observed between the activation level of Th2 and Th1 and Tc cells, it appears, that Th2 cell predominance further increases as

CD becomes more severe. Paired sample analysis of this group indicated that IFX has a clear effect on calcium influx characteristics of Th2 cells. This effect has never been mentioned before in the literature, and it is unclear which signaling pathway is responsible for the observed phenomenon.

We also investigated the effect of lymphocyte potassium channel inhibition on the samples of moderate severe, IFX treated subjects. We found that the decrease in intracellular calcium concentration induced by IFX cannot be further enhanced by the application of MGTX or TRAM. This seems to be logical, since the calcium homeostasis of T cells is regulated precisely, and a basal level of calcium is necessary for the proper function of resting lymphocytes, thus controlling biochemical mechanisms do not allow an extreme degree of alteration. Based on our results, the future potential of lymphocyte potassium channel inhibitors might be as simultaneous therapeutic agents besides IFX therapy, to lower the therapeutic dose of IFX. This approach might the possibility of decreasing both the cost and side-effects of IFX treatment.

3.1.3 Results from measurement of Kv1.3 expression

In healthy children, the voltage gated Kv1.3 potassium channel expression was comparable in Th1, Th2 and Tc subsets. In CD patients, Th2 cells express Kv1.3 potassium channel at a higher rate. In moderate-severe patients before the 1st IFX therapy, Th2 cells showed higher median value of expression than Th1 cells. After the 4th IFX therapy, this difference decreased to nearly equal levels.

3.2 Results from the study investigating the possibility of simultaneous observation of the four major T helper lymphocyte subsets

3.2.1 Prevalence of investigated lymphocyte subsets

The percentage of the four major subsets within CD4+ lymphocytes differs greatly. Th17 cells have the lowest percentage (4.7%) and Th2 cells are the most prevalent (27.2%). The percentage of Th1, Th17 and Treg cells is all significantly lower than Th2 cells.

3.2.2 Results from calcium kinetic measurements

Interestingly, our results indicate that the basal cytoplasmic calcium concentration is comparable across the investigated T lymphocyte subsets. Our results indicated that Th1 cells reach the highest maximal cytoplasmic calcium concentration (End value) following the addition of PHA from the investigated subsets. We detected the highest cytosolic calcium level (AUC value) in Th1 cells as well, while AUC values were decreased in the other three T helper subsets. Although not statistically significant, it is worth to notice that the lowest AUC value and the highest t50% value were found in the Treg subset, indicating that this subset shows the slowest and least reaction compared to other subsets upon an identical activating stimulus. This is in line with the physiological regulatory role of this subset controlling lymphocyte activation.

While MGTX unequivocally decreased calcium influx in Th1, Th17, and Th2 cells, the inhibitory effect of TRAM was not so homogenous. Calcium influx was decreased by TRAM to a similar extent as by MGTX in Th1 cells, to a lower extent compared to MGTX in Th17 cells, and showed no statistically significant decrease in Th2 cells. In case of Treg cells, the total amount of calcium influx (represented by AUC values) are not altered upon the inhibition of Kv1.3 and IKCa1 channels, however MgTX decreased the End value.

3.2.3 Results from measurement of Kv1.3 expression

Although the inhibitory effect of MGTX is similar in Th1, Th2, and Th17 cells, Kv1.3 channels are expressed at a considerably higher level in the Th2, and especially in the Th17 subset, raising the notion that their significance is smaller in balancing calcium influx in Th2 and Th17 compared to Th1 cells. All subsets expressed higher levels of Kv1.3 channels than Th1 cells.

4 Conclusions

1. In pediatric Crohn's disease the number of Th1 lymphocytes is elevated on the periphery.
2. In pediatric Crohn's disease the calcium influx of Th2 lymphocytes upon activation is higher than that of Th1 and Tc cells.
3. The *in vitro* administration of potassium channel inhibitors, namely margatoxin and TRAM-34 selectively and effectively decreases the elevated activation level of Th2 cells in pediatric CD.
4. Infliximab treatment also affects peripheral Th2 cells showing increased level of early T cell activation.
5. Calcium influx of Th2 cells normalized by infliximab can no longer be decreased by margatoxin and TRAM-34.
6. The altered expression of Kv1.3 channel can be partially responsible for the alterations of early T cell activation observed in pediatric Crohn's disease.
7. In healthy adults the calcium influx kinetics of Th1, Th2, Th17 and Treg cells differ significantly.
8. Potassium channels contribute to the activation of Th1, Th2, Th17 and Treg cells to different extent in healthy adults.
9. The method developed by our research group is suitable for the simultaneous detection of the calcium influx kinetics of Th1, Th2, Th17 and Treg cells without losing the ability to describe subtle differences.

5 List of publications

Publications related to the thesis

Cumulative impact faktor: 10.419.

1. Orbán C, Szabó D, Bajnok A, Vásárhelyi B, Tulassay T, Arató A, Veres G, Toldi G. (2016) Altered calcium influx of peripheral Th2 cells in pediatric Crohn's disease: infliximab may normalize activation patterns. Oncotarget, 7: 44966-44974. **IF: 5.008**

2. Orbán C, Szabó D, Bajnok A, Vásárhelyi B, Tulassay T, Arató A, Veres G, Toldi G. (2017) Altered activation of peripheral CD8+ T cells in pediatric Crohn's disease. Immunol Lett, 185: 48-51. **IF: 2.483**

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Cumulative impact faktor:18.859, as first author: 3.181.

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