SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

The functional role of Ca^{2+}-and voltage-gated potassium channels in activated human T cells and fibroblast-like synoviocytes

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The Examination takes place at the room 2.306, Department of Biophysics and Cell Biology, Faculty of Medicine, University of Debrecen
13.12.2017., 11 a.m.

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen
13.12.2017., 1 p.m.

I. INTRODUCTION

I.1. General characteristics of ion channels

The ion selective conductance of the cell membrane is mediated by ion channels and various carrier proteins. Ion channels are pore-forming transmembrane proteins that allow passive passage of ions through the cell membrane. Two key features distinguish ion channels from other transporters: the ion transport always follows the electrochemical gradient and the ion transport through the pore has a particularly high rate. Ion channels can be categorized based on the ions they conduct, their selectivity, their gating, the direction of ionic current and their structural similarities. This work mainly focuses on voltage- and Ca^{2+}-gated K^+ channels and the Ca^{2+} release- activated Ca^{2+} channel (CRAC), as well accessory subunits of ion channels, hence these will be introduced below.

I.2. Overview of potassium channels and their auxiliary subunits

K^+ channels have been classified into several families according to their amino acid sequences and they uniformely contain one or more pore (P-) loops that comprise a selectivity filter responsible for selective K^+ permeation. The channels containing 6 transmembrane helices include the voltage-gated K^+ channels (VGKC or Kv) as well as the Ca^{2+}-activated K^+ channels (KCa). These channels assemble as homotetramers around the central ion conducting pore in a fourfold symmetrical manner. This complex is also known as the α subunit of the channel, which can be associated to one or more ancillary β subunits. The polypeptide chains of the α-subunit contain typically six alpha transmembrane helices S1 through S6 with a membrane re-entrant pore loop intervening the S5 and S6 helices. The voltage-sensing domain is formed by the S1-S4 helices, with the S4 segment being highly conserved and containing multiple gating charges in a repeating RXXR motif. The ion conducting pore domain consists of the S5 and S6 segments of the four peptides and the pore loop between them.

Ca^{2+}-gated K^+ channels are structurally homologous to VGKC in their transmembrane domains and the selectivity filter, however they contain intracellular Ca^{2+}-sensing segments. The Ca^{2+}-sensitivity of KCa3.1 is conferred by the Ca^{2+}-binding protein calmodulin (CaM), whereas KCa1.1 α subunits contain two regulator of K^+ conductance domains (RCK1 and RCK2) which are accountable for calcium binding, Also, KCa1.1 is quite ubiquitously expressed, and its tissue-specific expression mainly relies on the regulatory subunits it is

associated with, whereas the KCa3.1 is expressed in peripheral tissues such as the pancreas, lung, placenta, erythrocytes and lymphocytes.

The accessory subunits of ion channels, traditionally labeled in the literature as Greek letters β, γ or δ are essential for proper physiological function, and their mutations cause severe human diseases. In total, there are more than 50 ion channel-specific regulatory subunits of ion channels known in the literature, and taking their numerous transcript variants into account they are the reason for the remarkable functional heterogeneity of ion channels. Structurally, regulatory subunits are usually profoundly smaller than their corresponding channels. Functionally, regulatory subunits can be interpreted as metaphoric reins on the neck of the channel horses. Through selective interaction with the channels, accessory subunits may modify the single channel conductance of the respective channels, and impair or enhance their voltage sensing, enabling a voltage-gated channel to function in both excitable and non-excitable cells. They can also influence the membrane expression of the channel, and can have a significant impact on channel kinetics.

Kv channels are known to have altogether nearly 20 auxiliary subunits. All members of this family are coupled to Shaker-type potassium channels, and have a conserved C-terminal domain. The minK (KCNE1-5) family are small proteins of utmost physiological relevance that mostly interact with members of the KCNQ family. The most prominent member of the family, KCNE1 is known to assemble with KCNQ1 to form the cardiac IKs channels that are responsible for cardiac repolarization. Moreover, other K^+ channel interacting proteins known as KChIP and DPPL can interact with the intracellular and extracellular domains of Kv4 channels, respectively, thereby forming gigantic molecular complexes.

In KCa1.1, four β subunits with distinct amino acid sequences have been described: β1 is found in smooth muscles, β2 is prevalent in the adrenal gland and brain, β3 is expressed mainly in the testis, and β4 is specifically expressed in the central nervous system. Also, KCa1.1 can be associated to four types of γ auxiliary subunits, that, similarly to β subunits have distinct expression profiles. The β1 and β4 subunits overall induce slowing of the macroscopic current kinetics and an increase in apparent calcium and voltage sensitivity. In addition, the β1, β2, and β4 subunits modulate membrane expression of the KCa1.1 conducting α subunit. The β2 and some splice variants of β3 subunits also cause rapid inactivation through their intracellular N-termini. Steroid compounds, such as lithocholic acid (LCA), selectively enhance KCa1.1 currents only if the channel contains the β1 subunit. In contrast, arachidonic acid (AA) amplifies currents in presence of β2 or β3, but not β1 or β4 subunits whereas the scorpion toxins iberiotoxin and ChTx fail to inhibit KCa1.1 in presence of the β4 subunit.

I.3. Modes of ion channel inhibition

Ion channel inhibitors or blockers are molecules that interact with ion channels in a manner that results in a net decrease of ionic current. Channel inhibitors can be used to assess the physiological function of ion channels, and due to their specific binding to the channel, they are also suitable for testing the structural properties of the binding site. The two major mechanisms of ion channel inhibition are pore block, where the inhibitor directly occludes the ion conduction pathway, and allosteric inhibition. Pore blockers occlude the passage of permeant ions through the channel and are the most commonly applied type of inhibitors. In contrast, allosteric inhibitors act by closing channels as they induce channel proteins to assume nonconducting conformations, such as an inactivated or closed state.

Quantification of ion channel inhibition is necessary to precisely describe the dose-response relationship. A commonly applied method to describe an ion channel inhibitor is to measure its K_d value. The K_d can be practically understood as the concentration where 50% of channels are blocked, and is sufficient to describe the dose-response of a system at equilibrium. Fractional inhibition of ion currents depending on the concentration of the blocker can be fit by a sigmoidal Hill equation that reaches saturating effect at very high concentrations. This means that if $10\times K_d$ concentration is applied, about 90% of the channels are blocked, whereas at $100\times K_d$ concentration more than 99% of the channels are inhibited.

For the peptide-channel interaction it is typical that the toxins bind to the extracellular region of the channels, consequently inhibiting ionic flux through the pore. ChTx, discovered in scorpion venom was the first polypeptide blocker of Kv1.3 with nanomolar affinity. Our workgroup is actively involved in characterizing and modifying polypeptide inhibitors, such as anuroctoxin (AnTx). With site directed mutations our workgroup could design and synthesize a variant of AnTx, which is a high affinity and selective inhibitor of the Kv1.3 channel.

Small molecule inhibitors have a simpler structure, and are quite heterogeneous in their blocking mechanisms as well as in their binding sites. These inhibitors are mainly hydrophobic or at least amphipathic, so they can diffuse through the cell membrane. One of the best characterized small molecule inhibitors of K^+ channels is tetraethylammonium (TEA) which has both an extra- and intracellular binding site in Shaker channels. However, the exact mechanisms of action of many allosteric small molecule inhibitors, e.g. paxilline remains a mystery. Recently, it has been shown that it acts almost exclusively on closed channels, but the exact location of the channel-blocker interaction still remains to be elucidated.

I.4. Ion channels as key regulators of the T-cell mediated immune response

Sustaining a proper immune response requires a coordinated interaction between several types of immune cells, including lymphocytes and antigen presenting cells (APCs). Like all other excitable and non-excitable cells, immune cells express ion channels to control their membrane potential (V_m) and regulate Ca^{2+} signaling as well as physiological cell functions, such as gene expression, proliferation, migration and differentiation. The following section focuses on ion channels that play key roles in T cell physiology and in various autoimmune reactions.

To date numerous ion channels have been discovered in T lymphocytes. These include potassium channels such as the Kv1.3 and KCa3.1, two-pore potassium leak channels such as the TASK1, TASK2, TASK3, calcium channels such as the CRAC channel, non-specific cation channels such as the TRPV2, TRPM2,TRPM7 channels, and the Cl_{swell} chloride channel. Among these channels. As CRAC, Kv1.3 and KCa3.1 channels co-localize in the immune synapse and are up-regulated in different T-cell subtypes, it is widely accepted that these channels are indispensable early factors in the Ca^{2+}-dependent activation pathways of the T cell.

In non-excitable cells, probably the most studied event involving ion channels is the Ca^{2+} signal occurring subsequently to the T cell receptor (TCR) activation. This initial signaling step of TCR activation results in the activation of phospholipase C-γ (PLC-γ). This enzyme catalyzes the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacyl glycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). DAG is responsible for the activation of protein kinase C (PKC), which in turn phosphorylates several intracellular substrates. The other crucial component of this signaling cascade is the two-phase elevation of the intracellular Ca^{2+} concentration. IP_3 is the key element in the initial Ca^{2+} influx: binding of IP_3 to its receptors on the ER results in Ca^{2+} release from the endoplasmic stores. This in turn activates STIM1 on the membrane of the ER, which translocates to a region of the endoplasmic membrane where it can activate Orai1 molecules. Orai1, as mentioned earlier forms the pore of CRAC channels, through which Ca^{2+} ions can flow into the cell, creating the second, prolonged increase in the cytosolic Ca^{2+} concentration.

One of the consequences of the increased $[Ca^{2+}]_i$ following TCR activation is membrane depolarization, which limits further Ca^{2+} influx by impairing the favorable electrochemical gradient to drive Ca^{2+} into the cytoplasm. Thus, lymphocytes as well as other immune cells require K^+ channels that, by the efflux of K^+, maintain a hyperpolarized membrane potential critical for sustaining the gradient for Ca^{2+} entry via Ca^{2+} release– activated Ca^{2+} (CRAC)

channels. CRAC channels are responsible for the generation of Ca^{2+} currents in these cells, and the negative membrane potential is ensured by the two potassium channels.

Increase in $[Ca^{2+}]_i$ may activate intracellular pathways involving calmodulin as well as additional secondary messengers such as calcineurin. This phosphatase removes the inhibitor phosphates from the nuclear factor of activated T-cells (NFAT), allowing its dimerization and subsequently, nuclear translocation. This transcription factor can then bind to the promoter region of target genes (e.g. IL-2 gene) involved in cytokine production and proliferation of T lymphocytes.

I.5. The mitogen-dependent T cell activation

The essential functions of adaptive immunity are to differentiate between the own antigens of the host and the foreign antigens through antigen presentation, to generate effective immune responses to counter pathogens and to create an effective immunological memory. T lymphocytes are highly potent cells of the adaptive immune system and are crucially important in the maintenance of immunological homeostasis.

Physiological T cell activation occurs upon contact with professional antigen presenting cells. However, this TCR-clone specific activation generates a low amount of active T lymphocytes that is not desired in *in vitro* systems, where scientists aim to activate the majority of a given T cell population. To overcome this obstacle, modeling of lymphocyte proliferation is possible by applying polyclonal T-cell activators, mitogens, which are capable of activating T-lymphocytes independently of TCR specificity. The most commonly used mitogens include plant lectins, activating antibodies, phorbol esters, and lipopolysaccharides.

Plant lectins, such as phytohemagglutinin (PHA) cross-link with the carbohydrate moieties of membrane glycoproteins (including the TCR and the CD3 molecule). Activator antibodies designed primarily against TCR and CD3, and combined with antibodies against costimulatory receptors such as anti-CD2 or anti-CD28, achieve complete and TCR-mediated T cell activation. Lastly, phorbol-myristoil-acetate (PMA) and the Ca^{2+} ionophore ionomycin activate the T cell independently of the presence of antigen. PMA is can penetrate the cell membrane and then intracellularly leads to protein kinase C activation. The ionophore permeabilizes the cell membrane and the ER for calcium, so its effect is on Ca^{2+} influx from the extracellular space and further intracellular Ca^{2+} increase through the CRAC channel activation through the store-operated calcium entry mechanism.

I.6. Ion channel inhibitors as therapeutic tools

As discussed ion channels including the CRAC, Kv1.3, KCa3.1, and KCa1.1 have been implicated to contribute to the differentiation and maturation of immune cells, recognition of foreign antigens, initiation of immune responses and even inducing apoptosis. Therefore, it is not surprising that both inhibitors of Kv1.3 and several KCa3.1 and CRAC inhibitors have been shown to have effects on T lymphocyte proliferation.

The anti-proliferative effects of different ion channel blockers on T cells have already been described in several experiments and reviews. However, there is an obvious variability in the results of previous studies related to this topic. For example, the average blocker concentration necessary for 50% inhibition of cell proliferation ranged from $1\times K_d$ concentration to $1000\times K_d$ in case of Kv1.3 channel blockers, or from $1.5\times K_d$ to $275 \times K_d$ in the case of the KCa3.1-blocker TRAM-34, where K_d is the drug concentration required to block half of the relevant channels in electrophysiological experiments. The underlying mechanism responsible for this variability has not been systematically addressed before, but must be largely due to the different methods of T cell stimulation and different doses of mitogens applied in these studies.

T lymphocyte activation can also be inhibited without profoundly affecting the Ca^{2+} signaling. Rapamycin, as mentioned in the previous chapter, binds to the mTOR (mammalian target of rapamycin) complex intracellularly through FKBP12 and inhibits its function.

I.7. Fibroblast-like synoviocytes and the KCa1.1 in rheumatoid arthritis

The previous sections have mainly covered ion channels in T cells and various immune cells. However, ion channels of tissue effector cells such as fibroblasts or other stromal cells can also be crucial for pathogenesis of certain autoimmune diseases. Rheumatoid arthritis (RA) affects nearly 1% of the western population and is associated with reduced quality of living, disability, and reduced survival. The hyperplastic synovial tissue in RA, also called pannus, has unique characteristics and like a cancer invades and destroys cartilage and bone. While the RA synovial tissue degeneration is incompletely understood, the joint destruction mediated by it correlates with increased disease severity and unfavorable outcome. Fibroblast-like synoviocytes (FLS) are normally nurturing the surrounding tissues and supporting the extracellular matrix can become highly invasive in RA. FLS in RA (RA-FLS) have been implicated in disease pathogenesis as they exhibit a transformed “tumor-like” phenotype with increased invasiveness and production of proteases and of various pro-inflammatory and pro-angiogenic factors.

In the last decades, management of RA has started to advance with the development of new treatments, however disease remission is rarely achieved and most patients only achieve mild to modest improvement. Therefore, novel therapeutic options that lead to pronounced improvement or remission without inducing immunosuppression are needed.

RA-FLS and FLS from arthritic rats express functional KCa1.1 as the major K^+ channel at their plasma membrane. Blocking the function of KCa1.1 pore-forming α subunits in these FLS with paxilline inhibits their invasiveness and stops disease progression in animal models of RA. However, paxilline is a lipophilic small molecule that blocks all KCa1.1 channels found in major organs, regardless of channel subunit composition, and can cross cell membranes as well as the blood-brain barrier; it therefore induces severe side effects such as tremors, incontinence, and hypertension. Therefore, if KCa1.1 channels are to be targeted with an inhibitor, care must be taken to prevent broad exposure of KCa1.1 channel containing tissues to drug during dosing. As the ancillary subunits of KCa1.1 are restricted to certain tissues, it may be possible to design inhibitors (e.g. antibodies) that only target the channel coupled to a certain regulatory subunit to minimize cross-reaction with different channel phenotypes in other tissues.

II. OBJECTIVES

As mentioned previously, voltage-gated ion channels play a key role in the regulation of different inflammatory cells, and thus in immune responses in general. We approached the functional aspects of potassium channels of the inflammatory cells from two directions.

Firstly, as detailed in the introduction, we approached the heterogeneity in the anti-proliferative effects of different ion channel blockers on T cells (Fig. 2.6.). The underlying mechanism responsible for this phenomenon was still unclear, although it could be accounted largely to the different methods of T cell stimulation. Therefore, our aim was to elucidate this phenomenon by comparing the anti-proliferative effects of ion channel blockers and rapamycin on lymphocytes cultured and activated under identical experimental conditions. Moreover, considering our results at various mitogen concentrations, we propose a theory to explain the underlying mechanisms of our observations.

Furthermore, we approached ion channel function in autoimmune diseases in regards of rheumatoid arthritis and more specifically RA-FLS. The RA-FLS express functional KCa1.1 as the major K+ channel at their plasma membrane. Paxilline, a lipophilic small molecule that blocks all KCa1.1 channels found in major organs, regardless of channel subunit composition, it therefore induces severe side effects such as tremors, incontinence, and hypertension. As detailed in section 2.4.2, despite the drawbacks of the unspecific block of KCa1.1 by paxilline, the channel remains an attractive target for therapy, partly because the regulatory subunits of KCa1.1 have restricted tissue distribution and affect channel pharmacology. To date, however, no KCa1.1 β subunit has been described in RA-FLS. Thus, our aim was to elucidate whether RA-FLS express any accessory β subunits, and whether expression of these subunits has any functional implications in these cells.

III. MATERIALS AND METHODS

III.1. Reagents

All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless stated otherwise.

III.2. Isolation and culture of peripheral blood mononuclear cells

PBMCs were isolated from heparin-treated (heparin from TEVA Pharmaceutical Industries Ltd., Debrecen, Hungary) peripheral blood of healthy volunteers. First, Hanks' Balanced Salt Solution (HBSS) was used to dilute the blood in 1:1 ratio, then the blood was centrifuged with the Ficoll-Hypaque density gradient (GE Healthcare Life Sciences, Little Chalfont, UK) at 1400 rpm for 30 minutes at room temperature. Next, the cloudy mononuclear cell layer was collected and washed two times using 50 ml HBSS, ultimately obtaining the PBMC population used in our experiments.

Following carboxifluorescein succinimidyl ester staining (CFSE staining, see in section 4.5.3.) and activation, cells were cultured in 24 or 96 well plates at a cell density of 10^6 cells/ml in standard RMPI-1640 medium (Sigma-Aldrich Co., Saint Louis, MO, USA) containing 15% HEPES buffer (Sigma-Aldrich Co., Saint Louis, MO, USA) at 37°C in humid atmosphere with 5% CO_2. In each experiment, every plate was incubated for 5 days and was supplemented with fresh culture medium after 72 hours. After harvesting, cells underwent propidium iodide (PI) staining and subsequent FACS analysis.

III.2.1. Selective stimulation of T lymphocytes

At the beginning of the study, we performed preliminary experiments regarding our preferred method of stimulation. Four widely used and well-known lymphocyte stimulation techniques were compared using CFSE dilution assay on PBMCs: PHA stimulation; PMA combined with ionomycin; soluble anti-CD3 antibody alone and in combination with anti-CD28. Moreover, we measured whole-cell K^+ currents, current density and biophysical characteristics of the Kv1.3 channel on representative populations of the stimulated T cells. We found the anti-CD3 and anti-CD28 stimulation was most reproducible and thus, we used this approach in our further experiments.

We applied 200 nM – 3 µM soluble anti-CD3 antibodies combined with a constant amount of 1 µg/ml soluble mouse anti-human CD28 in n=8 experiments for specific T cell

stimulation in the PBMC and lymphocyte cultures. We enhanced the rate of stimulation by adding the soluble antibodies to the bottom of the culture well, left it to bind to the plate surface for 30 minutes at room temperature, then cells were added to the wells in culture medium suspension. In n=8 experiments we used superparamagnetic bead-conjugated anti-CD3 and anti-CD28 monoclonal antibodies (Life Technologies Co., Waltham, MA, USA), which we found more user-friendly than the soluble antibodies. The pairwise comparison of soluble mitogens and bead-mediated stimulation Student's t-test showed no significant difference between the divided cell populations with the two methods of stimulation. The beads are also known to provide adequate cross-linking, thus inducing a relatively high level of activation (150), in contrast to stimulation with soluble anti-CD3 and anti-CD28, that resulted in a higher amount of variability in our measurements. The bead-to-cell ratio in these cases was 1:200 - 1:1.

III.2.2. Applying pharmacological inhibitors of T lymphocyte ion channels

To block the Kv1.3 channel, we used the peptide-type toxin AnTx, previously pharmacologically assessed by our lab. KCa3.1 channels were blocked using TRAM-34 and the CRAC channels were inhibited by 2-Apb. We used the ion channel inhibitors at two different concentrations: the lower was equal to the dissociation constant, or $1\times K_d$, of ion channel inhibition of the blockers and the higher was 10 times the K_d ($10\times K_d$). In the case of AnTx, we used 500 pM ($1\times K_d$) and 5 nM. In the case of 2-Apb, the K_d for lymphocytes is 5 μM, and the other concentration used was 50 μM ($10\times K_d$). Finally, the KCa3.1 blocker TRAM-34 was used in 20 nM ($1\times K_d$) and 200 nM ($10\times K_d$) concentrations. In the case of rapamycin the lowest concentration reported in the literature to inhibit T cell proliferation by 50% ($1xIC_{50}$ =20 pM) was used as the lower dose and 200 pM ($10\times IC_{50}$) was used as the higher dose.

III.3. CFSE dilution assay and PI staining

We applied the CFSE dilution essay, originally described in 1994 by Lyons et al, to measure the rate of cell proliferation. The staining procedure can be summarized as following: the membrane-permeable, but non-fluorescent carboxifluorescein diacetate succinimidyl ester (CFDA-SE) binds to structural proteins within the cell, and is subsequently cleaved by nonspecific esterases to become the membrane non-permeable and fluorescent CFSE. Upon cell division, the amount of CFSE is gradually halved in the daughter cells, thus the number of

division cycles the cells have undergone can be determined. In our case, the lymphocytes divided usually every 24-48 hours, leading to 4-6 measurable cycles at the end of our experiments.

The final concentration of CFDA-SE (CellTrace™ CFSE Cell Proliferation Kit, Life Technologies Co., Waltham, MA, USA) in our experiments was 1μM that led to a 100-to-1000-fold increase in the fluorescence intensity of the measured cells over the basal autofluorescence of unstained cells. After adding CFDA-SE, we incubated the PBMCs or T lymphocytes for 15 min at room temperature, then for 20 min at 37°C. Lastly, the cells were washed once with phosphate buffer solution (PBS). We took caution that the CFSE-stained cells remain hidden from excess light during our experiments. The cellular fluorescence after CFSE staining was ultimately recorded by flow cytometry.

PI staining was performed at the end of the 5-day incubation period. Therefore, we harvested and washed the cells once using HBSS, then added PI to the cell suspension to achieve 1 μg/ml final concentration. Cells were mixed gently with PI and then incubated in the dark for 5 minutes at room temperature.

III.4. Flow cytometry experiments

The flow cytometry measurements on PBMCs and T lymphocytes were performed on BD FACScan™ and Facs Array™ flow cytometers. We measured the light scatters, namely the forward scatter (FSC) and side scatter (SSC) and the fluorescence intensity on green and red channels. The sheath fluid consisted of 1x PBS. Lymphocytes were selected from mixed cell populations of PBMC by their light scatter profile on FACS analysis. Cell proliferation was measured based on the declining CFSE intensity in the green channel. Division index (DI) was used was the indicator of proliferation.

When performing the PI staining, the flow cytometer settings were adjusted to a negative control tube containing unstained cells. PI fluorescence intensity was measured in the red channel, because samples were co-stained with CFSE.

In case of RA-FLS, detection of the α subunit of KCa1.1 and of CD44, podoplanin, cadherin 11 and MMP-2 was performed as previously described using antibodies listed in Table 1. Cells were treated with brefeldin A (eBioscience, San Diego, CA, USA) for 6 h before detection of intracellular MMP-2. Cells were permeabilized with 0.5% saponin for detection of intracellular epitopes. Data was acquired by a Canto II flow cytometer (BD Biosciences, San Jose, CA) using BD FACSDiva and analyzed using FlowJo (Treestar, Ashland, OR).

Alternatively, live $CD44^{high}$ and $CD44^{low}$ cells were sorted under sterile conditions using a FACSAriaII flow cytometer (BD Biosciences) and immediately used for invasion assays.

III.4.1. Measurement of secreted cytokine concentration

Culture supernatants of human peripheral blood mononuclear cells (n=3) were harvested five days after application of mitogens and ion channel blockers. Cytokine sandwich enzyme-linked immunosorbent assay (ELISA) was used to specifically detect and quantitate the concentration of soluble cytokines, namely IL-10 and IFN-γ. The level of these cytokines in the supernatant was measured by OptEIA kits (BD Biosciences, Franklin Lakes, NJ, USA).

III.5. Culturing RA-FLS

FLS from 14 patients with RA and 4 patients with osteoarthritis (OA), defined according to the criteria of the American College of Rheumatology, were purchased from Asterand (Detroit, MI) or collected under the appropriate Institutional Review Board (IRB) approved protocols. Cells were harvested for future experiments between passages 4 and 11; the FLS were cultured in DMEM (Life Technologies, Grand Island, NY) supplemented with 10 IU/ml penicillin, 0.1g/ml streptomycin, 1 mM sodium pyruvate, 2 mg/ml L-glutamine, and 10% FBS.

III.6. Reverse transcription (RT) and quantitative polymerase chain reaction (qPCR)

Total RNA was isolated from approximately 5×10^5 cells using TRIzol (Life Technologies). Reverse transcription was performed with Superscript III reverse transcriptase and random hexamer primers (Life Technologies), according to the manufacturer's protocol. The resulting cDNA was used as a template for qPCR primers designed from the National Institutes of Health qPrimerDepot, or designed manually and tested by PrimerBlast. Amplicon sizes were between 70 and 250 bp in all cases. qPCR reactions were conducted in final volumes of 10 µL using the following components: diluted cDNA (1:10) 4 µL; and oligo forward and reverse primers (2.5 µM each) in 6 µL of iTAQ SYBR Green supermix (Bio-Rad, Hercules, CA). Reactions were run in a ViiA™ 7 Real-Time PCR System (Life Technologies), detecting the accumulation of the fluorescent signal. The cycling condition were: 20 s at 95°C, 40 cycles at 95°C for 1 s and 60°C for 20 s, 95°C for 15 s, 60°C for 1 min, and a gradient from 60°C to 95°C for 15 min. The results were analyzed using the ViiA™ 7 Software.

For data analysis, we attained the cycle threshold (cT) values, defined as the number of cycles required for the fluorescent signal to cross the background level. Therefore, a lower cT value indicates greater amount of target nucleic acid in the sample, and in our case greater gene expression. Each cT value was substracted by the cT of the loading control, yielding the ΔcT. Finally, for relative comparison of gene expression, we substracted each ΔcT value with the ΔcT of the reference housekeeping gene GAPDH, noted as ΔcT- ΔcT_{GAPDH}.

III.7. SDS-PAGE and Western blotting

RA-FLS and rat testes were lysed in RIPA buffer (Sigma, Saint Louis, MO) containing 1% protease inhibitors. Protein levels were measured using the Bradford assay. Equal amounts of protein (20 µg) were loaded and separated by SDS-PAGE (Life Technologies), then transferred onto nitrocellulose membranes (Bio-Rad), according to manufacturer's guidelines. Blots were incubated overnight in a blocking solution consisting of 4% Blotto non-fat milk (Santa Cruz Biotechnology, Santa Cruz, CA). Blots were probed using antibodies specific for the human KCa1.1 channel α and β subunits. Each primary antibody was diluted 1:500 in blocking solution. As loading controls, we used anti-actin antibodies. After overnight incubation and wash followed by 2 hours probing with IR-680 or IR-800 labeled secondary antibodies for 1 h at room temperature. The membranes were washed 3 times for 15 min with PBS + 0.1% Tween-20. Visualization was performed with a Li-Cor Odyssey Scanner, and data analyzed with ImageJ (National Institutes of Health).

III.8. Transfection of small interfering RNA (siRNA)

The mix of three different constructs of GAPDH, KCNMB1, or KCNMB3-specific small interfering RNA were mixed with DharmaFect transfection reagent (Dharmacon, Lafayette, CO) and added to 35 mm petri dishes containing cells at ≈80% confluency according to the manufacturer's instruction. RA-FLS were kept in serum-free medium for 24 h prior to siRNA treatment with DharmaFect siRNA transfection reagent. Cells were used 40-80 hrs following transfection of electrophysiological, flow cytometry, and transwell invasion experiments.

III.9. Transwell invasion assay

The *ex vivo* invasiveness of FLS was assayed in a transwell system using collagen-rich Matrigel-coated inserts (BD Biosciences) as described by the manufacturer and in the literature.

Briefly, at the start of the experiment, FLS -containing inserts in serum free medium were placed on 10% serum +DMEM containing wells. The FLS aimed to reach into the serum-rich environment, but had to digest the Matrigel along the way. Moreover, they needed to migrate actively through their environment to reach the bottom of the insert, that the cells were not able to penetrate. After 24 hours, we carefully discarded the Matrigel layer and stained the FLS using crystal violet and counted the cells on the bottom of the inserts.

III.10. Electrophysiology

III.10.1. Whole-cell patch-clamp

Standard whole-cell patch-clamp techniques were used in voltage-clamp configuration, as described previously. Pipettes were pulled from GC 150 F-15 borosilicate glass capillaries (Clark Biomedical Instruments, Pangboume, UK) to gain electrodes of 2-5 MΩ resistance in the bath. Series resistance compensation up to 85% was used to minimize voltage errors and achieve optimal voltage clamp conditions.

Cells were plated on glass coverslips or petri dishes and allowed to adhere. When indicated, cells were incubated for 15 min at room temperature with fluorophore-conjugated anti-CD44 antibodies, washed, and $CD44^{high}$ and $CD44^{low}$ cells were immediately assessed for K^+ currents. Total K^+ currents were recorded using the patch clamp technique in the whole-cell configuration, as described. When measuring Kv1.3 in lymphocytes, the internal solution contained 140 mM KF, 5 mM NaCl, 11 mM K_2EGTA, 2 mM $MgCl_2$, and 10 mM HEPES (pH 7.20, ~295 mOsm). When measuring KCa1.1, the internal solution contained 10 mM EGTA, 5 mM HEPES and 5 µM free Ca^{2+}, calculated using Maxchelator (http://maxchelator.stanford.edu/CaEGTA-TS.htm). The external solution contained 160 mM NaCl, 4.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES, pH 7.4 is both cases. Experiments were performed at room temperature (20-22°C).

Current density was calculated by dividing whole cell peak currents by the cell capacitance. Lymphocyte cell capacitances varied from 2-9 pF. Whole-cell measurements of T lymphocytes were carried out using Axopatch-200B amplifiers connected to personal computers using Axon Instruments Digidata 1440 data acquisition boards (Molecular Devices, Sunnyvale, CA). For data acquisition and analysis, the pClamp10 software package (Molecular Devices, Sunnyvale, CA) were used. RA-FLS cell capacitances ranged from 9 to 17 pF. Results were analyzed using Igor Pro software (WaveMetrics, Lake Oswego, OR) when measuring RA-FLS and OA-FLS.

III.10.2. Activation and inactivation kinetics of the K^+ current

To assess the activation time constants of the Kv1.3 and KCa1.1 channels, the membrane was depolarized to +50 mV for 15 ms and +140 mV for 50 ms, respectively, from a holding potential of –100mV. Current traces were fitted with a single exponential function rising to the maximum according to the Hodgkin-Huxley model ($I(t) = I_a \times (1-\exp(-t/\tau_a))^4 + C$, where I_a is the amplitude of the activating curve component; τ_a is the activation time constant of the current; C: current amplitude at –100 mV).

To study the inactivation of Kv1.3 and KCa1.1 channels, pulses were evoked to +50 mV for 2 s and +140 mV for 200 s, respectively, from a holding potential –100mV. The decaying part of the current traces was fitted with a single exponential function ($I(t) = I_0 \times \exp(-t/\tau_{in,i}) + C$, I_0: amplitude of current, $\tau_{in,i}$: inactivation time constant for different groups, C: steady-state current of the whole-cell record at the end of the depolarizing pulse) to attain the inactivation time constant.

III.10.3. Test substances

We used altogether four different substances to assess the pharmacological properties of the KCa1.1 channels. **LCA**, that enhances KCa1.1 currents only when the channel contains the β1 subunit; a stock of LCA (Sigma) was prepared in 1:10 solution of dimethyl sulfoxide (DMSO) and 70% ethanol. **AA**, that enhances KCa1.1 currents only when the channel contains β2 or β3 subunits; a stock of AA (Sigma) was prepared in DMSO. **Paxilline**, that blocks KCa1.1 channels regardless of β subunit expression; a stock of paxilline (Fermentek, Jerusalem, Israel) was prepared in DMSO. **ChTx**, that blocks all KCa1.1 channels unless they contain the β4 subunit; a stock of ChTx (Peptides International, Louisville, KY) was prepared in P6N buffer (10 mM $NaHPO_4$, 0.8% NaCl, 0.05% Tween-20, pH 6.0). The stock solutions were further diluted with electrophysiology bath solution immediately before use so that final DMSO concentrations did not exceed 0.05%.

III.11. Data analysis

Flow cytometry data were collected using the BD CellQuest software. For data analysis, we used the freeware program Cyflogic 1.2.1 and the Flowjo X software. The analyzed data was exported to Microsoft Office Excel. For statistical evaluation of our results we used the program SigmaPlot 12.0 and GraphPad Prism 5, where all data is represented as mean ±S.E.M. To compare different treatments to the control cell population, we applied one-way analysis of

variance (ANOVA) test and as post hoc analysis, the Holm-Sidak test. Also, when the data was not normally distributed, as was the case in the very heterogeneous RA-FLS population, we performed non-parametric ANOVA on ranks test (also known as Kruskal-Wallis test), followed by Dunn's post-hoc test, to calculate statistical significance of our results. We marked the level of significance with * if p was <0.05, with **, if p was <0.01, and with ***, if p was <0.001.

IV. RESULTS

IV.1. The anti-proliferative effect of cation channel blockers in T lymphocytes depends on the strength of mitogenic stimulation

IV.1.1. Dose-dependence of mitogen-induced proliferation

Firstly, we aimed to determine which mode of T lymphocyte activation creates the most homogenous cell population in regards of ion channel expression. Therefore, we measured Kv1.3 membrane expression by whole-cell patch clamp and subsequent calculation of current density after treatment of PHA; PMA and ionomycin; anti-CD3; anti-CD3 and anti-CD28 used in combination. In case of all treatments, cell division index was >80%. Our results indicate that both PHA and anti-CD3 and anti-CD28 stimulation lead to significant increase in Kv1.3 current density, however, in case of PHA, the population was extremely heterogeneous compared to other modes of stimulation in regards of capacitance as) well as current density. The biophysical characteristics of the Kv1.3 channel did not change significantly upon stimulation, only the inactivation kinetics slowed upon stimulation with combined anti-CD3 and anti-CD28.

After choosing our preferred mode of stimulation, our first step was to achieve multiple levels of selective T lymphocyte stimulation using anti-CD3 and anti-CD28 monoclonal antibodies. In our preliminary experiments, we did not find significant differences between $CD3^+$ T cells and PBMCs regarding the extent of proliferation or in the proliferation-inhibiting effect of AnTx at K_d and $10K_d$ concentrations. Therefore, we used whole PBMC population in our main experiments.

Fig. 1. shows that comparing the division indices (DI) of stimulated PBMC populations 5 days following mitogen stimulus, four levels of the mitogen effect could be distinguished: low concentration (200 ng/ml or 1 bead:200 cells) of the mitogen led to a relatively low amount of proliferation, while the medium (500 ng/ml or 1 bead:50 cells), high (1 µg/ml or 1 bead:10 cells) and very high concentrations (3 µg/ml or 1 bead:1 cells) resulted, as expected, in markedly higher rates of cell division. Pairwise comparison of the observed proliferation rates indicated a significant increase with each subsequent increase in mitogen concentration.

IV.1.2. Ion channel blockers and rapamycin alone and in combination inhibit lymphocyte proliferation

The effect of ion channel inhibitors on cell proliferation was tested at a concentration corresponding to the dissociation constant of the drug on the relevant channel ($1\times K_d$) and at ten times higher concentration ($10\times K_d$). Rapamycin was used at the lowest IC_{50} obtained from the relevant literature and at ten times higher concentration. Quantitative analysis using normalized DIs showed that AnTx at $1\times K_d$ and $10\times K_d$ concentration inhibited proliferation at low, but not at very high mitogen concentration.

The nearly superimposable fluorescence histograms in Fig. 5.3.B show that the KCa3.1 inhibitor TRAM-34, regardless of its concentration, caused only a minor reduction of the proliferation of T cells stimulated by low mitogen concentration. The statistical analysis of the DIs showed that TRAM-34 failed to inhibit cell proliferation both at $1\times K_d$ and at $10\times K_d$ concentrations regardless of the mitogen concentration used. At low mitogen stimulation the CRAC channel modulator, 2-Aminoethoxydiphenyl borate (2-Apb) applied at $10\times K_d$ blocker concentration inhibited cell proliferation whereas $1\times K_d$ blocker concentration was ineffective.

The mTOR inhibitor rapamycin, applied at both $1\times IC_{50}$ and $10\times IC_{50}$ concentrations markedly inhibits the proliferation of T cells stimulated with low mitogen concentration. As opposed to the ion channel blockers AnTx and 2-Apb, rapamycin alone inhibited proliferation even at very high mitogen concentration both at $1\times IC_{50}$ and $10\times IC_{50}$ doses. The combination of all ion channel blockers at $10\times K_d$ concentration led to a marked inhibition of cell proliferation, which did not differ from the blocking potential of $10\times$ IC_{50} rapamycin. The inhibitory effect of ion channel blockers combined with rapamycin proved to be the most effective treatment, resulting in the complete blockage of cell division. In the latter case proliferation was not significantly different from the negative control group, which was not stimulated by mitogens.

Our data indicates that the Kv1.3 blocker AnTx and the CRAC channel blocker 2-Apb interfered with T cell proliferation only if cells were stimulated at low mitogen concentration but were ineffective if cells were stimulated with very high mitogen concentration. To further explore this phenomenon, we measured the normalized DI at varying mitogen concentrations in the presence of $1\times K_d$ or $10\times K_d$ concentrations of AnTx. A marked inhibition of cell division was observed when the combination of low mitogen and $10\times K_d$ blocker concentration (black bar) was used. At medium, high and very high mitogen concentrations the inhibition of proliferation was not statistically significant as compared to the positive control, but a clear decreasing trend is seen in the effectiveness of the blockers with increasing mitogen

concentration. The same tendency could be observed if AnTx was applied at $1\times K_d$ concentration. The inhibition of proliferation was statistically significant only if low mitogen concentration was used whereas at medium, high, and very high mitogen concentrations the inhibition of proliferation did not prove to be significant.

IV.1.3. Cell viability is not affected by inhibitors of T cell proliferation

The reduced proliferation in the presence of the ion channel blockers and rapamycin might be induced by a decrease in the viability of the cells in the presence of these compounds. This was tested in the experiments shown using a

IV.1.4. Cytokine production of T cells can be reduced by ion channel blockers

We investigated the effect of channel blockers and rapamycin on the secretion of the anti-inflammatory IL-10 and the pro-inflammatory IFN-γ cytokines by ELISA. Increasing the mitogen concentration from low to very high induced approximately 4-fold and 10-fold increases in secreted IL-10 and IFN-γ levels, respectively. IFN-γ as well as IL-10 secretion was significantly inhibited at low mitogen stimulation by all inhibitors and combinations at both applied concentrations. At very high mitogenic stimulation IFN-γ secretion was inhibited by AnTx, rapamycin and combination treatments at $10\times K_d$ and by 2-Apb and the ion channel blocker combination at $1\times K_d$. IL-10 production was only inhibited by $10\times K_d$ of 2-Apb, rapamycin and combination treatments.

IV.2. Expression of Kv1.3 channels in CD4$^+$ T lymphocytes in hypercholesterolemia

IV.2.1. RA-FLS express multiple β subunits at the mRNA and protein levels

We found the marked expression of KCa1.1 α subunit in RA-FLS, described thoroughly in the literature. Analysis of mRNA levels by qPCR in RA-FLS showed expression of most KCa1.1 β subunits compared to the housekeeping gene GAPDH, albeit at very low levels for β2b and β3d. When compared to the expression levels of the KCa1.1 pore-forming α subunit, the highest relative mRNA expression levels were found for three splice variants of the β3 subunit (β3b, β3c, and β3e) and for β4 subunits.

Since mRNAs are not always translated into proteins, we used Western blotting to determine protein levels of the different β subunits. Analysis of the total cellular protein content shows variable amounts of all β subunits of KCa1.1 in different RA-FLS donors compared to the loading control actin. Whereas expression of β1, β2, and β4 was only detectable in the cell lysates from some donors, β3 subunits were consistently identified in all donors but one.

IV.2.2. RA-FLS express either β1 or β3b subunits at their plasma membrane

The pore-forming α subunits of KCa1.1 can be detected at the plasma membrane and in the nucleus of RA-FLS. Since our focus here is on the channels expressed at the plasma membrane and since co-expression of β subunits with α subunits affects the kinetics and pharmacology of K^+ currents through the KCa1.1 channel, we used patch-clamp electrophysiology to assess the expression of functional β subunits at the plasma membrane of RA-FLS. Of the 51 RA-FLS from 5 different donors patch-clamped, 47 (92%) exhibited a K^+ current as previously described. Addition of paxilline, a blocker of the KCa1.1 α subunits regardless of β subunit expression, completely blocked the K^+ current in all 14 cells tested, further confirming that the K^+ channel observed is KCa1.1, as previously demonstrated. In 70% of the cells analyzed, the current displayed little or no inactivation. Since β2a, β3a, β3c, and β3e subunits have all been shown to induce inactivation of KCa1.1, the lack of inactivation suggest these subunits are not involved in the KCa1.1 channel in RA-FLS.

To further identify the β subunits associated with the KCa1.1 channels in RA-FLS, we used KCa1.1 openers and blockers known to exert different effects on the channel depending on its β subunit composition. First, we tested the effects of the scorpion venom toxin ChTx on RA-FLS K^+ currents as it can only block KCa1.1 channels that do not contain the β4 subunit. ChTx inhibited the currents at 100 nM in all cells tested, demonstrating the absence of β4

subunits in KCa1.1 channels of RA-FLS. We next tested the effects of LCA, known to enhance currents through KCa1.1 channels only in the presence of β1 subunits. This increase in current was observed in only 36% of RA-FLS tested, demonstrating that KCa1.1 channels are formed of α and β1 subunits in approximately a third of RA-FLS. To identify the β subunit in the remainder of the RA-FLS, we used AA, known to increase KCa1.1 currents in the presence of β2 and β3 subunits. Such an increase was observed in 65% of cells tested.

Since the kinetics data had already eliminated the possibility of β2a, β3a, β3c, or β3e subunits, this result with AA suggests that the majority of RA-FLS express a β3 subunit, either β3b or β3d. To discriminate between these two splice variants of β3, we performed Western blot experiments using two types of antibodies. The first antibody is directed to a conserved region of the β3 subunit, common to all five splice variants, and leads to a band of the correct molecular weight. The second antibody used was raised against the N-terminus of β3 and therefore detects all splice variants of β3 other than β3b. Although this antibody detected a band of the correct molecular weight (about 32 kDa) in rat testis extracts, the only tissue with known β3 expression, it yielded no detectable band with RA-FLS extracts, suggesting that RA-FLS express the β3b subunit of KCa1.1. Since β1, but not β3b, subunits slow the activation kinetics (τ_{Act}) of KCa1.1 channels, we measured these kinetics in RA-FLS and found a spread in τ_{Act} with only 17 of the 54 cells assessed (31%) having a τAct > 4 ms and 69% of RA-FLS having a $\tau_{Act} \leq 4$ ms. These results reinforce the finding that different individual RA-FLS cells within a line express different β subunits.

IV.2.3. Expression of KCa1.1 β3b is associated with higher levels of KCa1.1 α and CD44

Since invasiveness is an important feature of aggressive FLS during RA, we wanted to test whether invasiveness is associated with a differential expression of KCa1.1 β subunits by the cells. In the absence of antibodies that recognize an extracellular epitope of either β1 or β3 subunits, we searched for a surrogate marker with an extracellular epitope to allow for isolation of live cells. Elevated expression of CD44, a type I transmembrane glycoprotein that binds hyaluronan and other extracellular and cell surface ligands, by FLS and other cells was observed in RA. Interestingly, an elevated CD44 expression is correlated with enhanced invasiveness of cancer cells. To determine whether expression of β1 or β3 subunits is correlated with CD44 expression levels and invasiveness in RA-FLS, we first showed a correlation between elevated expression of KCa1.1 α and of CD44. We next used flow cytometry to sort $CD44^{high}$ and

$CD44^{low}$ RA-FLS and performed invasion assays. $CD44^{high}$ RA-FLS were significantly more invasive than $CD44^{low}$ cells. Since FLS invasiveness has also been associated with expression of podoplanin, cadherin-11, and MMP-2, we assessed expression levels of these three markers within the $CD44^{low}$ and $CD44^{high}$ populations of RA-FLS. We observed an association between elevated expression of CD44 and all three markers.

We then stained cells for expression of CD44 and performed whole-cell patch-clamp to assess K^+ current densities and activation kinetics in $CD44^{high}$ and $CD44^{low}$ cells. $CD44^{high}$ RA-FLS exhibited higher current densities and faster activation rates than did $CD44^{low}$ cells. These results were further confirmed by assessing the effects of AA and LCA on the two cell subsets as $CD44^{high}$ cells displayed sensitivity to AA but not LCA whereas $CD44^{low}$ cells displayed sensitivity to LCA and not AA. As a control, we used minimally invasive FLS obtained from patients with OA. OA-FLS exhibited low current densities at 140 mV, fast τ_{Act}, and sensitivity to LCA but not AA, similar to $CD44^{low}$ RA-FLS.

IV.2.4. Knocking down the β3 subunit of KCa1.1 decreases cell surface expression of the pore-forming α subunit of KCa1.1

We used a pool of siRNA to selectively inhibit gene expression of the β3 subunit. In whole-cell patch-clamp assays, RA-FLS became less sensitive to treatment with the β3 agonist AA after transfection with β3, but not control siRNA, demonstrating the effectiveness of the siRNA. Neither control nor β3 siRNA affected the cells' response to LCA showing a lack of effect on β1 subunits. Flow cytometry measurements showed that β3 silencing induced a 20% reduction in expression of the KCa1.1 α subunit. Moreover, whole cell KCa1.1 current densities at voltages above 50 mV were significantly decreased after KCa1.1 β3 subunit silencing, suggesting lower surface expression of the α subunit of KCa1.1.

IV.2.5. Knocking down the β3, but not the β1, subunit of KCa1.1 attenuates the ex vivo invasiveness of RA-FLS

Reducing the expression or function of the α subunit of KCa1.1 inhibits the invasiveness of FLS; we therefore assessed the effects of silencing the β3 subunit of KCa1.1 on RA-FLS invasiveness using Matrigel invasion assays. We also used a pool of siRNA to inhibit the expression of the β1 subunit of KCa1.1 and used the channel's sensitivity to LCA to demonstrate the effectiveness of the siRNA as control siRNA did not affect RA-FLS response to LCA whereas β1 siRNA reduced it. Whereas β3 siRNA reduced the invasiveness of RA-FLS, silencing the β1 subunit of KCa1.1 did not affect this invasiveness.

V. DISCUSSION

V.1. The anti-proliferative effect of cation channel blockers in T lymphocytes depends on the strength of mitogenic stimulation

Altered T cell homeostasis is involved in the pathogenesis of autoimmune diseases such as MS. To maximize anti-proliferative effects and to reduce potential side effects, immunosuppressive drugs are commonly used in combinations. One group of the most promising candidates for future therapy is the family of Kv1.3 inhibitors, because this ion channel is found only in a few tissues and it can be inhibited selectively. Before applying ion channel blockers in therapy, it is crucial to investigate how they interact with other immunosuppressive agents. However, original research data about the pharmacodynamics of combinations of traditional immunosuppressive and novel drugs such as ion channel blockers are scarce and they usually lack functional comparison. Therefore, in our recent experiments, we approached this problem from multiple aspects and have found an additive interaction between rapamycin and the ion channel blockers when using them in combination.

The effects of ion channel blockers exerted on T cell functions have already been described. However, to the best of our knowledge, no data comparing the proliferative effects of Kv1.3, KCa3.1 and CRAC channel blockers applied alone or in combination at identical experimental conditions are currently available. Moreover, the synergy between the effects of ion channel inhibitors and the mTOR inhibitor rapamycin has not been investigated to date.

Our most intriguing finding in this research was that increasing the mitogen concentration markedly decreased the anti-proliferative effect of ion channel blockers that ultimately completely disappeared when cells were stimulated with very high concentration of the mitogens. A possible explanation may be that at low mitogen concentrations the few, initially highly localized Ca^{2+} signals are suppressed by the blocked ion channels in their immediate vicinity. However, at very high mitogen concentration when most TCRs are likely to be activated, the number of localized signaling loci is sufficiently high so that even a very low fraction of unblocked ion channels is sufficient to maintain the downstream activation cascade upon TCR activation. Moreover, it is reasonable to assume that lymphocytes redirect their activation pathways to other, Ca^{2+}-independent directions. As several intracellular signaling pathways, e.g. mTOR activation, do not essentially involve ion channels these processes may become overly active upon applying very high mitogen concentrations.

At very high mitogen concentrations we could achieve significant blockage of proliferation only by using rapamycin or its combination with the ion channel blockers acting

on a different pathway that ultimately leads to permanent changes in cellular signaling. This may indicate that co-treatment of T cells with rapamycin and ion channel blockers may be a more feasible therapeutic approach than using these drugs separately.

Since IL-10 and IFN-γ levels were affected in a qualitatively comparable manner by the inhibitors both at low and very high mitogen concentrations, it is safe to assume that these treatments did not alter the proportion of T cell subtypes specifically, but rather were affecting globally the entire T cell population. In summary, the greatest level of inhibition of T-cell proliferation and the production of selected cytokines could be achieved by rapamycin, and this effect could be further potentiated by using it in combination with cation channel blockers. This may indicate an additive effect of Ca^{2+}-dependent and Ca^{2+}-independent inhibitory mechanisms involved in T-cell activation. Finally, we found that upon increasing the concentration of the mitogenic antibodies, the anti-proliferative effect of ion channel blockers faded. The increased *in vitro* antiproliferative potency of rapamycin and ion channel blocker combination presented in this study urges for *in vivo* experiments whereby the therapeutic benefit of the combined treatment can be assessed.

V.2. Different Expression of β Subunits of the KCa1.1 Channel by Invasive and Non-Invasive Human Fibroblast-Like Synoviocytes

In the second part of this work we focused on the KCa1.1 channel, that has been proposed as a therapeutic target to treat RA. However, the wide tissue distribution of the pore-forming α subunit of the channel precludes the use of antagonists targeted to this subunit alone due to the risk of severe side effects in multiple organ systems. KCa1.1 does however remain an attractive target for therapy because the regulatory β subunits of KCa1.1 have restricted tissue distribution Here, we demonstrated that RA-FLS express functional β1 and β3 subunits of KCa1.1 at their plasma membrane and that expression of β3 is higher on $CD44^{high}$ RA-FLS and is associated with higher expression levels of KCa1.1 α. Silencing β3, but not β1, significantly reduced the invasiveness of RA-FLS. In addition, silencing β3 reduced the expression level of KCa1.1 α.

Analysis by qPCR showed expression of most β subunits in RA-FLS at both mRNA and protein levels. Determination of the kinetics and pharmacological profile of KCa1.1 currents of RA-FLS by single-cell electrophysiology demonstrated the expression of functional β1 and β3 subunits at the plasma membrane.

The majority of RA-FLS had characteristic whole cell KCa1.1 K^+ currents sensitive to paxilline, except for approximately 8% of the cells that displayed no K^+ currents under the conditions used, confirming our previous study. No single assay is sufficient to identify the β subunits associated with α subunits to form KCa1.1 channels; we have therefore used a combination of patch-clamp electrophysiology to measure activation and inactivation kinetics of the currents and test the effects of well-characterized pharmacological agents and of Western blots using antibodies raised against different epitopes of the β3 subunit. According to our results, we discovered a phenotype of non-inactivating KCa1.1 currents blocked by paxilline and ChTx, and potentiated by AA, but not by LCA, leads to the conclusion, that the majority of RA-FLS mainly express functional KCa1.1 α and β3 subunits. Western blots using antibodies specific to different β3 epitopes indicate that RA-FLS express the β3b isoform.

To our knowledge, an association between high expression levels of a potassium channels and CD44 has not directly been reported. However, CD44 expression has long been associated with cancer cell metastasis. Further work is warranted to determine whether CD44 and potassium channels share any signaling pathways leading to their concomitant upregulation. Additional work is also required to determine whether the switch from KCa1.1 β1 to β3b expression in RA-FLS is a consequence, an initiating event, or an independent event in the upregulation of CD44 by these cells. Finally, it will be interesting to establish whether KCa1.1 and CD44 play synergistic roles in regulating the invasiveness of RA-FLS.

The KCa1.1 channel formed of α and β3 subunits expressed by RA-FLS represents an attractive therapeutic target for RA. The mRNA for β3 is expressed only in very low quantity in most tissues, with the highest levels detected in the testis. The incidence of RA is higher in women than in men, in the majority of patients there would therefore not be a concern about male reproductive organ toxicity. Furthermore, the testes are protected by the blood-testis barrier that prevents access from many drugs. Therefore, it could be possible in the future to design KCa1.1 blockers that cannot cross this barrier.

VI. SUMMARY

Ion channels are key regulators of the inflammatory process, in regards T lymphocytes as well as fibroblast like synoviocytes in rheumatoid arthritis, RA-FLS. It is still necessary to deepen our understanding on these topics to be able to treat autoimmune diseases using ion channel inhibitors.

As a major concern, it was not clear how ion channel inhibition correlated with inhibition of cell proliferation as there was an obvious variability in the literature regarding this topic. Our results showed that ion channel blockers and rapamycin inhibit cytokine secretion and cell division in T cells in a dose-dependent manner, while not impairing cell viability. Our key finding was that upon increasing the extent of mitogenic stimulation, the anti-proliferative effect of the ion channel blockers diminished and at very high concentrations it disappeared. Also, this antiproliferative effect can be recovered by combining ion channel blockers with immunopharmacological agents such as the mTOR inhibitor rapamycin. Our findings thus may indicate synergy among the various activation pathways and wishes for *in vivo* experiments where the benefit of the combined treatment can be assessed in autoimmune diseases.

Furthermore, we aimed to improve our understanding considering novel therapeutic opportunities involving ion channel inhibition in rheumatoid arthritis. As RA-FLS, the major effector cells responsible for cartilage destruction in RA, express mainly KCa1.1 channels as potassium channels, these channels can be regarded as an attractive target for RA treatment. As the pore-forming subunit is ubiquitously expressed in the human body, tissue-restricted accessory subunits are the best targets in blocking these channels without any significant side effects. We identified KCa1.1 β1 and β3 regulatory subunits expressed in RA-FLS. KCa1.1 β3 subunits were expressed by in the vast majority of the cells and were associated with highly invasive $CD44^{high}$ RA-FLS, whereas minimally invasive $CD44^{low}$ RA-FLS and FLS from patients with osteoarthritis expressed either β1 or no regulatory β subunits. Furthermore, we showed that silencing the β3 but not the β1 subunit with siRNA reduces KCa1.1 channel density at the plasma membrane of RA-FLS and inhibits RA-FLS invasiveness. These findings suggest that the KCa1.1 channel, composed of α and β3 subunits in RA-FLS is an attractive therapeutic target for RA, and future efforts are desired to develop a specific KCa1.1 β3 inhibitor.

In conclusion, we lifted former mysteries involving the functional role of ion channels in both physiological and pathological conditions and by pointing out novel therapeutic opportunities we authenticated future efforts in the aspect of ion channels and disease.

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VII. PUBLICATIONS

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Candidate: Zoltán Pethő
Neptun ID: JOG5ED
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List of publications related to the dissertation

1. **Pethő, Z.**, Tanner, M. R., Tajhya, R. B., Huq, R., Laragione, T., Panyi, G., Gulko, P. S., Beeton, C.: Different expression of [béta] subunits of the KCa1.1 channel by invasive and non-invasive human fibroblast-like synoviocytes.
Arthritis Res. Ther. 18 (1), 103, 2016.
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IF: 4.121

2. **Pethő, Z.**, Balajthy, A., Bartók, Á., Bene, K., Somodi, S., Szilágyi, O., Rajnavölgyi, É., Panyi, G., Varga, Z.: The anti-proliferative effect of cation channel blockers in T lymphocytes depends on the strength of mitogenic stimulation.
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List of other publications

3. Pajtás, D., Kónya, K., Kiss-Szikszai, A., Džubák, P., **Pethő, Z.**, Varga, Z., Panyi, G., Patonay, T.: Optimization of the Synthesis of Flavone-Amino Acid and Flavone-Dipeptide Hybrids via Buchwald-Hartwig Reaction.
J. Org. Chem. 82 (9), 4578-4587, 2017.
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IF: 4.849 (2016)

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IF: 3.156

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Cell. Immunol. 281 (1), 20-26, 2013.
DOI: http://dx.doi.org/10.1016/j.cellimm.2013.01.004
IF: 1.874

Total IF of journals (all publications): 16,86
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The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

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