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

Contribution of voltage-gated cation channels to immunity: function and importance of localization within the membrane

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The Examination takes place at the library of the Department of Physiology, Faculty of Medicine, University of Debrecen
27. 01. 2014, 11 a.m.

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The PhD Defense takes place at the Lecture Hall of Bld. A of the Department of Internal Medicine, Faculty of Medicine, University of Debrecen
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1. INTRODUCTION

Obtaining an appropriate immune response requires a coordinated interplay between several immune cells. The reactivity of these cells greatly depends on activating signaling cascades, which are in part regulated by ion channels. Initially, the importance of ion channels has been shown on excitable cells, where they control the membrane potential, eventually creating action potentials. However, by now it is known, that ion channels can be found on every cell type, where they affect various cellular functions, through the regulation of the membrane potential and possibly through the interaction with various intracellular scaffolding proteins.

Dendritic cells (DC) are one of the major professional antigen presenting cell (APC) types. Upon antigen encounter immature dendritic cells (IDC) differentiate into mature DC (MDC), which present the processed antigen to T lymphocytes through major histocompatibility complex II (MHCII) molecules. Therefore it is crucial that the differentiation process is under strict control, since the lack of maturation or unwanted maturation could both cause severe immune deficiencies. A voltage-gated sodium channel, Nav1.7, has been described as the major sodium channel of human monocyte-derived immature dendritic cells. We have shown in this study, that it is expressed especially on one IDC subset, which can skew the immune response towards a more immunogenic direction. We propose that by the regulation of the membrane potential, this channel constrains IDC to only become activated at an appropriate signal intensity threshold, hence preventing unnecessary DC activation.

Antigen presentation requires the physical interaction of antigen presenting cells with T cells, termed the immunological synapse (IS). This supra-molecular cluster involves the rearrangement of several proteins in the cell membranes of both cells. Kv1.3, the voltage-gated potassium channel of T cells has been shown to translocate into the synaptic area. However, the exact mechanism and function of this redistribution was unknown, only suggesting its possible role in the regulation of the local membrane potential. We were able to show that this redistribution is controlled by a scaffolding protein from the membrane-associated guanylate kinase (MAGUK) family, PSD-95. This protein, originally reported in neurons, is also expressed in human T cells, where it regulates the localization of Kv1.3, and possibly allows the indirect interaction of several other proteins with the channel. We believe, that this finding may bring us closer to concluding what the exact role/roles Kv1.3 has at the IS and most importantly in immune response regulation.
2. SCIENTIFIC BACKGROUND

2.1 Dendritic cells, T lymphocytes

The first line of defense against pathogens is provided by the innate immune system, which is non-specific and only provides short-term immunity for the host. However, the innate immune system is crucial in the activation of the adaptive response, which features specialized cells against specific pathogens and creates immunological memory as well. Dendritic cells (DC) have an important role in linking innate to adaptive immunity: they are capable of capturing and processing antigens, they can migrate to lymphoid organs in order to initiate immunity, and they are able to differentiate, mature rapidly responding to different stimuli. CD1a is a type 1 membrane protein that is stabilized by captured self- or pathogen-derived modified lipids to activate CD1a-restricted T lymphocytes. In vitro differentiation of monocytes to CD1a⁻ and CD1a⁺ DC requires granulocyte-macrophage colony-stimulating factor (GM-CSF) to support cell survival and IL-4 driving cell differentiation. These DC subsets show immature tissue DC (IDC) features and can be further activated by stimulatory signals. It has been shown that CD1a⁻ and CD1a⁺ DC subsets exhibit different functional properties and are able to skew T cell polarization toward tolerogenic or immunogenic directions, respectively. IDC may undergo drastic functional and phenotypic changes upon antigen encounter and concomitant danger signals, resulting in the transition toward mature DC (MDC). When activated, MDC migrate to draining lymph nodes and act as professional antigen presenting cells (APC) to prime and polarize naïve antigen (Ag)-specific T cells towards inflammatory or tolerogenic directions.

The most important functions of adaptive immunity are to differentiate between the host’s own antigens and the foreign antigens through antigen presentation, to generate effective responses against pathogens and to create immunological memory. Different T lymphocyte subsets play a very important role in all of the above mentioned features. Following selection in the thymus, two major types of T cells can be distinguished according to the T cell receptors (TCR) expressed on their surface. Approximately 90% of the cells express αβ chains, while a minority of T cells expresses γδ chains in the TCR. αβ TCR T cells can be further sorted either according to their lineage markers or their functional activities. The two best known markers are CD4 and CD8 that separate two distinct T cell subsets with different functions. CD4⁺ cells produce various cytokines as effector T helper cells following the recognition of antigens presented on the surface of MHC class II molecules. Cytotoxic T lymphocytes on the other hand express CD8, and recognize antigenic peptides through MHC
class I molecules, which are present on every nucleated cell type. However, depending on the functional status of T cells, we can distinguish between naïve, effector and memory T lymphocytes. The fate (activation, maturation) of both dendritic cells and T cells depends in a great deal on different ion channels in their plasma membrane. The next sections will focus on ion channels in general, and on their functions in dendritic and T cells.

2.1.1 Ion channels in general

Ion channels are transmembrane, pore-forming proteins that can be found in every cell type. Two characteristics separate ion channels from other transporter molecules: the rate of ion transport through the pores is very high and the ion transport always follows the electrochemical gradient. Ion channels can be classified according to their selectivity (highly-, mildly- or non-selective) or their gating (e.g. voltage-, ligand-gated, mechanosensitive). Because this study focuses on a voltage-gated sodium and a voltage-gated potassium channel, these will be described in detail in the upcoming sections.

Voltage-gated sodium channels (VGSC) are made up of a single approximately 260 kDa polypeptide chain (α-subunit) that is associated with one or more auxiliary β subunits. The latter are not necessary for channel expression, however they modify the kinetics and voltage-dependence of channel gating. The α-subunit contains four homologous domains and each is made up of six alpha helices (segments, S) that span the plasma membrane. The voltage sensor is the S4 helix, containing positively charged amino acid residues. The central pore of the channels is formed by the S5-S6 segments of the four domains and the pore loop amidst them.

Voltage-gated potassium channels (VGPC) have a similar structure to VGSCs, except that the four homologous domains are formed by separate polypeptide chains that are held together by non-covalent bonds. The voltage sensor is again the S4 helix, which contains positively charged amino acids. The pore is formed by the extracellular loops between the S5 and S6 segments and contains the typical four-amino-acid motif (GYGD) that is responsible for K⁺ selectivity.

2.1.1.1 Ion channels in dendritic cells, the Nav1.7 channel

The above mentioned voltage-gated ion channels were first described in excitable cells, where they are involved in the electrogenesis of action potential. Since then several studies have shown the existence of ion channels in non-excitable cells as well, including immune cells. Numerous signaling pathways depend on the intracellular Ca²⁺ concentration in
DC, which is controlled by various ion channels: RyR1 receptors, P2Y receptors, L-type calcium channels, and the calcium release-activated calcium channel (CRAC). Voltage-gated potassium channels help to maintain a negative membrane potential required for efficient Ca\(^{2+}\) signaling in these cells. During immune cell differentiation the expression of VGPC may change, suggesting their inevitable effect on various cellular functions.

The Nav1.7 channel is a voltage-gated sodium channel that was originally described in neurons, but since then has been shown in DC as well. The biophysical properties of the channel have been investigated extensively: the voltage dependence of steady-state activation, which defines the distribution of the ion channels between the open and closed state at a given membrane potential, characterized with the \( V_{1/2} \) is approximately -20 mV, meaning that about 50% of the Nav1.7 channels are open at this potential. Nav1.7 shows fast inactivation kinetics between 1-2 ms (typical for Na\(^{+}\) channels), which is due to the fast inactivation gate. According to pharmacological studies Nav1.7 currents are tetrodotoxin (TTX, \( K_d \approx 25 \text{ nM} \)) sensitive.

Nav1.7 was characterized as a novel VGSC in monocyte-derived DC, which shows a developmental switch to VGPC Kv1.3 during \textit{in vitro} DC maturation. However, the exact role of VGSC and Nav1.7 in particular, in the regulation of Na\(^{+}\) flux to immune cells and their contribution to cellular functions are still poorly understood.

2.1.1.2 T lymphocytes and ion channels, the Kv1.3 channel

Ion channels expressed in T lymphocytes regulate Ca\(^{2+}\) signaling, thus have a crucial impact on cell activation and differentiation. CRAC channels are responsible for the generation of Ca\(^{2+}\) currents in these cells, and the negative membrane potential is ensured by two potassium channels. Intermediate-conductance Ca\(^{2+}\) -activated potassium channel (IKCa1, or also termed KCa3.1, \( KCNN4 \) gene encoded) is sensitive to the rise of intracellular Ca\(^{2+}\) concentration, opening of the channel leads to the hyperpolarization of the plasma membrane. On the other hand, a voltage-dependent K\(^{+}\) current has been described in T cells as well that can be attributed to the Kv1.3 channel (coded by the \( KCNA3 \) gene).

The Kv1.3 channel belongs to the \textit{Shaker} family of potassium channels. \textit{Shaker} type channels open quickly after a depolarizing impulse, after which they become inactivated. During activation the S4 transmembrane segment moves towards the extracellular space due to the positively charged amino acids in this region, thereby altering the conformation of the channel. The activation threshold potential for Kv1.3 channels is between -40-(-50) mV. The voltage dependence of steady-state activation can be characterized by the Boltzmann-
equation, which reaches its maximum between +20 - +30 mV, when the probability of channel opening is approximately 1. Kv1.3 shows slow inactivation which consists of the closing of the gate at the end of the pore (P-type inactivation), followed by conformational changes in this region that stabilizes the conformation of the voltage sensors and the non-conducting state of the channel as well (C-type inactivation).

2.2 In vivo T cell activation, the immunological synapse

Helper T cells are one of the key components in establishing adaptive immune response. The crucial step that triggers T cell activation and proliferation is the interaction between MHCII molecules of professional APC with the T cell receptor (TCR)/CD3 complex. This initial signaling step results in the activation of a signaling cascade, due to which Ca^{2+} flows into the cells through CRAC channels. This results in the opening of KCa3.1/IKCa1 and Kv1.3 channels in the plasma membrane, which ensures the optimal driving force for sustained calcium influx. The persistent, oscillating Ca^{2+} concentration elevation activates the phosphatase calcineurin through calmodulin. Calcineurin in turn dephosphorylates the transcription factor, nuclear factor of activated T-cells (NF-AT) in the cytoplasm, which then translocates to the nucleus, and activates the IL-2 gene. This signaling cascade in concert with other pathways induces the proliferation of T cells.

The first step of T cell activation is the interaction of MHCII molecules with the TCR/CD3 complex, forming the immunological synapse (IS), during which the antigen presentation may occur. During IS formation plasma membrane proteins of both the APC and T cell rearrange and segregate into the contact area between the two cells creating the so called supramolecular activation cluster (SMAC), consisting of two concentric rings of molecules. These separate rings were termed central supramolecular activation cluster (c-SMAC) and peripheral supramolecular activation cluster (p-SMAC), as they include different molecules. Later a third, distal supramolecular activation cluster (d-SMAC) has been recognized as well.

When expressed in cytotoxic T cells, the Kv1.3 channels concentrate between the T cell - target cell interface, namely at the cytotoxic IS. The same polarized distribution was shown for Kv1.3 and KCa3.1/IKCa1 channels at the IS between a T cell and an APC. Blocking Kv1.3 channels has no effect on molecular clustering, however the longer term stability of the IS may be affected. Nevertheless, the exact purpose and the mechanism of this redistribution are still unknown.
2.3 Ion channels and autoimmune diseases

It has been suggested that the exact localization of Kv1.3 channels during immunological synapse formation may affect auto-reactivity of T cells. Blocking Kv1.3 channel movement to the IS has no effect on IS formation itself, however, it results in a magnified Ca$^{2+}$ amplitude, implying the role of Kv1.3 channels in Ca$^{2+}$ response. The changes in Ca$^{2+}$ signaling could alter the regulation of transcription factors that are important in T cell activation and proliferation. Also, systemic lupus erythematosus (SLE) T cells show exaggerated response to Ag stimulation due to more sustained increase in intracellular Ca$^{2+}$ levels. Interestingly, resting T lymphocytes display a long-lasting recruitment of Kv1.3 channels in the IS, whereas pre-activated T cells show a different time course: Kv1.3 channels have a maximal recruitment at the first minute and progressively move out of the synapse by the 30th minute following IS formation. The latter was observed in resting SLE T cells as well, implying that the premature loss of Kv1.3 from the IS in these cells could result in improper Ca$^{2+}$ regulation. In line with this, it has been shown that the presence of Kv1.3 prevents the development of an exaggerated Ca$^{2+}$ response, and that sustained Ca$^{2+}$ signaling in SLE T cells highly correlates with short-lived Kv1.3 localization in the IS.

2.4 MAGUK proteins

Membrane-associated guanylate kinases (MAGUK) form a widely expressed scaffolding protein family that is essential in neurological synapse formation. Post-synaptic density protein-95 (PSD-95) and synapse-associated protein 97 (SAP97) belong to the DLG1-4 subfamily, and have been extensively studied in neurons. PSD-95 is mainly expressed in the post-synaptic density (PSD), while SAP97 can be found in the presynaptic and axonal regions. Both proteins have two isoforms: the α-isoforms have a shorter N-terminal that can be reversibly palmytoilated on cystein residues, allowing the association with lipid bilayers. β-isoforms on the other hand contain an N-terminal L27 domain that promotes homo- and/or hetero-oligomerization with multiple proteins. PSD-95 is expressed mostly in the α-isoform, while SAP97 can be found mainly in β-isoform. They both contain three type I PDZ (PSD-95/DLG/ZO1) domains in their N-terminal half, a src homology 3 (SH3) domain, and a guanylate kinase (GK)-like sequence (enzymatically inactive) at their C-terminal regions.
2.4.1 Voltage-gated potassium channels and DLG proteins

It has been shown, that DLG proteins, including PSD-95 and SAP97 interact with various ion channels, such as voltage-gated potassium channels in neurons. A Shaker-type potassium channel, Kv1.4, interacts with PSD-95 and SAP97 (through their first two PDZ-domains), which proteins affect its cellular localization. It was also shown, that when expressed in COS1 cells, PSD-95 is predominantly at the cell periphery, while SAP97 shows perinuclear localization. This dissimilarity may be due to the difference in isoform expression: as mentioned before, PSD-95 can be found mainly in the α-isof orm, containing two cystein residues as targets for palmytoilation that may result in the association with membranes. In contrast, the abundant β-isof orm of SAP97 lacks these residues, which could explain the perinuclear localization. Because PSD-95 is originally present close to the plasma membrane, it is possible that it anchors various Kv channels to the plasma membrane, rather than mediates their intracellular trafficking. The same applies for SAP97, except that this scaffolding protein may be responsible for the retention of the voltage gated potassium channels to the endoplasmic reticulum.

Kv1.3 also interacts with both PSD-95 and SAP97, and both scaffolding proteins are expressed in murine T cells. However it has not been shown so far if this interaction occurs through the C-terminal, PDZ binding domain of the channel. Because Kv1.3 has SH3 binding domains on its N- and C-terminal as well, the possibility that PSD-95 and/or SAP97 bind to the channel through this domain cannot be completely ruled out either. Taken together, it is an intriguing question whether these scaffolding proteins are also expressed in human T cells, where they could potentially affect ion channel (e.g. Kv1.3) clustering and distribution especially during immunological synapse formation, somewhat analogous to neuronal synapses.
3. AIMS OF THE STUDY

As mentioned previously, voltage-gated ion channels play an important role in the regulation of different immune cells, and thus in immune response in general. Nav1.7 channels have been shown to influence several neuronal functions, but their existence has also been described in immune, specifically dendritic cells. However, the question that which DC functions could be regulated by this channel has not been addressed so far. According to this, in the first part of our study we had the following objectives:

*Investigating the expression and function of Nav1.7 channels in immature dendritic cells:*

- Is the Nav1.7 expression dendritic cell subtype specific?
- Is this channel functional in DC?
- What DC functions may be affected by the channel?

Kv1.3 channels regulate the membrane potential of T cells where they relocate to the immunological synapse during antigen presentation, but how and why this translocation occurs is unknown. Several scaffolding proteins have been shown to affect the cellular localization of various voltage-gated potassium channels in neurons, but the presence and possible effect of these proteins on ion channels in immune cells still needs to be elucidated. Based on this, the aims of the second part of our study were as follows:

*Addressing the question of how Kv1.3 channels redistribute to the immunological synapse:*

- Do human T cells express PSD-95 and SAP97?
- Do these proteins interact with Kv1.3, and if yes, through which region of the channel protein?
- Does the binding site removal of Kv1.3 or the knock-down of PSD-95 or SAP97 affect the redistribution of the ion channel during immunological synapse formation?
4. MATERIALS AND METHODS

4.1 Reagents

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

4.2 Dendritic cells and cell culture

Human monocyte-derived DCs were generated from CD14+ blood monocytes at the Department of Immunology, where the phenotypic characterization and sorting of the dendritic cells were performed as well.

Jurkat and Raji cells (ATCC, Germany) were cultured in RPMI solution supplemented with 10% FBS, L-glutamine (2 mM), Penicillin (100 unit/liter) and Streptomycin (100 mg/liter). HEK-293T (human embryonic kidney-293.T), tsA-201 and CHO (chinese hamster ovary) cells (ATCC, Germany) were cultured in DMEM medium, which also contained 10% FBS, L-glutamine (2 mM), Penicillin (100 unit/liter) and Streptomycin (100 mg/liter). Cells were maintained at 37°C in a humidified atmosphere of 5% of CO2 and 95% air. Cells were passaged every 2-3 days.

4.3 Reverse transcription (RT) and polymerase chain reaction (PCR)

Total RNA was isolated from approximately 5×10⁶ Jurkat cells with RNA isolation kit (GenElute Mammalian Total RNA Miniprep Kit). Reverse transcription was performed with Revert Aid H Minus enzyme using the First Strand cDNA Synthesis Kit (Fermentas, Biocenter Ltd., Hungary). Coding sequences were amplified using the following forward and reverse primers, respectively: for PSD-95: 5’-CTAGAAGCCCCAGGATATGAGTTGC and 5’-CTGTCACTCAGGTAGGCTGG, for SAP97: 5’-AGATTTCCAGCCTTCCAAGCTCTAC and 5’-GCTGATTTCACAACCTCCAGCAAT (Bio-Science Ltd., Hungary).

4.4 Plasmids, cloning

PSD-95 and SAP97 containing plasmids were obtained from Prof. J.S. Trimmer, University of California. The various Kv1.3 channels containing a monomeric green fluorescent protein (mGFP) N-terminal tag were subcloned into the retroviral pBMN-LacZ vector (from Nolan’s Lab).
4.4.1 Transformation

Plasmids were added to 200 µl competent cells thawed on ice. After 20 min incubation on ice, 50-60 seconds heat shock was applied (42°C), then cells were replaced on ice for at least 2 min. 800µl SOC medium was added to the samples which were then cultured at 180 rpm and 37°C for 50 min. After that bacteria were spread onto LB agar containing 100 µg/ml ampicillin. Controls were spread onto LB agar with and without the antibiotic. Plates were incubated overnight, at 37°C.

4.4.2 Plasmid preparation

A single colony of plasmid containing bacteria was inoculated into LB medium containing antibiotic and was grown at 37°C, 180 rpm overnight. PureYield Plasmid Miniprep System (Promega Corporation, USA) was used to purify plasmid DNA. For larger scale plasmid preparation, the overnight culture was diluted 1:100 and further grown overnight and PureYield Plasmid Maxiprep System (Promega Corporation, USA) was applied.

4.5 Transfection of plasmids and small interfering RNA

To express PSD-95 and SAP97 in tsA-201 cells 2 µg DNA/10 µl Lipofectamine 2000 reagent (Invitrogen Corporation, Carlsbad, CA, USA) was mixed and added to the 35 mm petri dishes containing cells at ≈80% confluency according to the manufacturer’s instruction. Cells were used for further experiments 24 hours later.

The mix of three different constructs of Nav1.7-specific and control small interfering RNA (Applied Biosystems) was transfected into differentiating IDC on day three at a final concentration of 1 pM using the GenePulser X Cell electroporator and 0.4-cm cuvettes (Bio-Rad Laboratories, Hercules, CA, USA). Two days after the transfection the level of Nav1.7 mRNA expression was tested by Q-PCR.

4.6 SDS-PAGE and Western blotting

Protein samples were separated by 10-12% SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA) after electrophoresis. The membranes were blocked with milk powder, and immunoblotted with mouse-anti-PSD-95, mouse-anti-SAP97 (Antibodies Incorporated, Davis, CA) or rabbit-anti-actin (Sigma-Aldrich) primary and anti-mouse IgG HRP-linked or anti-rabbit IgG HRP-linked secondary antibodies (Cell Signaling
Technology, Inc., Beverly, MA), respectively. Blots were developed with ECL reagent (Thermo Scientific Inc., Vantaa, Finland).

4.7 GST pull-down assay

*Escherichia coli* BL21 transformed with pGEX-4T-2 containing glutathione S-transferase (GST) (obtained from the Department of Chemistry) or pGEX-4T-2 containing the N-terminal or C-terminal coding DNA sequence (N-terminal: first 184, C-terminal: last 94 amino acids) of Kv1.3 (from *KCNA3* gene, accession number: NM_002232) fused with GST were induced with 1 mM isopropyl β-D-thiogalactoside and grown at room temperature with shaking for 2 hours. Cells were harvested by centrifugation, sonicated in lysis buffer (50 mM Tris-HCl (pH 7.5), 0.1 % Tween 20, 0.2 % 2-mercaptoethanol, Protease Inhibitor Cocktail Set III (Calbiochem, Darmstadt, Germany)), and proteins were isolated by affinity chromatography on GST SpinTrap Purification Module (GE Healthcare, Piscataway, NJ) according to the manufacturer’s protocol. Jurkat cells were washed twice with 1x ice-cold PBS and lysed with sonication in lysis buffer. The Jurkat cell lysates were incubated with the Sepharose-bead-immobilized fusion proteins (GST, GST-Kv1.3-N-terminus, or GST-Kv1.3-C-terminus) overnight at 4°C. The beads were washed two times with 1x PBS and afterwards the GST fusion proteins were eluted with 10 mM glutathione. Elutes were tested for protein expression by SDS-PAGE and specific protein expression was confirmed by Western blotting.

4.8 Viral infection

Transduction protocol for CHO and Jurkat cells was obtained from the Nolan lab web page (http://www.stanford.edu/group/nolan/).

4.9 Immunological synapse formation and immunofluorescence

The Jurkat T cell line is specific for *Staphylococcus* enterotoxin E (SEE, Toxin Technologies, Sarasota, FL, USA). The B cell lymphoma Raji cells were used as APCs. Raji cells were pulsed with 10 µg/ml *Staphylococcus* enterotoxin E (SEE) for 30 min. Cell conjugates were formed by mixing Jurkat cells with Raji cells at a 1:1 ratio, and co-centrifugating them at 200 g for 1 min at 37°C. The mixtures were plated on poly-L-lysine coated coverslips and were incubated for 1, 5, 10, 20 or 30 min at 37°C in a humidified atmosphere of 5% of CO₂ and 95% air, and were then put on ice for labeling. The cells were washed once with 1×TBS, and fixed with 2% paraformaldehyde in 1 x TBS for 10 min. Between each step, the cells were rinsed three times with 1 x TBS. Cells were labeled with
primary CD3 antibody (Invitrogen Corporation, Carlsbad, CA, USA) followed by the appropriate secondary antibody (Alexa Fluor 647, GAMIG, Invitrogen Corporation, Carlsbad, CA, USA). Both antibodies were diluted in 1×TBS containing 1% BSA and incubated with the cells for 30 min. Coverslips were rinsed and mounted in Mowiol Antifade.

4.10 Confocal microscopy and evaluation

Confocal images were taken using Olympus FV1000 confocal microscope. To quantify the accumulation of Kv1.3 channels in the IS (judged by the CD3 polarization in the contact area of the T cell and the B cell) we used the following expression (accumulation ratio, AR):

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AR = \frac{(I_{IS} - I_{BG}) \cdot AREA_{IS}}{(I_{outside} - I_{BG}) \cdot AREA_{outside} - (I_{inside} - I_{BG}) \cdot AREA_{inside}}
\]

where \( I_{IS} \), \( I_{outside} \), \( I_{inside} \) and \( I_{BG} \) is the mean fluorescent intensity of mGFP (Kv1.3 channels) in the IS, outside the cell (including the membrane and intracellular region), inside the cell (only intracellular region) and background intensity detected at a cell-free area of the image, respectively. \( AREA_i \) denotes the area of the described sections. For analysis MacBiophotonics ImageJ software was used.

4.11 Electrophysiology

Standard whole-cell or outside-out patch-clamp techniques were used in voltage-clamp (current detection) or current-clamp (membrane potential measurement) configuration. The measurements were carried out using Multiclamp 700B and Axopatch-200A amplifiers. Pipettes were pulled from GC 150 F-15 borosilicate glass capillaries (Clark Biomedical Instruments, Pangbourne, UK) in five stages and fire-polished to gain electrodes of 2-5 MΩ resistance in the bath.

4.12 Statistical analysis

The statistical analysis and sample comparisons were made by unpaired t-test and ANOVA, the level of significance was set to 0.05. Chi-square test with Yates correction was used for correlation analysis.
5. RESULTS AND DISCUSSION

5.1 Nav1.7 maintains the membrane potential and regulates the cytokine production of a monocyte-derived immature dendritic cell subset

5.1.1 Nav1.7 is expressed predominantly in the CD1a⁺ subpopulation of monocyte-derived immature dendritic cells

Our workgroup has shown previously that Nav1.7 channels are expressed by monocyte-derived IDC and their expression is down-regulated upon differentiation into mature DC. CD1a⁻ and CD1a⁺ IDC subsets have been characterized previously by distinct functional activities hence we compared the Nav1.7 expression of these immature DC subsets. We detected a robust inward, rapidly activating and inactivating Na⁺ current in CD1a⁺ IDC. The incidence of Na⁺ current detection in CD1a⁻ IDC was significantly less frequent than in CD1a⁺ cells (Chi-square test with Yates correction: CD1a⁺ (n=25) and CD1a⁻ (n=19), p=0.021). We also found a considerable difference in the Na⁺ current density (CD) of CD1a⁻ and CD1a⁺ IDC: it was significantly higher in the CD1a⁺ subset (CD_{CD1a⁺}=-83 ± 7.6 pA/pF, CD_{CD1a⁻}=-43 ± 13.4 pA/pF (n=9, p=0.015). Still, there was no difference in the cell membrane capacitance of the two DC subtypes (11.9 ± 1.5 pF and 13.5 ± 1.5 pF for CD1a⁺ and CD1a⁻ cells, respectively, n=9, p=0.457). In addition the relative expression of Nav1.7 mRNA was also higher in the immature CD1a⁺ than in the CD1a⁻ subset, but this expression level dramatically decreased upon DC maturation. These results indicated that the role of the Nav1.7 channels is closely connected to the immature state of DC and acts primarily in the CD1a⁺ subset.

5.1.2 Intracellular Ca²⁺ elevation down-regulates the expression of Nav1.7

Several studies support the role of Ca²⁺ signaling in the activation of DC. Therefore, next we investigated whether sustained elevated Ca²⁺ concentration by itself could decrease Nav1.7 channel expression. The elevation of intracellular Ca²⁺ concentration induced either by ionomycin or by thapsigargin, was an effective inhibitor of Nav1.7 gene expression and had a comparable inhibitory capacity to that of an inflammatory cytokine cocktail. Applying 5mM extracellular EGTA had no effect on the inhibition of Nav1.7 mRNA expression induced by the cytokine mix however it partially or completely reversed the ionomycin- and thapsigargin-induced Nav1.7 mRNA expression inhibition. We also examined the channel expression of CD1a⁺ IDC after 24 h thapsigargin treatment using electrophysiology.
According to our expectations, the percentage of Nav1.7 expressing cells significantly decreased compared to the non-treated cells, and this decrease was partially reversed by EGTA. These results suggest that the increase of intracellular Ca\(^{2+}\) level may trigger Nav1.7 channel down regulation.

5.1.3 The membrane potential of CD1a\(^{+}\) IDC is Nav1.7 channel dependant

Even though the exact role of VGSC in immune cells is still unknown, they are thought to contribute to the maintenance of the resting membrane potential. Therefore next we analyzed the membrane potential of both CD1a\(^{+}\) and CD1a\(^{-}\) IDC together with the corresponding MDC. We found that the resting membrane potential of CD1a\(^{+}\) IDC is significantly depolarized (\(-8.7 \pm 1.5\) mV, \(n=14\)) as compared to CD1a\(^{-}\) IDC or MDC (between -40 and -60 mV, with an average of \(-47 \pm 6.2\) mV, \(n=7\)). We surmised that the measured differences in membrane potential of the different cell types could be the result of the presence of functional Na\(^{+}\) channels in CD1a\(^{+}\) IDC. In order to test this hypothesis we determined the extracellular Na\(^{+}\) sensitivity of the membrane potential in IDC. Transient exposure to a Na\(^{+}\) free extracellular solution hyperpolarized the membrane potential of CD1a\(^{+}\) IDC to values close to those recorded in IDC and MDC of both CD1a\(^{-}\) and CD1a\(^{+}\) subtypes. However, this Na\(^{+}\) free extracellular solution had no effect on the membrane potential of CD1a\(^{-}\) IDC.

To further study the Nav1.7 dependence of the membrane potential, Nav1.7 gene silencing was carried out. Transfection of the Nav1.7-specific siRNA into IDC decreased the number of Nav1.7 current-positive CD1a\(^{+}\) IDC to 13%, whereas 96% of CD1a\(^{+}\) cells transfected with negative control siRNA remained positive for Na\(^{+}\) current expression. According to the current-clamp recordings the membrane potential of Nav1.7-specific siRNA-transfected CD1a\(^{+}\) IDC were significantly more negative (-22.3 mV ± 1.5 mV, \(n=8\)) than in non-transfected CD1a\(^{+}\) IDC (\(p<0.001\)). In addition, there was no change in the membrane potential of SCN9A siRNA-transfected cells when exposed to a Na\(^{+}\)-free solution, similarly to the behavior of CD1a\(^{-}\) IDC lacking Nav1.7 channels.

During the current-clamp measurements we also applied an extracellular high K\(^{+}\) solution in order to see whether it has an effect on the membrane potential of the different dendritic cell subtypes investigated. Transient exposure of IDC to the solution depolarized the membrane potential to \(-0\) mV. This and the relatively negative membrane potential of CD1a\(^{-}\) IDC suggested the presence of K\(^{+}\) channels in the membrane. Since a previous study of our workgroup showed that IDC lack voltage-gated potassium channels we assumed the presence
of a non-voltage-gated K⁺ channel in the membrane of IDC. The current traces recorded in CD1a⁻ IDC indicated the expression of IKCa1, a Ca²⁺-activated K⁺ channel. The number of IKCa1 channels/cell was estimated for both the CD1a⁻ and CD1a⁺ IDC. We could not detect any significant difference between the two DC subsets (p=0.269). Therefore we concluded that the lack of IKCa1 channels cannot be responsible for the depolarized resting membrane potential of CD1a⁺ IDC.

5.1.4 Nav1.7 affects cytokine secretion of CD1a⁺ IDC

Based on the literature that the inflammatory nature of monocyte-derived MDC can be attributed to the CD1a⁺ subset, and our current finding that Nav1.7 channels are expressed primarily in this subpopulation of IDC, we hypothesized that the functional role of Nav1.7 in dendritic cells could be related to the maintenance of the immature state of the cells. Because of the elevated resting potential of CD1a⁺ IDC (due to the Nav1.7 channels) they need a larger stimulus for activation than CD1a⁻ IDC. TTX was used to inhibit Nav1.7 channel function and to test its possible role in fine-tuning CD1a⁺ IDC activation. According to our results the inhibition did not change the viability or the internalizing capacity of IDC nor did it affect the monocyte to IDC and the IDC to MDC differentiation pathway. On the other hand, CD83 expression was slightly elevated when cells were activated by optimal and suboptimal concentrations of the inflammatory cocktail for a short period, although statistically this effect was not significant. A similar sensitizing effect of TTX treatment on IDC could be shown when the production of various IDC-derived cytokines was tested after activation by the cytokine cocktail. Although the absolute amounts of cytokines varied among individuals due to different CD1a⁺ ratios in the donors, the increase of TNF-α (n=5; p=0.015) and IL-10 (n=5; p=0.036) secretion was 3–4 and 6–7 fold higher in TTX-treated samples as compared to untreated controls, respectively.

5.2 Protein interactions of Kv1.3 ion channel in the immunological synapse

5.2.1 Jurkat cells express PSD-95 and SAP97

As mentioned previously, PSD-95 and SAP97 are both scaffolding proteins expressed in neurons where they influence the localization of different Kv channels. To understand whether these proteins could have an effect on Kv1.3 channel distribution in human T cells (Jurkat) we needed to test if they are expressed in these cells as well. We were able to detect PSD-95 and SAP97 at the cDNA and the protein level as well, using reverse transcription
PCR and Western blot, respectively. Therefore we could conclude that Jurkat cells do express both PSD-95 and SAP97.

**5.2.2 PSD-95 and SAP97 interact with Kv1.3 through its C-terminal region**

The next question that we addressed was whether the Kv1.3 channel interacts with PSD-95 and/or SAP97, and if it does, which part of the channel is involved in this interaction. The potential interaction sites (PDZ and SH3 binding domains) can be found on the N- and the C-terminal region of the channel, therefore we created GST-tagged bacterial expression constructs that contained either the first 184 (GST-Kv1.3-N-term.) or the last 94 amino acids (GST-Kv1.3-C-term) of Kv1.3. According to our GST pull-down experiments, Kv1.3 interacts with both scaffolding proteins, however, this interaction occurs only through the C-terminal of the channel.

**5.2.3 Over-expression of mGFP-tagged Kv1.3 constructs and knockdown of PSD-95 or SAP97 in Jurkat cells**

Since in our future experiments we intended to investigate the localization of Kv1.3 under different circumstances with confocal microscopy, we needed Kv1.3 constructs that were tagged with a fluorescent protein that enabled the visualization of the channel. We chose the monomeric green fluorescent protein (mGFP) and added this tag not only to the wild type Kv1.3 (mGFP-Kv1.3-WT), but to a C-terminal truncated clone of the channel (mGFP-Kv1.3-ΔC) that lacks the potential binding site for PSD-95 and SAP97. We applied the retroviral transduction system, to introduce our Kv1.3 constructs into Jurkat cells. To further investigate the role of PSD-95 and SAP97 in T cells, we knocked down either of these proteins both in non-transduced and in mGFP-Kv1.3-WT over-expressing Jurkat cells using specific shRNA.

**5.2.4 Biophysical characterization of mGFP-tagged wild type and C-terminal deleted Kv1.3 channels**

Before using the above mentioned mutated ion channels for microscopy it was crucial to investigate whether the mGFP tag and/or the C-terminal truncation had caused any change in the biophysical characteristics of Kv1.3. For this we used the patch-clamp technique in outside-out patch configuration and compared the gating parameters of mGFP-Kv1.3-WT and mGFP-Kv1.3-ΔC channels that were expressed in CHO cells using retroviral transduction.

We found that the activation kinetics of mGFP-Kv1.3-WT and mGFP-Kv1.3-ΔC currents were identical. The activation time constants for the mGFP-Kv1.3-WT and the
mGFP-Kv1.3-ΔC constructs were not statistically different (\(\tau_a\) was 0.54 ± 0.04 ms for mGFP-Kv1.3-WT (n=10) and 0.56 ± 0.02 ms for the mGFP-Kv1.3-ΔC (n=9), \(p= 0.698\)).

The inactivation kinetics of the mGFP-Kv1.3-WT and mGFP-Kv1.3-ΔC currents were also found to be overlapping indicating that truncation of the C-terminal domain does not modify the inactivation kinetics. The inactivation time constants (\(\tau_{in}\)) were statistically not different for the mGFP-tagged WT and C-terminal deleted Kv1.3 channels (\(\tau_{in}\) was 271 ± 31 ms for mGFP-Kv1.3-WT (n=7) and 240 ± 23 ms (n=6) for mGFP-Kv1.3-ΔC, \(p=0.158\)).

Finally, the equilibrium parameters of the voltage-dependence of steady-state activation were determined for both channel constructs. The small leftward shift in the normalized conductance-test potential curves for mGFP-Kv1.3-ΔC channels was not statistically significant (slope factor was 14.2 ± 1 mV and 13.5 ± 1 mV for mGFP-Kv1.3-WT (n=7) and mGFP-Kv1.3-ΔC (n=5), respectively, \(p=0.458\); whereas \(V_{1/2}\) was −19.5 ± 1.2 mV for mGFP-Kv1.3-WT (n=7) and −25.4 ± 5.4 mV for mGFP-Kv1.3-ΔC (n=5), \(p=0.282\)). We believe, that since the biophysical characteristics of the mGFP-Kv1.3-WT and C-terminal truncated mGFP-Kv1.3-ΔC channels are identical, any difference in the redistribution of the channels upon IS formation cannot be attributed to the altered ion channel function of the truncated channels.

5.2.5 Immunological synapse formation between Jurkat and Raji cells

To reveal if PSD-95 or SAP97 influences Kv1.3 redistribution at the immunological synapse, we initiated IS formation between Jurkat T cells (expressing the different Kv1.3 constructs) and Raji B cells. The cells were fixed at five different time points (1, 5, 10, 20, and 30 min) after IS formation initiation in order to reveal the kinetics of Kv1.3 redistribution in the plasma membrane. Because it is well known that CD3 molecules rearrange into the immunological synapse, to verify synapse formation and to observe the physical boundaries of the IS, we used specific anti-CD3 immunofluorescent labeling as a positive control. All samples were analyzed by confocal microscopy.

5.2.6 C-terminal deletion of Kv1.3 or the knock-down of PSD-95 inhibits the accumulation of Kv.3 into the immunological synapse

In order to quantify the rearrangement of Kv1.3 channels upon IS formation we introduced the accumulation ratio (AR), which is proportional to the excess amount of Kv1.3 in the IS area compared to the even distribution of the channels in the membrane of
standalone cells. In order to rule out false positive data (due to the patchy distribution of Kv1.3 channels), we accepted Kv1.3 redistribution in the IS only at AR values being 1.5 or higher.

We analyzed at least 50 cells at each time point for all cell lines. According to our results mGFP-tagged wild-type channels accumulate at a high rate in the immunological synapse: the AR was higher than 1.5 in approximately 30% of the cells 1 min after IS formation, and the fraction of Kv1.3 polarized cells monotonically decreased until the 30th minute. On the other hand, the C-terminal truncated channels (mGFP-Kv1.3-ΔC) showed different accumulation kinetics: less than 10% percent of the cells showed Kv1.3 polarization at any given time point, nor did this parameter show any time dependence.

Deleting the whole C-terminal tail of Kv1.3 may inhibit the binding of any intracellular scaffolding protein to the channel. Therefore, in order to see the specific effect of PSD-95 and SAP97 on channel redistribution we also compared the Kv1.3 polarization of normal and PSD-95 or SAP97 knockdown Jurkat cells upon IS formation. We found that PSD-95 depleted cells were similar to the C-terminal truncated channel expressing cells: the ratio of Kv1.3 polarized cells was significantly lower after 1 min compared to the control cells, and the AR lacked any time-dependence up to the 30th minute. In contrast, deletion of SAP97 or the introduction of non-specific scramble control shRNA into our model cell line had no effect on Kv1.3 distribution compared to the non-knockdown cells. These results support our hypothesis that PSD-95 may be one of the key scaffolding proteins that influence Kv1.3 distribution in the T cell membrane during immunological synapse formation.

5.2.7 The expression level of the different mGFP-tagged Kv1.3 channels is identical in Jurkat cells

As we showed previously the mGFP-Kv1.3-ΔC channel shares identical biophysical properties with the mGFP-Kv1.3-WT channel. However, the quantitative analysis of Kv1.3 channel’s redistribution upon IS formation can also be fairly influenced by the expression level of Kv1.3 in the three different Jurkat cell lines: Jurkat-mGFP-Kv1.3-WT, Jurkat-mGFP-Kv1.3-ΔC and Jurkat-mGFP-Kv1.3-WT-KD-PSD95. Thus, using patch-clamping we measured the peak current and current density of Kv1.3 channels. We found that the amplitude of Kv1.3 current is statistically the same for all three Jurkat cell lines (mean peak current was 4702 ± 540 pA for Jurkat-mGFP-Kv1.3-WT (n=10), 3618 ± 444 pA for Jurkat-mGFP-Kv1.3-ΔC (n=12) and 4297 ± 800 pA for Jurkat-mGFP-Kv1.3-WT-KD-PSD-95 (n=12), p=0.631). In addition, the current density was also found to be the same in these cell
The mean current density was 1284 ± 329 pA/pF for Jurkat-mGFP-Kv1.3-WT (n=10), 1109 ± 314 pA/pF for Jurkat-mGFP-Kv1.3-ΔC (n=12) and 960 ± 229 pA/pF for Jurkat-mGFP-Kv1.3-WT-KD-PSD-95 (n=12), p=0.541. These data can exclude the possibility that the found difference in the accumulation of mGFP-tagged Kv1.3 channels in the IS could be due to the variation in the number of channels or in the channel density in the membrane of Jurkat cells lines.
6. SUMMARY

In this study we showed that CD1a+ monocyte-derived immature dendritic cells express the voltage-gated Nav1.7 channel. We found that this VGSC sets the membrane potential of these cells, thereby may modulate the threshold of DC activation, maturation. Inhibition of Nav1.7 channel by tetrodotoxin results in increased cytokine secretion of CD1a+ IDC upon suboptimal cytokine cocktail stimulation, which suggests that the channel has an important functional role during DC activation and differentiation. These facts confirmed our hypothesis that the presence of Nav1.7 channels in the plasma membrane of CD1a+ IDC keeps the membrane potential at a “depolarized state” (app. -10 mV), thus protecting the cell from unnecessary or harmful activation below an actively set threshold. Since the transition of IDC to MDC is a crucial step in triggering both innate and adaptive immunity, these findings shed light to a new regulatory mechanism, by which DC functions may be controlled.

We also investigated the association of a voltage-gated potassium channel, Kv1.3, with two different scaffolding proteins in human T cells. We found that Kv1.3 interacts with both PSD-95 and SAP97, and this interaction occurs only through the C-terminal of the channel. Deleting this region of Kv1.3, or knocking down PSD-95 in a human T cell line significantly decreased the fraction of T cells displaying Kv1.3 accumulation at the immunological synapse upon T cell conjugation to an APC. On the other hand, SAP97 had no effect on Kv1.3 redistribution, implying that PSD-95 could be responsible for the rearrangement of the channel during the formation of this supramolecular cluster. The exact function of Kv1.3 channels at the IS is still not fully understood, however, we believe that elucidation of the mechanisms by which these channels can be recruited to the IS may help us in resolving this issue.

In summary this work focused on two different immune cell types and their dominant voltage-gated ion channels, which are both crucial in triggering an adequate immune response to different stimuli. Understanding the appropriate function and regulation of these channels may bring us closer to discovering possible causes of, and even therapies for various immune diseases.
7. LIST OF PUBLICATIONS

1. **Szilágyi, O.**, Boratko, A., Panyi, G., Hajdu, P.: The role of PSD-95 in the rearrangement of Kv1.3 channels to the immunological synapse.
   *Pflugers Arch.* 465 (9), 1341-1353, 2013.
   DOI: [http://dx.doi.org/10.1007/s00424-013-1256-6](http://dx.doi.org/10.1007/s00424-013-1256-6)
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**List of other publications**

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8. ORAL AND POSTER PRESENTATIONS

Oral presentations:

Szilágyi Orsolya, Boratkó Anita, Panyi György, Hajdu Péter. A PSD-95 a Kv1.3 ioncsatorna immunológiai szinapszisban valófeldúsulásában (Ernst Jenő award), Veszprém, 2013, XXIV. Congress of the Hungarian Biophysical Society

Orsolya Szilágyi, Anita Boratkó, György Panyi, Péter Hajdu. The role of PSD-95 in the rearrangement of Kv1.3 channels to the immunological synapse, Mátraháza, 2013, Immune-related Pathologies: Understanding Leukocyte Signaling and Emerging therapies (IMPULSE)

Poster presentations:

Szilágyi Orsolya, Tóth Ágnes, Bartók Ádám, Krasznai Zoltán, Panyi György, Hajdú Péter: Fluoreszcens fehérjével konjugált Kv1.3 csatornák komparatív vizsgálata, Sümeg, 2008, Membrane Transport Conference

Szilágyi Orsolya, Papp Ferenc, Herceg Mónika, Gáspár Rezső, Batista C. V. F., Zamudio F. Z., Varga Zoltán, Possani L. D. Pharmacological and biophysical studies of a toxin from the scorpion Tytius stigmurus, Leuven, Belgium, 2008, IST Conference

Ágnes Tóth, Orsolya Szilágyi, Ákos Fábián, Zoltán Krasznai, György Panyi, Péter Hajdú. Functional Consequences of Kv1.3 Ion Channel Rearrangement Into The Immunological Synapse, Strbske Pleso, Slovakia, 2008, 8th EFIS-EJI Tatra Immunology Conference

Ádám Bartók, Ferenc Papp, Mónika Herceg, Orsolya Szilágyi, Rezső Gáspár. Pharmacological and biophysical studies of a toxin from the scorpion Tytius stigmurus, Sümeg, 2008, Membrane Transport Conference


Orsolya Szilágyi, György Panyi, Anita Boratkó, Péter Hajdú. The possible role of PSD-95/SAP90 in the rearrangement of Kv1.3 channels to the immunological synapse, Visegrád, 2011, Immune-related Pathologies: Understanding Leukocyte Signaling and Emerging therapies (IMPULSE)

Orsolya Szilágyi, György Panyi, Anita Boratkó, Péter Hajdú. The possible role of PSD-95/SAP90 in the rearrangement of Kv1.3 channels to the immunological synapse, San Diego, USA, 2012, Biophysical Society 56th Annual Meeting

Orsolya Szilágyi, György Panyi, Anita Boratkó, Péter Hajdú. The possible role of PSD-95/SAP90 in the rearrangement of Kv1.3 channels to the immunological synapse, Sümeg, 2012, Membrane Transport Conference

Domján Brigitta, Papp Ferenc, Szántó G. Tibor, Bartók Ádám, Szilágyi Orsolya, Bacsó Zsolt, Panyi György, Krasznai Zoltán. RF expozíció akut hatásai Nav1.5 ioncsatornák működésére, Sümeg, 2012, Membrane Transport Conference

Orsolya Szilágyi, Anita Boratkó, György Panyi, Péter Hajdu. The role of PSD-95 in the rearrangement of Kv1.3 channels to the immunological synapse, Saint Petersburg, Russia, 2013, FEBS Congress
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