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

T lymphocyte ion channels' activity under pathological conditions

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1. PREFACE

$CD8^+$ T cells play a role in tumor growth suppression and immunotherapy. T-lymphocyte ion channels such as Kv1.3, KCa3.1 and CRAC influence Ca^{2+}-dependent functions of T cells such as activation, proliferation, migration as well as other effector functions (cytokine/granzyme production, tumor killing) by regulating Ca^{2+} signaling. The Kv1.3 and KCa3.1 K^+ channels stabilize the negative membrane potential of T cells to maintain Ca^{2+} influx through CRAC (calcium release-activated channels) channels. As in T cells, these ion channels also play a prominent role in T cells with chimeric antigen receptor (CAR). Genetically modifying T cells to express CAR allows them to recognize a specific antigen on the tumor surface and then eliminate the tumor.

My thesis includes two topics, exploring the role of T lymphocyte ion channels in pathological conditions.

We examined the expressions of Kv1.3, KCa3.1 and CRAC in $CD8^+$ cells from ovarian cancer (OC) patients. Our aim was to characterize the activity of $CD8^+$ T lymphocyte ion channels isolated from the blood of patients with OC (malignant and benign), providing a potential biomarker for prognostics and diagnostics. We successfully demonstrated that Kv1.3 expression levels were higher in malignant tumor patients than in control or benign tumor groups, while the activity of KCa3.1 activity was lower in the malignant tumor group compared to the others. We showed that Ca^{2+} response was higher in malignant tumor patients compared to control groups.

We investigated the role of Kv1.3 and KCa3.1 channels in tumor infiltration and elimination by CAR-T cells under *in vitro* conditions in monolayer and 3D spheroid tumor models. We have successfully shown that CAR-expressing cells specifically eliminate tumor cells. Furthermore, the use of Kv1.3 (Vm24) and KCa3.1 (TRAM34) inhibitors significantly improved the tumor killing efficiency of both CEM-CAR and CAR-T cells in spheroids.

2. Introduction

2.1 Role and function of the immune system

The immune system destroys pathogens and abnormal internal structures. Two types of immune system have evolved over evolution: innate and acquired immunity, which work closely together to help maintain immune homeostasis. Lymphatic organs are called primary (thymus, red bone marrow) or secondary (lymph nodes, spleen, Peyer's plaque) lymphatic organs according to their function. Bone marrow hematopoietic stem cells develop into myeloid and lymphoid progenitors, which then give rise to immune cells. The myeloid development dendritic cells, granulocytes, macrophages and mast cells pathway gives rise to e.g. dendritic cells, granulocytes, macrophages and mast cells, while the lymphoid development pathway gives rise to differentiated lymphocyte precursors, which eventually give rise to B cells, T cells or natural killer (NK) cells.

2.2 Role of T cells in the immune response

T-cells are the prominent members in the adaptive immune response, which can be classified into two groups based on their function: cytotoxic ($CD8^+$) and helper ($CD4^+$) T cells. Helper T-cells regulate and control the immune response through the activation of other immune cells (e.g. B lymphocytes, macrophages), while cytotoxic T cells are able to recognize and eliminate infected and tumor cells. T lymphocytes recognize linear sequences of peptides derivered from protein antigens in the form of major histone compatibility complex (MHC) molecules bound to the membrane of antigen-presenting cells (APCs). Antigen presentation results in the initiation of several signaling pathways in the cell, such as Ca^{2+}-dependent signaling pathways, which cause the cell to proliferate and differentiate.

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2.3 Ovarian cancer

Ovarian cancer (OC) is a common gynecological malignancy that affects many women around the world. The incidence of OC is high, with a limited 5-year survival rate of <50%. The most commonly used biomarker for non-mucinous epithelial ovarian cancer is carcinoma antigen-125 as a serum marker, but it is not informative in mucinous cancers and germ cell tumors. Platinum-based drugs are available as first-line treatment but have not been shown to be effective in advanced stage patients.

2.4 Tumor microenvironment (TME)

Immune surveillance by the immune system often fails, as tumor cells are able to reprogram vital pathways to ensure their survival. $CD8^+$ T cells are unable to perform efficiently their effector functions and thus eliminate cancer cells due to various immunosuppressive mechanisms and barriers present in the tumor microenvironment (TME). TME impairs effector functions of $CD8^+$ T cells through the overexpression of metabolic (elevated extracellular K^+, H^+, ATP and adenosine levels, hypoxia and increased acidity) and cellular components (programmed cell death ligand 1 (PD-L1)). Immunosuppressive factors: myeloid-derived suppressor cells (MDSC), regulatory T cells, tumor-associated macrophages (TAM) or neutrophils (TAN), suppressive soluble factors and cytokines.

2.5 Ion channels

Ion channels are pore-forming proteins that ensure the controlled and selective passage of ions across the cell membrane in the direction of an electrochemical gradient. Based on the selectivity of ion channels, we can distinguish between high-selectivity (4 subunits), low-selectivity (5 subunits) and non-selective channels (6 subunits). Based on gating, we distinguish ligand-gated, intracellular (i.c.) messenger-gated, voltage-gated, mechanosensitive gated and background channels. The possible states of ion channels are open, closed and sometimes inactive, with different conformations.

2.6 Voltage-gated potassium channel, Kv1.3

Voltage-dependent potassium (Kv) channels regulate the membrane potential of the cell and various signaling processes. The Kv channels are composed of four subunits, with one subunit containing six transmembrane helices. The assembly of each subunit in the ER is facilitated by the tetramerization domain T1. The formation of a conserved pore region between transmembrane segments 5 and 6 (S5 and S6) and parts of segment S6 are responsible for the formation of a loop that allows ion conduction. This region contains the selectivity filter sequence responsible for potassium selectivity. The fourth transmembrane segment is considered as the voltage sensor for Kv channels. The Kv1.3 ion channel has been shown to be a key component of the pathway that regulates GrB production and secretion by $CD8^+$ T cells.

2.7 Calcium-gated potassium channel, KCa3.1

A medium conductance K^+ channel is the Ca^{2+}-activated KCa3.1 ion channel. The KCa3.1 ion channel is a transmembrane protein containing 4 alpha subunits, each containing 6 transmembrane segments (S1-S6). The pore region that allows ion conduction is located between S5 and S6. At the C-terminal is a calmodulin binding domain to which Ca^{2+}-activated calmodulin is attached, which will activate the channel. The activated channel contributes to the regulation of membrane potential by K^+ efflux and intracellular calcium levels. The KCa3.1 channel plays an important immunoregulatory role in various intracellular processes such as cell cycle progression, T cell proliferation, activation, migration and expression of genes that promote their effector function.

2.8 CRAC channel

Among the SOCE (store-operated calcium entry) channels, the most studied is the "calcium release activated calcium" (CRAC) channel. The CRAC channel is highly Ca^{2+} selective channel. The CRAC channel is composed of two subunits: the Orai1 and STIM1 subunits. Depletion of the intracellular Ca^{2+} store is sensed by the STIM1 subunit, which is able to bind directly to Orai1 after a conformational change, leading to activation of the CRAC channel, thereby allowing extracellular Ca^{2+} to flow in through CRAC channels. A key step in T cell activation and effector function is the influx of Ca^{2+} ions through CRAC channels. The interaction between CRAC and KCa3.1 channels is required for optimal target cell elimination.

2.9 The role of T lymphocyte potassium channels in T cell activation and effector functions

T-cell ion channels such as Kv1.3, KCa3.1 and Orai1 play a prominent role in T-cell activation, proliferation and tumor killing. Antigen recognition by T cells activates a signaling cascade that results in the release of inositol-1,4,5-triphosphate (IP_3), which binds to the IP_3 receptor (IP_3R) located in the ER membrane, thereby inducing Ca^{2+} efflux via IP_3R. The depletion of Ca^{2+}-store is sensed by the STIM1 subunit of the CRAC channel, which translocate to the Orai1 channel, thereby activating the CRAC channel, resulting in Ca^{2+} influx into the i.c. space. Elevated i.c Ca^{2+} levels depolarize the cell membrane, which activates the Kv.1.3 ion channel, while elevated calcium concentrations activate the Ca^{2+}-dependent KCa3.1 channel, thus maintaining the membrane potential at a sufficiently negative value (-50 - -60 mV for T cells) by potassium efflux, thus providing the driving force for proper Ca^{2+}-dependent signaling.

2.10 T cell subtype-specific expression of Kv1.3 and KCa3.1 channels

Naive $CD4^+$ and $CD8^+$ T cells express both CD45RA phosphatase and the chemokine receptor CCR7, the latter serving as a key receptor for entry into lymph nodes, where naive cells, upon encountering antigen, are activated and become "naive activated" or "naive effectors". The number of Kv1.3 and KCa3.1 channels expressed in T cells depends on the activation and differentiation state of the cell. At rest, CD4 and CD8 naive, T_{CM} ($CCR7^+CD45RA^-$) and T_{EM} ($CCR7^-CD45RA^-$) cells express significantly more Kv1.3 channels than KCa3.1 channels. Naive-effector and T_{CM}-effector cells express similar numbers of Kv1.3 and KCa3.1 channels. Activated $CD4^+$ and $CD8^+$ T_{EM} cells express high numbers of Kv1.3 channels and low numbers of KCa3.1 channels. A subset of $CD8^+$ T_{EM} cells differentiates into T_{EMRA} ($CCR7^-CD45RA^+$) cells, characterized by high Kv1.3 and low KCa3.1 expression.

2.11 The role of Ca^{2+}-optimum regulation in the fight against tumors

In CTL and NK cells, changes in i.c. ($[Ca^{2+}]_{int}$) and e.c. ($[Ca^{2+}]_{ext}$) Ca^{2+} levels can affect cell migration, proliferation and cytotoxic processes. At resting $[Ca^{2+}]_{int}$ values below 100 nM, cytotoxicity is slightly reduced, and their migratory capacity is severely limited. An increase in $[Ca^{2+}]_{int}$ (100-300 nM) is necessary to achieve optimal cytotoxic potential. Above 300 nM $[Ca^{2+}]_{int}$ is optimal for migration, but cytotoxicity is already slightly reduced. At very high $[Ca^{2+}]_{int}$ proliferation and apoptosis dominate.

2.12 CAR-T cell structure, generations

CAR T cells are T cells with a chimeric antigen receptor (CAR) created by genetic manipulation of T cells. CAR is composed of four main domains: an extracellular antigen-recognition ectodomain, a hinge region, a transmembrane domain and an intracellular endodomain. CAR-T cells can recognize specific antigens on the surface of tumor cells and then eliminate the tumor. Over the past years, the conformation of the CAR endodomain has been extensively modified for the activity, proliferation and therapeutic efficacy of CAR-T cells. Consequently, 5 different CAR generations can be distinguished today.

2.13 CAR-T cell therapy

CAR T-cell therapy represents a major advance in personalized cancer treatment. The FDA has already approved more than six CAR T-cell therapies for hematological malignancies such as large B-cell lymphoma. Despite the success of this therapy, however, many challenges remain to improve the efficacy and safety of the therapy for CAR T-cell depletion, toxicity and solid

tumors. The efficacy of CAR T cell therapy can be improved by combining it with various immunotherapeutic agents or other therapeutic modalities (radiotherapy or chemotherapy) that can promote tumor elimination.

2.14 Spheroid, *in vitro* tumor model

The use of 2D model systems is limited, while tumor xenotransplantation is a costly and time-consuming procedure. This problem could be solved by developing 3D tumor models that mimic native tumor and TME characteristics. Spheroids have many applications, they can be used to model TME and angiogenesis, as xenograft models and biobanks, for drug screening and to aid the development of therapeutic agents.

3. AIMS OF THE STUDY

In my PhD thesis, I investigated the role of T lymphocyte ion channels in pathological conditions using two different approaches:

3.1 Activity of Kv1.3, KCa3.1 and Orai1 channels in cancer.

T cell ion channels have different expression patterns in different tumors. In many cases, these channels may serve as biomarkers in cancer. Most of the research to date has investigated tumor infiltrating T lymphocytes (TILs) to explore this data. Our aim was to:

1. Characterize the biophysical parameters of $CD8^+$ T lymphocytes Kv1.3, KCa3.1 channels, isolated from peripheral blood of ovarian tumor (malignant and benign) patients and from healthy donor (control) blood using whole-cell patch-clamp method.
2. Investigate the Ca^{2+} response of $CD8^+$ cells from ovarian tumor (malignant and benign) patients and healthy donors (control) and thus characterize the biophysical properties of Orai1 channel using FURA-2 based Ca^{2+}-imaging.

3.2 The role of CAR-T cell ion channels (Kv1.3, KCa3.1, CRAC) during tumor infiltration and elimination.

It has been previously described that the Kv1.3, KCa3.1 and Orai1 channels play an important role in T cell migration and effector function by regulating Ca^{2+} signaling. Whether in T cells only, these ion channels may also play a prominent role in CAR-T cells. Our aim was to:

1. Expression of chimera antigen receptor (CAR) in activated T cells isolated from CCRF-CEM and human peripheral blood and validation of its functionality by Calcein-Red AM based 2D killing assay with CEM-CAR cells.

2. Determination and pharmacological identification of Kv1.3/KCa3.1 and CRAC ion channel expression of the generated CEM-CAR cells.

3. Investigate the role of ion channels (Kv1.3 and KCa3.1) of CEM-CAR and CAR-T cells in their tumor killing and infiltration capacity by Live-or-Dye based 3D spheroid assay using specific ion channel inhibitors.

4. MATERIALS AND METHODS

4.1 Human subjects

The OC studies were performed on peripheral blood from unidentified ovarian cancer patients aged 44 to 71 years (4 malignant and 3 benign tumor patients). The inclusion criteria for the study were a positive diagnosis of OC confirmed by tissue biopsy and no chemotherapy or radiotherapy prior to the date of blood collection. The tumor stage was T2 and T3 for patients, with nodal status of N0 and N1. The data on the study subjects were collected and managed at the University of Debrecen, Faculty of Medicine, Department of Gynecology and Obstetrics. Peripheral blood was also drawn from 5 healthy female donors (HDs) in the age range of 40 to 55 years. Informed consent was obtained from all OC patients and HDs. The study and informed consent forms were approved by the Regional and Institutional Research Ethical Board of the University of Debrecen (RKEB/IKEB No.: 5091-2018,6627-2023).

4.2 PBMC Isolation and Activation

PBMCs were isolated by centrifugation of whole blood using SepMate PBMC isolation tubes, according to a protocol downloaded from the STEMCELL Technologies website: (https://www.stemcell.com/products/brands/sepmate-pbmc-isolation.html/, download date: September 2021). PBMCs were cultured in RPMI medium supplemented with 10% FCS, 15 mM HEPES, 2 mM L-glutamine, 1 mM Na-pyruvate and 200 units streptomycin/penicillin. Activation was performed for 48 h in a 24-well cell culture plate with anti-human CD3 and CD28 antibodies (both 10 µg/ml). On day 2, T cell expansion was subsequently promoted by IL-7 (10 ng/ml) and IL-15 (5 ng/ml).

4.3 Cell culture

HEK293T and MCF-7 cells were cultured in DMEM medium supplemented with 2 mmol/l GlutaMAX and 10% FCS and antibiotics. Jurkat-E6-1, Raji were cultured in RPMI medium supplemented with 2 mmol/l GlutaMAX and 10% FCS and antibiotics. CCRF-CEM cells were cultured in RPMI-1640 medium supplemented with 10% FBS and antibiotics. Primary human T cells and CAR-T cells were cultured in RPMI medium supplemented with 25 mM Hepes free acid, 2 mmol/l GlutaMAX, 10% FCS and antibiotics. All cells and cell lines were maintained in a humidified atmosphere containing 5% CO_2 at 37 °C.

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4.4 Transformation

The encoding plasmids (10 ng) were added to 105 µl of TOP10 competent cells and incubated on ice for 20 min. Heat shock was applied at 42°C (60 s) and samples were again placed on ice. Cells were added to 800 µl of SOC medium and shaken at 37°C for 1 h at 200 rpm. Cells were disrupted on agar medium containing appropriate antibiotics. The cells in the petri dish were grown for 16 hours at 37°C.

4.5 Plasmid preparation

Bacterial colonies grown during transformation were inoculated into LB or TB culture medium containing the appropriate antibiotic and shaken at 200 rpm for 16 h at 37°C. To obtain larger amounts of plasmid DNA, the PureYield Plasmid Maxiprep System was used.

4.6 Monoclonal antibody adhesion of $CD8^+$ cells

Activated PBMCs were labeled with mouse anti-human CD8 primary antibody, and the labeled cells were incubated in bacterial-grade petri dishes coated with goat anti-mouse IgG until use.

4.7 Retroviral transduction, generation of $CD19^+$ MCF and CAR-T cell lines

For the retroviral transduction of CCRF-CEM, T and MCF-7 cells, we used the protocol downloaded from the Polyplus website (https://www.polyplus-sartorius.com/products/jetprime/, downloaded September 2022) and the Takara website (https://www.takara.co.kr/file/manual/pdf/T100A_B_e.v1705.pdf, downloaded September 2022). Plasmids used for transfection: CD19-specific CAR-encoding pBMN retroviral vector, human CD19 marker-encoding MSCV vector, PAX2 packaging plasmid and VSVG plasmid. For T cells, IL-7, IL-15 were added to the medium. After 72 h incubation, cells were used for further experiments.

4.8 Electrophysiological measurements

KCa3.1 and Kv1.3 currents in $CD8^+$ T cells and CAR-T cells were measured in the whole-cell voltage-clamp configuration. For the measurements, an aspartate external solution (145 Na-aspartate) was used and the pipette was filled with an internal solution containing 145 K-aspartate. Currents were elicited by a 200 ms ramp protocol ranging from −120 to +50 mV from a holding of −70 mV in every 15 s. The whole-cell conductance of KCa3.1 channels ($G_{KCa3.1}$) and Kv1.3 ($G_{Kv1.3}$) channels was calculated. For each cell, the conductance was calculated as the average of three subsequent measurements.

4.9 Pharmacological identification of ion channels

For pharmacological analysis, the following drugs were added to the cells: Vm24 (1 nM, specific Kv1.3 blocker) and TRAM34 specific KCa3.1 (200 nM). TRAM34 were dissolved in DMSO. For pharmacological measurements, a high K^+ solution/normal extracellular solution was used. Solutions were exchanged with a gravity-driven perfusion system. Vm24 were dissolved in aspartate bath solution. The composition of the pipette solution for pharmacological measurement was the same as for the patch-clamp measurements. TRAM34 was obtained from Heike Wulff (UC Camp Davis, USA) recombinant Vm24 was produced in our laboratory.

4.10 Intracellular Ca^{2+}-measurement

NT CEM, CEM-CAR, NT T and $CD8^+$ T-cells were loaded with 1 µM FURA-2-AM. The cells were perfused with 0 mM Ca^{2+} solution to check the cells' integrity and then 1 µM thapsigargin (TG) containing 0 mM Ca^{2+} solution to deplete of ER and then 2 mM Ca^{2+} containing 1 µM TG to activate intracellular Ca^{2+} elevation through CRAC. To test the effect of the ion channel inhibitors on the intracellular Ca^{2+} responses of NT T-cells we added 1 nM Vm24/ 1 µM TRAM34 to the 1 µM TG containing 2mM Ca^{2+} solution. The amplitude of Ca^{2+}-response for NT CEM, CEM-CAR, NT T cells was defined as the difference between peak and baseline of 340 nm/380 nm intensity ratio of FURA-2.

4.11 Flow cytometry

CD19-specific CAR expression was confirmed by sGFP positivity. For CD19 positivity, Alexa Fluor 647 anti-human CD19 antibody was used. Evaluation was performed using a NovoCyte 3000RYB flow cytometer and NovoExpress software.

4.12 Calcein Red killing assay

To test cytotoxicity target cells were resuspended in 1 µM Calcein Red AM solution. CEM-CAR effector cells and Raji, NT MCF-7 and CD19 MCF-7 target cells were co-incubated at 2:1 E:T ratio for 3 h. To detect Calcein Red staining of target cells confocal snapshots were taken with Nikon confocal microscope (excitation: He-Ne laser line of 561 nm, detection: LP filter 576 nm). The mean Calcein Red fluorescence intensity of cells was determined with ImageJ 1.54F software.

4.13 Agarose-based spheroid formation

For the generation of CD19-negative Jurkat spheroids, $CD19^+$ Raji and CD19 MCF-7 spheroids, we used the protocol downloaded from the ibidi website (https://ibidi.com/img/cms/downloads/an/AN32_Generation_of_spheroids.pdf, download date: April 2023).

4.14 Spheroid-based tumor infiltration and killing assay

Infiltration and killing capacity of CEM-CAR and CAR-T cells in three-dimensional cell cultures were determined by Live-or-Dye 640/662 incorporation assay. Spheroids were cocultured with effector cells for 24 hours. Ion channel inhibitors, such as TRAM34 (1 μM), Vm24 (10 nM) were added to the required samples. Spheroids incubated without effector cells served as controls to test the cytotoxic effect of TRAM34, Vm24 and DMSO. After 24 h, 3D cocultures were labeled with Live-or-Dye 640/662 stain, as detailed in manufacturer's protocol. The z-stack images of spheroids were taken at 10 μm thickness with Nikon-STORM confocal microscope. Using ImageJ software, we determined the total cell # (TC), the # of CEM-CAR or CAR-T (# of CAR T cells (CC)) cells and the # of dead cells (DC), and then from these cell counts, the infiltration rate (IR) (IR = CC / (TC-CC)), the kill rate (KR) (KR = DC / (TC-CC)) and the kill efficiency (KE) (KE = KR / IR) of CEM-CAR and CAR-T cells were determined. For the cytotoxicity assay, the percentage of dead cells (Live-or-Dye positive cells) was calculated from total cell and dead cell numbers. To monitor the effect of antagonist on the proliferation we calculated changes in TC and diameter (μm) of spheroids in the presence and absence of ion channel inhibitors (Vm24, TRAM34).

4.15 Statistical Analysis

GraphPad Prism 9.3.1 software was used for statistical analysis and plotting. One-way ANOVA or Mann-Whitney test was used to compare multiple groups. Differences were considered significant at $p<0.05$. Data are presented as mean ± standard error of the mean (SEM).

5. RESULTS AND DISCUSSION

5.1 $CD8^+$ malignant tumor cells have low KCa3.1 and high Kv1.3 conductance

Infiltration of $CD8^+$ T along with NK (natural killer) cells into the tumor is inevitable for elimination of tumor cells. We have shown before that KCa3.1 channel activity is lower in $CD8^+$ cells isolated from head and neck cancer (HNC) patients' blood: it could be attributed to the reduced calmodulin expression or inhibition of adenosine through adenosine receptor A_{2A}. Here, we demonstrated that KCa3.1 conductance is lower in $CD8^+$ T cells that are from donors with malignant ovarian tumor but not those with benign ones. KCa3.1 channels regulate the chemokine-induced chemotaxis as well as random walk in T cells, both of which are hindered by the addition of adenosine via the A_{2A}-PKA path. One may argue that these cells are suppressed by a relatively high concentration of adenosine produced by the tumor in the blood, however, this is not the scenario since adenosine's half-life is very short, up to a few seconds. Use of our "model system"—namely PBMCs of patients—clearly demonstrates that impairment of $CD8^+$ cell function does not happen only in the vicinity of the tumor: it can downregulate the KCa3.1 channels even at a distant location from the tumor to assure for itself survival. What is the benefit of KCa3.1 downregulation in $CD8^+$ cells for the tumor? With low KCa3.1 activity, the migration capacity of $CD8^+$ T cells is fairly reduced. This can explain why cytotoxic T cells are unable to infiltrate into the tumor: though they uncover the site of the tumor via a chemokine gradient, they are unable to migrate to the soma to eliminate the tumor's core

The immune responses of $CD8^+$ cells partly rely on the Ca^{2+}response, which is regulated by the Kv1.3 and KCa3.1 channels. Previously, it was shown by many groups that intra- and extracellular Ca^{2+} concentration definitely influence the target cell-killing efficiency of $CD8^+$ cells: a moderate elevation in cytosolic Ca^{2+} level is required. Here, we obtained that the CRAC-related response of mTTs is much higher compared to hTs or bTTs. We suppose that higher Ca^{2+} response in mTTs, and consequently the increased CRAC expression, counteracts their tumor cell-killing ability.

We suppose that our results can contribute to the understanding of $CD8^+$ T cell function and their ion channels' role in cancerous diseases, and these data clearly show a noteworthy connection between the malignancy of the tumor and the ratio of Kv1.3 and KCa3.1 conductance. Furthermore, our data provide functional information (unlike immunofluorescent techniques) on ion channels at the single-cell level in T cells, which can be more useful in the design of novel immune therapies relying on T cell engineering.

5.3 Inhibition of Kv1.3 and KCa3.1 ion channels in CAR-T cells increases tumor killing efficiency.

The use, development and optimization of CAR T-cell-based therapeutic systems has become increasingly popular in recent years. The CAR T-cell-based treatments have low efficacy against solid tumors, which could be attributed to the suppressive features of tumors preventing infiltration and effector function of these cells. T cell ion channels such as Kv1.3, KCa3.1 and CRAC channels are involved in the regulation of many T cell functions such as migration and tumor killing, that are pertinent in a cancer eradication. However, little is known about the role of these ion channels in the antitumor response of CAR-T cells, and thus in tumor cell recognition, tumor infiltration, and elimination of cancer cells.

First, we tested by electrophysiological and pharmacological tools whether our CD19-specific CEM-CAR cells express the Kv1.3 and KCa3.1 channels. Our results showed that CAR-T cells express both Kv1.3 and KCa3.1 ion channels, and the conductance level/channel number for both were comparable to those described in primary activated T cells. Consequently, the CEM cells were appropriate to model the behavior of CAR-expressing T cells in further experiments, however, we also included T cells in our study.

Here we obtained that Ca^{2+} influx is reduced in CAR-T cells compared to control NT CEM cells and a similar result is observed for CAR-T and NT T cells. As was reported before, the reduced Orai1 expression in CTLs facilitate target cell elimination. However, targeting Orai1 channels is not resolved due to the lack of high affinity and selective inhibitors, hence we could not test its role in CAR-expressing cells.

Next, we assessed the target killing affinity of our CEM-CARs in a monolayer, and we could show that CAR receptor specifically recognizes the target $CD19^+$ Raji, CD19 MCF-7 cells. Even during the short-term incubation, the CEM-CAR cells could eliminate target cells, however, the tumor defensive mechanism would prevent even cells migrating into the *in vivo* tumors. To model solid tumor vs. CAR-T cell scenario, we generated spheroids from $CD19^+$ Raji, CD19 MCF-7 ($CD19^+$ MCF-7 cell line) and $CD19^-$ Jurkat cells: they had necrotic core, and exhibited dense cell structure, which probably built up a TME-like milieu.

We reported here that CEM-CAR and CAR-T cells can infiltrate spheroids regardless of the CD19 cell surface marker presence but tumor killing only occurred in $CD19^+$ Raji spheroids: this clearly proves that the anti-tumor response of CEM-CAR and CAR-T cells occurred through specific antigen recognition. We introduced killing efficiency or KE, which is an estimate of the number of killed cells per CAR expressing cell, and it was extensively higher

for Raji spheroids as compared to Jurkat, which further underlines the specific response of CAR bearing CEM and T cells to the CD19 antigen.

Inhibition of the Kv1.3 by Vm24 and KCa3.1 by and TRAM34 significantly increased the killing efficiency of CD19-specific CEM-CAR and CAR-T cells in the spheroids (Raji, CD19 MCF-7). Previously it was reported for CTL and NK cells that changes in the intracellular and extracellular Ca^{2+} concentrations play a regulatory role in migration, proliferation and cytotoxic functions. In CTLs, the optimal cytotoxicity range is associated with low $[Ca^{2+}]_{int}$ (<300 nM), which correlates with the release of lytic granules containing granzyme B. However, at high $[Ca^{2+}]_{int}$ values (>300 nM) the migration speed and persistence are optimal, whereas cytotoxicity against target cells is already reduced. We suppose that the inhibition of Kv1.3 and KCa3.1 ion channels contributes to a shift of this calcium optimum into the cytotoxicity range, resulting in a more efficient anti-tumor response by CEM-CAR and CAR-T cells. Since inhibitors were added to the Raji/CD19 MCF-7 spheroids simultaneously with the CEM-CAR/CAR-T cells and we could not detect change in the infiltration of either CEM-CAR or CAR-T cells, we suppose that migration of these cells is not vastly hampered by channels' inhibition.

In the 24-hour spheroid killing assay 10 nM Vm24 was used (unlike in patch-clamping where 1 nM), since the Vm24 might be degradable peptide and due to its size (app. 4 kD) its diffusion could be fairly limited into the tumor spheroids. Hence, we used ca. 1000 times the K_d value, while for TRAM34 50-fold K_d, as this small molecule is more stable than those with peptidyl origin. Also, we should mention that this concentration of drugs had no effect on the viability of target cells, as well as that of CAR cells.

6. SUMMARY

In my dissertation, I investigated the ion channel activity of T lymphocytes under pathological conditions using two different approaches. In one approach, I have investigated samples isolated from different tumor patients, and in the other, I have investigated CAR-T cells that are able to specifically recognize and eliminate tumor cells.

In the first part of this thesis, we investigated the Kv1.3 and KCa3.1 ion channel activity of $CD8^+$ T cells isolated from peripheral blood of ovarian cancer patients, where we report that ovarian tumor type (benign vs. malignant) determines the ion channel expression pattern of $CD8^+$ T cells. We hypothesize that lower KCa3.1 and higher CRAC activity contribute to impaired $CD8^+$ effector function in tumor killing. Since the total K^+ conductance was identical in mTTs, the activation of these cells is not suppressed upon stimulation of non-tumor antigen. Finally, we hypothesize that the decreased KCa3.1 function activity of $CD8^+$ cells from malignant tumors in combination with increased Kv1.3/CRAC activity may be of diagnostic significance. We speculate that altered Kv1.3 and KCa3.1 activity of $CD8^+$ cells in OC may serve as biomarkers for diagnostics and that an enhanced $Ca2^+$ response via CRAC may contribute to impaired $CD8^+$ function.

In the second part, we successfully demonstrated that both KCa3.1 and Kv1.3 ion channels are expressed and functional in CD19-specific CEM-CAR cells. Reduction in Ca^{2+} response of CEM-CAR cells compared to NT control, which may be contributed by reduced Orai1 expression to facilitate target cell elimination. CEM-CAR and CAR-T cells specifically recognized and eliminated tumor cells in both 2D and 3D cultures, however inhibition of Kv1.3/KCa3.1 channels promotes more efficient tumor killing. Based on these results, we propose that modulation of ion channels in CAR-T cell therapy should be a novel approach to achieve better therapeutical outcome, which could be further improved with combination of immune checkpoint strategies targeting PD-1/PD-1L and/or CTLA4/B7. Since these two channels are also present in normal T cells, the systemic application of K^+ blockers in vivo is limited, however, appropriate genetic modulation of their expression could be a possibility in CAR-T cell therapy.

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List of publications related to the dissertation

1. **Jusztus, V.**, Szöőr, Á., Hajdu, P.: Role of CAR-T cell K+ channels in tumor infiltration and elimination.
J. Immun. "Accepted by Publisher", 2025.
DOI: http://dx.doi.org/10.1093/jimmun/vkaf084
IF: 3.6 (2023)

2. **Jusztus, V.**, Medyouni, G., Bagosi, A., Lampé, R., Panyi, G., Matolay, O., Maka, E., Krasznai, Z. T., Vörös, O., Hajdu, P.: Activity of Potassium Channels in CD8+ T Lymphocytes: diagnostic and Prognostic Biomarker in Ovarian Cancer?
Int. J. Mol. Sci. 25 (4), 1-8, 2024.
DOI: http://dx.doi.org/10.3390/ijms25041949
IF: 4.9 (2023)

List of other publications

3. Medyouni, G., Vörös, O., **Jusztus, V.**, Panyi, G., Vereb, G., Szöőr, Á., Hajdu, P.: Inhibition of K+ Channels Affects the Target Cell Killing Potential of CAR T Cells.
Cancers (Basel). 16 (22), 1-12, 2024.
DOI: http://dx.doi.org/10.3390/cancers16223750
IF: 4.5 (2023)

DEBRECENI EGYETEM Egyetemi és Nemzeti Könyvtár

Total IF of journals (all publications): 13
Total IF of journals (publications related to the dissertation): 8,5

The Candidate's publication data submitted to the Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

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