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Role of ion channels in CAR-T cells

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1. INTRODUCTION

1.1 Ion channels

1.1.1 General information about ion channels

Ion channels are widely expressed membrane proteins that form a hydrophilic pore in the cell membrane (including plasma membrane and intracellular organelle membranes), allowing the passage of various ions (Na^+, K^+, Ca^{2+}, Cl^-) according to their electrochemical gradient. The different behavior of ions channels (high speed, passive transport) versus thermo-dynamically uphill transport of pumps makes them a distinguished entity. The ion movement control by the ions channels is essential for life. Several cellular functions are mediated by them such as immune responses, muscle contraction, pH regulation and cell cycle. The classification of ions channels mostly relies on ion selectivity (e.g.Ca^{2+}, K^+, Cl^-, highly, mildly- or non-selective), ionic current direction (outward-, inward), gating mechanism (voltage-gated, signal-gated, ligand-gated, stretch-gated, etc.).In this study, K^+ and CRAC channels are our primary focus and this will be covered in more details bellow.

1.1.2 Potassium channels

More than 90 genes in the human genome code for principal units of potassium channels forming a large family in both excitable and non-excitable cells. These proteins are implicated in several crucial biological processes like cellular signaling in the nervous system, regulation of cell volume, proliferation and activation of T cells, apoptosis, calcium signaling. From a structure point of view, K^+ channels are composed of pore forming α-subunits with auxiliary β-subunits. Potassium channels can be categorized into 4 different subfamilies based on their subunits structure:(i) six TM (S1-S6) segments channels having one pore domain including voltage gated potassium channels (K_v),(ii)inwardly-rectifying K^+(K_{ir}) regulated by intracellular factors permitting the movement of K^+ into the cell ,(iii) two- pore four TM segments K^+ channels (K_{2P}) which their subunits assembles as dimers and (iv)K_{ca} calcium activated potassium channels 7TM/1P . Most of K^+ channels are tetramers formed by four identical subunits consisting in two transmembrane domains (TM)connected by a pore region. The selectivity of potassium channels is determined by a conserved sequence of amino acids referred to as the signature sequence T/SXGXGX located in the pore forming region. The understanding of potassium channels' properties is essential for developing novel therapeutic agents targeting ion channels disorders.

1.1.3 Voltage- gated Potassium channels (K_v)

Voltage-gated potassium channels form one of the largest and diverse signaling molecules encoded by 40 genes in the human genome and divided into 12 subfamilies. These subfamilies can be classified into three groups. The dominant group is this division is the Kv1 shaker that includes 8 voltage-gated K^+ channels (Kv1.1-Kv1.8). K_v channels consist of four α-subunits arranged as homotetramers or heterotetramers, each subunit contains six transmembrane domains: α-helical segments S1-S6. The voltage sensitivity is due to the voltage sensor domain (VSD) formed by the first 4 α-helices (S1-S4) due to the positively charged arginine residues, while the selectivity is formed by the region between S5 and S6 in the pore domain (PD) and the connecting pore loop (P). K_v channels have been thoroughly characterized. However, the established properties of K_v α-subunit channel can be modified by different possibilities of heteromultimerization of α-subunit, their association with intracellular β-subunits or furthermore due to phosphorylation, dephosphorylation. This molecular variety presents a challenge in terms of drug discovery, but it also offers the possibility to design specific modulators that bind to tissue-specific β-subunits or that differentiate between homotetramers or heterotetramers. In our current study, our main focus is Kv1.3 ion channel .It is located in the plasma membrane, nuclear membrane, cis-Golgi apparatus membrane, and in the inner mitochondrial membrane.Kv1.3 opens upon depolarization .One of the main distinctive property of Kv1.3 channel is the P/C inactivation or also called slow inactivation which consists on a time-dependent inactivation that develops during repetitive or prolonged depolarization .This type involves conformational changes in the pore (P) and C-terminal region hence its name .

1.1.4 K_{Ca} Calcium activated potassium channels

Calcium activated potassium channels are widely expressed in a variety of tissues such as muscles, neurons and immune cells. As their name states, they require an intracellular calcium signal to function. According to their conductance, 3 categories has been identified: small conductance (SK) 2–25 pS ,SK1, SK2, and SK3, which correspond to KCa2.1, KCa2.2, and KCa2.3, intermediate conductance (IK) 25–100 pS referred to as KCa3.1, SK4, or IK1. and large conductance with a range of 200-300pS that are not only regulated by calcium signal but also voltage referred to as BK channels. Besides the difference in conductance, these 3 classes differ significantly in terms of Ca^{2+} binding affinity and location. IK and SK channels have a high affinity of 0.1-0.4μM and 0.3-0.75μM respectively.BK channels has a low affinity of 1-

11µM.The opening of the channel of IK and SK is modulated via calmodulin-binding domain whereas it is directly through RCK structural domains for BK channels. In this research study we are focused on KCa3.1 channel .The intermediated-conductance Ca^{2+} -activated K^{+} channel KCa3.1(KCNN4) is mostly distributed in blood cells, liver ,lungs, placenta ,endothelial and vascular cell. They have less charges in their S4 than K_v channels .Therefore , they do not respond to voltage changes but to the calcium binding to the calmodulin inducing channel opening .In immune cells , KCa3.1 is upregulated in activated cells compared to resting T cells.It plays a crucial role in immunity and cancer that we will detail in other sections . According to its expression pattern, KCa3.1 modulators represent highly therapeutic candidates for autoimmune diseases, blocking proliferation of smooth muscle cells and fibroblasts in atherosclerosis, asthma.

1.1.5 CRAC channels

Calcium is a crucial second messenger that modulates various physiological responses. Activation of cell surface receptors leads to the production of inositol triphosphate (IP3), causing Ca^{2+} release from endoplasmic reticulum (ER) stores and subsequent calcium influx through store-operated channels (SOCs). The Ca^{2+} release-activated Ca^{2+} (CRAC) channel, a well-studied SOC in T lymphocytes, is characterized by low conductance and high specificity for $Ca^{2+.}$ The ER Ca^{2+} sensor, STIM1, detects ER store depletion, undergoes conformational changes, and activates Orai1, enhancing cytosolic Ca^{2+} levels. This process is vital for gene expression, mitochondrial metabolism, and cell growth. Abnormal CRAC channel activity is linked to conditions such as severe combined immunodeficiency disease (SCID), breast cancer, and allergies.

1.2 Ion channels and immunity

1.2.1 Brief review of the immune system:

Our immune system is one of the fascinating networks in our body. There are two fundamental ways of defending against pathogens: an innate response and an adaptive response. The first one uses mainly phagocytic cells like macrophages, monocytes, neutrophils, and natural killers. This response is also modulated via molecules such as the complement and cytokines like interferons. Adaptive responses require the proliferation of antigen-specific T and B cells. Antigen-presenting cells (APCs) like dendritic cells and macrophages display the antigen to lymphocytes through a transmembrane protein complex called MHC (major histocompatibility complex). This will lead to cell activation, proliferation, and differentiation. The effector

response could occur via antibodies released from the differentiated plasmacytes or T cytotoxic cells. Th $CD4^+$ cells are considered as the ones orchestrating the immune response via the recognition of foreign antigens and activating cell-mediated responses. The immune system reactions are complex and involve several parts. However, it is a major target for therapeutic development against infections, tumors, and autoimmune diseases.

1.2.2 Role of Kv1.3 and KCa3.1 in calcium-activated signaling of T cells:

In 1984, voltage-gated K^+ channels were found in human T lymphocytes, prompting research into their role in immunity, particularly the Kv1.3 channel. Further studies revealed that other immune cells express ion channels that regulate ion movement across membranes. Mutations in these channels can lead to immunodeficiencies and autoimmune diseases. Activation of T cells involves antigen binding to the T cell receptor (TCR) and subsequent signal transduction pathways, including the activation of PLCγ1, which generates IP3 and DAG. This cascade causes calcium release from the endoplasmic reticulum, leading to T cell activation via transcription factors like NFAT, NF-KB, and AP-1. The influx of calcium is vital for T cell activation, balanced by K^+ efflux maintained by Kv1.3 and KCa3.1 channels to prevent excessive depolarization. Differential channel expression is observed in T cells based on activation state, with memory T cells showing distinct patterns of Kv1.3 and KCa3.1 channels, which adapt during immune responses.

1.3 Ion channels and tumor microenvironment

Previous studies indicate that K^+ currents in circulating T cells from head-and-neck squamous cell carcinoma (HNSCC) patients are similar to those from healthy donors, while Kv1.3 currents in tumor infiltrating lymphocytes (TILs) are significantly lower, affecting their proliferation and cytokine production. Peripheral $CD8^+$ cells exhibit impaired Ca^{2+} sensor calmodulin (CaM) upregulation, limiting their ability to infiltrate tumors. Restoration of KCa3.1 function and migratory capacity in $CD8^+$ cells was achieved using the KCa3.1 activator 1-EBIO, suggesting that ion channel modulators could be therapeutic. Additionally, the tumor microenvironment (TME) plays a crucial role in tumor development and contains immune cells that suppress anti-tumor responses. Elevated adenosine levels in the TME, due to ATP degradation, create an immunosuppressive niche. Research shows that adenosine affects T cell function by inhibiting KCa3.1 through the cAMP/PKAI signaling pathway, impacting cell migration and IL2 secretion. These findings highlight the potential of targeting ion channels in cancer therapy.

1.4 The localization of Kv1.3 in the immunological synapse

The immunological synapse (IS) concept has evolved to encompass a dynamic reorganization of proteins and membrane domains, with the synaptic cleft forming between 15 nm and 100 nm apart. Early IS formation involving Ca^{2+} influx that induces T cell rounding and mitochondrial translocation to the plasma membrane. TCR-antigen binding elevates Ca2+ levels, causing ERM protein phosphorylation and actin cytoskeleton detachment. Key proteins, including LFA-1, TCR/CD3, CD28, and talin, exhibit selective localization within the IS. Notably, our laboratory confirmed the redistribution of Kv1.3 channels into the IS of cytotoxic T cells, which influenced Ca2+ responses and downstream pathways, suggesting potential avenues for T cell response modulation via ion channels.

2.CAR T cell Therapy

2.1 CAR structure and evolution

In the 1960s, the connection between immune cells and cancer led to the development of immunotherapy, specifically CAR T cell therapy, which introduced Chimeric Antigen Receptors (CARs) to T cells starting in 1989. CAR cells comprise four components: an extracellular recognition domain, a hinge, a transmembrane domain, and an intracellular signaling part. Over time, CAR T cell therapy has evolved through five generations. The first generation utilized single-chain variable fragments (ScFv) linked to costimulatory domain CD3 ζ. The second generation enhanced activity by adding costimulatory domains like CD28 and 4-1BB. The third generation further expanded these domains to bolster activation and anti-tumor effectiveness, particularly in hematological cancers, although challenges remained for solid tumors. Consequently, the fourth generation, known as TRUCK-T or "Armored CAR-T cells," incorporates cytokine production to improve T cell responses. The fifth generation aims for universality, allowing CARs to recognize multiple antigens using models like BBIR and SUPRA CARs, which separate the targeting and signaling functions, enhancing response capabilities against diverse antigens.

2.2 CAR T cells targeting Cancer

Recent advancements in CAR T therapy have shown significant promise for treating hematological malignancies. Pioneering studies demonstrated the successful use of CD19-

targeted CAR T cells in patients with chemotherapy-resistant follicular lymphoma and chronic lymphocytic leukemia. FDA has approved several CAR T products, including Kymriah® and Breyanzi®, which are primarily effective against blood cancers. However, applying CAR T therapy to solid tumors remains complex due to challenges like tumor heterogeneity and immunosuppressive tumor microenvironments. Innovations such as multi-antigen targeting and improved administration techniques are being explored to enhance effectiveness. Side effects, including hypogammaglobulinemia and cytokine release syndrome, present additional hurdles in the treatment landscape.

2. AIMS OF THE STUDY

CAR-T cell immunotherapy has proven to be a promising treatment for blood cancer patients. However, several challenges need to be overcome. It has been previously described that K^+ channels play an important role in T cell effector function. To date, no study reported about the role of ion channels in CAR-T cells. In the thesis we studied the role of CAR-T cell K^+ channels in target cell elimination with two distinct approaches. They were the following:

2.1 To unveil the functional role of Kv1.3 channel localization in Jurkat-CAR cells

The first step was the establishment of anti-CD19 CAR expressing Jurkat cell line (Jurkat-CAR) and validate the functionality of the CAR using Calcein-Red AM based killing assay. Second, we analyzed the biophysical properties of Kv1.3 in Jurkat-CAR cells and assessed the localization of Kv1.3 channel in standalone and target cell engaged Jurkat-CAR cells. Since the channel is involved in the Ca^{2+}-dependent activation pathway, our aim was to monitor the Ca^{2+}-response during the synapse formation between the target cells and Jurkat-CARs. To reveal the Kv1.3 channel role we unveiled the influence of Kv1.3 channel immobilization via antibody-crosslinking on the Jurkat-CAR Ca^{2+}-response and target cell killing.

2.2 Determine the expression and role of K^+ channels in anti-HER2 3rd generation CAR-T cells

In the second part of the study, we aim to characterize the expression of Kv1.3 and KCa3.1 in $CD4^+$and $CD8^+$ third-generation anti-HER2 CAR-T cells and evaluate the Ca^{2+}-response of $CD4^+$and $CD8^+$ of CAR T cells. Using ion channel inhibitors, we tested the role of ion channels in the killing potential of CAR-T cells.

3. MATERIALS AND METHODS

3.1 Cell culture

3.1.1 HEK 293T cell line

HEK-293T packaging cells were cultured in a DMEM medium (Sigma-Aldrich Ltd., Hungary), which contained 10% FBS, 1 mM Na-pyruvate, and 200 units of penicillin/streptomycin. Cells were maintained at 37 °C in a humid atmosphere of 5% CO2 and 95% air. Cells were passaged thrice in a week following 2-5 minutes incubation in 0.05% trypsin-EDTA solution.

3.1.2 Jurkat and Raji cell line

Jurkat and Raji cell lines were cultured in a RPMI medium (Sigma-Aldrich Ltd., Hungary) supplemented with 10% FBS, 1 mM Na-pyruvate, and 200 units of penicillin/streptomycin. Cells were maintained at 37 °C in a humid atmosphere of 5% CO2 and 95% air. Cells were passaged thrice in a week.

3.1.3 MDA-MB-468, N87and JIMT-1 cell lines

The triple-negative human breast cancer cell line MDA-MB-468 (abbreviated MDA in the text), and N87 human gastric cancer cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2 mmol/l GlutaMAX and 10% Fetal Calf Serum (FCS) and antibiotics. The JIMT-1 human breast cancer cell line was established in the laboratory of Cancer Biology, University of Tampere, Finland . These cells were cultured in a 1:1 ratio of Ham's F-12 and DMEM supplemented with 20% FCS, 300 U/L insulin, 2 mmol/l GlutaMAX, and antibiotics. MDA.ffLuc, JIMT-1.ffLuc, and N87.ffLuc were generated by single-cell cloning of the MDA-MB-468, JIMT-1, and N87 cell lines, respectively, after transduction with a retrovirus encoding eGFP.ffLUC to express an enhanced green fluorescent protein/firefly luciferase fusion gene.

3.1.4 Isolation of primary human T cells

The experiments were carried out on human samples by the Declaration of Helsinki and approved by the Regional and Institutional Committee for Research Ethics (RKEB.5378/2019). To isolate human T cells, human peripheral blood mononuclear cells were isolated by Ficoll gradient centrifugation and stimulated in non-tissue culture 24-well plates precoated with 1 µg/mL OKT3 (Thermo Fischer, Waltham, MA, USA) and anti-CD28 (R&D Systems,

Minneapolis, MN, USA) antibodies. On day 2, human interleukin-7 (IL-7; 10 ng/mL) and human interleukin-15 (IL-15; 5 ng/mL) (Miltenyi Biotec, Bergisch Gladbach, Germany) were added to cultures. The primary human T cells were cultured in the RPMI (Roswell Park Memorial Institute) medium supplemented with 2 mmol/l GlutaMAX and 10% FCS and antibiotics.

All the cells and cell lines were maintained in a humidified atmosphere containing 5% CO_2 at 37 °C and were routinely checked for the absence of mycoplasma contamination by PCR.

3.2 Retroviral transduction

3.2.1 Jurkat-CARs anti CD19

To generate Jurkat-CARs cells, 2×10^6 cells of HEK293T were plated in a 10 cm petri dish with 10 ml of DMEM medium supplemented with 10% FBS. To proceed, the cells were required to adhere to the plate and reach 70-80% confluence. The plasmids used (described in the table below) were mixed and diluted in 500µL of jetPRIME buffer followed by the addition of 20 µL of jetPRIME reagent (Polyplus ®,Illkirch, France). The mixture was incubated for 10 min at room temperature. As a last step, the transfection mixture was added to plated HEK293T cells and incubated for 48 hours in a humidified atmosphere containing 5% CO_2 at 37 °C.For evaluating the transfection efficacy HEK293T transfected cells with pBMN-sGFP-CAR were checked under the microscope for GFP positivity. Later, the viral supernatant was removed from the HEK293T cells and collected, filtered using a 0.45µM filter then added to the target Jurkat cells, along with the polybrene (10µg/ml). Jurkat transduced cells were incubated for 48 hours at 37 °C in a humidified atmosphere of 5% CO_2. The retroviral transduction was evaluated using with flow cytometry with non-transduced Jurkat cells as a control for the cells transduced with pBMN-sGFP-CAR. As for the cells transduced with the empty vector of pBMN, the antibiotic selection marker was utilized to select these cells.

3.2.2 CAR T cells

For the generation of HER2-specific CAR T cells, the RD114-pseudotyped retroviral particles were generated by transient transfection of the HEK 293T cells with the HER2-specific CAR-encoding pSFG retroviral vectors, the Peg-Pam-e plasmid coding MoMLV gag-pol, and the pMax.RD114 plasmid using jetPrime transfection reagent (Polyplus, Illkirch, France).

After human T cell isolation (explained above), T cells were transduced with retroviral particles on RetroNectin-coated (Takara, Kusatsu, Japan) plates on day 3 in the presence of IL-7 (10 ng/mL) and IL-15 (5 ng/mL). The expansion of the T cells was subsequently supported with IL-7 and IL-15. The OKT3/CD28-activated non-transduced (NT) T cells were expanded with IL-7 and IL-15 using the same protocol. Following 48 h incubation, the cells were used for further experiments.

3.3 Jurkat-CARs anti CD19 sorting

To get a homogeneous cell population, we proceeded with cell sorting using cytoFLEX Flow cytometer since our successfully transduced cells are GFP positive. At first, the 1×10^6 of the transduced cells were centrifuged and washed with PBS then re-suspended into 1% FBS-PBS. Jurkat non-transduced cells were used as a negative control. After sorting, cells were centrifuged for 5 minutes, 300×g at RT then re-suspended in RPMI medium and placed in the incubator to grow at 37 °C in a humidified environment of 5% CO_2 ,95% air, with the RPMI medium replenished every 2 days. Results were analyzed with FCS Express 6 software.

3.4 Single cell electrophysiology

3.4.1 Patch Clamp electrophysiology conditions

Whole-cell currents were measured using patch-clamp technique in voltage-clamp mode following standard protocols. All recordings were performed using Axopatch 200B amplifier connected to a personnel computer with Axon Digidata 1440 digitizer and for data acquisition ,Clampex 10.7 software was used (Molecular Devices, Sunnyvale, CA, USA).Micropipettes were pulled from GC150F-7.5 borosilicate capillaries (Harvard Apparatus, Kent,UK) with a tip resistance ranging from 3-5MΩ in the bath solution .Recordings were performed at a room temperature (20-25°C).

3.4.2 Solutions

For the performed measurement, the intracellular and extracellular solutions were prepared according to table 2 and table 3 below. The osmolarity of the intracellular solution used to measure Kv1.3 and KCa3.1 currents of CAR T cells was 290–310 mOsm, 1µM free Ca^{2+} concentration and ~295 mOsm for the one used for measuring Kv1.3 channel on Jurkat-CARs. pH of solutions was checked before performing the experiments.

3.4.3 Voltage Protocols

-Kv1.3 measurements of Jurkat-CARS

Jurkat-CARs were placed into cell culture petri dishes 35mm.Kv1.3 currents were recorded in whole-cell configuration using the solutions listed above. The activation kinetics of the current were characterized by fitting the Hodgkin-Huxley (HH) model ($I(t)=I_a \times (1-\exp(-t/\tau a))4+C$, where I_a is the amplitude of the activating current component; where I_a is the amplitude of the activating current component, is the activation time constant of the current; C: current at the beginning of the trace) to the rising phase of the current trace obtained by 15-ms-long depolarization to +50mV. The activation time constant characterizes the average of the time constants of a given cell upon three sequential depolarizations repeated every 15s.

The inactivation kinetics of the current were characterized by fitting a single exponential function ($I(t) =I_0 \times \exp(-t/\tau in)+C$, I_0: amplitude of the current, τ_{in}: inactivation time constant, C: the steady-state value of whole-cell current at the end of the pulse) to the decaying part of the current traces obtained by 2-s-long depolarization to +40mV from a holding potential of -120mV.The inactivation time constant was determined as for the activation, except that pulses were delivered every 60 seconds. The voltage dependence of steady-state activation was determined as follows. Jurkat-NT, Jurkat-CAR, and Jurkat-PBMN were held at -120mV holding potential and depolarized to various test potentials ranging from -70mV up to +50mV in 10mV steps at every 30s. Peak whole-cell conductance (G(V)) at each test potential was calculated from the peak current (I_p) at test potential V and the K^+ reversal potential (E_r=−85 mV) using $G(V)=I_p/(V-E_r)$. The G(V) values were normalized for the maximum conductance and plotted as a function of test potential and the Boltzmann function was fitted to the data points: $G_N=1/(1+\exp[-(V-V_{½})/k]$, where GN is the normalized conductance, V is the test potential, $V_{½}$ is the midpoint or half-maximal activation potential and k is the slope factor of the function.

-Kv1.3 and KCa3.1 measurements of CAR-T cells

$CD3^+$ CAR/NT T cells were plated onto a poly-l-lysine-coated 35 mm petri dish, while CD4 and CD8 CAR T and NT cells were adhered to a petri dish using the antibody adhesion protocol as described here. The Kv1.3 and KCa3.1 currents in the CAR T cells were measured in a whole-cell voltage-clamp configuration. The solutions used are described above. The currents were recorded by 200 ms ramp depolarization from −120 to + 50 mV from a holding potential

of −70 mV at every 10 s. KCa3.1 conduction was defined as the ratio of the linear fraction of the macroscopic current slope and the slope of the voltage-ramp stimulus after subtraction of the leak current, $G_{KCa3\,1}(nS) = I_{slope}\left(\frac{pA}{ms}\right) / V_{slope}\left(\frac{V}{ms}\right)$. The slope conductance was measured between −100 and −60 mV to avoid contamination by the Kv1.3 current. As for the Kv1.3 current, it was determined from the same ramp protocol at 50 mV after subtraction of the KCa3.1 current extrapolated by linear regression. The conductance for each channel type was normalized to whole-cell capacitance (which is proportional to the channel number per unit area) and was applied to define the membrane expression level.

3.5 Intracellular Ca^{2+} measurements

3.5.1 Ca^{2+} response of Jurkat-CARs and CAR-T cells standalone

To determine the effect of CAR expression on the CRAC-related Ca^{2+}-response, a FURA-2 Ca^{2+} -imaging technique was performed. For Jurkat-CARs, cells (Jurkat CAR/NT /PBMN) were plated in poly-l-lysine coated glass bottom petri dishes. For CAR-T cells, cells were labeled using CD4-Alexa488 or CD8-Alexa488 (Biolegend, San Diego, CA, USA). Then, the cells were suspended in 10% BSA in PBS for 30 min on ice, then washed 2 times with PBS, then resuspended up in phenol-red free media. Afterwards, CAR T cells were plated in poly-L-lysine-coated glass bottom Petri dishes as well. To track the Ca^{2+} response, cells were loaded with 1µM Fura 2-acetoxymethyl ester (Thermo Fisher Scientific, Budapest, Hungary) dissolved in DMSO and incubated for 30 min at 37 °C in phenol -Red free RPMI solution (Sigma-Aldrich Ltd., Budapest, Hungary), supplemented with 1% FBS, 2mM L-glutamine, 1mM Na-pyruvate and 200 units penicillin/streptomycin. Later, we washed the cells with 2 mM Ca^{2+} solution (see table 4 for the recipe of solutions) and placed them on a 37 °C stage of an inverted fluorescence microscope. Then, the Jurkat CAR cells were perfused with 2mM Ca^{2+}solution,0mM Ca^{2+} and then 1 µM Thapsigargin (TG) (Thermo Fisher Scientific) containing 0 mM Ca^{2+} solution was applied to deplete the Ca^{2+} stores via passive release from the endoplasmic reticulum (ER). After store depletion, the addition of the extracellular 2mM Ca^{2+}containing 1µM TG activated intracellular Ca^{2+} elevation through SOCE (store-operated calcium entry). Experiments with FURA-2 were done with an inverted NIKON ECLIPSE Ts2R microscope combined with a VisiChrome High-Speed Polychromator (Visitron Systems GmbH, Puchheim, Germany). FURA-2 dual excitation and emission were accomplished using 340nm and 380 nm excitation filters and a 510 nm emission filter. Digital images (200 ms

exposure) were recorded with a PCO Edge 4.2 sCMOS Camera at 10s intervals. Data acquisition and analyses were accomplished using VisiView® 4.0.0.11 imaging software. Only GFP-positive cells were selected for Jurkat CAR cells.

3.5.2 Ca^{2+} response during immunological synapse formation

To investigate the immunological synapse (later we refer to it as CAR-synapse) between the Jurkat CAR and the Raji cells, the synapse was formed at a ratio of 1:1. First the cells were mixed and co-centrifuged for 1 min, at 37°C, 200×g. The mix was dropped on poly-L-lysine treated coverslips and incubated for 60 minutes at 37℃. Then Cells were fixed with 4% FA for 10 minutes, washed 3 times with PBS, and blocked with 20% FBS-PBS for 45 minutes. Then Kv1.3 channels were labeled as detailed at colocalization, and the next day cells were permeabilized with 0.1% TritonX-100, and blocking was performed with 20% BSA-PBS for 45 minutes at RT. Cells were washed 3 times with PBS and labeled with the primary antibody of CD19 (HIB19 by Biolegend in 10 % BSA-PBS and incubated at 4℃ overnight. Afterwards, cells were washed again with PBS 3 times, and anti-mouse AlexaFluor-647 IgG was added (1:1000, 45 minutes, RT). Finally, the cells were washed with PBS, and coverslips were mounted onto microscopy slides using Fluoromount-G. The samples were examined by the confocal microscope Nikon-, with the excitation lasers set to 488, 561, and 647 nm, and the images were analyzed by Image J software. To evaluate the Kv1.3 accumulation ratio, we used the method described previously.

3.6 Cytotoxicity assays

3.6.1 Cytotoxic function validation of Jurkat-CAR

To evaluate the cytotoxic potential of our CAR cells and to confirm the model, we conducted a Calcein Red-AM based cytotoxicity assay. Initially, Raji cells (the target cells) were stained with Calcein Red-AM. A total of 3×10^6 cells were centrifuged for 5 minutes at 300 x g and subsequently resuspended in PBS. The cells were stained with 1 µM of the dye for 30 minutes at 37 °C. Following this, the cells were plated into an 8-well Ibidi chamber containing phenol red-free RPMI supplemented with 1% FBS. In the co-culture setup, the ratio of target to effector cells was maintained at 1:2. The cells were then incubated for 3 hours in a humidified cell culture incubator at 37 °C. The recorded images were analyzed using Image J software with the ROI tool to assess the intensity of Calcein Red-AM.

3.6.2 Cytotoxic validation of CAR-T cells

To examine the specific cytotoxic effects of CAR T cells, we employed a luciferase-based cytotoxicity assay. MDA, JIMT-1, and N87 cells that express eGFP/ffLUC were plated in 96-well flat bottom plates at a density of 3×10^4 cells per well in triplicate. After 24 hours, various effector cells were introduced at a 0.5:1 or 1:1 ratio of effector to tumor cells. The wells that did not contain effector cells acted as untreated controls. Following another 24 hours, luciferase activity was measured using a luciferase assay kit in accordance with the manufacturer's guidelines (Promega, Madison, WI, USA) and a Synergy HT luminometer (BioTek, Winooski, VE, USA).

3.7 CAR and Kv1.3 colocalization

Jurkat CAR cells were cultured on poly-L-lysine coverslips for 30 minutes at 37 °C (2×10^5 cells / coverslip) within a humidified chamber, followed by three washes with PBS at room temperature. The cells were then fixed using 1% formaldehyde for 10 minutes at room temperature. To prevent non-specific binding, 20% FBS in PBS was added to the cells for 30 minutes at room temperature. For the staining of Kv1.3, we employed the anti-Kv1.3 (KCNA3) extracellular antibody (Alomone Labs) Rabbit IgG at a dilution of 1:50 in 10% FBS-PBS, incubating overnight at 4 °C. Subsequently, the cells were washed with PBS, and the secondary antibody, Alexa 647 goat anti-rabbit, was applied at a dilution of 1:800 in PBS for 45 minutes on ice in the dark. The coverslips were washed three times with PBS and then mounted onto slides using Fluromount G. Images were captured using a Nikon N–STORM confocal microscope. To detect the signal, laser wavelengths of 488 and 647 nm were utilized based on the fluorescent intensity of GFP and Alexa Fluor 647. The recorded images were analyzed with Image J software utilizing the colocalization 2 plugin.

3.8 Immuno-staining for CAR-synapse

To explore the immunological synapse, which will be referred to as the CAR-synapse, between Jurkat CAR and Raji cells, a 1:1 ratio was utilized to form the synapse. Initially, the cells were combined and co-centrifuged for one minute at 37°C with a force of 200×g. The resulting mixture was then placed onto coverslips that had been treated with poly-L-lysine and incubated for sixty minutes at 37°C. Subsequently, the cells were fixed using 4% formaldehyde for ten minutes, washed three times with PBS, and blocked with a solution of 20% FBS-PBS for forty-five minutes. Following this, Kv1.3 channels were labeled as per the colocalization protocol,

and the next day, the cells were permeabilized using 0.1% TritonX-100, with blocking conducted using 20% BSA-PBS for forty-five minutes at room temperature. The cells were washed three times with PBS and labeled with the primary antibody CD19 (HIB19 from Biolegend) in 10% BSA-PBS, followed by incubation at 4°C overnight. Afterward, the cells were washed again three times with PBS, and anti-mouse AlexaFluor-647 IgG was introduced (1:1000 dilution, for forty-five minutes at room temperature). Finally, the cells were washed with PBS, and the coverslips were mounted onto microscopy slides using Fluoromount-G. The samples were analyzed using a Nikon confocal microscope, with excitation lasers set to 488, 561, and 647 nm, and the images were processed using Image J software. To assess the accumulation ratio of Kv1.3, we employed the method previously described

3.9 Ca^{2+} imaging in CAR synapse

The immune synapse was established between Raji and Jurkat CAR cells / Jurkat NT. To stain the target cells, we utilized the CMTPX cell tracker (ThermoFisher, C34552). A total of 1×10^6 Raji cells were centrifuged for 5 minutes at 300×g, after which 1μM of CMTPX was introduced into serum-free culture medium. The cells were incubated for 30 minutes at 37 °C in the absence of light. Subsequently, the cells were washed twice and resuspended in phenol red-free RPMI medium supplemented with 10% FBS. The labeled Raji cells were then plated in a petri dish alongside the Jurkat-CAR cells at a 1:1 ratio. The Ca^{2+}-response was recorded using FURA-2 as previously described, with the exception that images were captured every 30 seconds for one hour without altering the solutions or applying TG. The recording commenced at the moment the Raji cells were added. Additionally, the same experiment was conducted with crosslinking of Kv1.3 and the inclusion of only a secondary antibody (IgG isotype) as detailed below. Only the engaged cells were subjected to analysis.

3.10 Kv1.3 Crosslinking

Kv1.3 channels were immobilized using antibody complexes formed by rabbit anti-Kv1.3 antibody and anti-rabbit goat IgG. Initially, Jurkat-CAR cells were placed on ice for one hour and incubated with polyclonal anti-Kv1.3 antibody targeting an extracellular epitope at a ratio of 100:1 of antibody to Kv1.3 α subunit (0.8 μg antibody per 10 ml of cell suspension containing 2 million cells/ml). This concentration was established based on the average expression of approximately 400 Kv1.3 channels per cell, considering that each channel comprises four α subunits. Following the incubation with Kv1.3 antibody, a subsequent 30-

minute incubation with anti-rabbit IgG was conducted. Control cells underwent identical experimental procedures, with the addition of an isotype IgG, referred to as the Ab2 condition.

3.11 Statistical analysis

For our statistical analysis, we used the GraphPad Prism software version 8.0.1. One-way ANOVA or ANOVA tests were performed for the multiple comparison. As for the two groups' comparison, we conducted the unpaired t test or Mann–Whitney test. The value of $p < 0.05$ was set as a significant difference. The results were shown as the mean ± standard error of the mean (SEM).

4. RESULTS

4.1 Establishment of Jurkat-CARs anti-CD19

As the first step, our aim was to establish a 3rd generation CD19-CAR model targeting the CD19 molecule on the B- cell surface. The Jurkat T cell line generated stably expressing this CD19-CAR construct (Jurkat-CAR) via retroviral transduction was evaluated by fluorescence-activated cell sorting (FACS) for sGFP positivity, i.e. CAR presenting cells. In order to evaluate the functionality of this model, we performed a Calcein Red-AM-based killing assay adopted from Carsten Kummerow et al. Calcein Red-AM is a cell-permeant fluorescent probe. The cell vitality of the cells is proportional to the signal intensity of the Calcein Red. The increase in the nonspecific membrane permeability of target cells, here we used $CD19^+$ Raji B cell line as a target cell, leads to efflux of the dye which reports the irreversible phase of cell death. The results of the killing assay shown in reveal that the intensity of Calcein Red dropped when Raji cells were co-cultured for 3 hours with Jurkat-CARs, unlike for non-transduced Jurkat cells (NT-Jurkat): this concludes that Jurkat-CARs specifically eliminates the target cells even within this short period. Therefore, the generated CAR-expressing model cell line could be applied in the subsequent experiments related to the ion channel, specifically Kv1.3

4.2 Biophysical characterization of Kv1.3 channel on Jurkat-CARs

After the validation of the cell model used in the first step of this study, we attempt to investigate the effect of the introduction of CAR into the Jurkat T cell line on the kinetics of the voltage-gated potassium channel Kv1.3, which contributes to the current-activated pathway in T cells. Current of Kv1.3 channels in Jurkat-CARs, NT-Jurkats and Jurkat-PBMNs were analysed to determine the biophysical parameters of the channel. The activation and inactivation kinetics were the same in Jurkat-CARs compared to NT-Jurkats and Jurkat-PBMNs (τa for NT-Jurkat is 0.65 ± 0.04 ms, τa for Jurkat-PBMN is 0.64±0.04 ms,τa for Jurkat-CAR is 0.85 ±0.08 ms), (τi: NT-Jurkat: 201.3 ± 21 ms, Jurkat-PBMN: 168.2 ± 11.4 ms, Jurkat-CAR: 220.4 ± 20ms). Next, we also evaluated the equilibrium parameters of membrane potential dependence of activation: the test-potential vs. normalized conductance curves show that the slope factor (k) is the same for Jurkat-CARs/NT-Jurkats and Jurkat-CARs/ PBMN-Jurkats (slope: NT-Jurkat: 9.84 ±0.6 mV, Jurkat-CAR: 10.95 ±0.6 mV, Jurkat-PBMN: 13.80 ± 1mV, $p > 0.05$). The half-maximal voltage ($V_{1/2}$) was higher for the Jurkat-CARs than NT-Jurkats (V_{half} for NT-Jurkat: -29.24 ±3.2 mV, Jurkat-CAR: -20.37 ± 1.9mV) as indicated by the rightward shift of the steady-state activation curve. The comparison of the half-maximal

voltage between NT-Jurkats/Jurkat- PBMNs and Jurkat-CAR/Jurkat-PBMN was non-significant (V_{half} for Jurkat-PBMN: -23.43 ± 1 mV, $p>0.05$). These results clearly show that the Kv1.3 steady-state activation is slightly affected by the presence of the CAR receptor on the membrane and partially due to the viral transduction itself.

Driven by these discoveries, we subsequently conducted a Ca^{2+}-imaging assessment to gain an understanding of this pathway.

4.3 Jurkat-CARs has a lowered TG-induced Ca^{2+}- response

To understand the functional expression of CRAC channels, we conducted FURA-2 based Ca^{2+}-imaging experiments. Following stimulation with thapsigargin(TG) and the reintroduction of 2 mM extracellular Ca^{2+} even in the presence of TG, an increase in cytosolic Ca^{2+} was observed, attributed to the influx of Ca^{2+} through the pore created by CRAC channels.The control NT-Jurkat and Jurkat-PBMN cells had a typical Ca^{2+}-response , while the representative trace of the cytosolic Ca^{2+} level (average of 20-30 cells) displays that Jurkat-CARs had a significantly lower CRAC-related response, i.e. the SOCE is lowered. The ratio of intensity ratios detected in the presence of 2 mM Ca^{2+} over the baseline (0 mM Ca^{2+} solution with TG, just before addition of 2 mM Ca^{2+}) was significantly higher in NT-Jurkat compared to Jurkat-CARs and also significantly different between Jurkat-PBMN and Jurkat-CAR ($p=0.01$). These findings report that the effect observed on the Ca^{2+} response is linked to the CAR expression and not because of the retroviral transduction.

4.4 Kv1.3 channel is co-localized with the CAR receptor and present in the IS

The immune synapse (IS) represents a dynamic interaction between the target cell and the effector cell. Prior research has indicated that Kv1.3 channels relocate into the IS, suggesting the potential to modulate the activity of this channel to influence the signaling initiated within the IS. Consequently, we investigated the localization of CARs and Kv1.3 channels in both standalone (co-localization) and IS-engaged (Kv1.3 redistribution) Jurkat-CAR cells. Confocal images of Jurkat-CAR cells labeled with Kv1.3 antibody are presented, where the patchy fluorescence of Kv1.3 channels and the sGFP signal of CAR exhibit significant overlapping regions. Utilizing Image J software, we assessed the co-localization of Kv1.3 and CAR, discovering that the Pearson correlation coefficient (R-value exceeding 0.54) indicates the proximity of these two proteins within the cell membrane. No prior studies have reported the presence of the Kv1.3 channel in the immunological synapse formed between a CAR T cell

and a target Raji B cell (hereafter referred to as the CAR synapse or CS to distinguish it from the CD3/MHC related immune synapse). Therefore, we established a CAR synapse between Jurkat-CAR cells and Raji cells, evaluating the rearrangement of Kv1.3 channels within the contact region between the effector and target cells. To verify the formation of the CS, we monitored the accumulation of CAR in the contact area of the two cells .

4.5 The inhibition of the Kv1.3 recruitment to the synapse abolishes target cell elimination of Jurkat-CAR cells

The role of Kv1.3 in the CAR-synapse formed between Jurkat-CARs and Raji cells remains unclear. Our previous findings indicated that the use of the specific blocker Vm24 to inhibit Kv1.3 enhances the cytotoxic potential of CAR T cells. In this study, we aimed to investigate whether the inhibition of Kv1.3's relocation to the synapse affects the canonical function of CAR cells, specifically their ability to eliminate target cells. As in prior experiments, we assessed the killing capacity of Jurkat-CARs by obstructing the accumulation of Kv1.3 channels at the contact site through antibody cross-linking, as previously outlined, and subsequently co-incubated these modified cells with Raji target cells. The results illustrate that the immobilization of Kv1.3 channels diminished the target cell elimination capability of Jurkat-CARs unlike the effect with cells treated solely with the secondary antibody. These results indicate that the disruption of Kv1.3 channels' membrane redistribution to the contact site in Jurkat-CARs significantly impacts their target elimination efficiency.

4.6 Kv1.3 location affects the Ca^{2+}response of Jurkat-CARs

It has been previously reported that the redistribution and accumulation of the Kv1.3 channel within the immune synapse plays a role in shaping Ca^{2+}-signaling. Additional studies have indicated that the residency of ion channels (Orai1/CRAC and Kv1.3) at the immune synapse affects the Ca^{2+}-response of T cells. Therefore, we conducted Ca^{2+}-imaging measurements while forming a CAR-synapse between a Jurkat-CAR and a Raji cell to explore the impact of inhibiting the trafficking of Kv1.3 into the synapse. These experiments were structured to assess the Ca^{2+}-response of NT-Jurkat cells, Jurkat-CARs, Jurkat-CARs with cross-linked Kv1.3, and Jurkat-CARs in the presence of the isotype IgG (secondary antibody), referred to as the Ab2 condition. Upon interacting with the target cell, all NT-Jurkat cells exhibited, as anticipated, no alteration in the baseline Ca^{2+}-level, which remained constant throughout the entire recording duration. In contrast, Jurkat-CARs demonstrated a varied response in Ca^{2+}-level upon contact with a Raji cell: 1) the majority (47%) of cells exhibited an oscillatory

response until the conclusion of the recording period 2) a smaller proportion of cells (30%) displayed oscillatory Ca^{2+} levels with a decreasing baseline , and 3) a minority (23%) showed isolated Ca^{2+}-spikes without oscillation . In the case of Jurkat-CARs in the presence of Ab2, the calcium response was comparable to that observed in its absence. Similar to NT-Jurkat cells, the Kv1.3 trafficking-restricted Jurkat-CAR cells (where Kv1.3 was cross-linked prior to Fura-2 loading) did not exhibit any change in the intracellular Ca^{2+}-level upon interaction with a Raji cell. The variations observed in Ca^{2+}-signaling may be attributed to differences in downstream signaling pathways, underscoring the significance of Kv1.3 relocation in the initiation of cell death mechanisms. This may stem from the differences in immunoreceptor tyrosine-based activation motifs (ITAMs) between the TCR and CAR that initiate the phosphorylation cascade.

4.7 Kv1.3 and KCa3.1 expression on CAR T cells

The incorporation of the CAR construct into T cells can influence the expression of ion channels in the cell membrane. This alteration can significantly impact the Ca^{2+}-dependent cellular processes within T cells. Therefore, we initially examined whether the levels of the ion channels KCa3.1 and Kv1.3 in CAR T cells were affected by the transduction of the specific CAR protein. Utilizing the patch clamp technique, we assessed the expression levels of these two channels in third-generation CAR T cells compared to control cells (non-transduced or NT T cells). We implemented the ramp protocol (with 1 µM free Ca^{2+} in the pipette solution) to simultaneously record the currents of the KCa3.1 and Kv1.3 channels. The KCa3.1 level is indicated by the slope of the curve, where the Kv1.3 channels remain inactive (more negative than −40 mV), while the peak current of +50 mV, observed at the conclusion of the ramp after subtracting the KCa3.1 current, reflects the magnitude of the Kv1.3 current. Through this protocol, we established the expression levels of these two channels in both control/non-transduced (NT) and CAR T cells: Results demonstrate that the KCa3.1 whole-cell, capacitance-normalized conductance level was elevated in the third-generation CAR T cells compared to the NT T cells (0.31 nS/pF for NT versus 0.64 nS/pF for CAR T cells; $p = 0.009$). Conversely, the Kv1.3 level did not exhibit any significant difference when compared to the NT cells (1.19 nS/pF for NT versus 1.25 nS/pF for CAR T cells; $p = 0.77$).

4.8 Difference in Kv1.3 and KCa3.1 expression on $CD4^+$ and $CD8^+$ CAR T cells

The third-generation CAR T cells exhibited a significantly higher expression of KCa3.1 in comparison to the control group, while the expression levels of Kv1.3 remained unchanged. Subsequently, we aimed to determine whether there was a variation in the expression of the ion channels, KCa3.1 and Kv1.3, between the $CD4^+$ and $CD8^+$ CAR T cells, given that the cytotoxic efficiency differs between these two populations. Therefore, we assessed the conductance of KCa3.1 and Kv1.3 in these subsets of T cells. We utilized the same protocol as previously described, with the exception that we employed the antibody adhesion technique to isolate the $CD4^+$ or $CD8^+$ subpopulations. Our findings indicated that the $CD8^+$ CAR T cells demonstrated a higher level of KCa3.1 expression when compared to the CD8 NTs (0.42 nS/pF for $CD8^+$ NT and 0.895 nS/pF for $CD8^+$ CAR T cells; $p = 0.04$); however, no significant differences were observed in the KCa3.1-normalized conductance of the $CD4^+$ cells or in the Kv1.3 expression across all groups .

4.9 $CD8^+$ CAR T cells Ca^{2+} response is suppressed

As previously stated, the KCa3.1 and Kv1.3 channels are responsible for maintaining the negative membrane potential, which is crucial for facilitating the influx of Ca^{2+} through CRAC channels. The activation of CRAC initiates various pathways that are integral to the effector functions of T cells. Consequently, the Ca^{2+} response induced by thapsigargin (TG) in third-generation $CD4^+$/$CD8^+$ CAR and NT T cells was evaluated by measuring the changes in cytosolic Ca^{2+} using FURA-2 ratiometric imaging. Our results illustrate the time course of the Ca^{2+} measurements. The average traces presented indicate that all T cell types exhibit a similar baseline Ca^{2+} level, contrasting with the Ca^{2+} response observed during CRAC activation and the subsequent reintroduction of extracellular Ca^{2+}. Our findings reveal that the Ca^{2+} responses of NT $CD8^+$, NT $CD4^+$, and third-generation $CD4^+$ CAR cells are comparable, whereas the $CD8^+$ CAR cells displayed a significantly diminished response in comparison to the control cells. The 2 Ca^{2+} /0 Ca^{2+} ratio (peak over baseline), which reflects CRAC expression in the cells, was significantly lower in the third-generation $CD8^+$ cells relative to the $CD8^+$ NT control ($p = 0.02$). These findings may imply that the Ca^{2+} influx through the CRAC channel is influenced by the presence of the CAR in the membrane, potentially affecting their cytotoxic capabilities.

4.10 The suppression of Kv1.3 and KCa3.1 facilitate the killing potential of $CD8^+$ CAR T cells.

$CD8^+$ cytotoxic cells are likely to play a crucial role in the eradication of tumor cells, with K^+ ion channels contributing to the modulation of Ca^{2+} signaling. Subsequently, we examined the cytotoxic activity of HER2-specific $CD8^+$ CAR T cells following the administration of Vm24 (a specific Kv1.3 blocker at 1 nM) and TRAM-34 (a KCa3.1 antagonist at 1 μM) in a firefly luciferase (ffLuc) activity-based cytotoxicity assay, utilizing a 1:1 effector to target (E:T) ratio with ffLuc-modified MDA-HER2, JIMT 1, and N87 target cells. MDA cells lacking HER2 expression were utilized as HER2-negative controls. Our findings indicate that CAR T cell populations effectively recognized and eliminated HER2+ target cells, whereas NT T cells exhibited no cytolytic activity, thereby confirming their specificity. The introduction of the Kv1.3 inhibitor Vm24 significantly enhanced the target cell killing capacity of the $CD8^+$ CAR T cells, irrespective of the target cell type (MDA-HER: approximately 51%, JIMT-1: approximately 36%, and N87: approximately 55% reduction in luciferin intensity. Furthermore, the addition of TRAM-34 (the KCa3.1 blocker) also augmented the short-term killing efficacy of the CAR T cells in vitro against the HER2+ monolayer target cell cultures for each cell line evaluated (MDA-HER2: approximately 65%, JIMT-1: approximately 53%, and N87: approximately 46% decrease in luciferin intensity.

5. DISCUSSION

5.1 Investigate the functional role of Kv1.3 channel localization in Jurkat-CAR cell

In this part of the thesis, we sought to determine the role of Kv1.3 channel in Jurkat-CAR, which was reported previously as an adequate approach for characterization of CAR in T cells. By employing the whole-cell patch-clamp technique, we revealed the biophysical characteristics of the Kv1.3 channel in Jurkat-CAR model. Our findings indicate a slight shift in the steady-state activation curve towards depolarizing potentials (rightward) in Jurkat-CARs, which is likely to influence Ca^{2+}-signaling. Driven by this observation, we assessed the Ca^{2+}-response of Jurkat-CARs: the CRAC-dependent Ca^{2+}-amplitudes were reduced when compared to NT-Jurkat and Jurkat-PBMN cells. We hypothesize that the introduction of CAR may alter the expression of proteins that regulate thapsigargin-induced Ca^{2+}-uptake. Alternatively, the alteration in Kv1.3 activation could result in a decreased Ca^{2+}-level. Nevertheless, the optimal intracellular Ca^{2+}-level may vary for different functions, suggesting that this reduction could enhance the cytotoxic potential of Jurkat-CARs.

Furthermore, we demonstrated the co-localization of Kv1.3 with the CAR receptor, indicating a functional relationship between the CAR and the channel. On the other hand, we showed the accumulation of CARs alongside Kv1.3 channels at the synapse between Jurkat-CAR and Raji cells. Previous reports have indicated that the localization and persistence of Kv1.3 channels in T cells at the immunological synapse modulate the Ca^{2+}-response, which may contribute to T cell hyperactivity. The co-localization of Kv1.3 with CD3 has been reported in T cells, suggesting that their proximity could have functional significance, as proposed in earlier research. However, it is important to note that CD3 and exogenous Kv1.3 reside in different regions of the immunological synapse in CD4 cells. To investigate how the redistribution of Kv1.3 at the synapse affects effector functions, we inhibited Kv1.3 channel IS-trafficking in Jurkat-CARs as previously described.

The crosslinking experiment clearly demonstrated that the recruitment of the Kv1.3 channel is crucial for the effective performance of Jurkat-CARs: inhibiting Kv1.3 synapse redistribution partially diminished the killing capacity. We believe this can be explained by the followings: i) not all Jurkat cells express Kv1.3, allowing these cells to still induce cell death in target cells, ii) since Kv1.3 and CAR are likely located within the same membrane domain, the immobilization of Kv1.3 disrupts CAR's relocation to the synaptic area, resulting in no formation of encounters with target cells, iii) the Ca^{2+}-dependent pathway is not the sole mechanism for target cell elimination. The first point can be dismissed, as patch-clamp studies

revealed the presence of Kv1.3 current in nearly every Jurkat-CAR cell. The second point is also unlikely, given that the number of CARs significantly surpasses the number of Kv1.3 channels in Jurkat-CAR cells. We believe there is enough CARs that can migrate to the synapse and trigger cell death in target cells. It is possible that the activation independent of Kv1.3 and Ca^{2+}-influx through CD28 and 4-1BB could explain this nuanced situation.

To reveal how the crosslinking of Kv1.3 channels influences the Ca^{2+}-dependent pathway, we examined the Ca^{2+}-response in Jurkat-CAR cells. When NT-Jurkat cells got in contact with Raji-target cells, no Ca^{2+} signal was detected over an extended duration (up to 60 minutes), clearly showing the specificity of the CAR-CD19 interaction. In contrast, the Ca^{2+}-signaling in Jurkat-CARs (and Jurkat-CARs treated solely with the secondary antibody, Ab2 condition, exhibited significant differences: the Ca^{2+}-levels showed periodic/oscillatory fluctuations that persisted until the 60^{th} minute. Previously, we indicated that the Ca^{2+}-response in Jurkat cells could be characterized by a single-peak response, with a few oscillations occurring over a brief period when these cells were stimulated via the CD3/TCR complex. A distinction in Ca^{2+} signaling induced by TCR and CAR has been noted before; however, the flow cytometric analysis conducted here does not permit a suitable comparison. The crosslinking of Kv1.3 entirely eliminated the changes in Ca^{2+} levels during the formation of the Jurkat-CAR - Raji encounter. Nicolaou et al. previously reported that Ca^{2+}-signaling is also influenced by the localization of Kv1.3 at the immunological synapse, although their experiments involved T cells being activated through the CD3/CD28 pathway. Therefore, we can conclude that modulating Kv1.3 could serve as a potential target for achieving improved therapeutic outcomes. It is important to mention that Jurkat cell line do not express the KCa3.1 channel, which contributes as well to the Ca^{2+} activated pathway Via this model, we wanted to examine the role of the Kv1.3 channel alone. The next step would be to study the role of ion channels in primary T cells.

5.2 Determine the role of ion channels in the target cell killing potential of CAR T therapy

CAR T cell immunotherapy has emerged as a significant advancement in recent years. Despite impressive clinical successes, the ideal engineering of CAR cell constructs remains unresolved. Patients undergoing this treatment may experience life-threatening toxicities, and the limited effectiveness against solid tumors presents a challenge to address. Ion channels in T cells, such as Kv1.3, KCa3.1, and CRAC, have been identified as crucial in regulating various cellular functions. The inhibition or knockdown of these channels impairs the proliferation and effector functions of T cells. However, there is still limited understanding of how these ion channels contribute to anti-tumor immunity in eliminating cancer cells, particularly in CAR T cells.In this part of the study, we examined the functional expression of Kv1.3 and KCa3.1 in third-generation CAR T cells that target the HER2 protein found in specific breast cancer cell types. Initially, we assessed whether the expression of CAR on the surface of human T cells would induce changes in the whole-cell conductance of these two channels. Our results indicated that the conductance of KCa3.1 in third-generation CAR T cells was greater than that in control cells, while Kv1.3 conductance remained unchanged. These findings clearly demonstrate that the expression of this ion channel is not inhibited by the introduction of CAR into $CD3^+$ cells, suggesting that the effector function and Ca^{2+}-dependent signaling remain unaffected by significant alterations in the expression of these channels. Moreover, the heightened activity of KCa3.1 may enhance the chemokine-driven migratory ability of CAR cells, potentially aiding their penetration into tumors. It is generally recognized that $CD8^+$ CAR T cells exhibit greater killing efficacy due to their elevated lytic activity. Consequently, we evaluated the expression of ion channels in third-generation CAR T cells, specifically $CD4^+$ and $CD8^+$ populations: the $CD8^+$ CAR T cells displayed significantly higher levels of KCa3.1, whereas the $CD4^+$ cells showed no notable changes in the expression of either KCa3.1 or Kv1.3 when compared to the corresponding NT control group .Nonetheless, it is worth noting that KCa3.1 conductance was also increased in $CD4^+$ CARs relative to NT cells, although this difference was not statistically significant. Previous studies have indicated that KCa3.1 in $CD8^+$ T cells is involved in chemokine gradient-induced migration, which may direct them towards the tumor site. We believe that this enhanced activity of KCa3.1 could promote the infiltration of $CD8^+$ CAR T cells into solid tumors, resulting in a favorable therapeutic outcome. Additionally, it has been demonstrated that the anti-tumor cytotoxicity of CAR T cells correlates with the

cytokine/chemokine profile. We propose that ion channels may play a role in these activated pathways, underscoring the necessity for further research.

Subsequently, we examined the Ca^{2+}-response in CAR T cells: the cytosolic Ca^{2+} concentration is regarded as a vital trigger for regulating a variety of effector functions. The influx of Ca^{2+} through the CRAC channel is modulated by the coordinated activity of ion channels within the T cell membrane; therefore, we opted for thapsigargin-induced activation of CRAC, which circumvents the TCR/CD3 activation pathway and offers direct evidence of ion channel regulation of the Ca^{2+}-response. In our present research, we explored whether the expression of the anti-HER2 CAR would affect the Ca^{2+}-response. Our findings indicate that in the case of $CD8^+$ CAR T cells, the Ca^{2+} influx is diminished compared to $CD8^+$ NT cells. Conversely, $CD4^+$ CAR and NT T cells exhibited similar Ca^{2+} signaling triggered by the Ca^{2+} release from the ER. Given that the expression of KCa3.1 was elevated in CD8 CARs, potentially leading to increased SOCE, we hypothesize that the expression of CRAC (ORAI1 and STIM1) should be lower in $CD8^+$ CAR T cells. Finally, we investigated whether the inhibition of K^+ channels (Kv1.3 and KCa3.1) affects the efficacy of $CD8^+$ CAR T cells in killing target cells. Prior research indicated that TRAM-34 effectively inhibits both the native and cloned KCa3.1 channels in human T lymphocytes (K_d of 20–25 nM) and is 200 to 1500-fold more selective than other ion channels. Additionally, Vm24, with a K_d of approximately 3 pM, shows a high selectivity for Kv1.3 channel. Our findings revealed that a decrease in Kv1.3 channel conductance (achieved by nearly completely blocking the channels with an approximate 300-fold K_d of Vm24) enhanced the CAR T cells' killing efficiency. Furthermore, antagonizing KCa3.1 channels also aided in the elimination of $HER2^+$ cells. Although one might expect that blocking KCa3.1 and Kv1.3 channels would hinder CTL function, this assumption may not hold true for CTLs and NKs. Previous studies demonstrated that inhibiting KCa3.1 with TRAM-34 in natural killer (NK) cells leads to plasma membrane depolarization, which boosts their cytotoxicity and degranulation against target cells. Nevertheless, this blockade did not impact on their migratory capabilities or chemokine expression. Recent research has indicated that specific inhibition of KCa3.1/Kv1.3 channels, which reduce calcium entry (SOCE), enhances NK cell cytotoxicity against the T-ALL Jurkat cell line. It has also been reported that effective target cell killing necessitates an optimal intracellular Ca^{2+} concentration, which can be achieved by impairing the functional expression of CRAC. Given the availability of highly potent and selective blockers, as well as molecular biology tools to modulate the expression of these K^+ channels, we believe that their modification could pave the way for new approaches in CAR T cell therapy.

It is important to mention that our study has certain limitations. Firstly, the signaling pathway, which can be altered with the introduction of the CAR. This requires further investigation to understand the impact of CAR expression such as cytokine production and cell migration. Secondly, the effectiveness of the channel blockers used in this study needs to be assessed i*n vivo,* primary due to the inhibitory nature of the tumor microenvironment.

Here we showed that inhibition of Kv1.3 conductivity with a specific blocker (Vm24) could result in an increased killing rate for CAR-T cells derived from primary $CD8^+$ T cells (long (24-hour) period), which also express KCa3.1, another channel essential for Ca^{2+}-dependent functions. However, for Jurkat-CAR model we obtained that prevention of the Kv1.3 channel redistribution impaired the killing potential (short (3-hour) period). To explain this discrepancy, we must mention that Jurkat cells lack KCa3.1, which could otherwise compensate for the absence of Kv1.3 function in T cells; thus, the killing of target cells may be hindered, at least during the initial phase or short duration.

6. SUMMARY

T cell -Ion channels (Kv1.3, KCa3.1, and CRAC) regulate the activation and effector functions via modulating the Ca^{2+}-dependent pathway. Chimeric antigen receptor (CAR) T cell therapy showed a remarkable role in anti-tumor therapy, especially in the treatment of chemotherapy-resistant liquid cancers. Nevertheless, the mechanisms regulating CAR-T cell function as well as the side effects remain an area of active research.

In our research study, we assessed the expression and role of ion channels in CAR T cells via a Jurkat-cell model and in primary T cells.

Our results from molecular, electrophysiological and functional assays highlight that the KCa3.1 conductance in HER2-specific CAR T cells was higher compared to the non-transduced (NT, control) cells, which was more prominent in the $CD8^+$ population ($CD4^+$ cell also showed elevation). Conversely, the Kv1.3 expression level was the same for all cell types ($CD4^+$, $CD8^+$, CAR, and NT). Single-cell Ca^{2+} imaging revealed that thapsigargin-induced SOCE via CRAC is suppressed in $CD8^+$ CAR T cells, unlike for $CD4^+$ and $CD8^+$ NT cells. The use of specific antagonists (Kv1.3: Vm24; KCa3.1: TRAM-34): showed that the target cell elimination capacity of the $CD8^+$ CAR T cells was improved either by blocking KCa3.1 or Kv1.3. By means of our Jurkat-CAR cell line we could show that Kv1.3 channels is colocalized with CAR and redistributes into the synapse between a CAR and a target cell. The biophysical properties of Kv1.3 channel are not vastly affected by the introduction of CAR in the cells when Kv1.3 is the only expressed channel. The blockage of Kv1.3 channel lateral movement to the synapse affects the killing potential of CAR-T cells, likely through disruption of the Ca^{2+}-response upon IS formation. Overall, these data suggest that the manipulation of the Kv1.3 channel may contribute to the improvement of CAR-T immunotherapy and provide new insights for future clinical strategies.

7. APPENDIX

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Candidate: Ghofrane Medyouni
Doctoral School: Doctoral School of Molecular Medicine

List of publications related to the dissertation

1. **Medyouni, G.**, Vörös, O., Panyi, G., Hajdu, P.: Functional role of Kv1.3 localization in CAR-T cells. *J. Immunol. [Epub ahead of print]*, 2025.
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2. **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.
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List of other publications

3. 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.
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   IF: 4.9

Total IF of journals (all publications): 12,7
Total IF of journals (publications related to the dissertation): 7,8

DEBRECENI EGYETEM Egyetemi és Nemzeti Könyvtár

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.

15 September, 2025