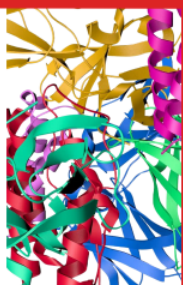


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Abstract

Genetically modified chimeric antigen receptor (CAR) T cells eliminate tumors by recognizing specific antigens on the cell surface. T cell ion channels (e.g. Kv1.3, KCa3.1) influence activation, proliferation, and effector functions such as target cell killing, through the regulation of Ca^{2+} signaling. We showed that CAR-expressing cells (Jurkat) specifically eliminate tumor cells (Raji B cells) in monolayer culture and inhibition of KCa3.1 by TRAM34 increased the tumor cell-killing ability of KCa3.1⁺ Jurkat CARs. Blockage of KCa3.1 facilitated the migration of KCa3.1⁺ Jurkat CARs (mean speed, displacement and distance). The application of TRAM34 lowered the baseline Ca^{2+} level in mCherry-KCa3.1⁺ Jurkat CARs. Finally, TRAM34 significantly reduced the time needed to eliminate tumor cells. We concluded that expression and modification of KCa3.1 ion channels shifts the intracellular Ca^{2+} concentration into the range where cytotoxicity dominates. Hence, modification of KCa3.1 channels could contribute to a more effective anticancer immunotherapy.

Keywords CAR T cell, cytotoxicity, immunotherapy, KCa3.1, migration

Introduction

Chimeric antigen receptor (CAR) T cells are T cells with a CAR created by genetic manipulation of T cells.^{1,2} Unlike endogenous T cell receptors (TCRs), these receptors are able to recognize antigens in the absence of major histocompatibility complex/HLA presentation.^{1,3} CARs consist of 4 main domains: an extracellular antigen-recognition ectodomain (scFv), which consists of variable light and variable heavy chains of an immunoglobulin, a hinge region, a transmembrane domain responsible for receptor stability and cell surface expression, and 1 or more intracellular endodomain that allows recruitment and phosphorylation of signal proteins.^{1,4} T cells expressing synthetic CAR are able to recognize the specific antigen on the tumor cell surface and then eliminate the tumor.⁵ First-generation CARs have only 1 CD3 ζ signaling domain, but the second generation contains a costimulatory (CD28/4-1BB/OX-40) region, which enhances T cell activation, proliferation, and survival.^{6,7} In the third generation, 2 costimulatory signaling domains (CD28-OX40 or CD28-41BB) were combined to generate the proinflammatory environment, rapid tumor elimination, and long-term survival of CARs.^{1,7,8} In the case of fourth-generation CARs (TRUCK), the endodomain contains NFAT (nuclear factor of activated T cells) specific cassettes, the activation of which can regulate cytokine production and subsequent cell activation, proliferation, and effector functions.^{3,6} The fifth-generation CARs

contain a truncated cytoplasmic IL-2 receptor β -chain domain that binds to the transcription factor STAT3, thereby inducing cytokine release.^{3,7} The advantage of CAR T cell therapy would be that it systematically and effectively kills tumor cells without damaging the surrounding healthy cells.⁸ The Food and Drug Administration has already approved in more than 6 CAR T cell therapies for hematological malignancies such as large B cell lymphoma.⁹ Despite the success of the therapy, several challenges remain in improving the efficacy and safety of CAR T cell therapy due to CAR T cell depletion, toxicity effects, and suppressive tumor microenvironment solid tumors.^{10–13} The efficacy of CAR T cell therapy can be improved by combining it with various immunotherapeutic agents or other therapeutic methods (radiotherapy or chemotherapy) that can promote tumor elimination.¹⁴ There are already developing CAR designs that are reversible on/off and equipped with universal CARs to increase safety and efficiency.^{15–18} Anti-PD-L1-expressing nanoparticles can enhance CAR T cell activity and can prevent CAR T cell exhaustion.¹⁹

T lymphocyte ion channels—voltage-dependent Kv1.3, calcium-dependent KCa3.1, and calcium release-activated channel (CRAC) ion channels—play a prominent role in T cell activation, proliferation, migration, and tumor/target cell killing.^{20,21} As a result of antigen recognition of T cells via the TCR/CD3 receptor complex, a signaling cascade is activated, resulting in the activation of phospholipase C_γ (PLC γ). The activated PLC γ converts PIP_2 into soluble

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IP₃. IP₃ binds IP₃-receptor on the endoplasmic reticulum (ER) membrane, thereby inducing Ca²⁺ efflux from the ER via IP₃R. The Ca²⁺ efflux is sensed by the STIM1 subunit of the CRAC, which undergoes a conformational change, translocate to the Orai1 subunit in the plasma membrane and CRAC channel gets activated, resulting Ca²⁺ influx through Orai1 into the intracellular space.^{22–26} As a consequence of increased intracellular Ca²⁺ levels, the cell membrane is depolarized, which is not favorable for Ca²⁺-dependent signaling.²⁷ Depolarization activates the voltage-dependent ion channel Kv1.3, while the elevated Ca²⁺ concentration activates the Ca²⁺-dependent KCa3.1 channel, thus decreasing the membrane potential sufficiently negative value (for T cells: –50 to –60 mV) by K⁺ efflux, thus ensuring the driving force for proper Ca²⁺-dependent signaling.^{28–31} The sustained Ca²⁺ level allows NFAT to translocate to the nucleus, thereby promoting the transcription of several genes (e.g. IL-2, granzyme B, perforin), which promotes T cell activation, proliferation, and effector functions.^{32–35}

The KCa3.1 channel plays an important immunoregulatory role in various cellular processes, such as cell cycle progression, T cell proliferation, activation, migration, and expression of genes that promote their effector function.^{27,36} KCa3.1 dysfunction of CD8⁺ cells from tumor cells limits their chemokine-induced migration, when cells are exposed to high levels of adenosine.³⁷ A key step in T cell activation and effector functions is Ca²⁺ ion influx through CRAC channels. For natural killer (NK) cells and cytotoxic T lymphocytes (CTLs), a recent study also reported that CRAC and KCa3.1 channels is required for optimal target cell elimination.^{38–40} We have previously shown that inhibition of KCa3.1 and Kv1.3 ion channels increases the tumor elimination efficiency of CAR cells in tumor spheroids and that the presence of CAR influences the Ca²⁺ response of cells.⁴¹

CTLs and NK cells play an important role in antitumoral response.^{42–44} Ca²⁺ is essential for lymphocyte function and cancer homeostasis. In CTLs and NK cells, intracellular ([Ca²⁺]_{int}) and extracellular ([Ca²⁺]_{ext}) Ca²⁺ levels may be important for tumor therapy, as these changes may influence cell migration, proliferation, and cytotoxic processes.⁴⁵ For a resting [Ca²⁺]_{int} value of <100 nM, the CTLs and NK cells have slightly reduced cytotoxicity, and their migratory ability is severely limited. An increase in [Ca²⁺]_{int} to 100 to 300 nM (slightly higher for NK cells) is associated with is required to achieve optimal cytotoxic potential. A [Ca²⁺]_{int} above 300 nM is optimal for migration, but cytotoxicity is already slightly reduced. At very high [Ca²⁺]_{int} cytotoxicity and migration are suboptimal, whereas proliferation and apoptosis is dominant.^{40,45}

In this study, we elucidated the role of KCa3.1 ion channels in migration and cytotoxicity of a CAR T cell line generated from a KCa3.1-deficient Jurkat E6-1 line. The Jurkat T cell line has been widely used as a prototypical in vitro model to dissect TCR-mediated signaling pathways and downstream functional responses such as Ca²⁺ influx, immunological synapse assembly, and cytokine production.^{46–48} Moreover, Jurkat cells express endogenous ion channels including voltage-gated Kv1.3 and CRAC components (STIM1/ORAI1), which have been extensively characterized using electrophysiological and pharmacological methods, enabling detailed analysis of channel behavior in a T cell-relevant context.⁴⁷ For these reasons, Jurkat T cells are used as a T cell model in numerous CAR T cell studies.^{49–51} With the use of primary T cell CAR T cells, the role of KCa3.1 channels

were further highlighted and supported our outcomes with Jurkat CAR cells. Our results pinpoint the conclusions that modification of KCa3.1 ion channels can contribute to a more efficacious CAR T cell therapy.

Methods

Cells and culture conditions

HEK 293 T packaging cells, T lymphoblast Jurkat-E6-1 cells (later Jurkat), and CD19⁺ B lymphoblastoid Raji cells were obtained from American Type Culture Collection. HEK 293 T cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 2 mmol/L GlutaMAX and 10% fetal calf serum (FCS) and antibiotics. Jurkat-E6-1 and Raji cells were cultured in RPMI (Sigma-Aldrich) medium supplemented with 2 mmol/l GlutaMAX and 10% FCS 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% CO₂ at 37 °C.

Peripheral blood mononuclear cell isolation and activation

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood as described previously.^{52,53} Briefly, PBMCs were isolated by centrifugation of whole blood using a SepMate PBMC isolation tube (STEMCELL Technologies) with the Ficoll-Paque (GE Healthcare Bio-Sciences) density gradient. PBMCs were separated from freshly drawn human blood, diluted 2-fold with Hanks' Balanced Salt Solution (Sigma-Aldrich; H4891, Ca²⁺- and Mg²⁺-free, 15 mM HEPES was added, pH = 7.4). We measured 15 mL of Histopaque-1077 into the SepMate tube. The diluted blood was added to the SepMate tube up to the Ficoll layer. After centrifugation, the opalescent part, which contained PBMCs, was transferred into a 50 mL tube and washed twice with Hanks' Balanced Salt Solution and resuspended in RPMI medium. PBMCs were maintained in RPMI medium supplemented with 10% FCS, 15 mM HEPES, 2 mM L-glutamine, 1 mM Na-pyruvate, and 200 units of streptomycin/penicillin. Activation was performed for 48 h in a 24-well cell culture plate coated with mouse anti-human CD3 and CD28 antibodies (both 10 µg/mL; BioLegend).⁵³ On day 2, the expansion of T and CAR T cells was subsequently supported with human IL-7 (10 ng/mL) and human IL-15 (5 ng/mL) (Miltenyi Biotec). The study and informed consent forms were approved by the Regional and Institutional Research Ethical Board of the University of Debrecen (RKEB/IKEB No.: 6627-2023).

Retroviral transduction

At 72 hours prior to transfection, 3.5 × 10⁵ HEK 293 T cells were seeded on a 10 cm culture dish in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (FBS) and 1% L-glutamine (Life Technologies). Viral vector particles were produced by transfection of HEK 293 T cells with the CD19-specific CAR-encoding pBMN retroviral plasmid (Addgene; #1734), the mCherry-KCa3.1 encoding pBMN plasmid (Addgene;

#1734), the PAX2 packaging plasmid (Addgene; #12259), and VSVG plasmid (Addgene; #14888) using jetPrime transfection reagent (Polyplus). The backbone of the CAR encodes CD19-scFv, CD8 transmembrane region, CD28, 4-1BB costimulatory and CD3 ζ intracellular signal domain, and sGFP (superfolder green fluorescent protein). Supernatants containing the retrovirus were collected after 48 h. The viral supernatants were added to the Jurkat, along with the polybrene (10 μ g/mL). For T cell transduction the virus binding plate was prepared by centrifugation. Primary T cells were transduced (2.5×10^4 cells/cm ^{2}), with retroviral particles (250 μ L/cm ^{2}) on RetroNectin-coated (Takara) plate, supplemented with IL-7 and IL-15 in case of T cells. Following 72 h incubation, cells were used for further experiments. The transduction efficacy was detected with flow cytometry using nontransduced (NT) T cells and Jurkat CAR cells as a control.

Cell sorting

To select the sGFP-positive (CAR-expressing) and mCherry-positive (KCa3.1-expressing) cells, we used the cytoFLEX Flow cytometer to sort the double positive mCherry-KCa3.1 $^+$ Jurkat CAR cells. At first, the 10×10^6 of the transduced cells were centrifuged and washed with phosphate-buffered saline (PBS) and then resuspended in 1% FBS-PBS. Jurkat CAR cells were used as a negative control. After sorting, cells were centrifuged and incubated in RPMI medium.

Flow cytometry

CD19-specific CAR expression was confirmed as described previously.⁴¹ For CD19 positivity analysis cells were spin (400 g, 5 min, 4 °C), fixed with 1% PFA and incubated for 10 min on ice and in the dark. Cells were spun and washed with 1 $\times$ PBS. For block of Fc receptors, we preincubated the cells with purified anti-CD16/CD32 antibody (1 μ g/10 ^{6} cells in 100 μ L volume 1 $\times$ PBS containing 10% bovine serum albumin) for 5 to 10 min on ice. Cells were spun (400 g), washed with PBS, and stained for 30 min on ice with the Alexa Fluor 647 anti-human CD19 (catalog no. 302220, HIB19; BioLegend) antibody. Analysis was performed on at least 10,000 cells per sample using a NovoCyte 3000RYB (ACEA Biosciences) instrument and NovoExpress software 1.6.2 (ACEA Biosciences).

Electrophysiology

The electrophysiological measurement were performed as described previously.⁴¹ The functional expression of KCa3.1 was measured in NT Jurkat, mCherry-KCa3.1 $^+$ Jurkat, mCherry-KCa3.1 $^+$ Jurkat CAR, and activated T cells using whole-cell patch-clamp configuration at room temperature (23–25 °C) using a Multiclamp 700 B/Axopatch 200 A amplifier connected with an Axon Digidata1440 digitizer (Molecular Devices) to the personal computers. The aspartate bath solution contained the following (in mmol/L): 145 Na-aspartate, 5 KCl, 2.5 mM CaCl $_2$, 1.0 MgCl $_2$, 5.5 glucose, and 10 HEPES (pH = 7.4). Patch-clamp pipette filling solution composition was as follows (in mM): 145 K-aspartate, 10 K $_2$ EGTA, 8.5 CaCl $_2$, 2 MgCl $_2$, 10 HEPES (free Ca $^{2+}$ concentration 1 μ M, pH = 7.2). Patch-clamp pipettes (4–6 M Ω) were pulled from GC150F-15 borosilicate capillaries (Harvard Apparatus). 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. pClamp 10.6 software (Molecular Devices) was used for current recording and subsequent analysis.

Intracellular Ca $^{2+}$ response measurement

Intracellular Ca $^{2+}$ responses were measured as described previously.⁵³ Briefly, the NT Jurkat, NT T, Jurkat CAR, and mCherry-KCa3.1 $^+$ Jurkat CAR cells were loaded with 1 μ M FURA-2 acetoxymethyl ester (AM) (Thermo Fisher Scientific). The cells were perfused with 0 mM Ca $^{2+}$ solution (143.3 mM NaCl, 4.7 mM KCl, 10 mM HEPES, 5.5 mM glucose, 1 mM MgCl $_2$, 0.1 mM EGTA, pH 7.35) to check the cells' integrity and then 1 μ M thapsigargin (TG) (Thermo Fisher Scientific) containing 0 mM Ca $^{2+}$ solution to deplete of ER and then 2 mM Ca $^{2+}$ (143.3 mM NaCl, 4.7 mM KCl, 10 mM HEPES, 5.5 mM glucose, 2 mM CaCl $_2$, 1 mM MgCl $_2$, pH 7.35) containing 1 μ M TG to activate intracellular Ca $^{2+}$ elevation through CRAC. mCherry-KCa3.1 $^+$ Jurkat CAR cells were perfused with 2 mM Ca $^{2+}$ solution containing TG together with 1 μ M TRAM34 (KCa3.1 inhibitor). The amplitude of Ca $^{2+}$ -response for NT Jurkat, Jurkat CAR, and mCherry-KCa3.1 $^+$ Jurkat CAR cells was defined as the difference between peak and baseline (calculated as the mean of ratios before addition of 2 mM Ca $^{2+}$ along with TG) of 340 nm/380 nm intensity ratio of FURA-2.

Intracellular Ca $^{2+}$ -baseline measurement

To test the effect of TRAM34 on the intracellular Ca $^{2+}$ baseline, Jurkat CAR and mCherry-KCa3.1 $^+$ Jurkat CAR cells were plated onto poly-L-lysine-coated glass-bottom petri dishes and were loaded with 1 μ M FURA-2 AM (Thermo Fisher Scientific) dissolved in dimethyl sulfoxide (DMSO) and incubated for 25 min at 37 °C in phenol red-free RPMI solution (Sigma-Aldrich) supplemented with 10% FBS, 2 mM L-glutamine, 1 mM Na-pyruvate, and 200 units penicillin/streptomycin. The cells were washed with phenol red-free RPMI solution and placed on a 37 °C stage of an inverted fluorescence microscope, and the Ca $^{2+}$ signal was recorded as described previously. After 45 min, the cells were perfused with 1 μ M TRAM34 containing phenol red-free RPMI solution. The Ca $^{2+}$ level was measured until the 90th minute after addition of TRAM34. For evaluation, the Ca $^{2+}$ signal in each cell was background corrected. Only sGFP- and mCherry-expressing cells were selected for evaluation.

Live-or-Dye-based killing assay

The killing capacity of mCherry-KCa3.1 $^+$ Jurkat CAR cells in monolayer cell cultures were determined by Live-or-Dye 640/662 (Biotium) incorporation assay. CD19 $^+$ Raji cells were cocultured with effector cells for 3 h. In the cocultures the effector-to-target (E:T) ratio was 2:1. Raji cells incubated with NT Jurkat cells served as a control. The ion channel inhibitor TRAM34 (1 μ M) and DMSO were added to the required samples. After 3 h, cocultures were labeled with Live-or-Dye 640/662 stain, as detailed in the manufacturer's protocol. The confocal images of cells were taken with Nikon-STORM confocal microscope. ImageJ (National Institutes of Health, 1.54F version) was used to calculate the incorporation of Live-or-Dye 640/662 stain, and presence of sGFP and mCherry positive Jurkat CAR cells. The total cell number, number of Jurkat CAR or mCherry-KCa3.1 $^+$ Jurkat CAR cells

(number of CAR cells), and number of Live-or-Dye-positive cells (number of dead cells) were determined in each sample. From the cell numbers, we calculated the killing rate (KR) ($KR = \text{number of dead cells} / (\text{total cell number} - \text{number of CAR cells})$) of Jurkat CAR and mCherry-KCa3.1⁺ Jurkat CAR cells. The KR was determined at least in 6 confocal image tiles per sample. The measurements were performed on 2 different days.

Calcein red AM killing assay

The measurements were performed as described previously.^{41,53} First, the target cells were loaded with 1 μM calcein red AM solution, followed by 30 min incubation at room temperature, in the dark. Cells were washed with 1 $\times$ PBS (400 g, 5 min, room temperature), and incubated in phenol red-free RPMI medium for 10 min. Cells were plated into 8-well ibidi chamber (80806; ibidi GmbH). Effector Jurkat CAR and target Raji cells were added at 2:1 E:T ratios and cocultured with target cells for 3 h in presence/absence of 1 μM TRAM34. 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.

Migration assay

Jurkat CAR and mCherry-KCa3.1⁺ Jurkat CAR cells were spun (1200 rpm, 5 min, room temperature) and resuspend in phenol red-free RPMI solution. The cells were placed into ibidi chamber (ibidi GmbH). For time-lapse imaging, a Nikon-STORM confocal microscope was used. The camera focus was set automatically. The period of frame acquisition was set to 16.30 s for 60 min. In the 30th minute, 1 μM TRAM34 was added to the sample. The heated stage of microscopy was set at 37 °C. To calculate the mean speed ($\mu\text{m/s}$), distance (μm), and track displacement (μm), of cells we used the Fiji TrackMate plugin.

Calcein violet AM killing assay

To test cytotoxicity, target cells were resuspended in 1 μM calcein violet AM solution. This is followed by 20 min incubation at room temperature, in the dark. Cells were spun (400 g, 5 min, room temperature), washed with 1 $\times$ PBS and then resuspended in phenol red-free RPMI medium and incubated for 10 min before assay. Effector mCherry-KCa3.1⁺ Jurkat CAR/CAR T cells and target Raji cells were added at 1:1 E:T ratios. Cells were plated into 8-well ibidi chamber (ibidi GmbH) in presence/absence of 1 μM TRAM34. To detect calcein violet staining of target cells and sGFP + mCherry/sGFP signal of CAR expressing cells time-lapse imaging was used with a confocal microscope (LSM 880 Airyscan; Zeiss). Based on the fluorescence spectrum of the calcein violet, sGFP, and mCherry, 405, 488, and 514 nm laser lines were used to detect the signals. The heated stage of microscopy was set at 37 °C. Camera focus was set automatically. The period of frame acquisition was set to 10.13 s, for 90 min. The mean calcein violet fluorescence intensity of Raji cells was determined using fluorescent signals by ImageJ 1.54F software. Killing time (KT) was calculated from the difference of the time of contact (CT) between E:T cell (the plasma membrane of target cell and

CAR cell got into contact) and the time of cell death (CDT) of Raji (sudden decrease of the calcein violet AM mean intensity and irreversible cell shape change), with the formula $KT = CDT - CT$. The calculation of the CT and CDT was based on individual and visual frame evaluation for each individual cell-cell encounter. The cytotoxic-effect of DMSO (0.1%) was tested on mCherry-KCa3.1⁺ Jurkat CAR and Raji cells' viability.

Statistical analysis

For statistical analysis and plotting, GraphPad Prism 9.3.1 software (GraphPad Software) was used. For multiple group comparison, 1- or 2-way analyses of variance tests were performed. Post hoc testing was performed with Holm-Sidak or Tukey method. Differences were considered significant at $P < 0.05$. Data was presented as mean $\pm$ SEM.

Results

Generation of KCa3.1 expressing Jurkat CARs

In this study, we used a third-generation CD19-CAR model targeting the CD19 cell surface antigen on B cells. The third generation sGFP-CD19-CAR and mCherry-KCa3.1 construct was packaged into VSVG pseudotyped retrovirus particles and transduced into Jurkat cell lines and primary T cells, thereby generating CD19-specific Jurkat CAR, mCherry-KCa3.1⁺ Jurkat CAR cell lines, and CAR T cells. The Jurkat E6-1 cell line was picked because it did not express KCa3.1 channels, hence its role in effector function could be evaluated separately. The transduction efficiency of mCherry was $\sim 57\%$ in the mCherry-KCa3.1⁺ Jurkat CAR group (Fig. 1A), as assessed by flow cytometry on day 3 post-transduction. Jurkat CAR cells were used as control. To achieve a higher CAR and KCa3.1 positive cell rate, we sorted the KCa3.1- and CAR-expressing Jurkat (GFP⁺ and mCherry⁺) cells and later we used this homogeneous cell population to establish various assays ($\sim 97\%$ GFP⁺ and mCherry⁺) (Fig. 1B). We confirmed the CD19 positivity of the target Raji cells using mouse anti-human CD19 conjugated with Alexa Fluor 647 (as we described previously).⁴¹ KCa3.1 plays a key role in various effector functions of T cells by influencing Ca²⁺ signaling. We used the patch-clamp method to test the mCherry-KCa3.1 construct in transduced Jurkat and Jurkat CAR cells. We used a ramp protocol (between -120 mV and $+50$ mV, holding was set to -70 mV) to record current voltage independence, and the K⁺ current was detected during the measurements (Fig. 1B, C). The conductance level was the same in activated T cells and in KCa3.1-expressing Jurkat cells (with or without CAR). This points out that the use of mCherry-KCa3.1⁺ Jurkat CAR cells can mimic CAR T cells from primary T lymphocytes, as far as ion channels are concerned.

KCa3.1 inhibition facilitates tumor cell elimination: An anomalous outcome

To test the target cell killing ability of CAR⁺KCa3.1⁺ Jurkat cells, we proceeded with Live-or-Dye-based killing assay in which Raji cells were incubated with Jurkat CAR and mCherry-KCa3.1⁺

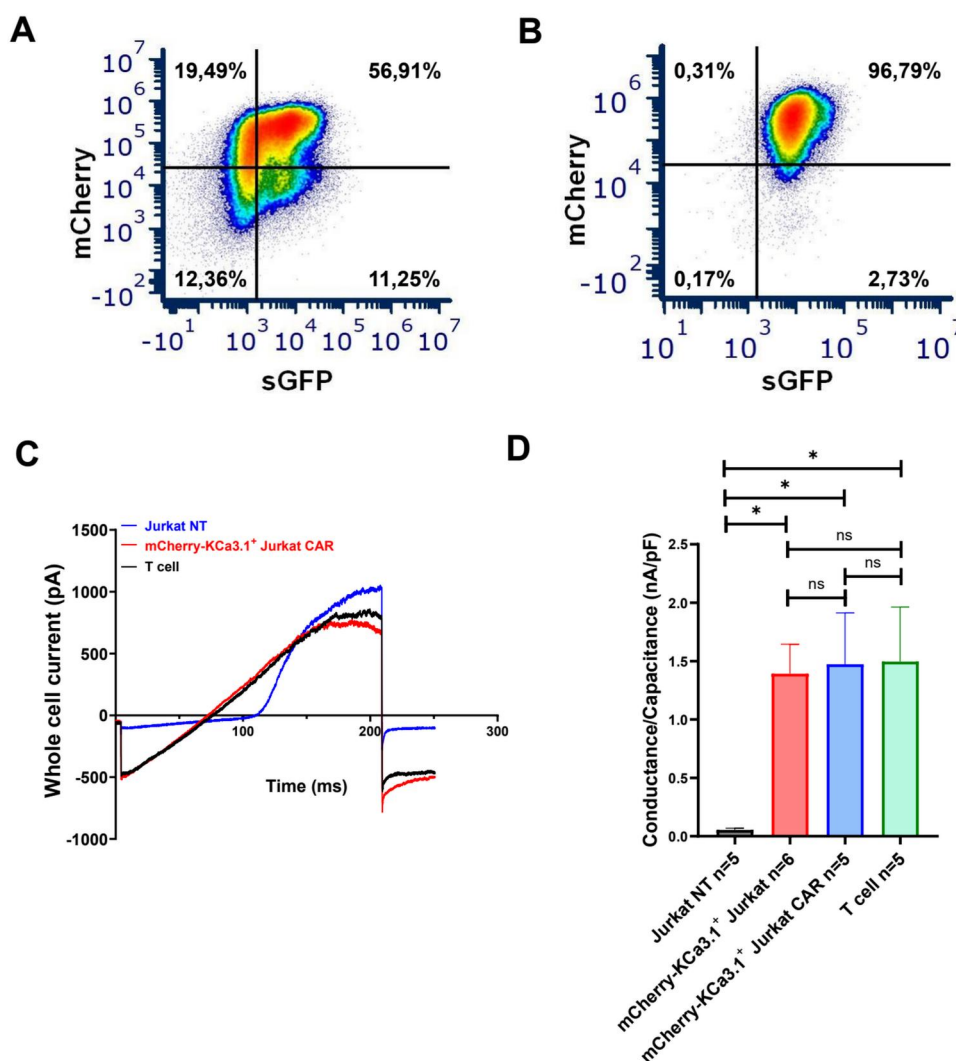
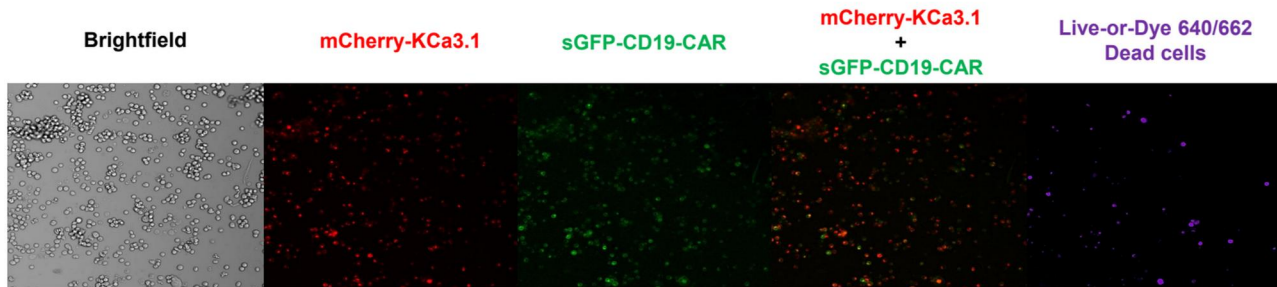


Figure 1 Representative flow cytometry histogram of CAR and KCa3.1 expression. sGFP-mCherry density plot of the CD19-CAR (sGFP) and KCa3.1 (mCherry) transduced Jurkat cells (A) before and (B) after sorting. (C) Whole-cell current trace recorded in mCherry-KCa3.1⁺ Jurkat CAR cells, Jurkat NT cells, and activated T cells upon a voltage-ramp protocol ranging from -120 mV up to $+50$ mV, holding potential was -70 mV. Blue: Jurkat NT cell; red: mCherry-KCa3.1-expressing Jurkat CAR cells; black: T cell. (D) The bar graph of whole-cell conductance of KCa3.1 channels in Jurkat NT (KCa3.1⁻, $n=5$), mCherry-KCa3.1⁺ Jurkat ($n=6$), mCherry-KCa3.1⁺ Jurkat CAR ($n=5$), and T cells ($n=5$). For each cell, the conductance was calculated as the average of 3 subsequent measurements. Data are plotted as mean $\pm$ SEM. One-way analysis of variance test was performed for multiple comparison. * $P < 0.05$. ns, not significant.

Jurkat CAR cells at a 2:1 E:T cell ratio for 3 h. Previously, we showed that blocking the KCa3.1 with the specific blocker TRAM34 facilitates the killing potential of CAR T cells in tumor spheroid model. Therefore, we wanted to examine the killing rate of mCherry-KCa3.1⁺ Jurkat CAR cells in monolayer culture in the presence of the $1 \mu\text{M}$ TRAM34 inhibitor. For controls, NT Jurkat and DMSO-treated (solvent of TRAM34) mCherry-KCa3.1⁺ Jurkat CAR cells were applied. After the incubation, we visualized the fluorescent cells with a confocal microscope (Fig. 2A). In each sample, the total cell count, the number of sGFP⁺/sGFP⁺mCherry⁺ cells (CAR and/or KCa3.1-expressing), and the number of dead cells incorporating the Live-or-Dye 640/662 stain was determined. The analysis showed that killing rate significantly increased in the presence of KCa3.1 expression ($P < 0.0001$, $n=8$); however, the addition of TRAM34 ($P < 0.0001$,

$n=8$) further improved the target cell elimination ability of mCherry-KCa3.1⁺ Jurkat CARs (Fig. 2B) compared with the controls (mCherry-KCa3.1⁺ Jurkat CARs [$n=8$] with or without 0.1% DMSO [$n=8$]). In our analysis, we determined that the overlay between mCherry-KCa3.1 + sGFP (CAR) (channels were merged) and Live-or-Dye 640/662 signal for cells (defined by the JaCoP plugin of Fiji), and we calculated the Pearson coefficient for these channels: 0.017 ± 0.009 ($n=6$) without TRAM34 and 0.0175 ± 0.016 ($n=6$) when TRAM34 was added. We suppose that this clearly indicates that Jurkat cells were not included as dead and we only detected dead Raji cells with Live-or-Dye killing assay. To test the killing ability of KCa3.1⁻ Jurkat CAR cells on Raji cells, the calcein red AM killing assay (with/without TRAM34) was performed, which demonstrated that TRAM34 has no effect killing ability of KCa3.1⁻ Jurkat CAR cells (Fig. S1).

A



B

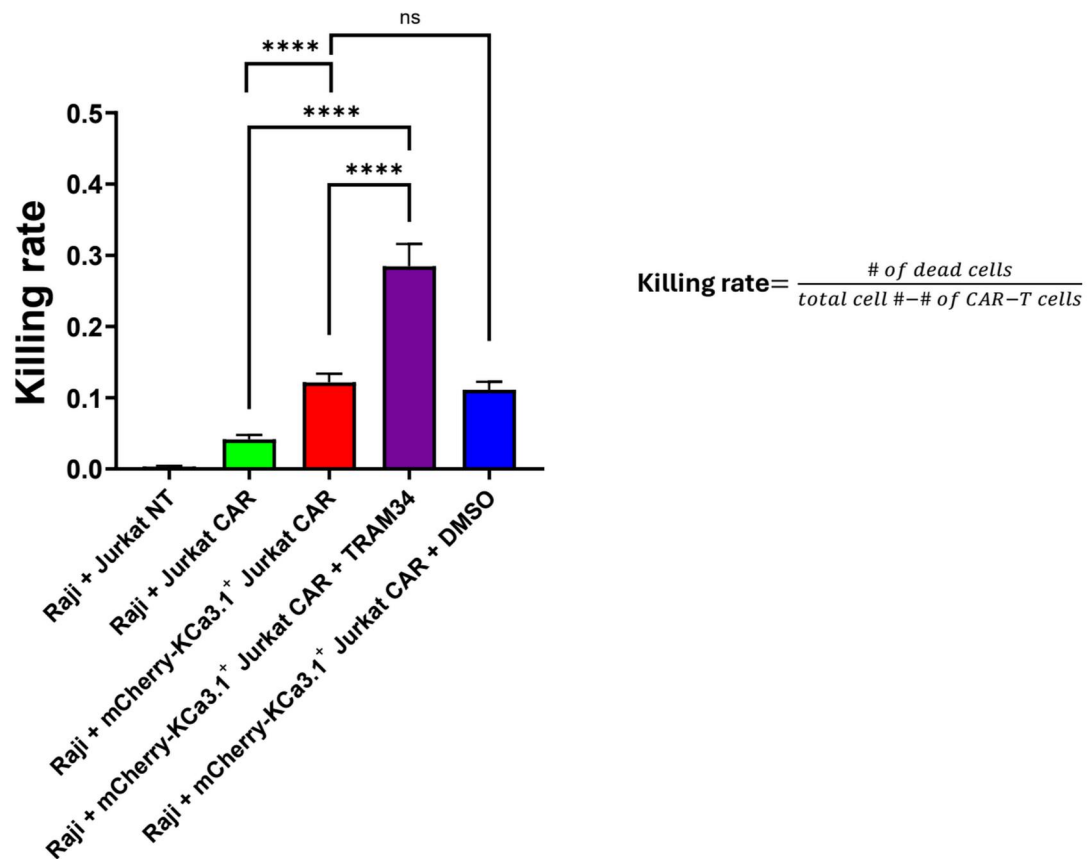


Figure 2 KCa3.1 inhibition increases target cell elimination. (A) Confocal images of Raji (CD19⁺) cells, mCherry-KCa3.1⁺ (red), and sGFP-CD19-CAR⁺ (green) Jurkat CAR, Live-or-Dye 640/662 positive (dead) target (Raji) cells after 3 h co-incubation. (B) Bar graph of killing rate values: Raji + NT Jurkat, Raji cells incubated with NT Jurkat cells (n = 6); Raji + Jurkat CAR, Raji cells incubated with Jurkat CAR cells (n = 8); Raji + mCherry-KCa3.1⁺ Jurkat CAR, Raji cells incubated with mCherry-KCa3.1⁺ Jurkat CAR cells (n = 8); Raji + mCherry-KCa3.1⁺ Jurkat CAR + TRAM34, Raji cells incubated with mCherry-KCa3.1⁺ Jurkat CAR cells in presence 1 μ M TRAM34 (n = 8); Raji + mCherry-KCa3.1⁺ Jurkat CAR + DMSO, Raji cells incubated with mCherry-KCa3.1⁺ Jurkat CAR cells in the presence of DMSO (same concentration as in 1 μ M TRAM34) (n = 6). The experiments were performed on 2 different days, for each sample nonoverlapping tiles (n $\geq$ 3; n > 1,000 cells per tile) was recorded. For multiple comparison, 1-way analysis of variance test was performed. Horizontal lines: mean $\pm$ SEM. ****P < 0.0001. ns, not significant.

KCa3.1 promotes migratory capacity of mCherry-KCa3.1⁺ Jurkat CARs

The Ca²⁺-activated KCa3.1 potassium channel is considered a significant regulator of proliferation and migration of T cells by mediating Ca²⁺ signaling.^{27,37,54,55} To untangle the role of

KCa3.1 in mCherry-KCa3.1⁺ Jurkat CAR cell migration, we conducted real-time imaging in presence of 1 μ M TRAM34, KCa3.1 inhibitor. From the confocal images (Fig. 3A) we determined the mean speed, distance between two frames (distance), and track displacement (displacement of the cell between first and last frame) of individual cells (3 or 4 independent experiments). We

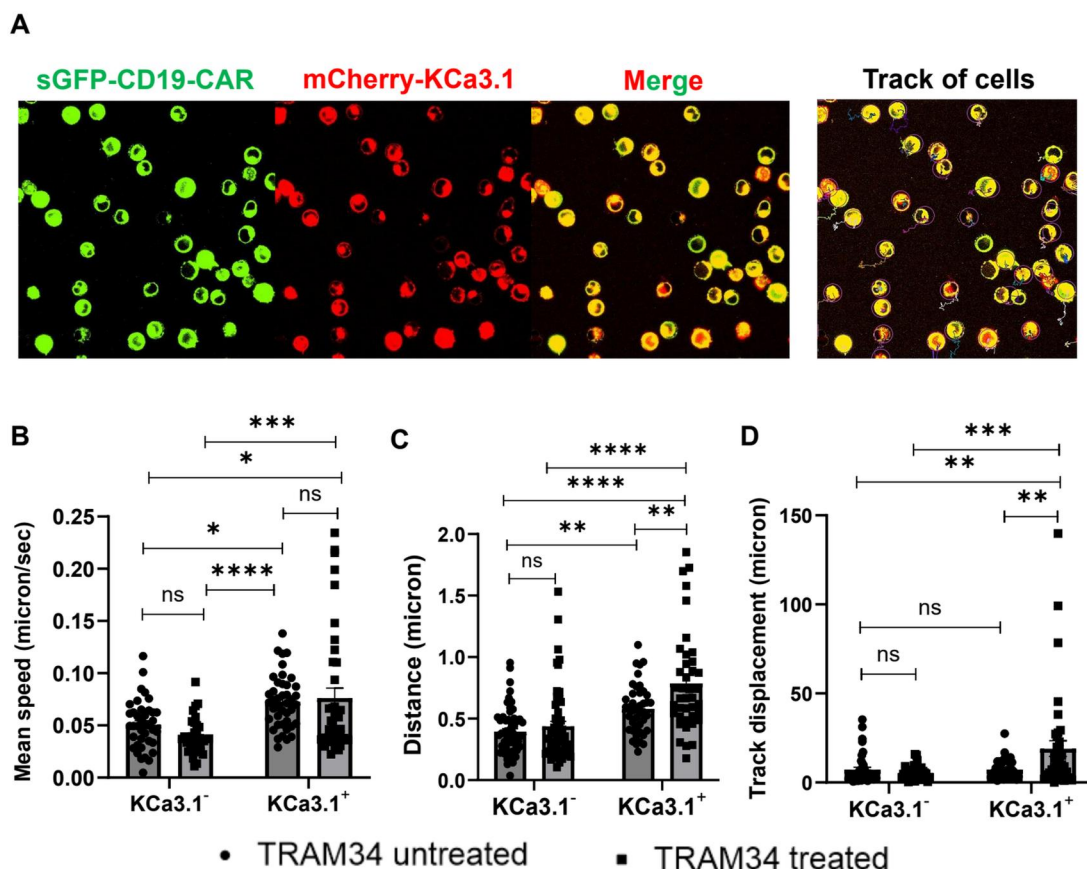


Figure 3 TRAM34 influences migration parameters. (A) Confocal images of sGFP-CD19-CAR⁺ (green) and mCherry-KCa3.1⁺ (red) Jurkat CAR, merge: fusion of green and red channels, and trajectories of mCherry-KCa3.1⁺ Jurkat CAR cells recorded for 60 min. (B) Mean speed ($\mu\text{m/s}$), (C) distance (μm), and (D) track displacement (μm) values in KCa3.1⁻ and KCa3.1⁺ Jurkat CAR in the presence or absence of 1 μM TRAM34: untreated Jurkat CAR ($n=39$); TRAM34-treated Jurkat CAR ($n=37$); untreated mCherry-KCa3.1⁺ Jurkat CAR ($n=42$); TRAM34-treated mCherry-KCa3.1⁺ Jurkat CAR ($n=40$). Data presented as mean $\pm$ SEM; each point represents the value for an individual cell. The experiments were performed on 3 different days. For multiple group comparison, 2-way analysis of variance test was performed. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. ns, not significant.

determined these parameters for the KCa3.1 expressing (mCherry-KCa3.1⁺ Jurkat CAR) and nonexpressing (Jurkat CAR) with/without TRAM34 addition (Fig. 3B–D). The analysis revealed that the expression of KCa3.1 significantly raised the mean speed ($n=40$; $P < 0.05$) (Fig. 3B) and distance ($n=40$; $P < 0.01$) (Fig. 3C) of migration compared with the KCa3.1 negative Jurkat CAR cells ($n=39$). The administration of TRAM34 for KCa3.1 expressing cells elevated the distance ($n=40$; $P < 0.01$) (Fig. 3C) and track displacement ($n=40$; $P < 0.01$) (Fig. 3D) compared with the untreated mCherry-KCa3.1⁺ Jurkat CAR cells ($n=42$). In the absence of KCa3.1, TRAM34 did not affect the migration capacity Jurkat CARs (Fig. 3B–D).

KCa3.1 introduction into Jurkat CARs elevates Ca^{2+} baseline level and CRAC-dependent Store-Operated Calcium Entry (SOCE)

The changes of intracellular Ca^{2+} levels in NK and CTL can influence cell migration, proliferation and cytotoxic

processes.^{38,40,45} To measure the CRAC-related intracellular Ca^{2+} -response and baseline we performed FURA-2 Ca^{2+} -imaging, which could help to outline the effect of KCa3.1 on changes of Ca^{2+} homeostasis. In the presence of TG, an increase in cytosolic Ca^{2+} was observed in a 2 mM extracellular Ca^{2+} solution, which is due to the Ca^{2+} influx through pores formed by CRAC channels. Figure 4A reports that in Jurkat CAR cell (KCa3.1⁻; $n=25$) the peak value of CRAC-specific response was significantly lower compared with the Jurkat NT ($P < 0.0001$; $n=19$) and mCherry-KCa3.1⁺ Jurkat CAR ($P < 0.0001$; $n=21$) cells (as we described previously).^{41,50} In mCherry-KCa3.1⁺ Jurkat CAR cells, the peak value was significantly higher compared with the control Jurkat NT cells ($P < 0.01$). The inhibition of KCa3.1 with 1 μM TRAM34 in mCherry-KCa3.1⁺ Jurkat CAR cells ($P < 0.05$; $n=23$) significantly decreased the Ca^{2+} peak compared the untreated mCherry-KCa3.1⁺ Jurkat CAR cells. Figure 4B displays that the presence of KCa3.1 significantly augmented the amplitude of the FURA-2 340 nm/380 nm intensity ratio in mCherry-KCa3.1⁺ Jurkat CARs (untreated: $P < 0.0001$; TRAM34-treated: $P < 0.0001$) cells regardless of the presence of the TRAM34, compared with KCa3.1 negative Jurkat

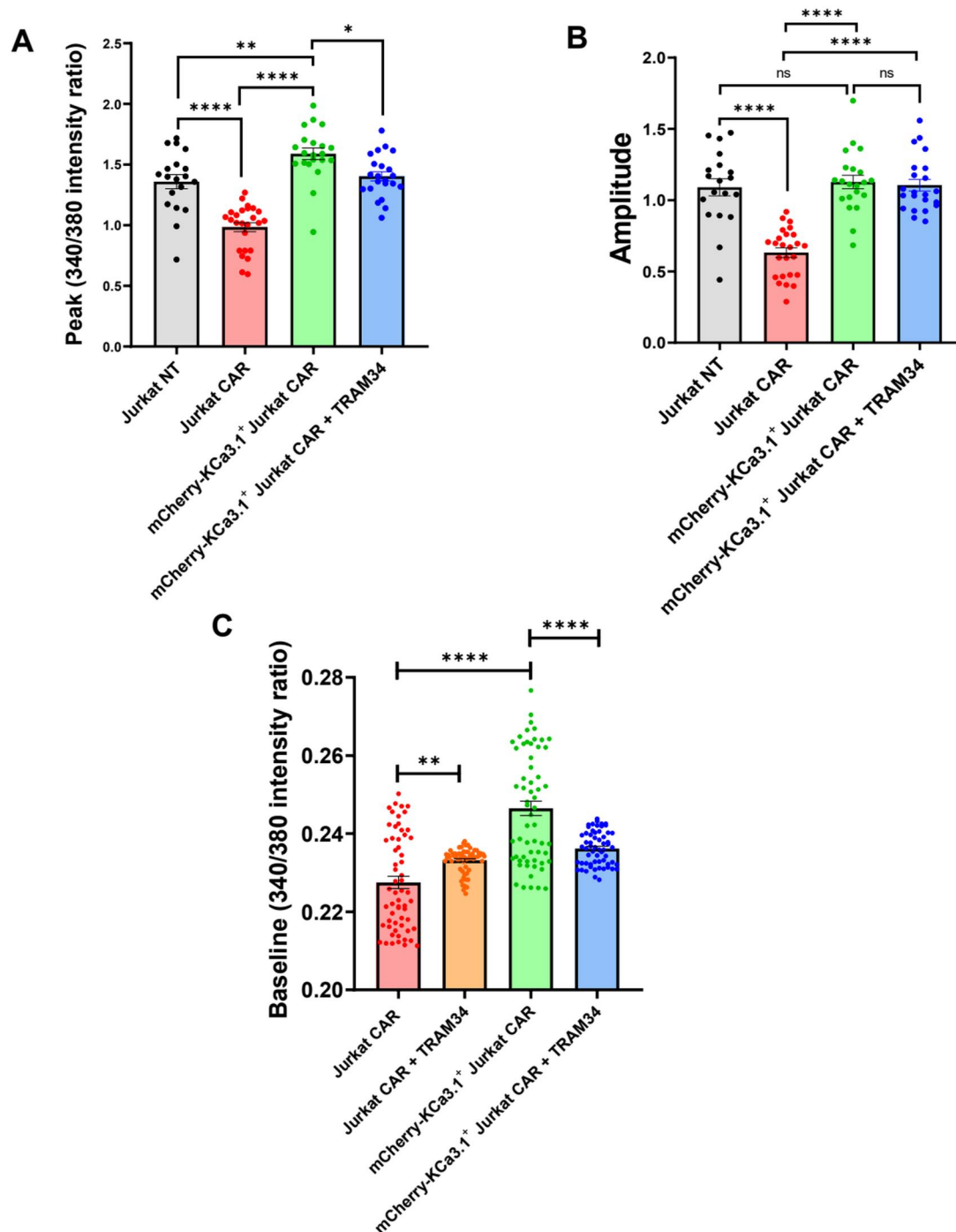


Figure 4 Presence of KCa3.1 channel alters the TG-induced Ca^{2+} response in mCherry-KCa3.1⁺ Jurkat CAR cells. Bar graph of TG-induced Ca^{2+} (A) peak and (B) amplitude of FURA-2 340 nm/380 nm intensity ratio in Jurkat NT (gray, $n=19$), Jurkat CAR (red, $n=25$), mCherry-KCa3.1⁺ Jurkat CAR (green, $n=21$), and 1 μM TRAM34-treated mCherry-KCa3.1⁺ Jurkat CAR (blue, mCherry-KCa3.1⁺ Jurkat CAR + TRAM34, $n=22$) cells. (C) Bar graph of baseline Ca^{2+} intensity ratios for Jurkat CAR (red, $n=60$), 1 μM TRAM34-treated Jurkat CAR (orange, Jurkat CAR + TRAM34, $n=60$), mCherry-KCa3.1⁺ Jurkat CAR (green, $n=60$), and 1 μM TRAM34-treated mCherry-KCa3.1⁺ Jurkat CAR (blue, mCherry-KCa3.1⁺ Jurkat CAR + TRAM34, $n=60$) cells. Each point represents a single-cell value, and data are represented as mean $\pm$ SEM. For multiple group comparison, 1-way analysis of variance test was performed. ns, not significant. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$.

CAR. There was no difference in amplitudes between Jurkat NT and mCherry-KCa3.1⁺ Jurkat CAR group ($P = 0.9944$). Moreover, the intracellular Ca^{2+} baseline measurement in medium used for killing and migration assay experiments (Fig. 4C) demonstrated that KCa3.1 expression causes an altered baseline level in

mCherry-KCa3.1⁺ Jurkat CAR ($P < 0.0001$, $n=60$) compared with Jurkat CAR ($n=60$). Application of TRAM34 in presence of KCa3.1 decreased the baseline level ($P < 0.0001$, $n=60$), while the absence of KCa3.1 increased the baseline level ($P < 0.01$, $n=60$) compared with untreated Jurkat CAR.

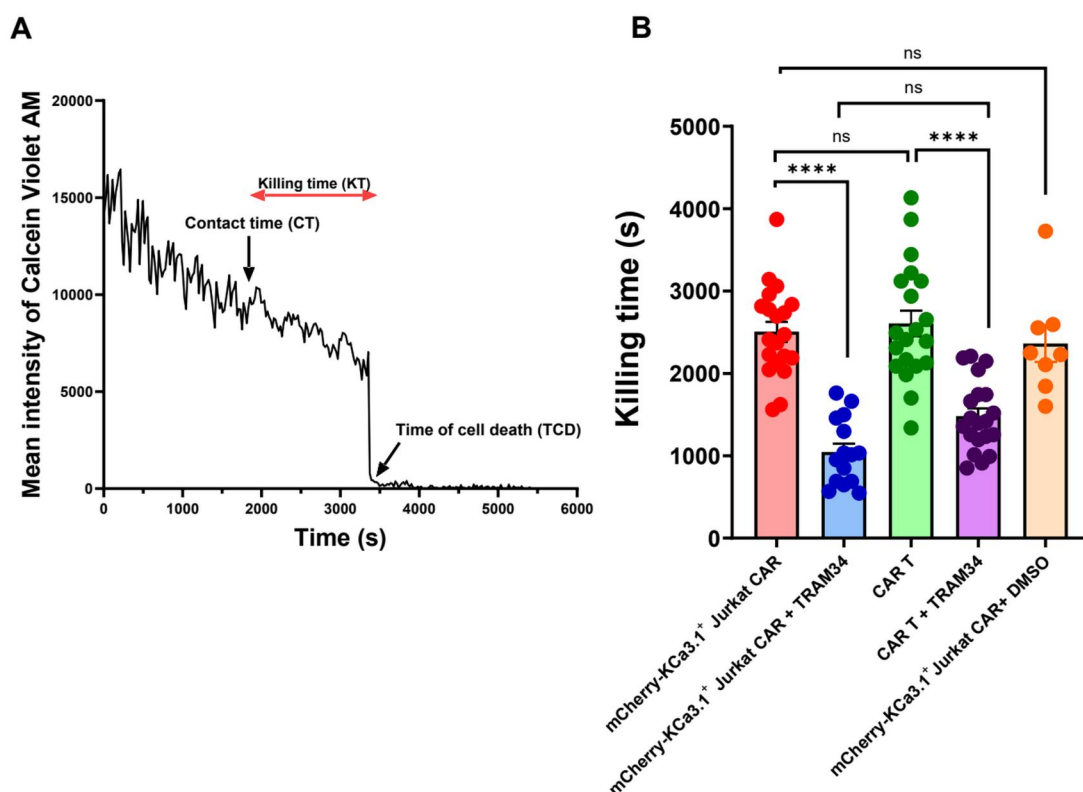


Figure 5 KCa3.1 inhibition decreases tumor cell elimination time of KCa3.1⁺ Jurkat CAR/CAR T cells. (A) Representative traces of the change of calcein violet AM mean intensity in Raji cells during contact (at the 1,854th second) and killing (at the 3,414th second) by mCherry-KCa3.1⁺ Jurkat CAR cells. (B) Bar graph of the KT values defined as the difference of contact and the KT: mCherry-KCa3.1⁺ Jurkat CAR (red, n = 20), 1 μ M TRAM34-treated mCherry-KCa3.1⁺ Jurkat CAR (blue, mCherry-KCa3.1⁺ Jurkat CAR + TRAM34, n = 15) cells, CAR T cell (green, n = 20), 1 μ M TRAM34-treated CAR T cell (purple, CAR T + TRAM34, n = 20), DMSO-treated mCherry-KCa3.1⁺ Jurkat CAR cells (orange, mCherry-KCa3.1⁺ Jurkat CAR + DMSO, n = 8). Each symbol represents the the KT value for 1 cell. The E:T ratio was 1:1; measurements were performed on 3 different days. For multiple group comparison, 1-way analysis of variance test was performed. **** P < 0.0001. ns, not significant.

KCa3.1 inhibition boost the “killing machinery” in KCa3.1-expressing Jurkat CARs and CAR T cells

We have previously demonstrated that inhibition of the KCa3.1 ion channel significantly increases the tumor elimination capacity of CAR T cells/cell lines.⁴¹ Therefore, we decided to investigate how TRAM34 affects the duration of contact between E:T cells, which could be a characteristic of tumor cell elimination and how long it takes for effector CAR cells to destroy target cells in the presence of TRAM34. The CD19⁺ positive target Raji cells were loaded with calcein violet AM and co-incubated with effector mCherry-KCa3.1⁺ Jurkat CAR or CAR T cells at 1:1 E:T ratios for 90 min. Time-lapse confocal imaging was used to calculate the KT from values of calcein violet AM mean intensity, in which in the encounter the cell expressed both sGFP (CAR) and/or mCherry (KCa3.1 for Jurkat cells, T cells have endogenous KCa3.1). Figure 5A exhibits the representative changes of calcein violet AM mean intensity in a Raji cell, before and after formation a contact with an effector mCherry-KCa3.1⁺ Jurkat CAR cell. The difference in the CT and CDT, in which the calcein violet intensity dropped to the baseline, was defined as the KT (Fig. 5A). Figure 5B details that the addition of TRAM34 significantly decreased the KT (mCherry-KCa3.1⁺ Jurkat CAR: P < 0.0001; n = 15; CAR T: P < 0.0001; n = 20) as

compared with the corresponding untreated controls (mCherry-KCa3.1⁺ Jurkat CAR: n = 20; CAR T: n = 20). DMSO alone did not influence the KT parameter for Raji cells co-incubated with mCherry-KCa3.1⁺ Jurkat CAR cells (n = 8) (Fig. 5B). The E:T cell encounter formation was calculated from time-lapse confocal images of calcein violet killing assay, and we obtained that encounter formation was significantly higher in presence of TRAM34 (56,7%) in case of mCherry-KCa3.1⁺ Jurkat CAR cells compared with the untreated samples (38%) (Fig. 5S).

Discussion

In recent years, numerous developments have been implemented in the structure of CARs with the aim of improving their efficacy and safety in therapeutic applications. The role of ion channels in CAR T cell functionality remains an unexplored area, but based on our research to date, Kv1.3, KCa3.1, and CRAC ion channels could play a key role in effector functions and migration in CAR T cells, as they do in T cells. In our research, we created third-generation CD19-specific Jurkat CAR cells expressing the KCa3.1 ion channel using retroviral transduction and CAR T cells. Jurkat cells can be considered a typical T cell model, but they do not express the KCa3.1 channel; hence, we decided to express this channel in this cell line. As the capacitance

normalized KCa3.1 conductance in mCherry-KCa3.1⁺ Jurkat CAR cells was the same as in primary T cells, the overexpression could not lead to incorrect conclusions.

First, we tested the tumor killing ability of KCa3.1 expressing Jurkat CAR cells using a Live-or-Dye-based killing assay. Our results showed that the presence of the KCa3.1 channel increased the killing ability of CAR cells, but the use of the TRAM34 inhibitor further facilitated the killing rate. From these results, we assumed that inhibition of KCa3.1 shifts the intracellular Ca²⁺ level to a range in which cytotoxicity and migration are also dominated/optimal, as described previously.^{40,41,45}

Subsequently, we determined parameters of the migration of KCa3.1⁺ and KCa3.1⁻ Jurkat CAR cells in the presence and absence of the TRAM34. Our results clearly underline that in KCa3.1-expressing Jurkat CAR cells the mean speed and distance (between two frames), were significantly higher than in Jurkat CARs. Surprisingly, the inhibition of KCa3.1 with TRAM34 in KCa3.1⁺ Jurkat CARs resulted in higher distance and displacement parameters as compared with controls, however, the mean speed was not influenced by the reduction in KCa3.1 conductance. These data attest that KCa3.1 is necessary for boosting the migratory pace and regulates the directionality of the movement.

Furthermore, we observed that the presence of KCa3.1 significantly increases the TG-induced Ca²⁺ response (peak but not amplitude) via the CRAC channel compared with KCa3.1⁻ Jurkat CAR cells, which was only slightly affected by the TRAM34 inhibition. Moreover, we obtained that baseline of Ca²⁺ concentration was elevated in KCa3.1⁺ Jurkat CARs, which further establishes the role of KCa3.1 in SOCE. These data indicate that expression of CRAC is not modified, though the total Ca²⁺ influx is higher in KCa3.1-expressing cells. This could be beneficial to the cells: an increased Ca²⁺ level in T cells can induce proliferation and a higher number of effector cells even in therapy. The elevated intracellular Ca²⁺ promotes chemokine induced migration, which is essential in tumor infiltration, and stimulates gene transcription of lytic enzymes such as granzyme B. Using confocal microscopy (Fig. S3), we assessed the colocalization of the KCa3.1 (mGFP tagged) and Orai1 (mCherry conjugated) channels in the membrane in Jurkat cells, and we found that the mean Pearson's coefficient was 0.75 ± 0.03 (n = 10). These results further support that introduction of KCa3.1 into Jurkat cells leads to the KCa3.1-targeting into Orai1 domains, which could guarantee a more efficient regulation of Ca²⁺ uptake through CRAC.⁵⁶

In the FURA-2-based intracellular Ca²⁺ baseline measurements, the Ca²⁺ level was increased in presence of TRAM34 inhibitor in KCa3.1⁻ Jurkat CAR cells, which we believe is due to the off-target effect of the inhibitor. Some studies reported that cytochrome P450 (CYP) is involved in Ca²⁺ signaling: it had been demonstrated that CYP inhibition affected Ca²⁺ entry into the ER, for example, in rat pancreatic acinar cells treated with imidazole antifungal agents, or in chromaffin cells with ketoconazole and clotrimazole, the latter can inhibit this Ca²⁺ influx into the ER.^{57,58} Furthermore, it has been shown that TRAM34, as a clotrimazole analogue, can inhibit several rats and human CYP isoforms.⁵⁹ However, it can be clearly seen that in KCa3.1⁺ Jurkat CAR cells, the use of TRAM34 reduces the intracellular Ca²⁺ baseline level, which could underline their role in regulation of intracellular Ca²⁺ concentration.

Using a calcein violet AM killing assay, we assessed the time period needed for CAR T cells and mCherry-KCa3.1⁺ Jurkat CAR cells to abolish a Raji target cell using time-lapse confocal imaging. Our data demonstrated that KCa3.1 inhibition significantly reduces the the KT of CAR T and mCherry-KCa3.1⁺ Jurkat CAR cells (not an artifact due to bleaching [see Fig. S4]: intensity decreases quasi exponentially over time). Our results clearly exhibited that inhibition of KCa3.1 enables CAR-expressing effector cells to recognize (directed migration as indicated by the increased displacement) and kill target cells more quickly.

We suppose that the enhanced target cell elimination stems from the following: the introduction of KCa3.1 into the Jurkat CAR cells restores and even overloads the Ca²⁺ in the cells (the KCa3.1 level is not drastically higher as compared with T cells [see Fig. 1]).³⁷ Indeed, as described in Kaschek et al.,⁴⁵ this shifts the Ca²⁺ regime in cells, which benefits the migration of Jurkat and partly cytotoxicity. This is accentuated by the fact that mean speed increased upon introduction of KCa3.1, and no change upon TRAM34 addition. When KCa3.1 activity is suppressed with TRAM34, the “resting/baseline” intracellular Ca²⁺ concentration drops to a lower level where the cytotoxicity is enhanced as the directional migration parameters (distance and displacement). Furthermore, the reduction in Ca²⁺ upon KCa3.1 block both in Jurkat CAR and CAR T cells shortened the time required for elimination of the Raji target cell, which was also reported in NK cells.^{38,39} Here, we should mention that Alshwimi et al.⁶⁰ reported that in NK cells from head and neck cancer patients and healthy donors, the KCa3.1 inhibition hindered chemotaxis and cytotoxicity. However, our model system (Jurkat CAR and CAR T cells) are not comparable to “native” cells, and presence of CAR can rewire the signaling processes in CAR-expressing cells.⁶¹ For migration experiments, we obtained that TRAM34 administration seem to guide the cells as if they drifted along a chemical gradient, which is supported by our previous finding.⁴¹ We suppose that these results, coincident with outcomes of killing assay experiments, namely, the increased speed and guided trajectory, contribute to the increased probability in effector-target cell encounter formation (Fig. S5). Because NK cells could be applied in CAR therapy as a donor-independent approach and NK-92 cells (an NK cell line) also showed optimized cytotoxic effect upon KCa3.1 inhibition, we propose that KCa3.1 in NK-CAR (or macrophage CAR cells, which also have KCa3.1) could be a target of forthcoming studies besides the well-established CAR T cells.

In summary, we could show that the presence of KCa3.1 in CAR T cells (1) enhances the migration (mean speed) of cells, (2) elevates the resting Ca²⁺ level and restores SOCE via CRAC channels, and (3) through inhibition of the channel, promotes target cell elimination through reduction in the time needed to destroy it. We suppose that our data further emphasize the importance of ion channels in CAR T cell physiology, and they should be considered as a modulatory target to improve the outcome of cell-based immune therapies utilizing T cells, NK cells, and macrophages.

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Author contributions

V.J.: writing—original draft preparation, formal analysis, data curation, methodology, investigation, visualization; M.H.: investigation; P.H.: investigation; G.P.: conceptualization; P.H.: conceptualization, writing—original draft preparation, writing—review & editing, project administration, resources, formal analysis, validation, funding acquisition. All authors have read and agreed to the published version of the manuscript.

V.J. (Data curation [Equal], Formal analysis [Equal], Investigation [Equal], Methodology [Equal], Visualization [Equal], Writing—original draft [Equal]), M.H. (Investigation [Equal]), P.H. (Investigation [Equal]), and P.G. (Conceptualization [Equal]), P.H. (Conceptualization [Equal], Formal analysis [Equal], Funding acquisition [Equal], Project administration [Equal], Resources [Equal], Validation [Equal], Writing—original draft [Equal], Writing—review & editing [Equal])

Supplementary material

[Supplementary material](#) is available at *The Journal of Immunology* online.

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Conflicts of interest

The authors declare no conflicts of interest.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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