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

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List of abbreviations:

CRAC: Ca^{2+}-release activated Ca^{2+} channel

CD: Cluster of differentiation

PI: Propidium iodide

mTOR: Mammalian target of rapamycin

FKBP: FK506 binding protein

Antx: Anuroctoxin

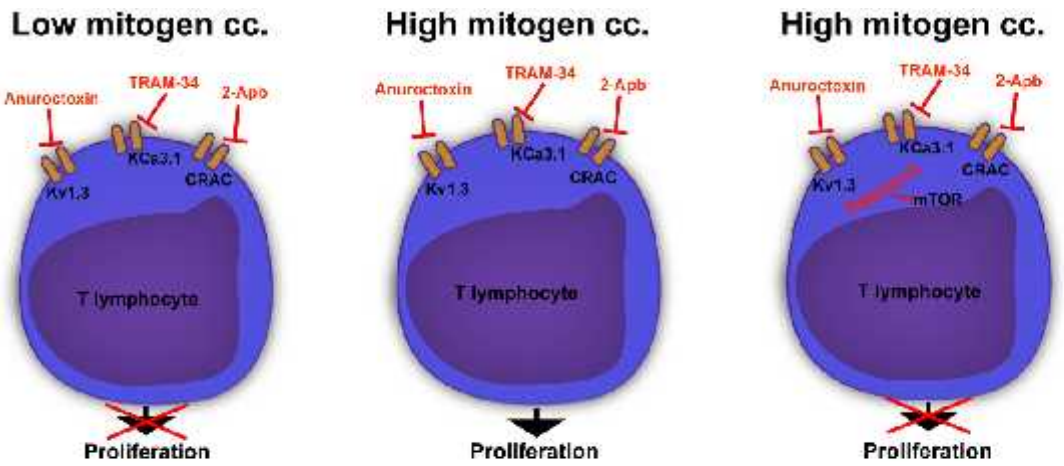
2-Apb: 2-Aminoethoxydiphenyl borate

DI: Division index

PBMC: Peripheral blood mononuclear cell

CFSE: Carboxifluorescein succinimidyl ester

Graphical Abstract



Ion channel blockers inhibit T lymphocyte proliferation at low mitogen concentrations. This effect diminishes upon using higher mitogen concentrations, but the antiproliferative effect can be recovered by combining ion channel blockers with other immunopharmacological agents such as the mTOR inhibitor rapamycin.

Abstract

Ion channels are crucially important for the activation and proliferation of T lymphocytes, and thus, for the function of the immune system. Previous studies on the effects of channel blockers on T cell proliferation reported variable effectiveness due to differing experimental systems. Therefore our aim was to investigate how the strength of the mitogenic stimulation influences the efficiency of cation channel blockers in inhibiting activation, cytokine secretion and proliferation of T cells under standardized conditions.

Human peripheral blood lymphocytes were activated via monoclonal antibodies targeting the TCR-CD3 complex and the co-stimulator CD28. We applied the blockers of Kv1.3 (Anuroctoxin), KCa3.1 (TRAM-34) and CRAC (2-Apb) channels of T cells either alone or in combination with rapamycin, the inhibitor of the mammalian target of rapamycin (mTOR). Five

days after the stimulation ELISA and flow cytometric measurements were performed to determine IL-10 and IFN-γ secretion, cellular viability and proliferation.

Our results showed that ion channel blockers and rapamycin inhibit IL-10 and IFN-γ secretion and cell division in a dose-dependent manner. Simultaneous application of the blockers for each channel along with rapamycin was the most effective, indicating synergy among the various activation pathways. Upon increasing the extent of mitogenic stimulation the anti-proliferative effect of the ion channel blockers diminished. This phenomenon was unknown to date but may be important in understanding the fine-tuning of T cell activation.

1. Introduction

T lymphocytes are highly potent cells of the adaptive immune system and are crucially important in the maintenance of immunological homeostasis. Rapid and specific activation through the TCR and its co-receptors CD4 and/or CD8 lead to the recruitment of numerous down-stream pathways, that ultimately result in T cell activation and proliferation, and subsequently lead to the differentiation into effector or memory cells [1,2].

Physiological T cell activation occurs upon contact with professional antigen presenting cells. The consequence of antigen presentation depends on the age and the stage of differentiation of the T cell, and also on the intensity and the duration of the stimulus [3]. It is well established that the co-localization of different signaling molecules forms an immunological synapse, which enhances the subsequent cellular response [4,5]. The molecules forming the immunological synapse on the T cell side involve the TCR-CD3 complex together with co-activator molecules such as CD28 [6], CD40 ligand [7] or the IL-2R [8].

Considering that the underlying mechanisms of lymphocyte stimulation are necessary for understanding the ensuing immune responses, various methods were designed to mimic *in vivo* activation pathways. These methods include monoclonal antibodies targeting the TCR-CD3

complex and other co-activator molecules [9,10]; cross linking of cell surface glycoproteins via mitogenic lectins such as PHA [11] and bypassing T cell Ca^{2+}-signaling by ionomycin and the diacylglycerol-analog PMA [12]. As these methods are not epitope-specific, they result in a high degree of cytokine secretion, such as the anti-inflammatory cytokine IL-10 and the inflammatory cytokine IFN-γ, and eventually in T cell activation and mitosis [13]. The pro-inflammatory IFN-γ is secreted by a wide array of cells, such as natural killer cells, Th_1 CD4 and CD8 cytotoxic T cells and even macrophages. On the other hand, anti-inflammatory IL-10 is mainly secreted by Th_2 T cells and regulatory $CD4^+/CD25^+/FoxP3^+$ T_{reg} cells [14].

Ion channels are important in cellular signaling, even in electrically non-excitable cells, such as immune cells. Since 1984, when electric signals from lymphocytes were first recorded [15,16], it has been suggested that ion channels are involved in the regulation of the immune system. To date numerous ion channels have been discovered in T cells (summarized in [17,18]): the Ca^{2+}-release activated Ca^{2+} channel (CRAC) [19]; the Shaker-type voltage-gated K^+ channel Kv1.3 [20], the Ca^{2+}-activated K^+ channel, KCa3.1, formerly known as IKCa1 [21,22]; the non-selective TRPM7, that is suggested to be involved in the magnesium homeostasis of the cell; TRPM2, important in T cell activation and proliferation [23], and finally the swelling-activated chloride channel Cl_{swell}, encoded by the SWELL 1 gene [14,24]. As CRAC, Kv1.3 and KCa3.1 channels co-localize in the immune synapse and are up-regulated in different T-cell subtypes [5,25], it is widely accepted that these channels are indispensable early factors in the Ca^{2+}-dependent activation pathways of the T cell [26]. Increase in $[Ca]_i$ may activate pathways involving the calcium-calmodulin complex and other secondary messenger molecules such as calcineurin. This phosphatase dephosphorylates the nuclear factor of activated T-cells (NFAT), allowing its dimerization and nuclear translocation. This transcription factor can then bind to the promoter region of target genes involved in cytokine production and proliferation of T lymphocytes [20,26].

As ion channels are key players in T lymphocyte activation, their blockade can decrease the array of pathological immune responses *in vivo*. Kv1.3 is an excellent candidate for immunotherapy, as it is expressed predominantly in astrocytes, T lymphocytes and oligodendrocytes [27] in contrast to CRAC and KCa3.1 channels, that are widely distributed and thus their blockers may have more side effects. Successful experimental trials employing Kv1.3 blockers have already been performed in animal models of autoimmune diseases such as multiple sclerosis [25], type 1 diabetes mellitus or rheumatoid arthritis [28].

Besides the Ca^{2+}-dependent mechanisms, other signaling pathways also participate in T cell activation that do not involve NFAT signaling. Such pathways include the mammalian target of rapamycin (mTOR), which contributes to the activation of both translational and metabolic pathways, and allows DNA synthesis [29,30]. The mTOR can be blocked indirectly using rapamycin (also known as sirolimus), which inhibits the FK506 binding protein (FKBP12), that interacts with mTOR. Rapamycin is a highly effective immunosuppressive drug, that is currently widely used in the treatment of kidney graft rejection or graft versus host disease [31].

The anti-proliferative effects of different ion channel blockers on T cells have already been described in a number of experiments and reviews. However, there is an obvious variability in the results of previous studies related to this topic. For example, the average blocker concentration necessary for 50% inhibition of cell proliferation ranged from $1\times K_d$ concentration to $1000\times K_d$ in case of Kv1.3 channel blockers, or from $1.5\times K_d$ to $275 \times K_d$ in the case of the KCa3.1-blocker TRAM-34, where K_d is the drug concentration required to block half of the relevant channels [25,32-35]. Moreover, TRAM-34 inhibition alone had no effect on the proliferation of mixed T cell populations [36]. The underlying mechanism responsible for this variability has not been systematically addressed before, but must be largely due to the different methods of T cell stimulation and different doses of mitogens applied in these studies. Therefore our aim was to elucidate this phenomenon by comparing the anti-proliferative effects of ion

channel blockers and rapamycin on lymphocytes cultured and activated under identical experimental conditions. Moreover, considering our results at various mitogen concentrations, we propose a theory to explain the underlying mechanisms of our observations.

2. Materials and Methods

2.1 Isolation and cultivation of mononuclear cells

PBMCs were isolated from heparinized (heparin from TEVA Pharmaceutical Industries Ltd., Debrecen, Hungary) peripheral blood of healthy volunteers. First the blood was diluted with Hanks' Balanced Salt Solution (HBSS; from Sigma-Aldrich Co., Saint Louis, MO, USA) in 1:1 ratio, and then centrifuged using the Ficoll-Hypaque density gradient (GE Healthcare Life Sciences, Little Chalfont, UK) at 1400 rpm for 30 minutes at room temperature. Next, the opaque layer of mononuclear cells was collected and washed two times with 50 ml HBSS. In n=4 experiments we used purified $CD3^+$ T lymphocytes obtained by negative selection using RosetteSep™ (Stem Cell Technologies™, Vancouver, Canada) technique according to the description in the manual. We did not find significant differences between $CD3^+$ T cells and PBMCs regarding the extent of proliferation ($p=0.785$) or in the proliferation-inhibiting effect of Antx at K_d ($p=0.667$) and $10K_d$ ($p=0.333$) concentrations. Therefore, we used the PBMC population in the majority of our experiments.

Following carboxifluorescein succinimidyl ester staining (CFSE stining, see below) and activation, cells were cultured in 24 or 96 well plates at a cell density of 10^6 cells/ml in standard RMPI-1640 medium (Sigma-Aldrich Co., Saint Louis, MO, USA) containing 15% HEPES buffer (Sigma-Aldrich Co., Saint Louis, MO, USA) at 37°C in humid atmosphere with 5% CO_2. In every experiment all plates were incubated for 5 days and were supplemented with fresh culture

145 medium on day 3. After harvesting, the cells underwent pripodium iodide (PI) staining and
146 subsequent FACS analysis.

147

148 **2.2 CFSE dilution assay and PI staining**

149 We applied the CFSE dilution essay, originally described by Lyons et al [37-39], to
150 measure the rate of proliferation. The staining procedure briefly was the following: the
151 membrane-permeable, but non-fluorescent carboxyfluorescein diacetate succinimidyl ester
152 (CFDA-SE) binds to structural proteins within the cell, and is subsequently cleaved by
153 nonspecific esterases to become the membrane non-permeable and fluorescent molecule CFSE.
154 Upon cell division, the amount of CFSE is gradually halved in the daughter cells, thus the number
155 of division cycles the cells have undergone can be determined. In our case, the lymphocytes
156 divided usually every 24-48 hours, leading to 4-6 measurable cycles at the end of our
157 experiments.

158 The final concentration of CFDA-SE (CellTrace™ CFSE Cell Proliferation Kit, Life
159 Technologies Co., Waltham, MA, USA) in our experiments was 1µM that provided a 100-to-
160 1000-fold increase in the fluorescence intensity of the measured cells over the autofluorescence
161 of unstained cells. After adding CFDA-SE, we incubated the PBMCs or T lymphocytes for 15
162 min at room temperature, then for 20 min at 37°C. Lastly, the cells were washed once with
163 phosphate buffer solution (PBS). We took care that the CFSE-stained cells remained hidden from
164 excess light during our experiments. This CFSE staining can be ultimately recorded by flow
165 cytometry.

166 PI staining was performed at the end of the 5-day incubation period. Therefore, we
167 harvested and washed the cells once using HBSS, then added 1 µl PI to the cell suspension. Cells

were mixed gently with PI and then incubated in the dark for 5 minutes at room temperature. The flow cytometer settings were adjusted to a negative control tube containing unstained cells. PI fluorescence intensity was measured in the red channel, because samples were co-stained with CFSE.

2.3 Selective stimulation of T lymphocytes

At the beginning of the present study, we performed preliminary experiments regarding our preferred method of stimulation. Four widely used and well-known lymphocyte stimulation techniques were compared using CFSE dilution assay on PBMCs: PHA stimulation [11]; PMA combined with ionomycin [12]; soluble anti-CD3 antibody alone and in combination with anti-CD28 [9,10]. Moreover, we measured whole-cell K^+ currents and current density on representative populations of the stimulated T cells. We found the anti-CD3 and anti-CD28 stimulation was most reproducible (data not shown) and thus, we used this approach in our experiments detailed in this article.

We applied 200 nM – 3 µM soluble anti-CD3 antibodies combined with a constant amount of 1 µg/ml soluble mouse anti-human CD28 (Sigma-Aldrich Co., Saint Louis, MO, USA) in n=8 experiments for specific T cell stimulation in the PBMC and lymphocyte cultures. We enhanced the rate of stimulation by adding the soluble antibodies to the bottom of the culture well, left it to bind to the plate surface for 30 minutes at room temperature, then cells were added to the wells in culture medium suspension. In n=8 experiments we used superparamagnetic bead-conjugated anti-CD3 and anti-CD28 monoclonal antibodies (Life Technologies Co., Waltham, MA, USA), which we found more user-friendly than the soluble antibodies. The pairwise comparison of soluble mitogens and bead-mediated stimulation Student's t-test showed no significant difference between the divided cell populations with the two methods of stimulation ($p=0.336$). The beads are also known to provide adequate cross-linking thus inducing a relatively

high level of activation [10], in contrast to stimulation with soluble anti-CD3 and anti-CD28, that resulted in a higher amount of variability in our measurements [9]. The bead:cell ratio in these cases was 1:200 - 1:1 (see Results).

2.4 Application of ion channel blockers and rapamycin

To block the Kv1.3 channel we used the peptide-type toxin Antx [40]. KCa3.1 channels in were blocked using TRAM-34 (Sigma-Aldrich Co., Saint Louis, MO, USA) [34] and the CRAC channels were inhibited by 2-Apb (Sigma-Aldrich Co., Saint Louis, MO, USA) [41]. We used the ion channel blockers at two concentrations: the lower was equal to the dissociation constant, or $1\times K_d$, of ion channel inhibition of the blockers and the higher was 10 times the K_d ($10\times K_d$). In the case of Antx, we used 500 pM ($1\times K_d$) and 5 nM [40]. In the case of 2-Apb the K_d for lymphocytes is 5 μM, and the other concentration used was 50 μM. Finally, the KCa3.1 blocker TRAM-34 was used in 20 nM ($1\times K_d$) and 200 nM concentrations. In the case of rapamycin the lowest concnetration reported in the literature [42,43] to inhibit T cell proliferation by 50% ($1xIC_{50}$ =20 pM) was used as the lower dose and 200 pM ($10\times IC_{50}$) was used as the higher dose.

2.5 Flow cytometry experiments

The flow cytometry measurements were performed on a BD FACScan™ and Facs Array™ flow cytometers. We measured the light scatters, namely the forward scatter (FSC) and side scatter (SSC) and the fluorescence intensity on green and red channels. Gate setting for lymphocytes is shown in Fig. 1A, and the gating of viable cells is represented on Fig. 2A and B. The sheath fluid consisted of 1x PBS. Lymphocytes were selected from mixed cell populations of PBMC by their light scatter profile on FACS analysis. Cell proliferation was measured based

on the declining CFSE intensity in the green channel (Fig. 1B.). Division index (DI) was used was the the indicator of proliferation and was calculated by this formula

$$DI = \left(\sum_{k=1}^{n} A_k\right) / \left(\sum_{k=1}^{n} A_k\right)$$

where k is the division cycle number (i.e. generation number) of cells, and A_k is the cell number in the k^{th} division cycle according to Fig. 1B.

2.6 Measurement of cytokine concentration

Culture supernatants of human peripheral blood mononuclear cells (n=3) were harvested five days after application of mitogens and ion channel blockers, and the concentration of IL-10 and IFN-γ was measured using OptEIA kits (BD Biosciences, Franklin Lakes, NJ, USA), according to manufacturer's instructions, using duplicates.

2.7 Data evaluation and statistics

Flow cytometric data were collected using BD CellQuest™ 12.1. For data analysis we used the freeware program Cyflogic 1.2.1. The analyzed data was exported to Microsoft Office Excel™ 2007. For statistical evaluation of our results we used the program SigmaPlot™ 12.0, where we applied one-way ANOVA test and as post hoc analysis, Holm-Sidak test versus the positive control cell population. We marked the level of significance with * if p was <0.05, with **, if p was <0.01, and with ***, if p was <0.001. Data are represented as mean ±S.E.M.

3. Results

3.1 Dose-dependence of mitogen-induced proliferation

As stated in the introduction, our first step was to achieve multiple levels of selective T lymphocyte stimulation using anti-CD3 and anti-CD28 monoclonal antibodies. Fig. 1. shows that comparing the division indices (DI) of stimulated PBMC populations 5 days following mitogen stimulus, four levels of the mitogen effect could be distinguished: low concentration (200 ng/ml or 1 bead:200 cells) of the mitogen led to a relatively low amount of proliferation, while the medium (500 ng/ml or 1 bead:50 cells), high (1 µg/ml or 1 bead:10 cells) and very high concentrations (3 µg/ml or 1 bead:1 cells) resulted, as expected, in markedly higher rates of cell division. The mean DI are 30.6 ± 4.8%, 60.8 ± 4.5%, 72.7 ± 1.5% and 85.3 ± 2.4%, respectively. Pairwise comparison of the observed proliferation rates indicated a significant increase with each subsequent increase in mitogen concentration (p=0.0036; p=0.044; p=0.0042, respectively).

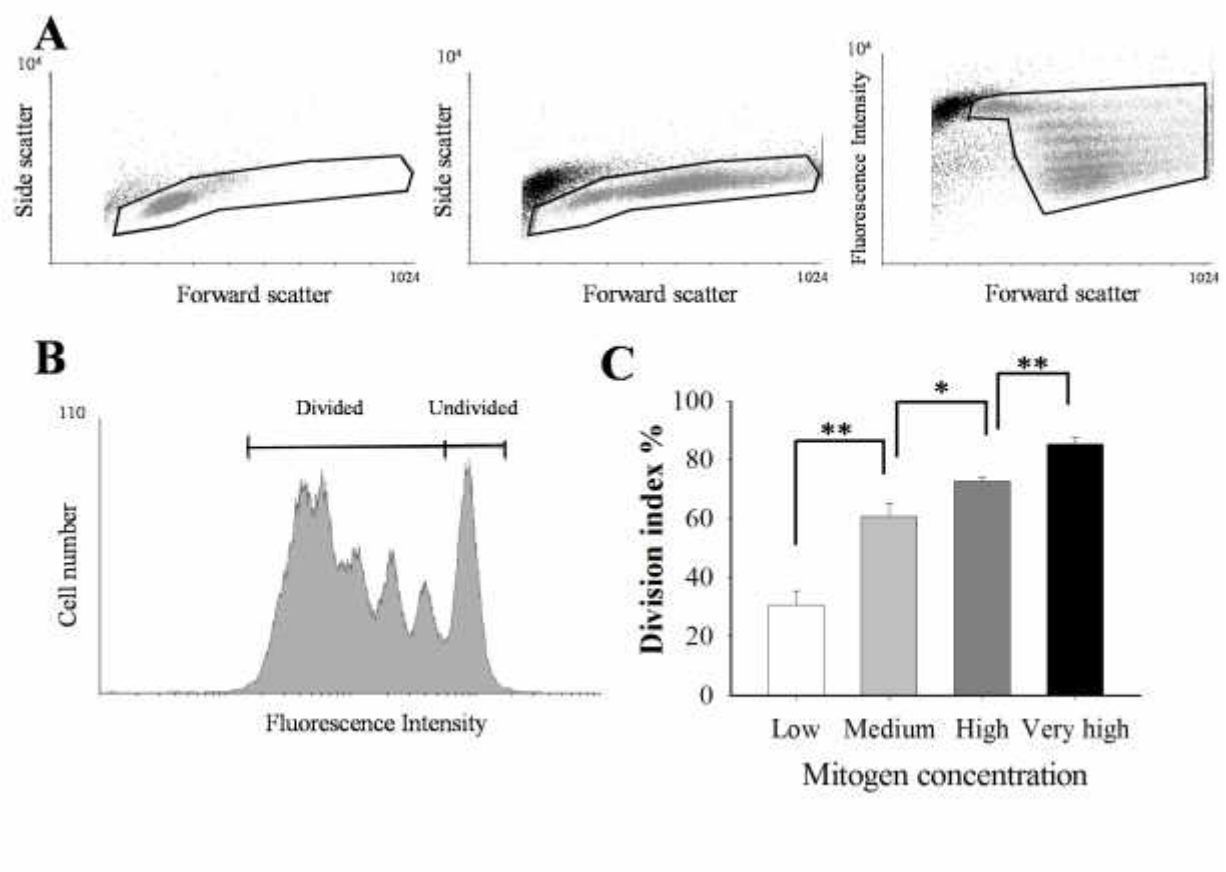


Figure 1. Cell proliferation at different mitogen concentrations. A) The left and middle dot plots show stimulated PBMC populations stimulated by 1 µg/ml anti-CD3 and anti-CD28 monoclonal antibodies on day 0 and day 5, respectively. Solid black line polygons indicate the position of the gates. Activated T cells on day 5 (middle) having higher light scatter properties and mainly dead cells outside the gate are clearly distinguishable. The dot plot on the right shows the FSC/ Fluorescence intensity of the same population as on the middle dot plot, where the gradual decline in the CFSE intensity as a consequence of cell division can be observed. B) The fluorescence intensity histogram shows CFSE fluorescence intensity obtained from the gated population of the dot plot on the right in panel A. The marker above the histogram indicates the divided and the undivided cell populations, and the ratio of divided cells to all gated cells was calculated yielding the division index (DI, see Materials and Methods). C) DI of the cells stimulated by anti-CD3 and anti-CD28 at various mitogen concentrations (low: 200 ng/ml or 1 bead:200 cells; medium: 500 ng/ml or 1 bead:50 cells; high: 1 µg/ml or 1 bead:10 cells; and very high concentration: 3 µg/ml or 1 bead:1 cells). The DIs of these populations are shown in Fig 2 as positive controls (Pos.) and the DI of inhibitor treatments were normalized to this data.

3.2 Ion channel blockers and rapamycin alone and in combination inhibit lymphocyte proliferation

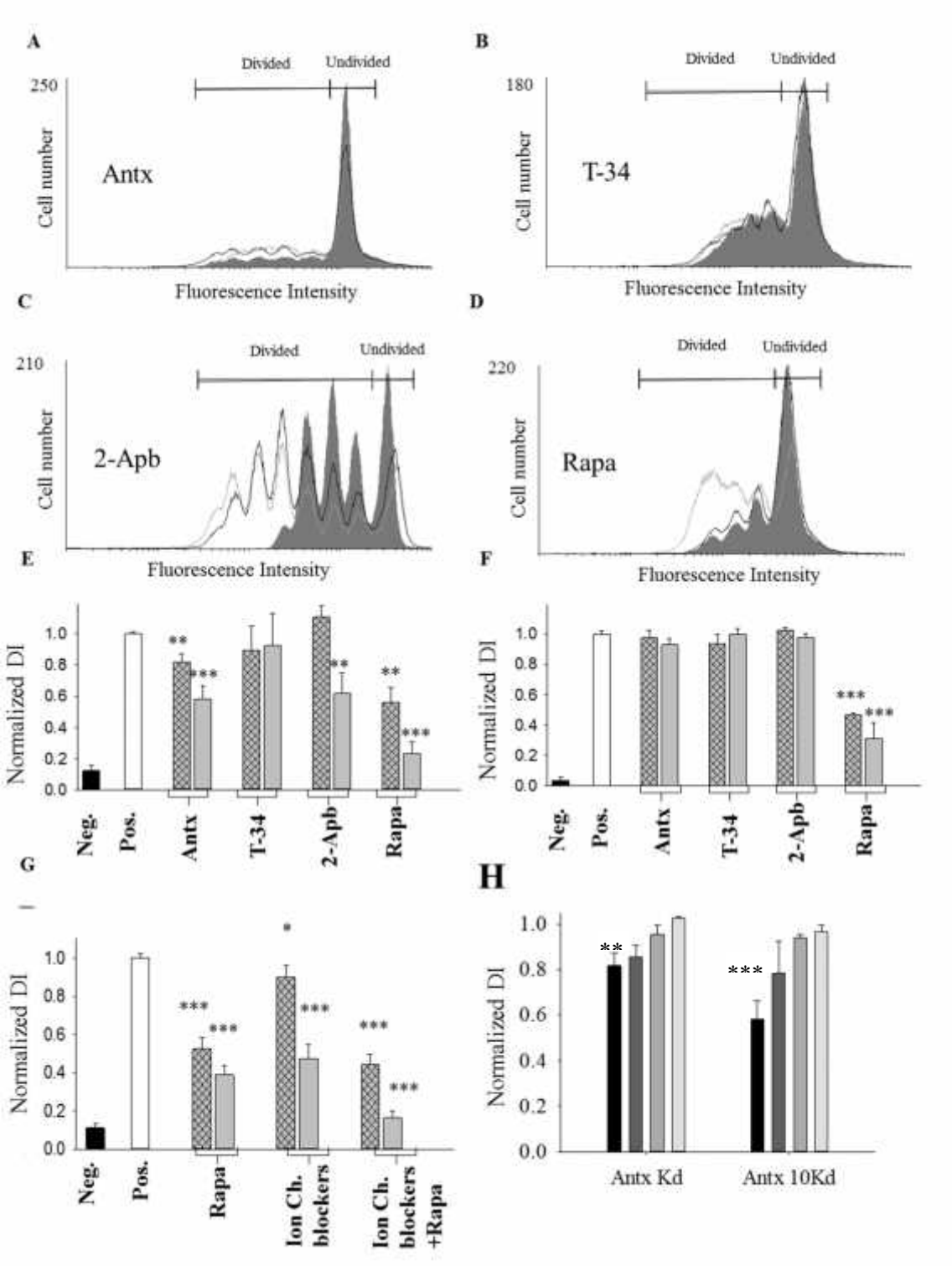
The effect of ion channel inhibitors on cell proliferation was tested at a concentration corresponding to the dissociation constant of the drug on the relevant channel ($1\times K_d$) and at ten times higher concentration ($10\times K_d$). Rapamycin was used at the lowest IC_{50} obtained from the relevant literature [42,43], and at ten times higher concentration. Figs. 2A-2D show representative fluorescence histograms of the CFSE dilution assay recorded in the absence or in the presence of the blockers in two concentrations. The markedly reduced peaks of the gray-shaded histogram relative to the control light-gray line in Fig. 2A qualitatively show that the Kv1.3 K^+ channel blocker anuroctoxin (Antx) at $10\times K_d$ concentration inhibited proliferation

when the cells were stimulated at low mitogen concentration. Quantitative analysis using normalized DIs (Figs 2E and F) showed that Antx at $1\times K_d$ and $10\times K_d$ concentration inhibited proliferation at low ($p=0.004$, $p<0.001$, respectively), but not at very high ($p=0.930$) mitogen concentration. The nearly superimposable fluorescence histograms in Fig. 2B show that the KCa3.1 inhibitor TRAM-34, regardless of its concentration, caused only a minor reduction of the proliferation of T cells stimulated by low mitogen concentration. The statistical analysis of the DIs (Figs 2E and F) showed that TRAM-34 failed to inhibit cell proliferation both at $1\times K_d$ and at $10\times K_d$ concentrations regardless of the mitogen concentration used (at $10\times K_d$ TRAM-34 concentration $p= 0.489$ and $p=0.993$ for low and very high mitogen concentrations, respectively). The gray-shaded histogram in Fig. 2C shows qualitatively that at low mitogen stimulation the CRAC channel modulator, 2-Aminoethoxydiphenyl borate (2-Apb) applied at $10\times K_d$ blocker concentration inhibited cell proliferation whereas $1\times K_d$ blocker concentration was ineffective. This was confirmed by statistical analysis in Fig. 2E ($p=0.694$ for $1\times K_d$ and $p<0.001$ for $10\times K_d$). Fig. 2F shows that at very high mitogen concertation 2-Apb did not inhibit T cell proliferation even at $10\times K_d$ concentration.

The representative histograms in Fig. 2D shows that the mTOR inhibitor rapamycin, applied at both $1\times IC_{50}$ and $10\times IC_{50}$ concentrations markedly inhibits the proliferation of T cells stimulated with low mitogen concertation. This effect was confirmed by the statistical analysis shown in Fig 2E ($1\times IC_{50}$: $p=0.003$; $10\times IC_{50}$: $p<0.001$). As opposed to the ion channel blockers Antx and 2-Apb, rapamycin alone inhibited proliferation even at very high mitogen concentration ($p<0.001$) both at $1\times IC_{50}$ and $10\times IC_{50}$ doses. As shown in Figure 2G, using the combination of all ion channel blockers at $10\times K_d$ concentration led to a marked inhibition of cell proliferation ($p<0.001$), which did not differ from the blocking potential of $10\times IC_{50}$ rapamycin ($p=0.113$). The inhibitory effect of ion channel blockers combined with rapamycin proved to be the most effective treatment, resulting in the complete blockage of cell division ($p<0.001$ compared to

control, mean DI=0.163). In the latter case proliferation was not significantly different from the negative control group (mean DI=0.110), which was not stimulated by mitogens (p=0.515).

Data above showed that the Kv1.3 blocker Antx and the CRAC channel blocker 2-Apb interfered with T cell proliferation only if cells were stimulated at low mitogen concentration but were ineffective if cells were stimulated with very high mitogen concentration. To further explore this phenomenon, we measured the normalized DI at varying mitogen concentrations (low, medium, high and very high, see above) in the presence of $1\times K_d$ (Fig. 2H left panel) or $10\times K_d$ (Fig. 2H right panel) concentrations of Antx. As shown in Fig. 2H right panel a marked inhibition of cell division was observed when the combination of low mitogen and $10\times K_d$ blocker concentration (black bar) was used ($p<0.001$). At medium, high and very high mitogen concentrations the inhibition of proliferation was not statistically significant as compared to the positive control (p=0.089, 0.372 and 0.742, respectively), but a clear decreasing trend is seen in the effectiveness of the blockers with increasing mitogen concentration. The same tendency could be observed if Antx was applied at $1\times K_d$ concentration (Fig. 2H left panel). The inhibition of proliferation was statistically significant only if low mitogen concentration (p=0.004) was used whereas at medium, high, and very high mitogen concentrations the inhibition of proliferation did not prove to be significant (p=0.365, 0.955 and 0.964 at medium, high and very high mitogen concentrations, respectively).



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315 **Figure 2. Effect of ion channel blockers and rapamycin on cell proliferation.** A-D) The
316 representative fluorescence histograms corresponding to the CFSE dilution assay (see methods)
317 show the effect of four inhibitors on T cell proliferation. Light gray lines in the histograms

indicate the positive control population (Pos.) treated solely with mitogens, black lines and gray filled histograms indicate data obtained in the presence of a blocker at $1\times K_d$ and $10\times K_d$ concentrations, respectively (A: Antx, 500pM ($1\times K_d$) and 5 nM; B: TRAM-34 (T-34), 20 nM ($1\times K_d$) and 200 nM; C: 2-Apb, 5 µM ($1\times K_d$) and 50 µM; D: rapamycin (Rapa), 20 pM ($1\times IC_{50}$) and 200 pM ($10\times IC_{50}$)). Cells were stimulated with low mitogen concentration in each case (200 ng/ml anti-CD3 and 1 µg/ml anti-CD28 or 1 bead:200 cells). E-F) Proliferation, represented by DI (see Figure 1.), at low (Fig. 2E, 200 ng/ml anti-CD3 and 1 µg/ml anti-CD28 or 1 bead:200 cells) or very high (Fig. 2F, 3 µg/ml and 1 µg/ml anti-CD28 or 1 bead:1 cell) mitogen concentrations in the presence of $1\times K_d$ (cross-hatched bars) and $10\times K_d$ (gray) blocker concentrations (for concentrations, see above). Neg. indicates the negative control population, where cells were not stimulated by mitogens, but were stained with CFSE. The DIs of samples treated with different blockers were normalized to the average DI of the Pos. sample. G) Comparison of the effectiveness of treatment combinations as compared to the positive control (Pos.). During the combined treatments each blocker was applied at its $1\times K_d$ (cross-hatched) or $10\times K_d$ (gray) concentration, data obtained for different mitogen concentrations were pooled for the analysis. H) Mitogen-dependence of the inhibition of cell proliferation. The DIs were determined in the presence of Antx at $1\times K_d$ (left) or $10\times K_d$ (right), at low (black), medium (dark gray), high (gray) and very high (light gray) mitogen concentrations (see legend to Fig. 1). Error bars indicate SEM, asterisks indicate significance (* if p was <0.05, with **, if p was <0.01, and with ***, if p was <0.001)

3.3 Cell viability is not affected by inhibitors of T cell proliferation

The reduced proliferation in the presence of the ion channel blockers and rapamycin (see above, Fig. 2) might be induced by a decrease in the viability of the cells in the presence of these

compounds. This was tested in the experiments shown in Fig. 3 using a propidium iodide uptake assay. The dot-plots in Fig. 3A show the threshold discriminating viable and non-viable cells using the combination of forward scatter and PI fluorescence. The corresponding fluorescence histograms in Fig 3B show that stimulation of the cells increased the proportion of the viable cells. The proportion of viable cells was not altered either by the ion channel blockers or rapamycin alone, or in their various combinations regardless of the concentration of the compounds (Fig. 3C, $1\times K_d$: p=0.903, 0.902, 0.652 and 0.508 for Antx, TRAM-34, 2-Apb and rapamycin, respectively; or $10\times K_d$: p=0.871, 0.867, 0.740 and 0.225 for Antx, TRAM-34, 2-Apb and rapamycin, respectively; p=0.244 for their combination).

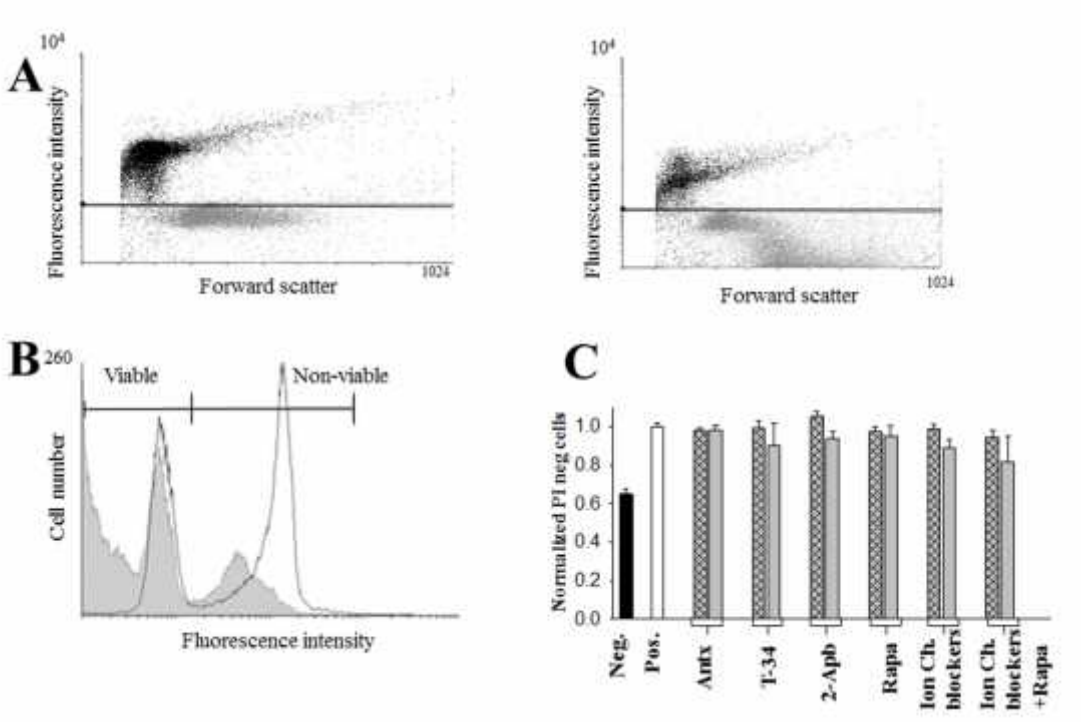

Figure 3. Effect of ion channel blockers and rapamycin on the cellular viability. A) The dot plots show the standard gating strategy for propidium iodide (PI) staining. The left panel indicates non-stimulated (Neg.), the right panel shows the stimulated population (Pos.) after 5 days of incubation with superparamagnetic bead-conjugated anti-CD3 and anti-CD28 monoclonal antibodies at 1 bead:1 cells ratio. The horizontal black line indicates the threshold for

discriminating live and non-live cells. B) The fluorescence histograms were generated from the dot plots shown in A, the bar and the markers indicate the PI negative viable and the PI positive non-viable cells in the Neg. (black line) and Pos. (gray fill) samples. C) Viability of the cell populations in the presence of inhibitors. The number of PI negative cells in the presence of various compounds was normalized to that of the untreated, but activated cells (Pos. sample). Cross-hatched and gray bars show data obtained in the presence of $1\times K_d$ and $10\times K_d$ concentrations of the indicated compounds alone or in mixtures (T-34: TRAM-34, Rapa: rapamycin). Mixtures of ion channel blockers contained each blocker at $1\times K_d$ or $10\times K_d$ (Ion Ch. blockers) whereas in the Ion Ch. Blockers+Rapa samples the ion channel blocker mixture is supplemented with the corresponding concentrations of rapamycin ($1\times IC_{50}$ or $10\times IC_{50}$). Error bars indicate SEM.

3.4 Cytokine production of T cells can be reduced by ion channel blockers

We investigated the effect of channel blockers and rapamycin on the secretion of the anti-inflammatory IL-10 and the pro-inflammatory IFN-γ cytokines by ELISA. Increasing the mitogen concentration from low to very high induced approximately 4-fold and 10-fold increases in secreted IL-10 and IFN-γ levels, respectively (Fig. 4. A and B, Pos.). IFN-γ as well as IL-10 secretion was significantly inhibited at low mitogen stimulation by all inhibitors and combinations at both applied concentrations (Fig. 4. A and B, left panels) (IFN-γ: $p<0.001$ for all inhibitors; IL-10: Antx $1\times K_d$ $p=0.046$, $10\times K_d$ $p=0.024$, TRAM-34 $1\times K_d$ $p=0.048$, $10\times K_d$ $p=0.008$, 2-Apb $1\times K_d$ $p=0.039$, $10\times K_d$ $p=0.007$, rapamycin $1\times K_d$ $p=0.009$, $10\times K_d$ $p<0.001$, ion chann blockers $1\times K_d$ $p=0.002$, $10\times K_d$ $p<0.001$, ion channel blockers together with rapamycin $1\times K_d$ and $10\times K_d$ $p<0.001$). At very high mitogenic stimulation IFN-γ secretion was inhibited by AnTx, rapamycin and combination treatments at $10\times K_d$ and by 2-Apb and the ion channel

blocker combination at $1\times K_d$ (Fig. 4A right panel)(Antx $10\times K_d$ p=0.041, 2-Apb $1\times K_d$ p=0.049, rapamycin $10\times K_d$ p<0.001, ion channel blockers $1\times K_d$ p=0.002, ion channel blockers together with rapamycin p<0.001). IL-10 production was only inhibited by $10\times K_d$ 2-Apb, rapamycin and combination treatments (Fig 4B right panel) (2-Apb $10\times K_d$: 0.004, rapamycin$10\times K_d$:0.002 , ion channel blockers p=0.004, ion channel blockers together with rapamycin: p<0.001).

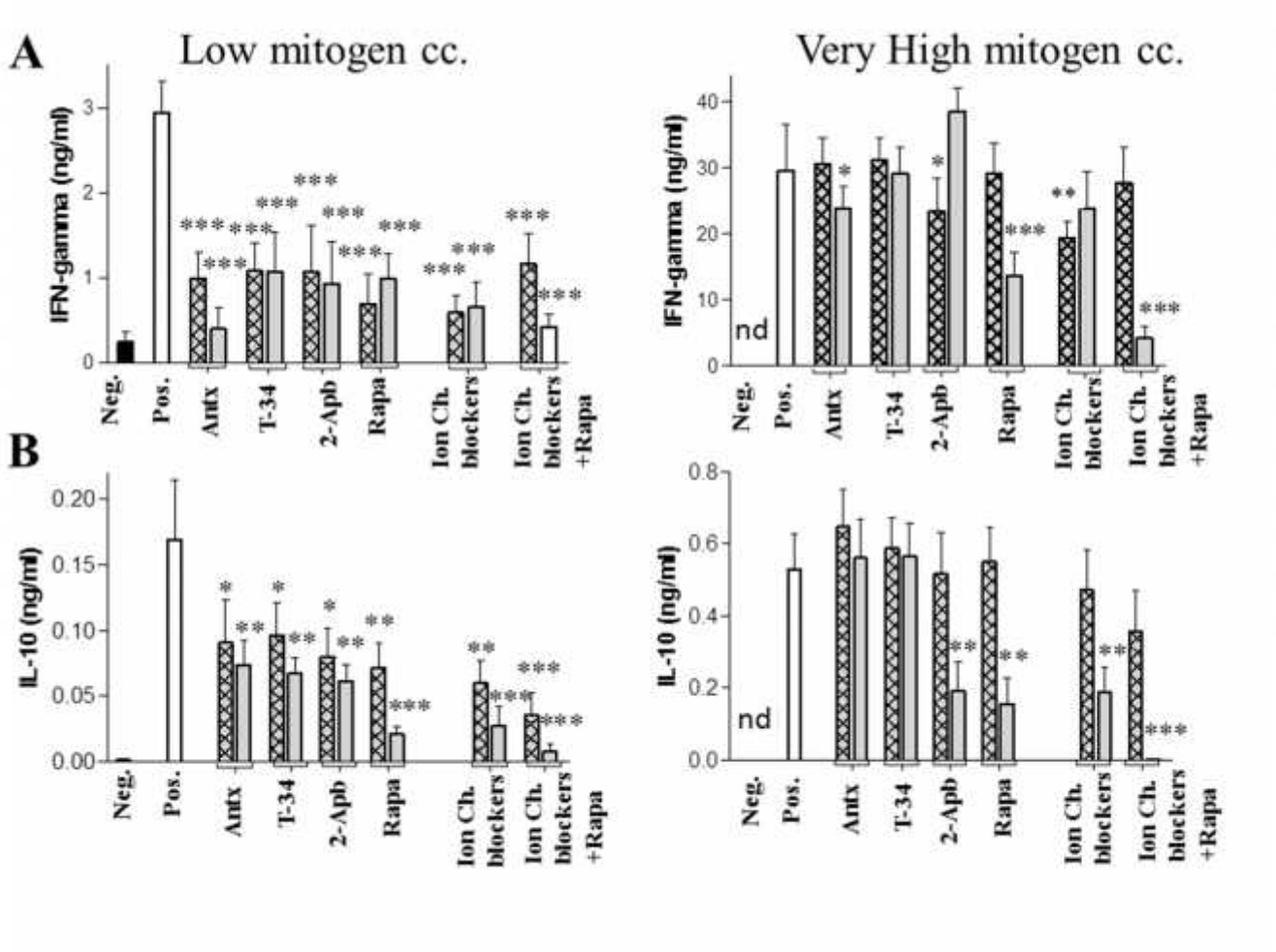

Figure 4. Inhibition of cytokine secretion by ion channel blockers and rapamycin

A) IFN-γ and B) IL-10 secretion were measured using ELISA (OptEIA kit) at different mitogen concentrations, respectively. Left and right panels refer to data obtained at low and very high mitogen concentrations. Neg. indicates the unstimulated control cell population whereas Pos. indicates the mitogen-stimulated cell population in the absence of ion channel blockers and/or

rapamycin. The blockers are represented at $1\times K_d$ (cross-hatched), $10\times K_d$ (gray) or in case of rapamycin $1\times IC_{50}$ and $10\times IC_{50}$ concentrations, respectively. Error bars indicate SEM, asterisks indicate significance (* if p was <0.05, with **, if p was <0.01, and with ***, if p was <0.001), and n.d. indicates “not detectable” where values are too low to be shown.

Discussion

Altered T cell homeostasis is involved in the pathogenesis of autoimmune diseases such as multiple sclerosis [44] and systemic lupus erythematosus [45]. To maximize anti-proliferative effects and to reduce potential side effects, immunosuppressive drugs are commonly used in combinations [46]. One group of the most promising candidates for future therapy is the family of Kv1.3 inhibitors, because this ion channel is found only in a few tissues and it can be inhibited selectively [28]. Before applying ion channel blockers in therapy it is crucial to investigate how they interact with other immunosuppressive agents. However, original research data about the pharmacodynamics of combinations of traditional immunosuppressive and novel drugs such as ion channel blockers are scarce and they usually lack functional comparison. Therefore, in our recent experiments, we approached this problem from multiple aspects and have found an additive interaction between rapamycin and the ion channel blockers when using them in combination.

The effects of ion channel blockers exerted on T cell functions have already been described [17]. However, to the best of our knowledge, no data comparing the proliferative effects of Kv1.3, KCa3.1 and CRAC channel blockers applied alone or in combination at identical experimental conditions are currently available. Moreover, the synergy between the effects of ion channel inhibitors and the mTOR inhibitor rapamycin has not been investigated to date. In the present study we are the first to describe the mitosis-inhibiting effect of Antx, a high affinity scorpion toxin blocker of Kv1.3 [40] both at high ($10\times K_d$) and low ($1\times K_d$) concentrations. The observed effect of 2-Apb correlated well with past literature, as it was proposed that 2-Apb has a bimodal effect. At low blocker concentrations (K_d), 2-Apb promotes Ca^{2+} signaling, which ultimately results in enhanced cell proliferation, whereas at higher concentrations, in our case at $10\times K_d$, it effectively inhibits the CRAC channel, ultimately blocking cellular proliferation [47].

Even low mitogen concentrations, which corresponded to 1:200 bead:cell ratio produced an unexpectedly high amount of polyclonal lymphocyte proliferation, as over 30% of the cells have undergone cell division. This phenomenon may be explained by the fact that there is a large number of anti-CD3 and anti-CD28 molecules on a single bead, and that lymphocytes form a rosette-like structure around beads. Therefore, numerous lymphocytes are activated simultaneously by a single bead, and this effect could be further enhanced by autocrine and paracrine cytokine secretion of the activated T cells [48,49].

Our most intriguing finding in this research was that increasing the mitogen concentration markedly decreased the anti-proliferative effect of ion channel blockers that ultimately completely disappeared when cells were stimulated with very high concentration of the mitogens. A possible explanation may be that at low mitogen concentrations the few, initially highly localized Ca^{2+} signals are suppressed by the blocked ion channels in their immediate vicinity [50]. However, at very high mitogen concentration when most TCRs are likely to be activated, the number of localized signaling loci is sufficiently high so that even a very low fraction of unblocked ion channels is sufficient to maintain the downstream activation cascade upon TCR activation. Moreover, it is reasonable to assume that lymphocytes redirect their activation pathways to other, Ca^{2+}-independent directions. As several intracellular signaling pathways, e.g. mTOR activation, do not essentially involve ion channels [29,30], these processes may become overly active upon applying very high mitogen concentrations. However, to the best of our knowledge no study has ever addressed this question and thus it warrants further experiments.

At very high mitogen concentrations we could achieve significant blockage of proliferation only by using rapamycin or its combination with the ion channel blockers acting on a different pathway that ultimately leads to permanent changes in cellular signaling. This may indicate that co-treatment of T cells with rapamycin and ion channel blockers may be a more feasible therapeutical approach than using these drugs separately.

Previous studies have shown that blocking Kv1.3 channels without affecting the KCa3.1 channels inhibited IFN-γ expression in a subset of T-cells with effector memory phenotye (T_{EM}) [51]. Moreover, the blockage of CRAC channels with SKF 96365 decreased both IL-10 and IFN-γ production [52]. In line with these studies our data showed that treatment of T-cells with various inhibitors (Fig. 4) significantly decreased both anti-inflammatory IL-10 and inflammatory IFN-γ cytokine production but only at low mitogenic stimulation. In accordance with the literature TRAM-34 strongly suppressed cytokine production despite the fact that it did not not inhibit proliferation [14,34]. At very high mitogen concentration the effect of the ion channel blockers on cytokine production diminished. Although some of them caused statistically significant reductions in cytokine production, these changes are not likely to be biologically relevant as the remaining concentration of IFN-γ still remained in the ng/ml range and therefore was sufficient to promote cell proliferation, so division rate was unaffected. In contrast, rapamycin and the combination treatments applied at $10\times K_d$ concentration caused a more robust decrease, which was also reflected in the suppressed proliferation of these cells.

Since IL-10 and IFN-γ levels were affected in a qualitatively similar manner by the inhibitors both at low and very high mitogen concentrations, it is safe to assume that these treatments did not alter the proportion of T cell subtypes specifically (i.e. Th_1 CD4 and CD8 cytotoxic T cells vs. Th_2 T cells and regulatory $CD4^+/CD25^+/FoxP3^+$ T_{reg} cells), but rather were affecting globally the entire T cell population.

In summary, the greatest level of inhibition of T-cell proliferation and the production of selected cytokines could be achieved by rapamycin, and this effect could be further potentiated by using it in combination with cation channel blockers. This may indicate an additive effect of Ca^{2+}-dependent and Ca^{2+}-independent inhibitory mechanisms involved in T-cell activation. Finally, we found that upon increasing the concentration of the mitogenic antibodies, the anti-proliferative effect of ion channel blockers faded. This phenomenon can be due to a yet unknown

mechanism in intracellular signaling of activated T cells, which is to be elucidated in the future. The increased *in vitro* antiproliferative potentcy of rapamycin and ion channel blocker combination presented in this study urges for *in vivo* experiments whereby the therapeutic benefit of the combined treatment can be assesed.

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5. Conflict of interest

The authors declare no commercial and financial conflict of interest regarding this project.

6. Vitae

Zoltan Petho, graduate student at the University of Debrecen, MD:

He studies the ion channels of the immune system and of cancer cells. During his medical studies his main interest were the voltage-gated ion channels of T-cells and their potential functional implications. Currently he is also focused on the ion channels of invasive cells and their roles in tumor metastasis. He is skilled in proliferation assays, flow cytometry, functional studies such as migration and invasion assays and patch-clamp electrophysiology.

Andras Balajthy, graduate student at the University of Debrecen, MD:

His main interest is to study the interaction of cholesterol and voltage gated ion channels. Currently he studies the Kv1.3 ion channels of lymphocytes isolated from Smith-Lemli-Opitz syndrome. He is a well-trained electrophysiologist, but also familiar with flow-cytometry and confocal microscopy.

Adam Bartok: postdoctoral fellow at the Thomas Jefferson University, Philadelphia, PhD:

He is experienced in K+ channel electrophysiology. During his graduate research in Gyorgy Panyi's lab, he focused mainly on the pharmacology of peptide toxins using patch-clamp technique. Moreover, he is skilled in measurements of mitochondrial calcium signaling, recombinant protein synthesis, fluorescence measurements, confocal microscopy and electron microscopy.

Krisztian Bene, predoctor at the University of Debrecen:

He is predictor at the research group headed by Éva Rajnavölgyi in the University of Debrcen and focuses on the biology of human dendritic cells (DC). He studies the effects of microbial antigens on inflammation and T lymphocyte activation mediated by different DC populations. He plays also part in analyzing the role of cell signaling pathways related to pro-inflammatory cytokine and type I. interferon production in DC.

Sandor Somodi, assistant professor at University of Debrecen, MD, PhD

Sandor Somodi completed his PhD in 2007 at the University of Debrecen. During his graduate research he studied inactivation kinetics and pharmacology of the Kv1.3 channel. Recently, he works at the Internal Medicine Department focusing on metabolic diseases and mainly lipid dysorders. He is skilled in ion channel pharmacology and patch clamp electrophysiology.

Orsolya Szilagyi, postdoctoral fellow at the National Institute of Health, PhD

Orsolya Szilagyi completed her PhD in 2014 in molecular medicine at the University of Debrecen in 2014. During her PhD she spent three months at the University of Cincinnati as a visiting scientist. Currently, her scientific interests include elucidating the function of various voltage sensor proteins both in the immune and the nervous system by the means of biophysical methods such as electrophysiology (patch clamp, voltage clamp fluorometry) and confocal microscopy.

Eva Rajnavolgyi, Professor of Immunology at the University of Debrecen, PhD, DSc:

She has a long lasting experience in studying the interplay of innate and adaptive immune responses with a focus to DC subtypes/subsets driving anti-microbial defense. Currently, she is interested in identifying cellular mechanisms regulating the collaboration of signaling pathways driving anti-viral immune responses. She is also involved in uncovering the impact of retinoic acid induced gene-I (RIG-I) and mesenchymal stromal cells (MSC) on the outcome of DC-induced immune responses. Recently, her research group described the role of mTOR in the regulation of monocyte-derived and CD1c+ DC functions.

Zoltan Varga, Senior research fellow, Head of the MTA-DE-NAP B Ion Channel Structure-Function Research Group, RCMM, University of Debrecen, PhD, DSc:

He is experienced in studying the gating of voltage-gated ion channels including voltage-sensor movements. Moreover he uncovered many aspects of the pharmacology of voltage-gated K^+ channels, especially Kv channel and characterized multiple blocking scorpion toxins. Moreover he is also involved in C-type inactivation of ion channels and the effect of ionic conditions and pH on channel gating.

Gyorgy Panyi Professor of Biophysics at the University of Debrecen, M.D., Ph.D., D.Sc.:

Gyorgy Panyi is engaged in unveiling the functional and biophysical characteristics of voltage-gated ion channels. His research projects focus on two types of ion channels of T-lymphocytes, the voltage-gated K+ channel, Kv1.3, and the Ca2+-activated K+ channel, KCa3.1. Understanding the biophysical properties of Kv1.3 and KCa3.1 and their regulation may be critical to our comprehension of T-cell physiology and immune responsiveness.

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