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Original Paper

Kv1.3 Current Voltage Dependence in Lymphocytes is Modulated by Co-Culture with Bone Marrow-Derived Stromal Cells: B and T Cells Respond Differentially

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Key Words
Kv1.3 channel • Voltage dependence • B lymphocytes • T lymphocytes • Mesenchymal stem cells

Abstract
Background/Aims: Kv1.3 channel is the only voltage-dependent potassium channel in plasma membrane of human lymphocytes. Bearing in mind a rather steep voltage-dependence of Kv1.3 activation and inactivation, its modulation by B and T cells activation and by co-culture with stromal bone-marrow cells was addressed. ***Methods:*** Patch-clamp technique in the whole cell mode was applied to human resting and activated human B and T cells, in monoculture and co-culture with stromal OP9 cells. ***Results:*** Polyclonal activation of B and T cells in monoculture caused Kv1.3 current in B cells to activate at more negative and in T cells at more positive potentials, whereas the inactivation of Kv1.3 current in resting T cells occurred at more negative voltages. Co-culture with OP9 cells abolished the shift of voltage dependence upon the polyclonal activation but fixed the substantial difference between B and T cells, resting or activated, with both activation and inactivation negatively shifted by 15 mV for T lymphocytes. However, activated B cells displayed an incomplete inactivation, which was augmented by the co-culture. Neither activation nor co-culture caused substantial changes in the Kv1.3 current density. ***Conclusion:*** The combination of activation and inactivation processes yields the fraction of steady-state Kv1.3 current (window current), which was higher in activated B cells, partly due to an incomplete inactivation. A relatively smaller window current in resting B cells and resting T cells in co-culture correlated with a more depolarized resting membrane potential. Rather than insignificant changes in the Kv1.3 channels functional expression, the modulation of their voltage dependence by activation and co-culture with bone-marrow stromal cells was essential for the control of membrane potential.

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Introduction

Bone marrow mesenchymal stem cells (BMSC) are multipotent non-hematopoietic cells usually found as a very small population in bone marrow. BMCS possess capability to differentiate in multiple cell lineages, and recirculating BMSC can migrate to damaged tissues and participate in their reparation. Additionally, numerous reports evidenced immunomodulatory properties of BMSC. In particular, BMSC were shown to suppress T and B cell responses to antigen stimulation. Due to this feature, BMSC were suggested to be used for the treatment of autoimmune diseases and graft-versus-host disease. Different mechanisms of action were reported for BMSC-related immunosuppression, including requirement of direct contact with immune cells and/or secretion of soluble factors, such as nitric oxide (NO), tumor growth factor (TGF-β), prostaglandin E2 (PGE2) [1-4]. Modulatory effects of aforementioned bioactive compounds on ion channel expression/function were reported for different cellular models [5-9]. Ion channels are important players in signaling events during lymphocyte proliferation, but possible BMSC effects on the expression and biophysical properties of lymphocyte plasma membrane ion channels were not addressed, except a single study where no significant difference in Kv1.3 current between human B cells in monoculture and co-culture with BMSC was reported, whereas possible modulation of the Kv1.3 voltage dependence by a co-culture with BMSC was not explored [10].

Electrical potential difference across the plasma membrane controls a variety of transport and signaling events, as well as cellular processes, including the progression of the cell cycle [11-12]. In quiescent lymphocytes, voltage-dependent K^+ channels of the Kv1.3 type are principle controllers of membrane potential. Inhibition of Kv1.3 channels in the mitogen-activated T cells induces membrane depolarization, suppresses Ca^{2+} influx via calcium release- activated channel (CRAC), and decreases IL2 production and proliferation [13-14]. Activation of B or T cells, except specific subsets of $CD8^+$ effector memory T cells, hardly causes an increase of a number of active Kv1.3 channel copies but of Ca^{2+}-activated K^+ ones, KCa3.1 [15]. At the same time, both Kv1.3 and KCa3.1 channels in T cells are recruited to the immunological synapse (IS) during antigen presentation [16-17]. Importantly, STIM1 and Orai1 subunits, which aggregate to form CRAC channels, are also re-localized to the IS [18]. All three channels seem to contribute to the localized Ca^{2+} influx, providing therefore a long-term stability of the IS [13].

Posttranslational modification and modulation by microenvironment need to be considered to understand the Kv1.3 channels' function. In T-lymphocytes, Kv1.3 channels form a part of a large protein complex, which includes membrane bound β1-integrin and cytosolic proteins as auxiliary Kvβ2 subunit (mediating the redox sensing), connected via ZIP to Lck-kinase, which, in turn, interacts with the T cell receptor (TCR) complex. There is ample evidence that Kv1.3 channel activity is suppressed by phosphorylation with Src-family tyrosine-kinase $p56^{lck}$ [19-22]. Moreover, this phosphorylation shifts the Kv1.3 activation by ~10 mV to more positive potentials [23]. Kv1.3-containing signaling complex is situated within a lipid raft, whose disruption causes a shift of the voltage activation curve up to by 11 mV, whereas voltage dependence of inactivation suffers a lesser change [24-26]. In contrast, hypoxia and oxidative stress mainly affects the inactivation, causing a positive shift of its voltage-dependence by ~10 mV [27-28].

Kv1.3 channels display a very sharp dependence of the activation and inactivation processes on the membrane voltage. Thus, their steady-state activity is defined by a juxtaposition of these two processes, which generates so called "window current", i.e. current through a relatively small population of Kv1.3 channels being activated and remaining non-inactivated at the same time [29-30]. Thus, the position of the activation voltage threshold and a *relative* position of activation and inactivation curves may result in a change of Kv1.3 channels activity by an order of magnitude, following moderate shifts of the voltage dependence by 10-12 mV. Changes of this magnitude were indeed documented (see above). In this study we wish to address the following unanswered questions. First, the voltage dependence of Kv1.3 current is relatively poor documented for human B lymphocytes in contrast to T ones.

The present work pretends to fill this gap. Next, we are wondering whether there are any significant changes in Kv1.3 voltage dependence upon lymphocytes activation and if so, whether the responses of B and T cells are similar or dissimilar? Finally, we have co-cultured B or T lymphocytes, extracted from healthy donors, with bone marrow- derived stromal OP9 cells and compared the Kv1.3 current voltage dependence with that in monocultures.

Materials and Methods

Isolation of CD4⁺ T and CD19⁺ B cells from peripheral blood

Heparinized freshly isolated blood samples from healthy volunteers (males and females younger than 35 years) were diluted 1:1 with cold PBS. Peripheral blood mononuclear cells (PBMC) were separated by centrifugation in Ficoll (17-144002, Ficoll-Paque 1.073, GE Healthcare) gradient (1:1.5 blood/Ficoll ratio, 1000x*g*, 30 min, RT). PBMC were collected from the interphase and carefully washed in PBS. Finally, cells were resuspended in a fresh RPMI 1640 medium, supplemented with 10% of fetal bovine serum (FBS) and incubated overnight for cell recovery at 37°C in a humidified atmosphere of 5% of CO_2. Culture medium, FBS, PBS and antibiotics were from GIBCO (Thermo Fisher Scientific, Waltham, MA, USA). Next day, PBMC were used to isolate specific populations of lymphocytes. CD4⁺ T Cell Isolation Human kit (Miltenyi Biotec, Bergisch Gladbach, Germany) or human CD19⁺ B Cell Isolation kit (Miltenyi Biotec) were used following the manufacturer's specifications. The purity of enriched populations was checked by flow cytometry (BD FacsCantoII cytometer, Franklin Lakes, NJ, USA), staining aliquots with either anti-CD4⁺ antibodies conjugated with PE (BioLegend 357404) or anti-CD19⁺ antibodies conjugated with FITC (BioLegend 302206). Purity of 96-98% was achieved.

Polyclonal activation of CD4⁺ T and CD19⁺ B lymphocytes

Polyclonal activation of the CD4⁺ T cells was performed using mouse-antihuman monoclonal anti-CD3e and anti-CD28 antibodies (BD 555336 and BD 555725, respectively). The wells of 48 well plates were pretreated with anti-CD3 antibodies (5 μg/mL) overnight at 4°C. Prior to use, the plate was incubated for 1 hr at 36°C with 5% of CO_2. Finally, the wells were washed with PBS, and CD4⁺ T lymphocytes (0.5-0.8x10^6 in 1 mL of complete RPMI medium) were seeded in presence of anti-CD28 antibodies (2 μg/mL).

Polyclonal activation of CD19⁺ B cells was performed in 48-well plates. Briefly, 0.5-0.8x10^6 of B cells were placed in 1 mL of complete RPMI medium additionally supplemented with 500 ng/mL ionomycin, 1 ng/mL PMA, 20 ng/mL IL-4, and 1 μg/ml CD40L (Thermo Fisher Scientific).

The activation efficiency was evaluated at 24 h of incubation by CD69 expression using flow cytometry. For this assay, the samples were stained with Bv421-conjugated anti-CD69 antibodies (BD 562884). Typically, 90-95% of activated T lymphocytes and 70-80% of activated B lymphocytes were obtained. Cells were incubated 72 h (37°C, humidified atmosphere of 5% of CO_2) and used for further experiments.

Co-culture with mesenchymal stromal cells

Lymphocytes were co-cultured with bone-marrow derived OP9 mesenchymal stem cells [31-33]. OP9 cells (2X10^5) were seeded onto round (8 mm diameter) coverslips placed in 48-well plates, in 1 mL of α-MEM medium (Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 10% FBS. At 24 hrs of incubation, CD4⁺ or CD19⁺ lymphocytes (resting or activated during 72 h) were added in relation 10:1 (lymphocytes/ stromal cells). The co-cultures were incubated for next 48 h (in case of polyclonal activation, corresponding supplements were added). Finally, the coverslips were transferred to the patch-clamp chamber for recording. Lymphocytes were easily distinguished from stromal cells by their appearance and much smaller size.

Electrophysiology

Patch pipettes were fabricated from borosilicate capillaries (Kwik-Fil 1B150F-4, World Precision Instruments, Houston, TX, USA) using the P-97 programmed puller (Sutter Instruments, Novato, CA, USA) and heat polished using the LPZ 101 microforge (List Medical, Germany). The resistance of the electrodes filled by standard solution was 3-5 MΩ.

Pipette solution was (in mM): 134 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 10 EGTA, 10 HEPES-KOH (pH 7.4) (7.5 nM free Ca^{2+}, evaluated with the WINMAXC32 v2.50 program). The bath solution contained (in mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 2.5 $CaCl_2$, 10 HEPES-NaOH (pH 7.4) (Sigma Aldrich, St. Louis, MO, USA). For all solutions, the osmolality was adjusted to 300 mOs, as verified with the OSMOMAT 030-D cryoscopic osmometer (Gonotec GmbH, Berlin, Germany). Margatoxin (1 nM) (TOCRIS, Bristol, UK) was used as a specific blocker for Kv1.3 [34].

Current records were made in a whole cell configuration under voltage clamp conditions and the current was measured by means of the AXOPATCH 200A amplifier (Molecular Devices, San José, CA, USA), using a PC connected to a Digidata 1200 (Molecular Devices). Data acquisition and analysis was done with the pClamp 6.0 software (Molecular Devices). To measure the resting cell potential, the measuring configuration was briefly switched to I=0 clamp mode.

Voltage protocols are described in detail by Valle-Reyes et al. (2018). Activation curves were constructed based on the relative magnitude of the chord conductance $I(V)/(V-E_{K+})$, where I(V) is a peak current at given potential and E_{K+} is equilibrium potential for K^+, about -84 mV at these ionic conditions. The data were adjusted to Boltzmann equation (Eq. 1) in the following way:

$$\frac{G}{G_{(max)}} = \frac{1}{1 + e^{-\frac{Z \times F \times (V - V_{1/2})}{R \times T}}}$$

where $V_{1/2}$ is the voltage for the activation of 50% of the Kv channels, Z is the gating charge, F, R and T have their usual meaning.

For the adjustment of the inactivation curves, a modified Boltzmann equation was used (Eq. 2):

$$\frac{I_{+50}}{I_{+50\,(max)}} = off + \frac{1 - off}{1 + e^{-\frac{Z \times F \times (V - V_{1/2})}{R \times T}}}$$

in this case the degree of inactivation was evaluated with a post-pulse at +50 mV, measuring the relative magnitude of the evoked current (subtracting the leakage current, estimated by a pre-pulse from -100 to -80 mV); offset current, *off*, has been introduced to indicate the fact that there may be a small but significant fraction of non-inactivated Kv channels at any test potential.

Statistical analysis

All results are expressed as the mean ± the standard error (n = total number of tested cells). Parametric tests of no paired assays were performed by means of ANOVA one-way, followed by a post hoc test of Tukey. Statistical tests were performed using the GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). p-value of ≤ 0.05 was considered statistically significant.

Results

Potassium-selective Kv1.3 channels are sole voltage-dependent K^+ channels in human lymphocytes and as such act as principle controllers of membrane potential, with important impact on the lymphocyte physiology [13, 29, 35]. To study Kv1.3, from a holding potential of -80 mV voltage was stepped to depolarized potentials up to 0 mV in 10 mV increment for 5 s to ensure the steady state inactivation. At the end of each record, the voltage was switched to +50 mV, to determine the fraction of non-inactivated channels (Fig. 1A, left). The same record was used to estimate the voltage-dependent activation, by measuring the amplitude of peak of the current transient at the pre-pulse. Alternatively, activation was evaluated by the application of brief (35 ms, to minimize the progress of inactivation) depolarizing pulses, ranging from of -60 to +20 mV (Fig. 1A right). In all cases, either with B or with T cells, resting, activated and/or co-cultured, measured voltage-dependent current was completely abolished by 1 nM margotoxin (MgTx), a high affinity blocker of Kv1.3 channels [34].

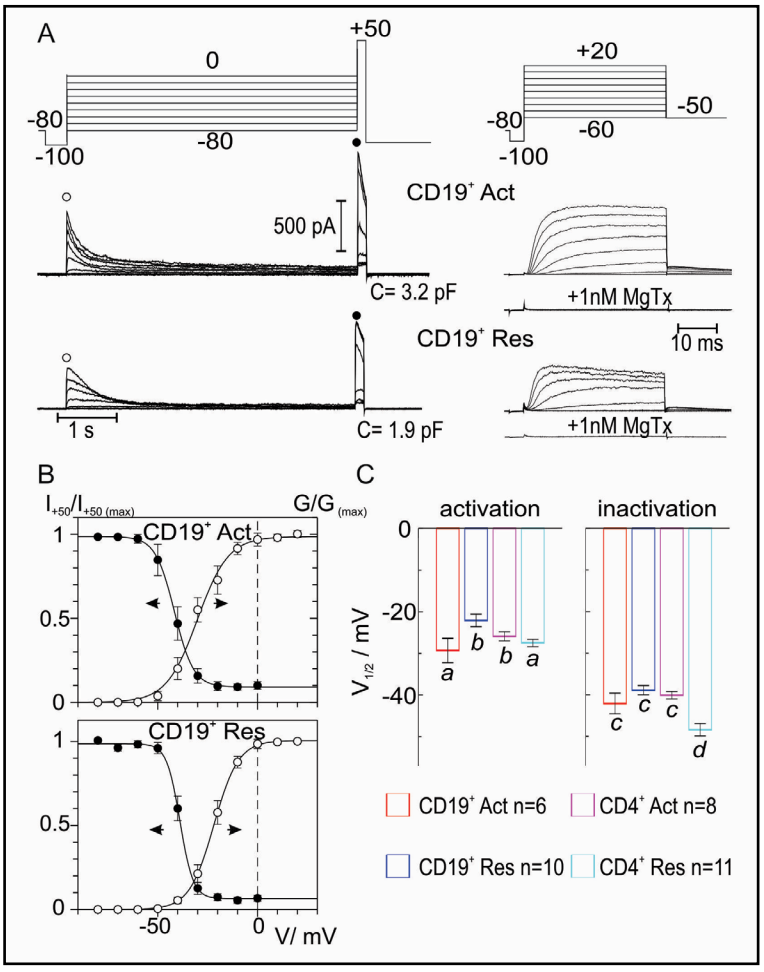

Fig. 1. Voltage dependence of activation and inactivation of Kv1.3 current in resting and activated lymphocytes. A) Time course of whole cell Kv1.3 current in response to voltage protocols, designed to reveal the inactivation (left) and activation (right) processes in B cells. B) Activation (hollow circles) and inactivation (filled circles) curves, relative Kv1.3 conductance and current, respectively, as a function of voltage in B cells. C) Midpoint potential values for Kv1.3 current in activated and resting B cells as compared to those obtained with T cells. For each cell sample, activation and inactivation curves have been fitted individually and the values of fitted parameters have been averaged for each condition. Data are mean ± SE, n is a total number of tested cells from 3 to 5 different donors for each condition. Different symbols are for significantly different values, significance range from $p<0.05$ (CD19+ activated vs CD4+ resting) to $p<0.0014$ (CD19+ resting vs CD4+ resting) for inactivation process and $p<0.013$ (CD4+ activated vs CD4+ resting) to $p<0.0001$ (CD19+ resting vs CD4+ resting or vs CD19+ activated) for the activation one.

Voltage dependence of both activation and inactivation tended to shift to more negative potentials during whole cell recording, especially notable in case of activated B cells (Fig. 2A). This phenomenon is very notable also in T cells [29-30]. Corresponding shift exceeded 10 and 15 mV in resting and activated T cells, respectively [36]. Thus, to minimize this effect and to extrapolate the situation to that in intact lymphocyte, we have initiated the first voltage protocol as soon as possible, 1-2 min after gaining into the whole cell configuration. In continuation, only the fits of voltage dependence originated from these initial records were considered. Quantitative analysis of activation and inactivation voltage dependence revealed the following differences between resting and activated B-cells (Fig. 1B): *i*) activation is shifted to more negative potentials in activated B cells; *ii*) the voltage dependence of the inactivation was quite similar, but the inactivation was incomplete, especially notable in activated B cells, with about 9% of channels remaining non-inactivated at depolarized potentials. Previously an incomplete inactivation was reported for human B lymphoma cells [37]. A combination of these two factors resulted in a larger time-averaged current ("window current") through a Kv1.3 channels in the activated $CD19^+$ cells, which may be roughly estimated from the area below cross of activation and inactivation curves.

Similar experiments on human $CD4^+$ T cells displayed a contrasting trend, with the Kv1.3 current in resting cells activated at more negative voltages than in activated ones (Fig. 1C). Voltage dependence of the inactivation was shifted to more negative potentials in resting T cells, with no significant difference between resting and activated B cells.

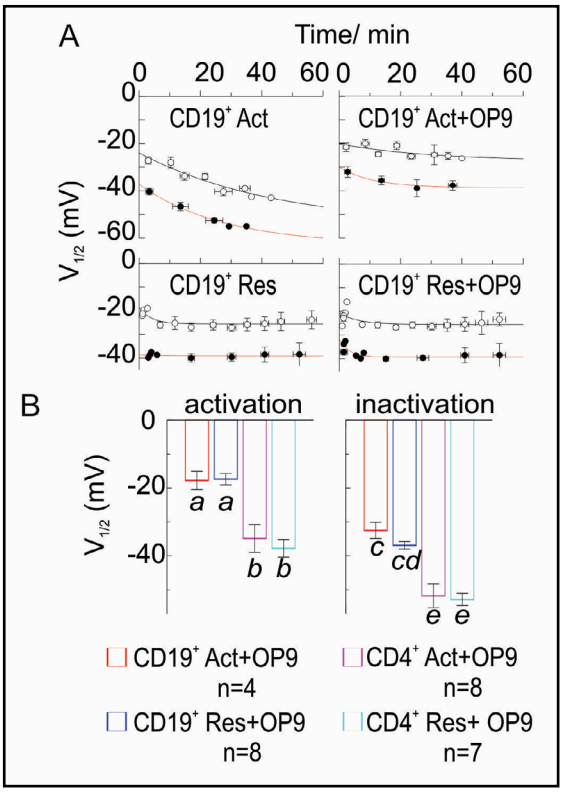

Fig. 2. Inactivation and activation of Kv1.3 current in B cells is shifted to more positive potentials as compared to T cells as a result of co-culture with stromal cells. A) Negative shift of midpoint potentials for activation (black line, hollow circles) and inactivation (red line, filled circles) of Kv1.3 current in B cells during recording in whole cell configuration, in mono- and co-cultures. B) Summary of midpoint potential values for Kv1.3 current activation and inactivation in resting and activated B and T lymphocytes, co-cultured with OP9 cells. Only the initial ones from the first record in whole cell configuration were considered. For each cell sample, activation and inactivation curves have been fitted separately and the values of fitted parameters have been averaged for each condition. Data are mean ± SE, n is a total number of tested cells from 2 to 3 different donors for each condition. Different symbols are for significantly different values, significance range from $p<0.003$ (CD19+ resting vs CD4+ activated) to $p<0.0001$ (CD19+ resting vs CD4+ resting) for the inactivation and $p<0.05$ (CD19+ activated vs CD4+ activated) to $p<0.0002$ (CD19+ resting vs CD4+ resting) for the activation process.

Co-culture with BMSC cells, OP9 [31] modulated the voltage dependence of Kv1.3 current, with contrasting behavior for B and T cells. First, upon gaining into the whole cell configuration, midpoint potentials for activation and inactivation remained relatively stable in time in B cells (Fig. 2A). Similar behavior was also observed with T cells (result not shown). Initial midpoint potential values for voltage dependence hardly changed in resting B cells as compared to the monoculture but suffered more than 10 mV positive shift in activated ones (Fig 1C, 2B). The difference in midpoint potential values between resting and activated B cells became statistically insignificant. Contrary, for T cells, the voltage dependence in activated ones is shifted negatively, approaching the values for resting cells (Fig. 2B). Consequently, the difference between the voltage dependence of Kv1.3 current between B and T cells, either activated or resting, became very significant, with activation and inactivation curves shifted to positive potentials by 16-20 mV as compared with T cells in the co-culture.

The functional expression of Kv1.3 channels (expressed as specific current density, pA/pF) changed insignificantly upon T or B cells activation or co-culture with mesenchymal stromal cells (Fig. 3).

Discussion

T and B cells displayed a significant difference in the Kv1.3 voltage dependence, when co-cultivated with BMSC (Fig. 2B). A straightforward interpretation of the positive shift of voltage dependence in B cells may be a proposition that they should also display more positive resting potential values as compared to T cells. However, one needs to take into the account not only the position, absolute and relative ones, of activation and inactivation curves, but also the completeness of the inactivation process. We have calculated "window current", which is time-averaged current via single Kv1.3 channel as P_{act} x (1-P_{inact}) x I_{single}, where P_{act} and P_{inact} are probabilities to be activated and inactivated, respectively, calculated using from fitted parameters of voltage dependence as in the example in Fig. 1B, whereas voltage

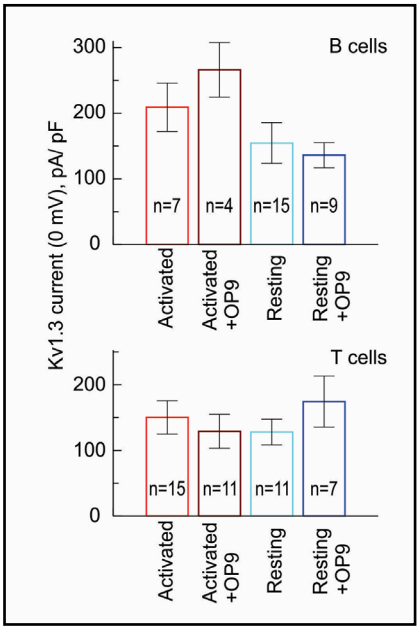

Fig. 3. Specific Kv1.3 density in lymphocytes insignificantly changes upon activation or co-culture with OP9 cells. Peak voltage- and time-dependent Kv1.3 current was measured at zero voltage and taken relative to whole cell capacitance, proportional to cell membrane surface (1 pF~ 100 μm^2). Data are mean ± SE, n is a total number of tested cells for each condition. No significant difference was observed as a result of activation or co-culture.

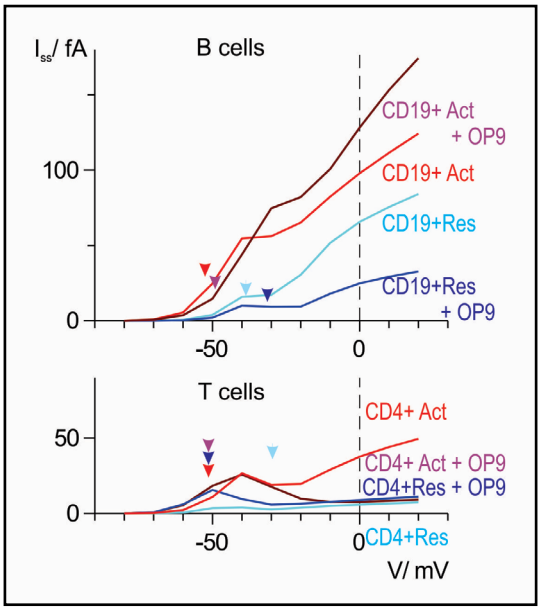

Fig. 4. "Window" Kv1.3 current in B and T cells. Window current corresponds to the fraction of unitary current through a Kv1.3 channel, which, with a certain probability, is activated by voltage and remains non-inactivated at steady state. It is a product of voltage dependent steady state open probability, multiplied by unitary current through an open Kv1.3 channel at the same voltage. Steady state open probability was calculated as a product of respective values, taken from activation and inactivation curves (see Fig. 1. B for an example) at given voltage. Arrowheads indicate mean cell resting potential values for each condition, directly measured by clamping membrane current to zero, I=0 mode. SE in all cases was about the symbol size, <2.5 mV, omitted for clarity.

dependence of I_{single} was calculated assuming Ohmic behavior, single channel conductance of about 13 pS and E_{K+} = -84 mV [29]. Results for all eight models are plotted in Fig. 4. An incomplete inactivation was crucial in case of B cells, which generated 3- and 10-times larger time-averaged K^+ current as compared to T cells in activated and resting states, respectively. Again, this difference was entirely relayed on the difference in voltage dependence, especially that of inactivation process (previously not reported for B cells) and not on the number of functionally expressed Kv1.3 channels, which was comparable for these models (Fig. 3). The latter result was also consistent with the previous data by Szabo et al. [10] who demonstrated that the co-culture of healthy human B cells with BMSC did not change the Kv1.3 current magnitude in the former significantly.

The effect of BMSC on the inactivation of Kv1.3 in B cells depended on their activation status. The percentage of non-inactivating channels decreased in resting but increased in activated cells. The latter apparently compensated for a positive shift of the voltage depen-

dence. So, measured resting potential was about -50 mV in activated B cells, either from monoculture or co-culture and is shifted to depolarized values (~-30 mV) in resting B cells, co-cultured with BMSC. For T cells inactivation was almost complete (less than 1% of Kv1.3 channels remaining non-inactivated) for co-cultures and monoculture of resting T cells. It was incomplete (about 4% channels remained non-inactivated) for activated T cells in monoculture, which compensated for a positive shift of the voltage dependence as compared to resting T cells in the monoculture. Consequently, T cells in the co-culture and activated T cells in monoculture display resting potentials about -50 mV. Quiescent T cells were strongly, above -30 mV, depolarized. Overall, resting B cells in co-culture and resting T cells in monoculture, which display the lowest conductance for balancing K^+ current via Kv1.3 channels (Fig. 4), were the most depolarized ones.

Why membrane potential control by Kv1.3 channels is important for the lymphocyte function? It is long known that potassium channel blockers depolarize the membrane potential in T cells from -50 up to -25 mV, exerting therefore an antiproliferative effect and reducing the interleukin IL-2 production [38-40]. Similarly, in B cells K^+ channel blockers arrest the cell cycle progression at the G1 phase [41]. It is generally accepted that in proliferating cells membrane potential changes in a cyclic mode, from hyperpolarized values at the G1/S transition, associated with a high K^+ channels activity, to depolarized ones at the G2/M transition, where K^+-specific conductance is down-regulated [12]. Mesenchymal stem cells are able to inhibit B cells proliferation by arresting the cell cycle at the G1 phase (see [4] and references therein). This may help to understand the result presented in Fig. 4A, when the co-culture with BMSC cells caused a suppression of Kv1.3- mediated steady state current and depolarization of resting B-cells. Indeed, to pass the G1 phase, cell membrane potential needs to be hyperpolarized, which can only be achieved via K^+ efflux. It should be noted also that in the activated B cells Kv1.3 channels open at more negative potentials as compared to resting ones (Fig. 1B, C), while co-culture with BMSC abolished this difference (Fig. 2B). It is perhaps more difficult to interpret the apparently opposite effect of BMSC on the Kv1.3 voltage dependence in T cells, especially that in resting ones (Fig. 4B). However, owing a large plasticity in immunomodulation by BMSC in T cells, when, depending on the context, either suppression or stimulation was observed [2], such a contrasting effect may not be surprising. On the other hand, because the G2/M transition requires a depolarization [12], whereas co-culture with OP9 clamps the membrane to more negative value (Fig. 4B), the cell cycle may be arrested either, albeit at the later phase. Indeed, too strict clamping of membrane voltage at a hyperpolarized level efficiently blocks the mitogenesis [12].

Our data imply that rather than a variation of a mean membrane density of functionally active Kv1.3 channels, which hardly changed upon the lymphocyte activation or co-cultivation with stromal cells, the modulation of their voltage dependence may be considered as a principle mechanism for the control of the Kv1.3 channels activity in B and T cells. The latter appears to underly the differences in the resting potential, observed in human lymphocytes under different environmental conditions.

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Author contributions

All authors participated in conceptual and experimental design. SV-R conducted all, and IP participated in some experiments. SV-R and IP analyzed the data and composed the figures. SV-R performed the statistical analysis. IP and OD have written the draft. All authors performed a critical reading and approved the final version of the manuscript.

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Statement of Ethics

All healthy volunteers were given a brief explanation of objectives, risks and benefits of the study. Each of them was asked to sign the informed consent, in accordance to the Declaration of Helsinki. The protocols were reviewed and approved by the Bioethics and Biosecurity Committee of the Biomedical Research Centre and the Faculty of Medicine of the University of Colima, in agreement with the federal laws (Artículo 100, Ley General de Salud). The procedure of isolation of the $CD4^+$ and $CD19^+$ populations was performed in a laminar flow biosafety cabinet (class II), ensuring the integrity of the samples and operator´s protection.

Disclosure Statement

The authors have no conflicts of interest to declare.

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