Immunology Letters 171 (2016) 60–69

Contents lists available at ScienceDirect

Immunology Letters

journal homepage: www.elsevier.com/locate/immlet

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

Zoltan Petho[a], Andras Balajthy[a], Adam Bartok[a], Krisztian Bene[b], Sandor Somodi[c], Orsolya Szilagyi[a], Eva Rajnavolgyi[b], Gyorgy Panyi[a,*], Zoltan Varga[a,d]

[a] Department of Biophysics and Cell Biology, Faculty of General Medicine, University of Debrecen, Debrecen, Hungary
[b] Department of Immunology, Faculty of General Medicine, University of Debrecen, Debrecen, Hungary
[c] 1st Department of Internal Medicine, University of Debrecen, Debrecen, Hungary
[d] MTA-DE-NAP B Ion Channel Structure-Function Research Group, RCMM, University of Debrecen, Debrecen, Egyetem tér 1, H-4032, Hungary

ARTICLE INFO

Article history:
Received 8 December 2015
Received in revised form 29 January 2016
Accepted 4 February 2016
Available online 6 February 2016

Keywords:
Immune regulation
T cells
Cellular proliferation
Cytokine secretion
Ion channel
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 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 downstream 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 colocalization 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

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, carboxyfluorescein succinimidyl ester.

* Corresponding author at: Nagyerdei krt. 98, H-4032 Debrecen, Hungary. Fax: +36 52 532 201.
E-mail address: panyi@med.unideb.hu (G. Panyi).

http://dx.doi.org/10.1016/j.imlet.2016.02.003
0165-2478/

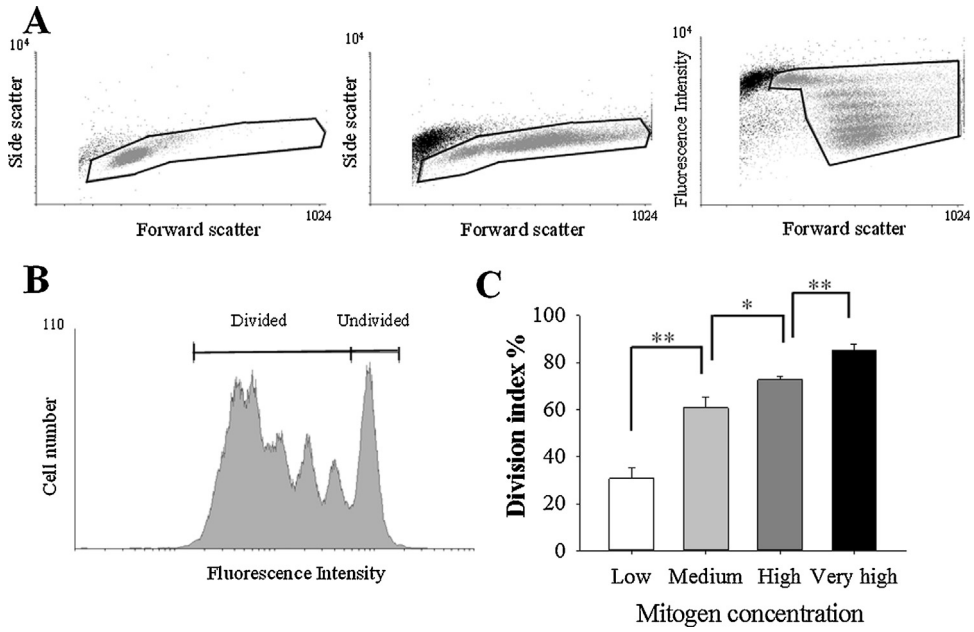

Fig. 1. Cell proliferation at different mitogen concentrations. (A) The left and middle dot plots show stimulated PBMC populations stimulated by 1 μg/ml anti-CD3 and anti-CD28 monoclonal antibodies on day 0 and day 5, respectively. Solid black line polygons indicate the position of the gates. Activated 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 Section 2). (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.

[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 cells, cytotoxic T cells and even macrophages. On the other hand, the anti-inflammatory IL-10 is mainly secreted by Th_2 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 Refs. [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 $[Ca^{2+}]_i$ 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 blockage 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 [28,29] type 1 diabetes mellitus or rheumatoid arthritis [30].

Besides the Ca^{2+}-dependent mechanisms, other signaling pathways also participate in T cell activation that do not necessarily 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 [31,32]. 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 [33].

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 [25,34–37]. Moreover, TRAM-34 inhibition alone had no effect on the proliferation of mixed T cell populations [38]. The underlying mechanism responsible for this variability has not been systematically addressed before,