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Inhibition of T Cell and Stimulation of B Cell Proliferation by Restraint Stress Mediated by Voltage-Gated Potassium Channel 1.3 Expression

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Abstract

Our previous study has showed that restraint stress inhibits T cell proliferation. Kv1.3 plays a key role in the lymphocyte activation process. Here, we investigate the effects of restraint stress on murine splenic T and B cell proliferation and the role of Kv1.3 in the process. ^{3}H-TdR incorporation is used to determine changes in splenocyte proliferation stimulated by Con A or LPS between control and restraint stress groups. The data shows that restraint stress inhibits T cell and enhanced B cell proliferation. Data from RT-PCR and Western blotting shows that Kv1.3 gene and protein levels are downregulated in T cells and upregulated in B cells in stressed mice. To examine a possible cause-and-effect relationship between Kv1.3 and stress-affected lymphocyte proliferation, we employ various Kv1.3 specific blockers (quinine, 4-AP and TEA) to determine K^+ channel function under restraint stress. The data shows that Kv1.3 blockers reverse the decreased T cell proliferation and increase B cell proliferation induced by restraint stress. These results indicate that Kv1.3 mediates restraint stress-induced modulation of T/B lymphocyte proliferation.

Keywords

Restraint Stress, Kv1.3, Proliferation, T/B Cell

1. Introduction

A wide variety of physical, chemical, and psychological stimuli induces a complex, physiological response in

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mammals [1] [2]. Many studies have reported that serious stress responses, including restraint stress, can cause immunosuppression [1] [3]-[6] and other types of immune system dysfunction [4] [7] [8]. Our previous work suggests that the immunosuppression is mediated by the central nervous system (CNS) [9]. The levels of neurotransmitters, hormones, cytokines and other stress proteins are increased or decreased in the peripheral circulation during most stress responses. Thereby immunocytes are exposed to increased or decreased concentrations of these mediators. One or more cell types in the immune system have receptors for these mediators. So the stress can either activate the immune system by providing danger signals or downregulate immune and inflammatory responses [7] [10]. Clearly, the neuroendocrine system can modify the immune system function. And the interactions are complex.

It has been reported that the stress primarily inhibits cell-mediated immunity [9] [11]-[14]. But how the stress affects humoral immunity has not been well established. Again, although the stress has profound effects on immunocyte function, how immunocytes respond and adapt to the stress is poorly understood.

T or B lymphocyte activation involves 2 similar intracellular signal pathways: the calcium dependent and independent cascades [15]-[17]. Stimulation of each pathway alone can trigger different events, and both are required for complete lymphocyte activation [15]. K^+ channels are essential for lymphocyte activation [16] [18] [19]. Two types of K^+ channels are expressed on lymphocytes: voltage-dependent (Kv) and calcium-activated (KCa) K^+ channels. Mouse splenic T lymphocytes mainly express 2 types of Kv channels: Kv1.3 and Kv3.1. Mouse B lymphocytes express only Kv1.3 and intermediate-conductance Ca^{2+}-activated K^+ (IKCa) channels [16]. Kv1.3 and IKCa expressions are enhanced during lymphocyte activation [19]-[21]. Kv1.3 channels are also important in regulating cell volume and lymphocyte adhesion and migration [16] [22]. It is also reported that Kv1.3 channel can be a therapeutic target for T cell-mediated autoimmune diseases [23] [24].

Although many studies have revealed the mechanisms of lymphocyte adaptation to the stress and shown that K^+ channels are effective modulators of lymphocyte proliferation, no data is available on the modulation of K^+ channels by restraint stress. In the present study, we investigate the effects of restraint stress on both T cell and B cell proliferation and the possible role of Kv1.3 in this process. It indicates that Kv1.3 channel mediates, at least in part, the changes of lymphocyte function induced by restraint stress.

2. Methods

2.1. Animals

Male BALB/c mice (20 ± 2 g) were provided by the Animal Center of Peking University Health Science Center (Beijing, China). The experimental protocols adhered to the guidelines of the Beijing Municipal Science & Technology Commission and were approved by the Institutional Authority for Laboratory Animal Care (Certificate No. SYXK(JING)2002-0002). Investigations were carried out under conditions delineated in the Guidelines, which comply with the "European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes".

2.2. Physical Restraint Stress Procedure

BALB/c mice were subjected to an established physical-restraint stress protocol [25] with some modifications. Briefly, mice were placed in supine position in a specially-made cylindrical cage with multiple punctures to allow ventilation for 12 h at room temperature under a natural light/dark cycle. Control littermates were kept in their original cage without food and water for 12 h. After physical restraint, mice were sacrificed and the spleens were harvested.

2.3. Cell Purification

Splenocytes were collected from mouse spleens and single cell suspensions were prepared by passing cells through a nylon mesh after the removal of erythrocytes by ACK lysing buffer (155 mM NH_4Cl, 0.1 mM $EDTA{\cdot}Na_2$, and 10 mM $KHCO_3$, pH 7.5). T and B cells were purified by positive immunomagnetic cell sorting using CD90 microbeads or CD19 microbeads (Miltenyi Biotec, Germany) respectively with standard protocols. The sorted cell populations were more than 98% pure on FACS analysis. For FACS assay, sorted cells were fluorescently stained with CD90-PE (Miltenyi Biotec, Germany) for T cells or CD45R (B220)-APC (Miltenyi Biotec, Germany) for B cells respectively. Cell debris and dead cells were excluded from the analysis based on

scatter signals. Stained cells were analyzed by FACScan flow cytometry with Cell QuestPro software (BD Biosciences, USA).

2.4. Cell Cultures

Splenocytes were cultured in RPMI 1640 medium (GIBCO BRL Lab., Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 U/ml streptomycin, and 120 U/ml penicillin. The cultures were incubated at 37˚C in a humidified atmosphere of 5% CO_2 in 96-well culture plates. For lymphocyte proliferation assays, 3.0×10^5 splenocytes from stressed and nonstressed mice were cultured in 200 μl per well in the presence of different concentrations of Con A or LPS for 48 h. All cultures were performed in triplicate. ^{3}H-TdR (1 μCi/well) was added to each culture in the last 6 h of culture, and the cells were then harvested onto glass filter paper. Radioactivity was assayed with use of a liquid scintillation counter and presented as CPM.

Quinine, 4-AP, TEA and TMA were respectively added into the cultures to examine the effects of K^+ channel blockers on T or B lymphocyte proliferation.

2.5. Cell Viability Assay

Cell viability was determined by trypan blue exclusion using a hemocytometer. Cell viability (percentage) was determined as the ratio of total viable cells (unstained) to total cells (stained and unstained). The data reported are the averages of separate experiments, each in triplicate.

2.6. Preparation of Polyclonal Antibody against the GST-Kv1.3 Fusion Protein

Kv1.3 cDNA was synthesized by RT-PCR from total RNA extracted from mouse splenocytes. The Kv1.3 primer sequences were as follows: sense, CGCGGATCCAACGTGCCCATCGACATCTT, and antisense, CCGCTCGAGCTTAGGATGGCCAGCGACAT. The RT-PCR product confirmed by DNA sequencing was ligated into the *BamH*I-*Xho*I sites of the pGEX-4T-2 vector after digestion with *BamH*I and *Xho*I. The resulting plasmid, pGEX-Kv1.3, was transformed into *E. coli* BL21 competent cells. The induction and purification of the fusion protein were performed using the GST Gene Fusion System (Pharmacia Biotech) according to the manufacturer's instruction.

Antibodies against the GST-Kv1.3 fusion protein were prepared using GST-Kv1.3 as an immunogen. The antiserum was purified by ammonium sulfate precipitation and chromatography over a protein G column (Pharmacia). After removal of GST-specific antibodies, the antibody against the Kv1.3 cDNA product was stored at −80˚C for further experiments.

2.7. RT-PCR

Total RNA was isolated with TRIzol® Reagent (Invitrogen) according to a protocol recommended by the manufacturer. cDNA was synthesized by the extension of dT primers with M-MLV reverse transcriptase (Invitrogen) in a mixture containing total RNA digested by RNase-free DNase (Ambion). PCR was used to amplify cDNA prepared from murine splenocyte RNA. Amplification of GAPDH served as a control. PCR products were visualized on 1% ethidium bromide-stained agarose gels, and bands were scanned and quantified by use of Totallab software (NonLinear Dynamic Ltd.). The Kv1.3 primer sequences were as follows: sense, CGCTGGCCATCCTAAGAG, and antisense, TACGGTTGCCAATTCTGTGC. The GAPDH primer sequences were sense, GGTCGGAGTCAACGGATTTG, and antisense, ATGAGCCCCAGCCTTCTCCAT.

2.8. Western Blot Analysis

Cells (4.0×10^7) were sonicated in 0.1 ml of ice-cold ionic detergent buffer (10 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM PMSF, 1% Triton X-100, 0.42 M NaCl, and 0.5 mM DTT). Protein concentrations were measured by the Lowry method [26]. Cell lysates (50 μg/lane) were subjected to SDS-PAGE and electrotransferred onto PVDF membrane. After being blocked in 5% skim milk and TBS (0.15 mM NaCl and 20 mM Tris-HCl, pH 7.6) at 4˚C overnight, the membrane was incubated with the antibody against Kv1.3 for 2 h in GENT solution (0.15 mM NaCl, 5 mM EDTA, pH 8.0, 50 mM Tris-HCl, pH 7.6, 0.25% gelatin, and 0.05% Triton X-100) and then with a horseradish peroxidase-labeled secondary antibody against rabbit IgG (Zymed Laboratories Inc., San

Francisco, CA). Kv1.3 and its derivatives blotted onto the membrane were visualized by use of an enhanced chemiluminescene detection system (Amersham Biosciences). eIF5 was used as a control protein in these samples. The anti-eIF5 antibody was obtained from Santa Cruz Biotechnology.

2.9. Statistical Analysis

All data are reported as the means ± S.E. unless otherwise stated. Statistical analysis of different treatments was performed using Student's unpaired *t*-test for two-way comparison. Analysis of variance (ANOVA) was used for differences between means (F test), with differences considered significant at $p < 0.05$.

3. Results

3.1. Restraint Stress Inhibited T Cell Proliferation and Promoted B Cell Proliferation

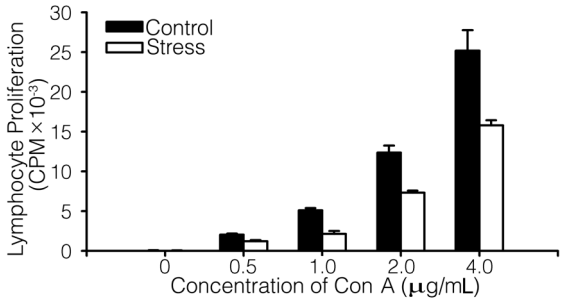
Our previous studies have demonstrated that restraint stress inhibits T lymphocyte proliferation stimulated by Con A [9] [12]. In this study, to examine the effects of stress on T cell and B cell function in the same mouse, we examined splenocyte proliferation stimulated by the T cell mitogen Con A and the B cell mitogen LPS at different concentrations. The data showed that T cell proliferation was significantly inhibited at Con A concentrations of 0.5, 1, 2 and 4 μg/ml (**Figure 1(a)**), but B cell proliferation was enhanced at LPS concentrations of 5, 10, 20 and 40 μg/ml (**Figure 1(b)**). In addition, there were similar trends for the effects of restraint stress on the T and B cell proportions in spleen. The T cell proportion was decreased from 47.6% ± 1.16% to 32.3% ± 0.48%, whereas the B cell proportion was increased from 44.7% ± 0.065% to 56.5% ± 0.95% by the stress (**Figure 1(c)**). These data indicate that restraint stress inhibits T cell but enhances B cell function.

3.2. Kv1.3 Gene and Protein Levels Were Downregulated in T Cells and Upregulated in B Cells by Restraint Stress

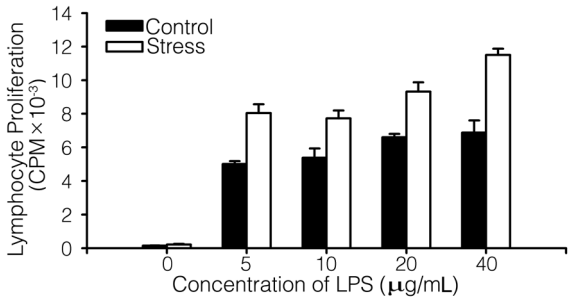
Kv1.3 plays an important role during lymphocyte proliferation [15] [16]. T and B cells are the major component of splenocytes [27]. Therefore, we investigated whether Kv1.3 expression was affected by restraint stress specifically in T or B cells. Data from semiquantitative RT-PCR experiments revealed that Kv1.3 mRNA in T cells purified from restraint-stressed animals was significantly downregulated compared with that in control animals (**Figure 2(a)**). The mean expression in stressed mice was decreased to 65.7% ± 1.8% (**Figure 2(b)**). Western blotting results paralleled those of RT-PCR: T cell Kv1.3 protein expression in the stressed mice was greatly downregulated compared with that in control mice (**Figure 2(c)**). We then investigated the effect of restraint stress on Kv1.3 expression in B lymphocyte. Interestingly, the semiquantitative RT-PCR results demonstrated that Kv1.3 mRNA level in B lymphocytes from stressed mice was increased to 261.2% ± 35.2% (**Figure 2(d)** and **Figure 2(e)**). Kv1.3 protein expression was also upregulated in B cells of stressed mice (**Figure 2(f)**). Therefore, restraint stress downregulated Kv1.3 protein in T lymphocytes and upregulated it in B lymphocytes, which was at least partly due to the changes in gene expression.

3.3. Diverse K^+ Channel Blockers Reversed the Downregulated T Cell Proliferation Induced by Restraint Stress

The above results demonstrated that Kv1.3 expressions in T and B cells were modified by restraint stress. Furthermore, we explored whether there is an association between Kv1.3 function and T or B lymphocyte proliferation under the condition of restraint stress. Quinine, 4-amino pyridine (4-AP) and tetraethylammonium (TEA) are well known to block the current of voltage-gated Kv1.3 channels. The three drugs were used in our experimental system, and the data showed that these inhibitors reversed the decreased mitogen-induced T cell proliferation (**Figures 3(a)-(c)**) in a dose-dependent manner. Tetramethylammonium (TMA), the negative control of TEA, had no significant effect on the alteration of T cell proliferation. Up to 40 mM, TMA failed to affect the cell proliferation (**Figure 3(d)**), which indicated that the TEA effect mentioned above was not due to the higher osmolarity of the medium. The difference in T cell proliferation decreased between control and stressed mice with increasing blocker concentrations (**Figure 3(e)**). **Table 1** showed that quinine, 4AP, TEA or TMA did not affect the viability of T cells as assessed by trypan blue dye exclusion.



(a)



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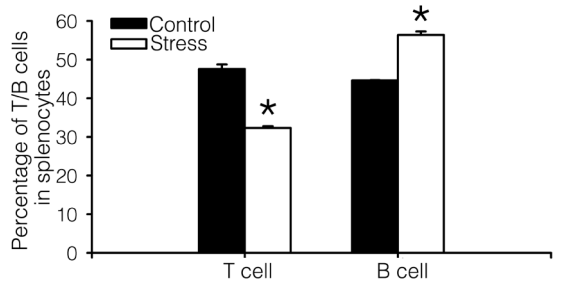

(c)

Figure 1. Restraint stress down regulated T cell activation stimulated by Con A and upregulated B cell activation stimulated by LPS. Lymphocyte proliferation of stressed or control mice was stimulated with Con A (a) or LPS (b) at different concentrations. (c) Percentages of T and B cells in splenocytes in stressed and control mice were analyzed by FACS. An asterisk ("*") indicates $p < 0.05$ compared with control, $n = 6$.

3.4. Diverse K^+ Channel Blockers Reversed the Upregulated B Cell Proliferation Induced by Restraint Stress

Figure 4 showed the effects of 3 K^+ channel blockers—quinine, 4-AP and TEA—on B lymphocyte proliferation stimulated by LPS in control and stressed mice. The 3 blockers reduced the difference in proliferation between the two groups in a dose dependent manner (**Figures 4(a)-(c)**). But the negative control, TMA, had no effect on B cell proliferation (**Figure 4(d)**). Similar with what was found in T cells, the difference in B cell proliferation decreased between control and stressed mice with increasing blocker concentrations (**Figure 4(e)**). As assessed by trypan blue dye exclusion, quinine, 4AP, TEA or TMA did not affect the viability of B cells (**Table 1**).

4. Discussion

The stress can downregulate various types of immune activity, primarily cell-mediated immunity [14] [28]. T

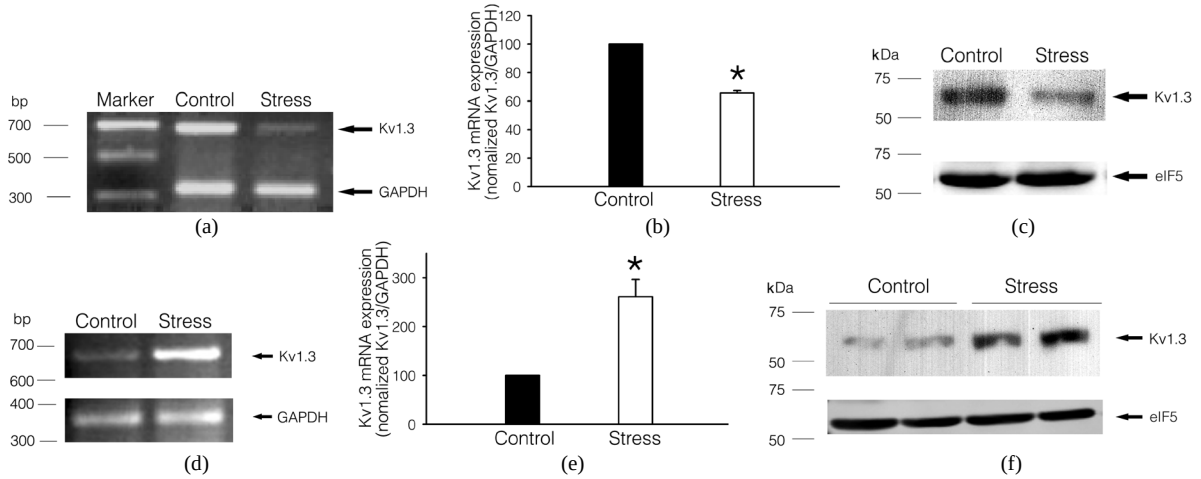

Figure 2. Downregulation of Kv1.3 gene and protein expressions in T lymphocytes and upregulation in B lymphocytes by restraint stress. T (a)-(c) or B (d)-(f) lymphocytes were separated from splenocytes of stressed and control mice. (a) and (d): Kv1.3 gene expression was determined by PCR after cDNA concentration was normalized to GAPDH gene expression. The bands are indicated by arrows. (b) and (e): Relative intensity analysis of PCR bands in (a) and (d), respectively. An asterisk indicates $p < 0.05$ compared with control, $n = 6$. Western blot analysis of Kv1.3 expression in T (c) or B (f) lymphocytes in control and stressed mice. A total of 50 μg total T cell lysate was loaded in each lane. eIF5 was used as a constitutive (control) protein in these samples.

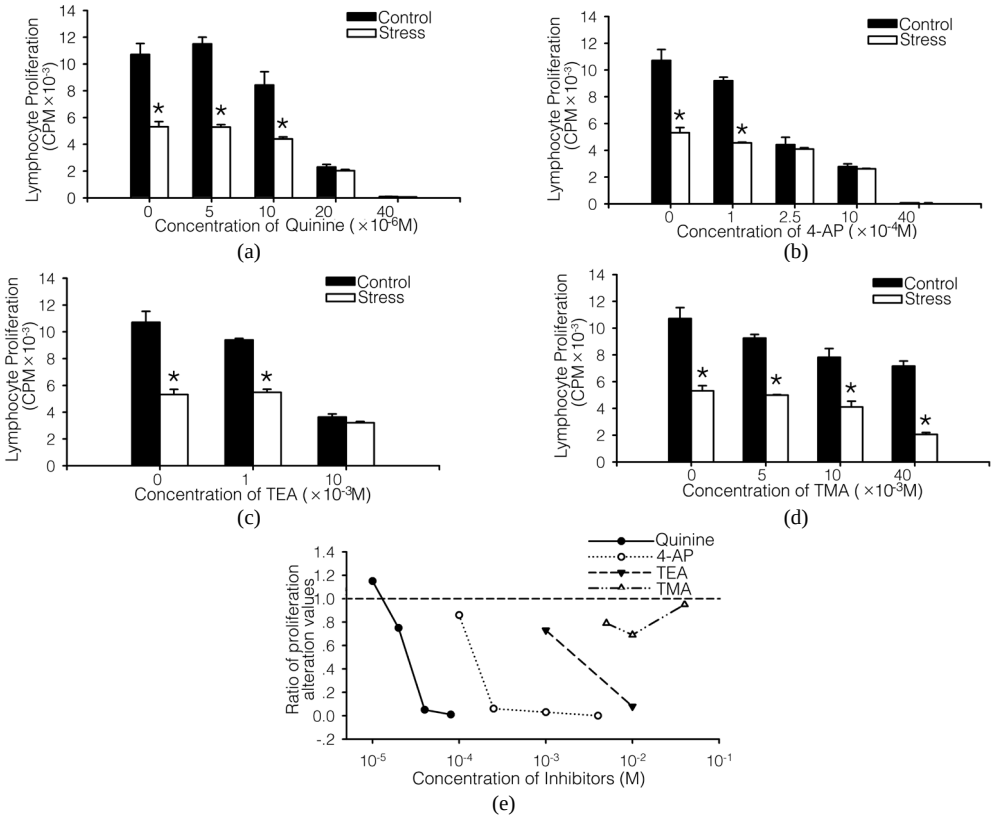

Figure 3. Abilities of diverse Kv1.3 channel blockers to eliminate the difference in T cell proliferation between the control and stressed mice. The effects of quinine (a), 4-AP (b), TEA (c) or TMA (d) on Con A-induced ^{3}H-TdR uptake by T lymphocytes in control and stressed mice after 48 h incubation ($n = 6$ for each). (e) The alteration values of T lymphocyte proliferation between control and stressed mice with different concentrations of Kv1.3 blockers and negative control compared with the standard (1.0, without any Kv1.3 blockers treatment).

Table 1. Viability of lymphocytes exposed to Kv1.3 channel blockers.*

Mitogens	Kv1.3 blockers (Concentration)	Viability (%)	
		Control	Stress
None	None	91.3 ± 2.1	96.9 ± 0.6
Con A	None	94.8 ± 0.3	83.2 ± 0.5
	Quinine (4.0×10^{-5} M)	90.3 ± 1.4	87.3 ± 3.4
	4-AP (4.0×10^{-3} M)	90.8 ± 0.0	87.2 ± 1.7
	TEA (1.0×10^{-2} M)	94.3 ± 0.6	85.4 ± 1.3
	TMA (4.0×10^{-2} M)	93.2 ± 1.1	83.5 ± 2.6
LPS	None	83.4 ± 1.3	89.4 ± 2.0
	Quinine (4.0×10^{-5} M)	92.8 ± 1.4	83.8 ± 0.9
	4-AP (4.0×10^{-3} M)	94.0 ± 1.8	84.8 ± 1.3
	TEA (1.6×10^{-2} M)	91.2 ± 2.0	84.0 ± 1.0
	TMA (1.6×10^{-2} M)	92.3 ± 1.5	87.7 ± 1.3

*Splenocytes stimulated with Con A or LPS were incubated in culture medium with or without different doses of drugs for 48 h. The viability was assessed by Trypan blue dye exclusion (n = 6 for each).

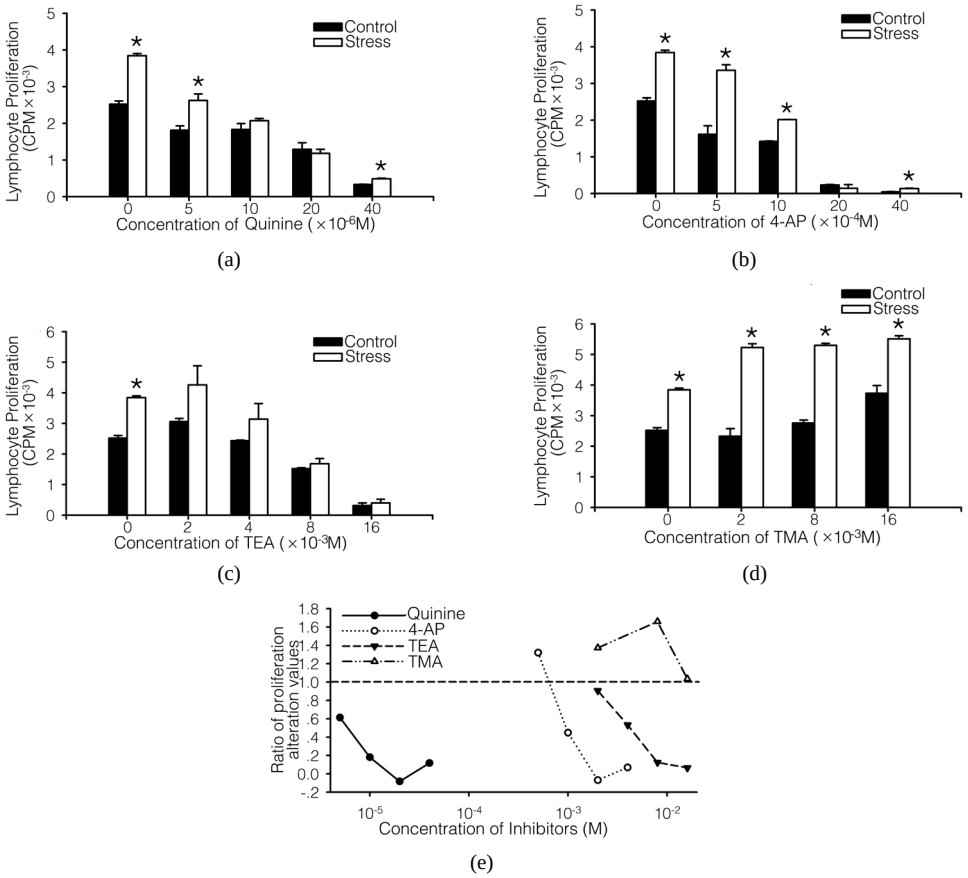

Figure 4. Abilities of diverse Kv1.3 blockers to eliminate the difference in B cell proliferation between control and stressed mice. The effects of quinine (a), 4-AP (b), TEA (c) or TMA (d) on LPS-induced ^{3}H-TdR uptake by B lymphocytes in control and stressed mice measured after 48 h incubation (n = 6 for each). (e) The alteration values of B lymphocyte proliferation between control and stressed mice with different concentrations of Kv1.3 blockers and negative control compared with the standard (1.0, without any Kv1.3 blockers treatment).

lymphocyte mitogenesis, cell number and NK cell activity are greatly suppressed by the stress [11] [29]. However, how resistant stress affects B cell function is only starting to be explored at present. Different groups have employed various animal models and stress periods, but their conclusions are conflicting [30] [31]. The findings presented in this study support the viewpoint that restraint stress inhibits T lymphocyte proliferation stimulated by Con A (**Figure 1(a)**) and enhances B lymphocyte proliferation stimulated by LPS (**Figure 1(b)**). Based on our previous work [9] [12], which demonstrates the inhibition of T lymphocyte proliferation in stressed mice, we show here for the first time that restraint stress causes different changes in T and B cell proliferation in the same mouse. Under restraint stress, T cell function is decreased, whereas B cell function is enhanced. The stress can either enhance or suppress immune functions depending on a variety of factors. Chronic stress has been demonstrated to exert a significant suppressive effect on immune function. The mechanisms by which restraint stress adversely affects humoral immunity in the present study need to be investigated further. The latter may be a compensatory regulation to balance the immune function.

Furthermore, the results show that restraint stress decreases the population of T lymphocytes and increases that of B lymphocytes in spleen (**Figure 1(c)**). Modification of population size of immune cells accommodates special needs for different conditions. Loss of cells, for example, acts to eliminate potentially autoreactive lymphocytes and prevents excessive cellular proliferation during immune responses [32] [33]. An acute or short-term stress response induces a rapid and significant redistribution of immune cells among different body compartments. And regulated redistribution of immune cells among different body compartments is essential for effective immune surveillance and salubrious immune function [34]. Our results yield insight into a new mechanism by which chronic stress regulates immune functions. But the significance of the T- and B-cell redistribution induced by restraint stress in the present study needs to be explored further.

In addition, T cells including Th1, Th2, Th17, natural killer T cells, and regulatory T cells (Tregs), may contribute to the development and/or progression of diseases. An imbalance between different T cells may lead to reciprocal and mutual amplification of the innate and adaptive immune responses. It has been reported that restraint stress does not alter the number of Tregs in the intestine, but compromise their immune suppressor functions [35]. A shift in the type-1/type-2 cytokine balance towards a type-2 response is induced by restraint stress [36] [37]. However, the reasons for the imbalance/shift are still poorly studied.

The Kv1.3 channel plays an important role in lymphocyte proliferation. The expression of Kv1.3 is upregulated during lymphocyte activation and its contribution in establishing the resting potential correlates well with their expression level [20] [38]. To study the direct effects of restraint stress on the function of Kv1.3 in T and B lymphocytes, we attempt to determine the I_k in mouse lymphocytes by patch-clamp technique. Unfortunately, no K^+ current is recorded in depolarized lymphocytes in control or stressed mice (data not shown). This may be due to the low quantity of Kv1.3 channels in quiescent T lymphocytes from mice (approximately 10 per cell), although some laboratories successfully recorded I_k in human lymphocytes or mouse thymocytes [16]. On the other hand, the lymphocyte membrane has high electrical resistance (10 - 20 GΩ), which allows the V_m to be regulated by a small number of ion channels, even the opening of individual ion channel [39]. It suggests that although the K^+ current is too low to record in lymphocytes by patch clamp, alterations of K^+ channel function and expression still affect lymphocyte activation. As expected, our findings demonstrated that Kv1.3 mRNA and protein levels in stressed mice were greatly downregulated in T lymphocytes but upregulated in B lymphocytes (**Figure 2**). Downregulation of Kv1.3 in T cells may reduce K^+ efflux, which could lead to the depolarization of V_m and, in turn, depress the driving force for secondary messenger calcium entry, and finally result in the inhibited T cell proliferation (**Figure 1(a)**). Similarly, upregulation of Kv1.3 expression in B cells (**Figures 2(d)-(f)**) may lead to promoted B cell proliferation in mice subjected to restraint stress (**Figure 1(b)**).

Because restraint stress has the potential to affect various physiologic functions in mammals, we employed various Kv1.3 channel specific blockers (quinine, 4-AP and TEA) to examine a possible cause-and-effect relationship between Kv1.3 and stress-affected lymphocyte proliferation and to further determine K^+ channel function in restraint stress. The pharmacological studies (**Figure 3** and **Figure 4**) have demonstrated that Kv1.3 blockers attenuated the differences between stressed and control mice in proliferation of both T and B lymphocytes. The difference in Kv1.3 channel function caused by its expression change between control and stressed mice was ameliorated by increasing Kv1.3 blocker concentrations, and as a result, the difference in lymphocyte proliferation between the 2 groups was also decreased. In addition, the results supported the notion that restraint stress modulated T and B cell proliferation mediated by Kv1.3. Recently, it has been reported that TLR9 [37],

IL-10/STAT3 pathway [40], and oxidative stress [41] also play an essential role in chronic stress-induced immune suppression. The potential cross talk between these signal pathways should be investigated.

Previous evidences suggest that expression of Kv1.3 channels during T cell activation is regulated through a posttranscriptional mechanism [42] [43]. Our data in this study provided a different conclusion (**Figure 2**). The difference in mechanism may be attributed to the different biologic species or stress modes. Some other studies on Kv1.3 in B lymphocytes have shown that proliferation is triggered by elevated Kv1.3 expression [16], which is consistent with our results (**Figure 1(b)** and **Figure 2**).

In conclusion, our data demonstrated that restraint stress decreased T cell proliferation and enhanced B cell proliferation, which was due to differential Kv1.3 expression patterns induced by stress in T and B lymphocytes. It was the first report that stress produced the different effects on T and B lymphocytes via the same membrane protein (Kv1.3). Our results provided a novel insight on the mechanism by which stress regulated lymphocyte activation.

Acknowledgements

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Conflict of Interest

The authors report no conflicts of interest.

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