Blockade of Kv1.3 potassium channel inhibits CD8⁺ T cell-mediated neuroinflammation via PD-1/Blimp-1 signaling

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Abstract
Kv1.3 potassium channel is considered as a target for the treatment of autoimmune diseases such as multiple sclerosis (MS), since Kv1.3 blockade suppresses memory T cell activation including cytotoxic CD8⁺ T cells. However, the underlying signaling pathway related to autoimmune CD8⁺ T cell inhibition by Kv1.3 channel in neuroinflammatory diseases remains unclear. We found that ImK, a selective Kv1.3 blocker, reduced auto-reactive CD8⁺ T cell infiltration in the spinal cords of experimental autoimmune encephalomyelitis (EAE) rats, an animal model of MS. ImK suppressed transcriptional factor Blimp-1 expression and reduced the cytotoxicity of CD8⁺ T cells on neuronal cells. Furthermore, ImK upregulated co-inhibitory molecule PD-1 to inhibit B lymphocyte-induced maturation protein (Blimp-1) in an IL-2 independent way. In addition, PD-1 inhibitor impaired the suppression of ImK on CD8⁺ T cells and accelerated EAE progression. Our study demonstrated a novel regulatory mechanism of Kv1.3 blockade on modulating CD8⁺ T cell differentiation through PD-1/Blimp-1 signaling. This work expands the understanding of Kv1.3 channel for modulating neuroinflammation.

KEYWORDS
Blimp-1, CD8⁺ T cells, EAE, Kv1.3 channel, PD-1

Abbreviations: APC, antigen presenting cell; BBB, blood-brain barrier; Blimp-1, B lymphocyte-induced maturation protein; CNS, central nervous system; CSF, cerebrospinal fluid; EAE, experimental autoimmune encephalomyelitis; MOG, myelin oligodendrocyte glycoprotein peptide; MS, multiple sclerosis; WT, wild type.
Multiple sclerosis has been considered as a CD4⁺ helper T cells-mediated autoimmune disease in the central nervous system (CNS). The activated circulating CD4⁺ Th1 cells and Th17 cells, produce pro-inflammatory cytokines like IFN-γ and IL-17A, cross and trigger CNS autoimmune-inflammation. It has become clear that CD8⁺ T cells also take part in the induction, progression, and pathological injury of MS. The severe cytotoxic CD8⁺ T cell infiltration has been identified in the cortex of MS patients. Moreover, the depletion of CD8⁺ T cells alleviates MS lesions and relapse. Therefore, identifying the mechanisms modulating CD8⁺ T cell cytotoxicity is important for developing potential therapeutic and vaccination strategies for MS.

CD8⁺ effector memory T (T EM) cells constitute an essential part of memory T cell phenotypes for their rapid and strong immune response in chronic viral infections and autoimmune diseases. To differentiate them from naïve T cell, effector memory CD8⁺ T cells upregulate Kv1.3 potassium channel for activating calcium-dependent signal pathway, which suggests Kv1.3 channel plays an important role in CD8⁺ T EM cell immunoregulation. Elevated expression of Blimp-1 promotes effector CD8⁺ T cell maturation and excessive cytokine release, but whether it can reduce autoimmune CD8⁺ T cells cytotoxicity and the underlying mechanism remains unknown.

Blimp-1 (encoded by prdm1) is a zinc-finger-containing transcriptional repressor perhaps best known for governing fate decisions in memory B cell differentiation. Blimp-1 also has an important role as a transcriptional regulator of CD8⁺ T cell exhaustion. Elevated expression of Blimp-1 leads to T cells differentiation, proliferation, and cytokine release. Co-inhibitory molecule PD-1 has been reported to suppress T cell differentiation, proliferation, and cytokine release, but whether it can reduce autoimmune CD8⁺ T cells cytotoxicity and the underlying mechanism remains unknown.

Blimp-1 regulates PD-1 expression through transcription inhibition. Meanwhile Blimp-1 can also regulate PD-1 expression through transcription inhibition. However, little is known about the relationship between Blimp-1 and PD-1 in autoimmune diseases.

Previously, we have reported that the Kv1.3 peptide blocker, ImKTx88, can reduce T EM cells levels and alleviate clinical severity of EAE rats. Here, we found the number of cytotoxic CD8⁺ T cells decreased in the central nervous system of ImK-treated EAE rats. Specifically, ImK upregulated co-inhibitory receptor PD-1 expression and reduced Blimp-1-induced inflammation in CD8⁺ T cells. Knockdown of Blimp-1 impaired CD8⁺ T cell cytotoxicity and removed the suppression of CD8⁺ T cells by ImK, while PD-1 inhibitor treatment aggravated the clinical score in EAE rats, suggesting the Kv1.3 blocker, ImK inhibits CD8⁺ T cell function via modulating PD-1/Blimp-1 signaling.

**2 | MATERIALS AND METHODS**

**2.1 | Kv1.3 peptide blocker**

ImKTx88 peptide was expressed and purified as described previously. Plasmid pGEX-6p-1-ImK was transfected into *Escherichia coli* Rosetta (DE3) cells. After overnight incubation, protein expression was induced by IPTG at 28°C for 4 hours. The fusion protein from the cell extract was purified through GSH affinity chromatography and then lyophilized.

**2.2 | Induction of EAE model**

Female Sprague-Dawley (SD) rats at 6-8 weeks of age were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. EAE was induced with an emulsion of rat brain and spinal cord white matter homogenate in phosphate-buffered saline (PBS, 50% w/v) mixed with an equal volume of complete Freund’s adjuvant (Sigma-Aldrich) supplemented with 5 mg/mL Mycobacterium tuberculosis. Each rat was immunized by subcutaneous injection of antigen emulsion at a dose of 0.75 ml/kg into its footpad, followed by an auxiliary injection of 10 μg/kg diluted in 0.5 ml pertussis toxin (List Biological Laboratories) in the dorsum of the foot. The second immunization was administered 7 days after the first immunization. Clinical signs of EAE were monitored daily. Beginning on the day when the first neurological signs appeared, animals were subcutaneously injected with ImKTx88 (100 μg/kg) or PBS once daily.

After immunization, the rats were observed in a double-blind manner every day and given clinical scores with the following criteria: 0, no clinical signs; 0.5, partial loss of tail tone; (a) affected tail tonus, (b) paresis of hind legs, (c) complete paralysis of the hind legs, (d) complete hind leg paralysis and foreleg paresis, and (e) death due to EAE. These criteria were established and modified according to previous clinical scale systems.

Rats were anesthetized at the peak of EAE according to the clinical scores (Day 11-15 of modeling). Anticoagulant peripheral blood was collected for lymphocytes isolation and flow cytometry analysis. Cerebrospinal fluid (CSF) from foramen magnum was used for cytokine detection. Paraformaldehyde-fixed spinal cords were sliced for immunohistochemistry analysis.
2.3 | PBMC isolation and stimulation

Blood was collected from abdominal veins of anesthetized rats. Lymphocytes were diluted with PBS, followed by enrichment based on 70% of Percoll (GE) gradients and centrifugation for 30 minutes at 800g. For stimulation, lymphocytes from WT rats were treated with 100 μg/mL ConA (Sigma-Aldrich), and lymphocytes from EAE rats were treated with 100 μg/mL MOG35-55 (Sigma-Aldrich) for 48 hours. A 100 nM ImKTx88 was added 1 hour before stimulation.

2.4 | CD8+ T cells isolation and stimulation

Plates were incubated with 1 μg/mL CD3 functional antibody (Ebioscience) in advance. After lymphocytes were isolated from rat blood, they were incubated with rat CD8 antibody conjugated magnetic beads (Miltenyi Biotec) diluted in the MACS buffer (Miltenyi Biotec) for positive selection. Then MACS separation columns were used to separate CD8+ T cells. Separated CD8+ T cells were plated and stimulated with 1 μg/mL CD28 functional antibody (Ebioscience) for 48 hours. A 100 nM ImKTx88 was added 1 hour before stimulation.

2.5 | Blimp-1 knockdown and PD-1 inhibitor

SiRNA of Blimp-1 (GenePharma) was transfected into CD8+ T cells with INTERFERin (DAKAWE). For the animal model, 5 mg/kg PD-1 inhibitor 3 (Selleck) was injected subcutaneously daily. 100 μg/mL PD-1 inhibitor 3 was added 1 hour before stimulation for cell experiment.

2.6 | Flow cytometry

Lymphocyte suspensions of rat blood were prepared by ACK lysis buffer or collected from 6-well culture plates. The surface antigen was stained first, and then the intracellular protein was stained after the membrane was fixed and broken. The following Abs were used for cell surface staining and intracellular staining: PE-CD8 (1 μg/mL, mouse, BD), Blimp-1 (1 μg/mL, goat anti-rat, Novus), PD-1 (1 μg/mL, rabbit anti-rat, Abcam), GrB (1 μg/mL, rabbit anti-rat, Abcam), FITC anti-goat (1 μg/mL, donkey, BD), APC anti-rabbit (1 μg/mL, donkey, BD), Fluo-3/AM (1 μM, Beyotime Biotechnology), and CFSE (1 μM, Beyotime Biotechnology). The cells were analyzed on a FACS Aria III flow cytometer (BD Bioscience, Breda, The Netherlands) from Research Center for Medicine and Structural Biology, Wuhan University.

2.7 | ELISA (enzyme-linked immunosorbent assay)

Cerebrospinal fluid samples were separated from foramen magnum at the peak of EAE. Cellular supernatants were collected after centrifugation. The gradient concentrations of standard cytokines IL-1β (DAKAWE), IL-6 (DAKAWE), and GrB (Mlbio) were prepared. The same volume of sample supernatants was used for the measurements of cytokines according to the manufacturer’s instructions. The levels of cytokines in each sample were calculated by comparing to the standard protein concentration curve. The absorbance values were read by a Microplate Reader (Biotek).

2.8 | Cytotoxicity assay

Purified cultures of oligodendrocytes and neurons were prepared as previously described. To promote proliferation, isolated oligodendrocyte progenitor cells (OPCs) were seeded in 96-well plates in Neurobasal media (Gibco) with 20 ng/mL PDGF-AA (PeproTech), 20 ng/mL bFGF (PeproTech), 2% B27 (Gibco), and 1% penicillin/streptomycin at a density of 2 × 10^4/well. After 2 days, cells were switched to Neurobasal media with 40 ng/mL triiodothyronine (T3) (Sigma-Aldrich) and 2% B27 to promote differentiation for 7 days. Then, oligodendrocytes and neurons were co-cultured in a dish. Mononuclear cells were collected from spleen after grinding and stimulated with primary neuronal cells culture supernatant containing fragments of tissue. A 100 nM ImKTx88 was added 1 hour before stimulation. Then, sorted CD8+ T cells were isolated to co-culture with neuronal cells for 48 hours. LDH assays were used for the cytotoxicity in the supernatant.

2.9 | QPCR (quantitative polymerase chain reaction)

Total RNA was isolated using TRIzol reagent, according to the manufacturers’ protocols. Then, cDNA was synthesized using a SuperScript VILO cDNA Synthesis Kit. Quantitative PCR was performed using the StepOnePlus Real-Time PCR System (Life Technologies). The levels of gene expression were normalized to that of GAPDH. The following specific primers were used: gapdh (forward primer, GCCGTATCGGACGCCCTGGTT; reverse primer, GCCCTTAGCCTGCGCTTCAG), gzmB (forward primer, CCCCTCAAGAACCTGAGCAAT; reverse primer, TGGGGAAAATAGCAGAGAATCT), il2 (forward primer, ACTTCAAGCCCTGGAAAGA; reverse primer, TTGCTAGAGTGTGGCCCTGCT), prdm1 (forward primer, CGTACCAGAGAGGGAGCCGC; reverse primer, AACCGGAGTTACACTTGGG), bcl6
controlled by a PULSE software (HEKA Elektronik). For measuring voltage-gated K⁺ channel currents, the internal pipette and external solutions were prepared according to previous procedures. The internal solution contained KCl 140 mM, MgCl₂ 1 mM, HEPES 5 mM, and EGTA 1 mM and the pH was adjusted to 7.2 with KOH. The external solution contained NaCl 140 mM, MgCl₂ 1 mM, KCl 5 mM, Glucose 10 mM, CaCl₂ 2 mM, and HEPES 10 mM and the pH was adjusted to 7.4 with NaOH. The currents were elicited by depolarizing the voltage steps of 200 ms from the holding potential −80 mV to 50 mV. ImK was dissolved in an external solution containing 0.01% BSA for toxin application in electrophysiological experiments. A multichannel microperfusion system MPS-2 (INBIO Inc, Wuhan, China) was used to exchange the external recording bath solution.

Sorted CD8⁺ T cells were plated into the slides incubated with 1 μg/mL CD3 functional antibody (Ebioscience). After stimulation with 1 μg/mL CD28 functional antibody (Ebioscience), slides were placed in a dish with the external solution and placed on the instrument for Kv1.3 current detection.

2.12 | Statistical analysis

Data are presented as the mean ± SEM. For experiments with two comparisons, a two-tailed Student’s t test was used for statistical analysis. For experiments with multiple comparisons, the level of significance was determined using ANOVA with Newman-Keuls test. The clinical scores curves in Figure 6 were statistically analyzed by the generalized equation. All analyses were performed using GraphPad Prism 5 (GraphPad Software). P values less than .05 were considered statistically significant. For all figures, *P < .05, **P < .01, ***P < .001.

3 | RESULTS

3.1 | Kv1.3 blockade reduced inflammatory cells infiltration in EAE rats

We have previously demonstrated that the Kv1.3 peptide blocker ImK can reduce CNS inflammation and increase oligodendrocytes survival in EAE rats. The defining feature of EAE is infiltration of multiple kinds of immune cells in CNS lesions with blood-brain barrier (BBB) breakdown. Here, we investigated which types of accumulated immune cells were inhibited by ImK in the spinal cords of EAE rats. The immunostaining data showed that the number of infiltrated peripheral immune cell neutrophils (Figure 1A), macrophages (Figure 1B) and cytotoxic T lymphocytes (Figure 1C) were significantly reduced in the ImK-treated group, compared with EAE group. Also, the enrichment of

2.10 | Immunohistochemistry

For immunohistochemistry, the lumbar segment of spinal cords from EAE or control rats were obtained from surgery, fixed in neutral-buffered formalin solution, and embedded in paraffin. A 5-μm sections were mounted and rehydrated in xyline, descending alcohol concentrations, and distilled water. For antigen retrieval, slides were microwaved for 8 minutes in 10 mmol/L Tris and 1 mmol/L EDTA buffer, pH 9, and equilibrated in PBS. Endogenous peroxidase activity was quenched by immersion in 1% H2O2/MeOH for 10 minutes. Non-specific antibody binding was blocked with 5% nonfat milk in PBS for 30 minutes, and slides were incubated in primary antibody diluted in 1% BSA in PBS overnight at 4°C. After washing in PBS, slides were immersed for 30 minutes in the corresponding ImmPRESS HRP Polymer Detection solution (Vector Laboratories). Antibody complexes were visualized by brief incubation in chromagen DAB/SG HRP substrate (Vector Laboratories). Slides were rinsed in distilled water, counterstained in hematoxylin, dehydrated via an ascending alcohol gradient and xyline, and mounted in a mixture of distyrene, plasticizer, and xyline. The whole sections of white and gray matter were imaged using a high-resolution digital Axio Scan.Z1 slide scanner and associated Zen software (ZEISS). Images were analyzed using Image Pro-Plus. Primary and secondary antibodies used were as follows: mouse anti-rat myeloperoxidase (1 μg/mL, Abcam), mouse anti-rat GFAP (1 μg/mL, Invitrogen), mouse anti-rat CD68 (1 μg/mL, Invitrogen), goat anti-rat Iba-1 (1 μg/mL, Abcam), mouse anti-rat CD8 (1 μg/mL, Abcam), goat anti-rat Blimp-1 (20 μg/mL, Santa Cruz), rabbit anti-rat GrB (1 μg/mL, Abcam), rabbit anti-rat PD-1 (5 μg/mL, Abcam), and immunohistochemical secondary antibody (DAKO).

2.11 | Patch clamp

Kv1.3 channel current of CD8⁺ T cells was measured using the whole-cell patch-clamp technique at room temperature. Current measurements and data acquisition were performed with an EPC 10 patch-clamp amplifier (HEKA Elektronik)
native immunocompetent cells in the CNS such as microglia (Figure 1D) and astrocytes (Figure 1E) were also relieved by ImK treatment. These results suggested an extensive inflammation inhibition of Kv1.3 blockade by ImK treatment in EAE rats. ImK also reduced pro-inflammatory cytokines IL-1β, IL-6, and killer cells- secreting granzyme B (Figure 1F) concentrations in the CSF, which relate to inflammatory cytotoxicity on neuronal cells in EAE rats. These findings
demonstrate the general immunoregulation effect of ImK in the CNS of EAE rats.

### 3.2 Kv1.3 blockade suppressed CD8\(^+\) T cell activation and alleviated cytotoxicity on neurons

In EAE or MS, cytotoxic CD8\(^+\) T cells are involved in the disturbance of neurological functions by attacking oligodendrocytes and causing neuroaxonal injuries.\(^6,9\) Kv1.3 blockade directly suppressed effector memory T cells function since their activation correlates to Kv1.3 overexpression.\(^14\) After CD3/CD28 activation, the Kv1.3 current of CD8\(^+\) T cells was significantly increased, and ImK can reduce the Kv1.3 current (Figure 2A). When the Kv1.3 channel was blocked in CD8\(^+\) T cells, the influx of Ca\(^{2+}\) was disturbed, so that subsequent activation and proliferation are suppressed.\(^31\) Our data showed that ImK decreased intracellular Ca\(^{2+}\) concentration labeled by Fluo-3 (Figure 2B) and inhibited CD8\(^+\) T cell proliferation (Figure 2C). Furthermore, ImK reduced inflammatory cytokine IFN-γ and cytotoxicity factor GrB release (Figure 2D,E). Furthermore, to mimic the damage CD8\(^+\) T cells cause to the CNS in MS, we used the oligodendrocyte specific antigen, myelin oligodendrocyte glycoprotein peptide (MOG\(_{35-55}\)), to induce antigen-specific stimulation of CD8\(^+\) T cells isolated from EAE rats, and then, co-cultured the stimulated cells with neuronal cells to determine neuronal cell damage. The results showed CD8\(^+\) T cells exhibited an obvious cell-killing effect after activation, while ImK reduced the cytotoxicity of CD8\(^+\) T cells since there was no effect on oligodendrocytes (Figure 2F). These results demonstrate ImK inhibits CD8\(^+\) T cell activation by...

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**FIGURE 2** Kv1.3 blockade suppressed CD8\(^+\) T cell activation, proliferation, cytokine release, and cytotoxicity. A, The Kv1.3 current of CD8\(^+\) T cells before and after ImK treatment were recorded by whole-cell patch clamp. B,C, The intracellular calcium concentration (B) and proliferation (C) of CD8\(^+\) T cells were quantified from stimulated PBMCs with or without ImK pretreatment. D,E, IFN-γ (D), GrB (E) in the supernatant of stimulated PBMCs were detected using ELISA. F, The cytotoxicity was measured in the supernatant of co-cultured CD8\(^+\) T cells and oligodendrocytes by LDH assays. Data are presented as mean ± SEM (n = 3 for each group in Figure B-F, three independent experiment repeats. \(* P < .05, ** P < .01, *** P < .001\)
blocking Kv1.3 current and suppresses the cytotoxic function of CD8+ T cells on oligodendrocytes.

3.3  |  Kv1.3 blockade suppressed CD8+ T cell stimulation by downregulating Blimp-1

IL-2 signaling, which is reported to be suppressed by Kv1.3 blockade, plays a crucial role in CD8+ T cell activation, proliferation, and survival. We confirmed the inhibition of ImK on IL-2/STAT3 expression and found ImK regulates the downstream gene, prdm1 (encoding Blimp-1) during CD8+ T cell activation (Figure 3A). It has been reported that Blimp-1 mediates CD8+ T cell terminal differentiation in viral infection. Specifically, we found that the Kv1.3 blockade decreased the mRNA level of prdm1 and had no influence on the expression of Bcl-6, an antagonist to Blimp-1 (Figure 3A). Besides, ImK reduced the ratio of Blimp-1+ CD8+ T cells after ConA stimulation (Figure 3B), and also lowered Blimp-1+ CD8+ T cell numbers in the blood of EAE rats (Figure 3C). Furthermore, Kv1.3 blockade prevented GrB+ or Blimp-1+ CD8+ T cell infiltration into the spinal cords of EAE rats (Figure 3D). In addition, ImK decreased the numbers of GrB+Blimp-1+ CD8+ T cells, demonstrating a suppression on cytotoxic CD8+ T cells by Kv1.3 blockade (Figure 3E). Therefore, we transfected siRNA of Blimp-1 (siBlimp-1) into CD8+ T cells to study the role of Blimp-1 on regulating cytotoxic function, and found siBlimp-1 reduced Blimp-1 protein level in CD8+ T cells after ConA stimulation (Figure 3F). Our data showed that knockdown of Blimp-1 lowered the ratio of GrB+ CD8+ T cells (Figure 3G) so that the death rate of neuronal cells decreased significantly when they were co-cultured with activated CD8+ T cells (Figure 3H). Meanwhile, the inhibition of ImK on CD8+ T cells cytotoxicity was not enhanced by Blimp-1 knockdown, suggesting Kv1.3 blockade suppressed stimulated cytotoxic CD8+ T cells by downregulating Blimp-1.

**FIGURE 3** The downregulation of Blimp-1 by Kv1.3 blockade reduced cytotoxic CD8+ T cell differentiation. A, The mRNA expression of Il2, Stat3, Prdm1, and Bcl6 was detected by real-time PCR in CD8+ T cells after stimulation with or without Kv1.3 blockade. B-C, The Blimp-1 expression of CD8+ T cells from stimulated PBMCs (B) or EAE rats (C) were measured by flow cytometry (FCM). D-E, The co-located expression of Blimp-1 and GrB were detected by immunohistochemistry in EAE rats’ spinal cords (D) and by FCM in restimulated CD8+ T cells (E). F-H, After Blimp-1 knockdown (F), the expression of GrB was examined with or without Kv1.3 blockade (G), and the cytotoxicity of CD8+ T cells on oligodendrocytes was measured by LDH assay (H). Data are presented as mean ± SEM (n = 3 for each group in Figure A, B, E, G and H, three independent experiment repeats. n = 6 for each group in Figure C and D, two independent experiment repeats. *P < .05, **P < .01, ***P < .001)
3.4 Kv1.3 blockade promoted PD-1 expression to reduce CD8+ T cells cytotoxicity

To illustrate the potential mechanism of cytotoxicity inhibition on CD8+ T cells by Kv1.3 blockade, we further investigated whether the suppression of Blimp-1 by Kv1.3 blockade was dependent on IL-2 signaling. In a high concentration of IL-2 medium, prdm1 showed a great increase after ConA stimulation, and ImK could still reduce its expression (Figure 4A). These data indicated that Kv1.3 blockade may inhibit CD8+ T cell activation through an IL-2 independent pathway. Since the co-inhibitory molecule PD-1 regulates CD8+ T cells terminal differentiation to exhausted cells, we examined whether PD-1 was involved in Kv1.3 blockade. Indeed, we found the gsk3β/pdcd1 expression were both increased significantly by ImK treatment after CD8+ T cell activation, which is reported to participate in Blimp-1 mediation (Figure 4B). Furthermore, ImK upregulated PD-1+CD8+ T cells and suppressed Blimp-1+CD8+ T cells after ConA stimulation (Figures 4C, S1). The immunochemistry data showed that ImK treatment increased PD-1+ cells in the spinal cords of EAE rats (Figure 4D). To identify the participation of PD-1 in Kv1.3 blockade regulation, we used a PD-1 inhibitor33 and found it does not affect Kv1.3 channel (Figure S2). By giving the PD-1 inhibitor, the Blimp-1+ CD8+ T cells levels raised again after stimulation even with ImK treatment (Figure 4E). In addition, the PD-1 inhibitor treatment increased Blimp-1+GrB+ CD8+ T cells (Figure 4F), promoted their cytotoxicity on neuronal cells (Figure 4G), and impaired the rescue effect of ImK, suggesting the PD-1 upregulation by Kv1.3 blockade suppressed Blimp-1 function in CD8+ T activation. While the PD-1 inhibitor decreased PD-1 expression, it had but had no effect on Blimp-1 expression and cytotoxicity after Blimp-1 knockdown (Figure S3).

3.5 Kv1.3 blockade inhibited Blimp-1 mediated cytotoxicity by PD-1 upregulation in CD3/CD28 antibody stimulated CD8+ T cells

Kv1.3 blockade can suppress CD4+ T cell function, which mediated CD8+ T cell activation. To prove whether ImK

![Figure 4](image-url)
can directly suppress CD8+ T cell activation, we isolated CD8+ T cells and stimulated them with CD3/CD28 antibody. Consistent with the results of ConA stimulation, ImK still increased PD-1 protein levels and decreased Blimp-1 expression in CD8+ T cells after CD3/CD28 antibody stimulation (Figure 5A). Furthermore, PD-1 inhibitor also upregulated Blimp-1 and IFN-γ release (Figure 5B) and promoted individually stimulated MOG35-55 stimulated CD8+ T cell cytotoxicity (Figure 5C). Besides, Blimp-1 knockdown removed the effects of ImK and PD-1 inhibitor on CD8+ T cells (Figure 5D). These results suggested Kv1.3 blockade can directly suppress CD8+ T cells functions through PD-1/Blimp-1 signaling.

3.6 | PD-1 inhibitor disturbed the rescue of Kv1.3 blockade in EAE model

PD-1 inhibitors are used for tumor immunotherapy to enhance CD8+ T cell cytotoxicity. However, little is known about their influence on autoimmune diseases. Here, we developed an EAE rat model and evaluated the effect of PD-1

FIGURE 5  Kv1.3 blockade suppressed Blimp-1-related cytotoxicity in a PD-1 dependent way in CD3/CD28 antibody stimulated CD8+ T cells. A, The expression of Blimp-1 and GrB in CD8+ T cells was detected by FCM after stimulation, with or without treatment of Kv1.3 blocker and PD-1 inhibitor. B,C, The concentration of IFN-γ was detected by ELISA (B), and cytotoxicity on oligodendrocytes was measured by LDH assay (C) in CD8+ T cells. D, The expression of Blimp-1 and GrB in CD8+ T cells was detected by FCM after Blimp-1 knockdown, with or without treatment of Kv1.3 blocker and PD-1 inhibitor. Data are presented as mean ± SEM (n = 3 for each group in Figure A-D, three independent experiment repeats. *P < .05, **P < .01, ***P < .001)
inhibitor treatment in EAE rats. The results showed that the PD-1 inhibitor accelerated the disease progression, but reached the same level of clinical scores as EAE group at the end of the experiments (Figure 6A,B). The clinical score of ImK+PD-1 inhibitor group did not change compared with EAE group, while it was higher than the ImK group, suggesting that PD-1 inhibitor disturbed the rescue effect of ImK treatment (Figure 6A,B). Our results in EAE model suggested that PD-1 inhibition alleviates the rescue effect of Kv1.3 blockade in EAE rats.

4 | DISCUSSION

MS is an autoimmune disease that presents with infiltrated peripheral immune cells as well as immunocompetent cells in lesions of the CNS. In previous work, we proved that ImK, a Kv1.3 channel blocker, can effectively suppress CD4+ T cells activation and alleviate the development of disease in an EAE rat model. In this study, although ImK showed an extensive inhibition on various immune cells (Figure 1), we focused on CD8+ T cells for further research, due to the high expression of Kv1.3 channel when CD8+ T cells were stimulated, as well as increased GrB in the CSF, suggesting that CD8+ T cells can be direct targets of Kv1.3 blockers, and the effects of other immune cells might be indirect through immune cell interaction.

Kv1.3 blockers can reduce the IL-2 concentration by suppressing calcium signaling, which is an essential pathway for CD8+ T cell activation and proliferation. Blimp-1 is a transcription factor for CD8+ T cell terminal differentiation and exhaustion, which is regulated by IL-2/STAT3 or IL-2/STAT5. The absence of Blimp-1 in CD8+ T cells correlates with the reduced cytolytic capacity including a decreased expression of GrB in viral infection. In addition, the major target of Kv1.3 blockers is TEm cells, appearing in chronic inflammation. The above suggests the inhibition of Kv1.3 blockers on CD8+ T cells may connect with Blimp-1. In this study, we found the expression of Blimp-1 was correlated with GrB in CD8+ T cells activation. The Kv1.3 blocker ImK can decrease Blimp-1 expression in stimulated CD8+ T cells, and protect against the cytotoxicity on neuronal cells. The inhibition of Blimp-1 expression by Kv1.3 blockade was verified in the rat EAE model, suggesting Kv1.3 blockade can regulate CD8+ T cell differentiation by Blimp-1 suppression.

Previous studies on Blimp-1 mostly focused on CD8+ T cell exhaustion. We found a link of PD-1 upregulation with Blimp-1 inhibition in CD8+ T cells by Kv1.3 blockade. It is reported that the co-inhibitory molecule PD-1 can negatively mediate T-bet expression, an upstream signal molecule of Blimp-1. Regulated by GSK-3, PD-1 expression is upregulated on the surface of exhausted CD8+ T cells and limits the cell-killing function. In addition, Blimp-1 can also repress PD-1 expression in CD8+ T cells, using a feed-forward transcriptional circuit during acute viral infection, but their relationship in autoimmune diseases remains unclear.

IL-2 is confirmed to regulate Blimp-1, and we investigated whether Kv1.3 blockade can inhibit Blimp-1 by other pathways. In an environment of high IL-2 concentration, Kv1.3 blocker ImK can also suppress Blimp-1 expression in CD8+ T cells, suggesting there was an IL-2 independent pathway participating in the Blimp-1 regulation by Kv1.3 blockade. Indeed, we found ImK can increase the numbers of PD-1+ CD8+ T cells. Besides, the PD-1 inhibitor reversed the suppression of ImK on Blimp-1, demonstrating that ImK can upregulate PD-1 expression in CD8+ T cells to suppress Blimp-1.

PD-1 is a hot target in tumor immunology, however, PD-1 inhibitors are reported to induce autoimmune diseases. In this work, we found that PD-1 expression can be upregulated by Kv1.3 blockade in stimulated CD8+ T cells, and PD-1 inhibitor accelerated the EAE progression and disturbed the treatment of Kv1.3 blocker ImK. The spinal cords of PD-1 inhibitor-treated EAE rats became infiltrated with many cytotoxic Blimp-1+ CD8+ T cells, suggesting that PD-1 inhibitors may trigger autoimmune diseases in animal models.
Our study demonstrated that Kv1.3 blockade upregulated PD-1 expression and further decreased Blimp-1 expression to suppress CD8+ T cells differentiation and cytotoxicity. This work reveals mechanisms of modulating CD8+ T cells through Kv1.3 channel in neuroinflammatory diseases.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS
Y. Zhao, S. Han, and X. He performed the experiments and analyzed the data. W. Qiu and J. Liu performed the EAE experiments. X. Yuan carried out the tissue immunohistochemistry. W. Mao expressed the Kv1.3 peptide blocker ImKTx88. B. Peng assessed the electrophysiological experiments of ImKTx88. W. Liu analyzed FACS data. J. Yin made the figures, and created and revised the manuscript. All authors contributed to and approved the final version of the manuscript.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the Supporting Information section.

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