

# Potent Suppression of Kv1.3 Potassium Channel and IL-2 Secretion by Diphenyl Phosphine Oxide-1 in Human T Cells

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## Abstract

Diphenyl phosphine oxide-1 (DPO-1) is a potent Kv1.5 channel inhibitor that has therapeutic potential for the treatment of atrial fibrillation. Many other Kv1.5 channel blockers also potently inhibit the Kv1.3 channel, but whether DPO-1 blocks Kv1.3 channels has not been investigated. The Kv1.3 channel is highly expressed in activated T cells, which is considered a favorable target for immunomodulation. Accordingly, we hypothesized that DPO-1 may exert immunosuppressive and anti-inflammatory effects by inhibiting Kv1.3 channel activity. In this study, DPO-1 blocked Kv1.3 current in a voltage-dependent and concentration-dependent manner, with  $IC_{50}$  values of 2.58  $\mu$ M in Jurkat cells and 3.11  $\mu$ M in human peripheral blood T cells. DPO-1 also accelerated the inactivation rate and negatively shifted steady-state inactivation. Moreover, DPO-1 at 3  $\mu$ M had no apparent effect on the  $Ca^{2+}$  activated potassium channel ( $K_{Ca}$ ) current in both Jurkat cells and human peripheral blood T cells. In Jurkat cells, pre-treatment with DPO-1 for 24 h decreased Kv1.3 current density, and protein expression by  $48 \pm 6\%$  and  $60 \pm 9\%$ , at 3 and 10  $\mu$ M, respectively (both  $p < 0.05$ ). In addition,  $Ca^{2+}$  influx to  $Ca^{2+}$ -depleted cells was blunted and IL-2 production was also reduced in activated Jurkat cells. IL-2 secretion was also inhibited by the Kv1.3 inhibitors margatoxin and charybdotoxin. Our results demonstrate for the first time that that DPO-1, at clinically relevant concentrations, blocks Kv1.3 channels, decreases Kv1.3 channel expression and suppresses IL-2 secretion. Therefore, DPO-1 may be a useful treatment strategy for immunologic disorders.

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## Introduction

Diphenyl phosphine oxide-1 (DPO-1) represents a novel class of Kv1.5 blocker that has been documented to selectively prolong atrial action potential duration (APD) without exerting significant effects on ventricular APD [1], [2]. *In vivo*, DPO-1 has been shown to increase the atrial effective refractory period and terminate atrial arrhythmias within 60 s in dog [3], [4]. However, in cholinergic atrial fibrillation (AF) models in pig, DPO-1 at 0.5  $\mu$ M did not terminate AF in a majority of experiments [5]. Recently, we have found that DPO-1 preferentially blocks the open state of the Kv1.5 channel by binding with several key residues in the S5-pore loop-S6 domains [1]. Interestingly, all of these Kv1.5 key residues are also highly conserved in the Kv1.3 channel [6], [7], which provides a molecular structural basis for why many Kv1.5 blockers (e.g., AVE0118 and S0100176) also potently inhibit the Kv1.3 channel [8], [9]. Therefore, the structural homology among these channel subtypes suggested that DPO-1 may block Kv1.3 channels as well.

Kv1.3 is the predominant voltage-gated potassium channel expressed in T cells, and shows C-type inactivation similar to other *Shaker* isoforms (Kv1). In human T cells, Kv1.3 regulates cell membrane potential and promotes sustained  $Ca^{2+}$  influx required

for T-cell receptor-mediated cell activation, migration, proliferation, and IL-2 secretion [10]. Therefore, Kv1.3 channel blockers have immunosuppressive and anti-inflammatory properties [11], [12]. Moreover, autoimmune disease-related effector memory T cells ( $T_{EM}$ ) expressed significantly higher levels of Kv1.3 channel after activation in multiple sclerosis [13], rheumatoid arthritis [14], type-1 diabetes [15], and psoriasis [16]. Selective inhibition of Kv1.3 channels resulted in the down-regulation of  $T_{EM}$  activities, and ameliorated autoimmune diseases in animal models [16], [17], [18]. Therefore, developing Kv1.3 channel blockers could be useful treatment strategy for immunologic disorders.

To date, no studies have evaluated the ability of DPO-1 to block Kv1.3 channels. *Xenopus* oocytes or mammalian cell lines (human embryonic kidney 293 and Chinese hamster ovary cells) have been widely used to characterize the electropharmacological properties of Kv1.3 channel blockers [19], [20]. However, use of these cell types does not reflect the true physiological environment of the Kv1.3 channel in human T cells and does not allow the immunomodulatory effects of Kv1.3 channel blockers to be evaluated. In the present study, we compared the effects of DPO-1 on Kv1.3 channels in the Jurkat cell line and in human peripheral blood T cells, and further investigated the effects of DPO-1 on  $Ca^{2+}$  influx, and IL-2 secretion in Jurkat cells. In the

current study we demonstrate for the first time that DPO-1 blocks Kv1.3 currents, decreases Kv1.3 channel expression, attenuates  $\text{Ca}^{2+}$  influx, and inhibits IL-2 production.

## Materials and Methods

### Ethics statement

In this study, the study protocol with human blood samples was approved by the Ethics Committee of Tongji Medical College of Huazhong University of Science and Technology. Human blood samples were taken from healthy blood donors, who were provided written informed consent for the collection of blood and subsequent T cells isolation and analysis.

### Cell preparation and culture condition

The human leukemia T-cell line, Jurkat E6-1, was obtained from the American Tissue Culture Collection (ATCC, Rockville, MD, USA). Human peripheral blood T cells were separated from whole blood samples using Ficoll gradients and purified by negative selection using CD4<sup>+</sup> T Cell Isolation Kit (Miltenyi Biotec, Bergisch-Gladbach, Germany). All cells were grown in culture medium consisting of RPMI 1640 supplemented with 10% heat-inactivated FBS, 10 mM HEPES, 2 mM glutamate, 100 U/mL penicillin/streptomycin and were maintained at 37°C in a humidified 95% air and 5% CO<sub>2</sub> atmosphere. Jurkat cells were stimulated with 50 ng/mL phorbol ester (PMA, Sigma-Aldrich, St Louis, MO) and 5 µg/mL phytohemagglutinin (PHA, Sigma-Aldrich). DPO-1 (Tocris Biosciences, Bristol, UK) at 3 µM or 10 µM was added at the onset of stimulation or 30 minutes prior to stimulation. Margatoxin (MgTX; 10 nM) and charybdotoxin (ChTX; 100 nM) were used as positive controls to block Kv1.3 channels (both from Alomone Laboratories Ltd, Jerusalem, Israel).

### Electrophysiological recordings

All currents were recorded using a whole-cell patch configuration at room temperature. The external Ringer solution was (in mM): 137 NaCl, 4 KCl, 1.8 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 glucose, and 10 HEPES, adjusted with NaOH to pH 7.4. For Kv1.3 current measurements, the pipette solution (in mM) consisted of 130 KCl, 1 MgCl<sub>2</sub>, 5 EGTA, 5 Mg-ATP, 10 HEPES, adjusted to pH 7.2 with KOH. Kv1.3 currents were elicited by depolarizing pulses ranging from -80 mV to +60 mV in 10 mV increments from the holding potential of -80 mV. The membrane potential was measured in zero current ( $I=0$ ) mode using whole-cell patch technique.  $\text{K}_{\text{Ca}}$  currents were elicited by using 200 ms voltage ramps from -120 mV to +60 mV, applied every 30 s from the holding potential of -40 mV. For  $\text{K}_{\text{Ca}}$  measurements, the pipette solution contained (in mM): 130 potassium aspartate, 10 EGTA, 8.55 CaCl<sub>2</sub>, 2.08 MgCl<sub>2</sub>, and 10 HEPES, adjusting to pH 7.2 using KOH, with a calculated free  $[\text{Ca}^{2+}]$  of approximately 1 µM. For some experiments recording  $\text{K}_{\text{Ca}}$ , the bath solution was replaced by a K<sup>+</sup> Ringer solution, which featured identical ingredients, except that all NaCl was substituted by KCl.

The pCLAMP 9.0 and Origin 8.5 (OriginLab Corporation, Northampton, MA, USA) software were used for data acquisition and analysis. Membrane conductance ( $G$ ) was defined as  $I/(V-E_{\text{rev}})$ , where  $I$  is the peak amplitude,  $V$  is the test voltage, and  $E_{\text{rev}}$  is the reversal potential (-90 mV) of Kv1.3 currents [21]. The steady-state inactivation curve was studied by using a double-pulse protocol, in which the test voltage was stepped to +40 mV, 100 ms long, and preceded by 30 s preconditioning pulses from -80 to 0 mV in 10 mV steps. The data for activation and steady-state inactivation were fitted with the Boltzmann equation to get the half-maximum voltage of activation or inactivation voltage ( $V_{1/2}$ )

and slope factor. The percent inhibition (% inhibition) of the current was defined as  $[(I_{\text{control}} - I_{\text{drug}})/I_{\text{control}}] * 100\%$ . The concentration required for 50% inhibition of the current ( $\text{IC}_{50}$ ) was determined by fitting the data with the Hill equation.

### Western blotting

Jurkat cell total lysate was prepared according to standard procedures. Cells were washed twice in cold PBS, homogenized with PMSF buffer (Roche, Basel, Switzerland), and centrifuged at 12,000 rpm for 20 min. Protein concentrations of the supernatants were measured using a BCA kit (Pierce, Rockford, IL, USA). Samples containing 40 µg of total protein were boiled in SDS loading buffer, separated on a 10% SDS-PAGE gel, and transferred to nitrocellulose membranes. The membranes were blocked in 5% dry milk, supplemented with 0.3% Tween-20 TBS, for 1 h at room temperature and probed with anti-Kv1.3 (diluted 1:500, Abcam, MA, USA) or anti-β-actin (diluted 1:1000, Santa Cruz Biotechnology, Inc., CA, USA) overnight at 4°C. The membranes were washed three times and incubated with anti-rabbit or anti-mouse peroxidase-conjugated IgG (diluted 1:3000, Antigenex, Wuhan, China) for 1 h at room temperature. Bands were visualized using West Pico Chemiluminescent Substrate (Thermo Scientific Inc., Bremen, Germany) and quantified by densitometric analysis using Quantity One software (Bio-Rad Laboratories, Hercules, CA).

### Single cell $\text{Ca}^{2+}$ measurements

Jurkat cells were loaded with 2 µM fluo-3 AM (Invitrogen, Carlsbad, CA) at room temperature for 30 min and washed twice with  $\text{Ca}^{2+}$  free Ringer's solution. Cells were allowed to adhere to laminin-coated glass coverslip chambers (Sigma-Aldrich) on the stage of a Nikon (Tokyo, Japan) A1SI laser confocal microscope equipped with a 40× (Uplan/Apo, N.A. 1.0) objective and illuminated at 488 nm. Fluorescence emissions at 530 nm were captured with a charge-coupled device camera, digitized, and analyzed using the NIS-Elements microscope imaging software program. Images were recorded at intervals of 10 s.  $\text{Ca}^{2+}$  release was induced by 1 µM thapsigargin (TG, Alomone) in  $\text{Ca}^{2+}$  free Ringer solution. After 5 min, normal Ringer solution (2 mM CaCl<sub>2</sub>) was reintroduced. The fluorescent index  $\Delta F/F_0$  was calculated as the average  $\text{Ca}^{2+}$  response ( $\Delta F = F - F_0$ ,  $F_0$  is the mean value of background fluorescence).

### IL-2 secretion measurements

IL-2 production in Jurkat cells was measured using an ELISA kit (eBioscience, San Diego, CA, USA) following the manufacturer's instructions. Cells were centrifuged at 1500 rpm for 10 min, and the supernatants were collected to measure IL-2 concentrations. Reactions were performed in 96-well plates coated with the capture antibody and stopped with phosphoric acid (1 M). Absorbance was measured with an automatic plate reader at 450 nm. Each experiment was repeated at least three times in duplicate.

### Statistical analysis

Results are reported as mean ± SEM. The significance of any differences before and after drug treatment was evaluated using a paired *t*-test. Comparisons between groups were carried out by analysis of variance with Turkey's post-test. Significance was set at  $P < 0.05$ .

## Results

### DPO-1 blocks Kv1.3 channel currents in human T cells

We first defined the Kv channels expressed in Jurkat cells and human peripheral blood T cells. Kv currents were elicited by 300 ms pulses at test potentials ranging from  $-80$  to  $+60$  mV with 10 mV steps, from a holding potential of  $-80$  mV. The outward currents at potentials positive to  $-40$  mV were characterized by rapid activation and slow inactivation as reported previously (Figure 1A and 1B) [22], [23]. After application of 1 nM MgTX, a selective Kv1.3 channel blocker [24], the currents were nearly completely abolished (Figure 1C), confirming that the Kv1.3 current was the predominant component of the outward current in Jurkat cells and human peripheral blood T cells.

We then examined the concentration-dependent inhibitory effects of DPO-1 on Kv1.3 currents in Jurkat cells and human peripheral blood T cells. Cells were perfused externally with different concentrations of DPO-1 from 0.3 to 30  $\mu$ M. The peak current amplitude generated by stimulation at  $+40$  mV was used as the index of inhibition (Figure 2A and 2B). A nonlinear least-square fit of the Hill equation to the concentration response data yielded an  $IC_{50}$  of  $2.58 \pm 0.33$   $\mu$ M in Jurkat cells and  $3.11 \pm 0.07$   $\mu$ M in peripheral blood cells. The Hill coefficient was  $1.22 \pm 0.13$  in Jurkat cells and  $1.02 \pm 0.03$  in peripheral blood T cells (Figure 2C and 2D). The Hill coefficient was very close to 1, which suggests that DPO-1 blocks the channel in a 1:1 stoichiometric ratio.

We further evaluated the biophysical effects of DPO-1 on Kv1.3 in Jurkat cells. Figure 3A shows the current density-voltage relationships, before and after the addition of DPO-1 and after the washout of DPO-1 in Jurkat cells. DPO-1 at 3  $\mu$ M reduced the Kv1.3 current for the entire voltages range over which this current was activated, and the blocking effect of DPO-1 was completely reversible after washout. Further, the membrane potential was depolarized from  $-41.07 \pm 1.93$  mV to  $-25.99 \pm 2.91$  mV ( $P < 0.01$ ) and recovered to  $-41.54 \pm 1.60$  mV after washout.

To investigate the voltage-dependent block of DPO-1, the percent inhibition of DPO-1 was plotted as a function of membrane potential together with the activation curve. In the presence of DPO-1, inhibition increased steeply between  $-40$  and 0 mV, from  $7 \pm 7\%$  to  $54 \pm 5\%$ , corresponding to the voltage range for channel opening. This inhibition reached a steady state

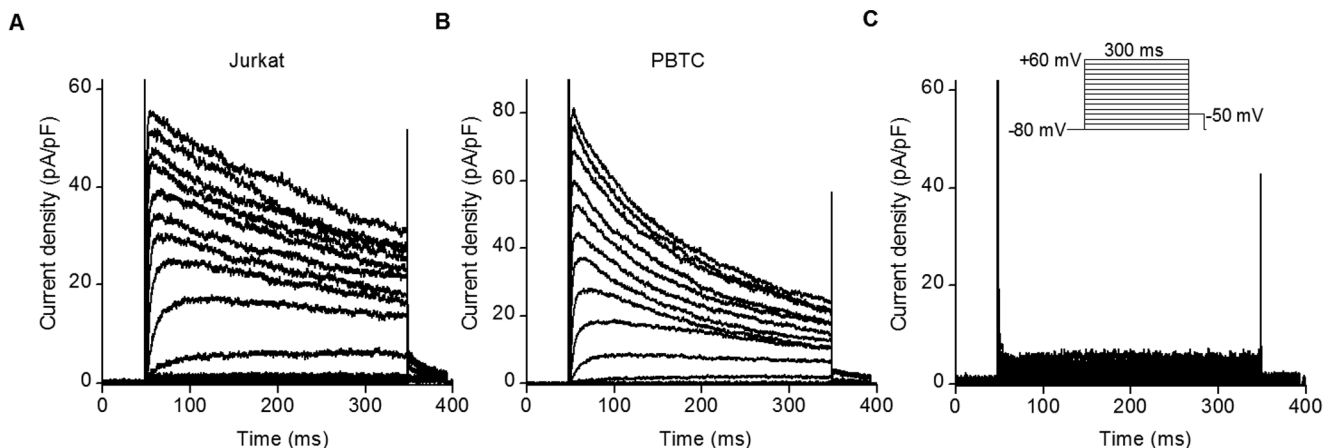
between  $+10$  and  $+60$  mV, when the Kv1.3 channels were fully activated (Figure 3B). These results suggested that blocking Kv1.3 by DPO-1 treatment is dependent on the voltage of the depolarizing pulse in a manner that indicates an open channel block.

To further determine whether blocking Kv1.3 channels with DPO-1 requires channel opening, we recorded currents in Jurkat cells before and after a pulse-free period of incubation with the compound. Control currents were elicited by repetitive application of depolarizing step pulses at  $+40$  mV. DPO-1 (3  $\mu$ M) was applied to the bath while the membrane potential was held at  $-80$  mV without any depolarization pulses. After an interval of 8 min to assure a steady-state condition, consecutive 250 ms pulses were applied every 10 s. Figure 3C and 3D show that DPO-1 did not affect the amplitude peak current density in the pulse-free conditions, where the amplitude peak Kv1.3 current density at pulse 1 was  $62.73$  vs.  $66.35$  pA/pF under control conditions, but significantly accelerated the inactivation of Kv1.3 channels (the time constants of inactivation were  $196 \pm 2$  ms under control and  $132 \pm 1$  ms at pulse 1). The onset of blockade occurred rapidly once the channel was opened with depolarization pulses (the amplitude peak current at pulse 17 was  $35.98$  vs.  $66.35$  pA/pF at pulse 1). Moreover, the current recovered quickly upon washout even in pulse-free conditions. These results indicate that DPO-1 preferentially binds to the open state of the Kv1.3 channel.

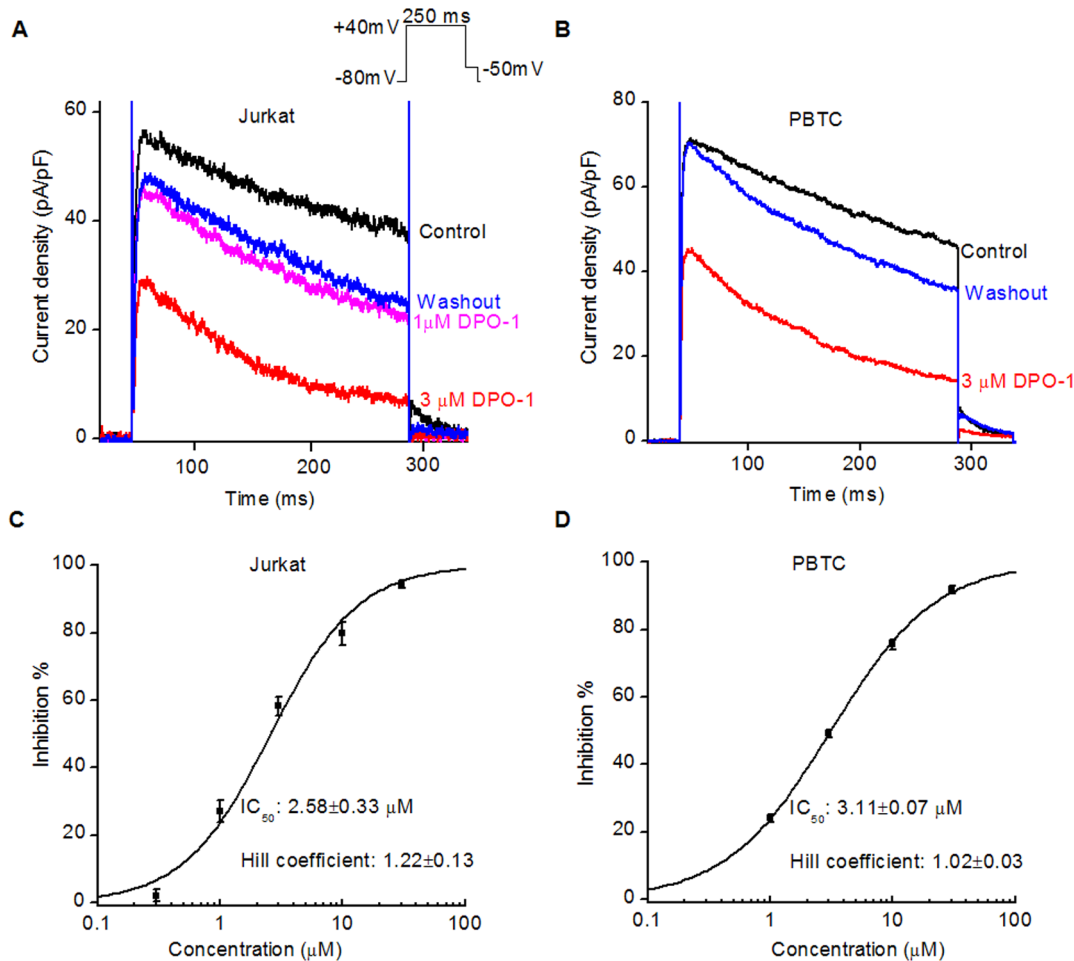
A double-pulse protocol was used to investigate the effects of DPO-1 on voltage-dependent of steady-state inactivation of Kv1.3 currents in Jurkat cells. DPO-1 at 3  $\mu$ M caused a negative shift of the steady-state inactivation curve (Figure 3F). The half inactivation voltage  $V_{1/2}$  were  $-43 \pm 1$  mV and  $-53 \pm 1$  mV for control and 3  $\mu$ M DPO-1, respectively ( $P < 0.001$  vs. control). The decay phase of the Kv1.3 current at  $+40$  mV was well fitted to a single exponential equation, yielding a time constant of  $234 \pm 28$  ms in control, and  $167 \pm 17$ ,  $95 \pm 12$ , and  $66 \pm 7$  ms in 1, 3, and 10  $\mu$ M DPO-1, respectively ( $P < 0.001$  vs. control for all of the concentrations) (Figure 3E). Thus, DPO-1 accelerated the channel inactivation rate in a concentration dependent manner.

### DPO-1 did not block $K_{Ca}$ channel currents in human T cells

T cells also express the calcium activated potassium channel  $K_{Ca}$ , which plays a major role in regulating the membrane



**Figure 1. Jurkat cells and human peripheral blood T cells (PBTC) express Kv1.3 currents.** Original current traces were elicited by 300 ms depolarizing pulses from a holding potential of  $-80$  mV to test potentials between  $-80$  mV and  $+60$  mV, with 10 mV steps in Jurkat cells (A), in peripheral blood T cells (B) and after application of 10 nM MgTX (C), a Kv1.3 blocker in Jurkat cells. doi:10.1371/journal.pone.0064629.g001



**Figure 2. Concentration-dependent blockade of Kv1.3 channels by DPO-1 in human T cells.** Currents were elicited by 250 ms depolarizing pulses from a holding potential of  $-80$  mV to  $+40$  mV every 30 s. Representative current traces were superimposed in Jurkat cells (A) and human peripheral blood T cells (B) before (control, in black), during perfusion with DPO-1 (pink and red curves), and after washout (in blue). Summarized data from Jurkat cells (C) and human peripheral blood T cells (D) showing the dose-response curve fitted with the Hill equation. Data were expressed as mean  $\pm$  SEM from 6 cells for each concentration. doi:10.1371/journal.pone.0064629.g002

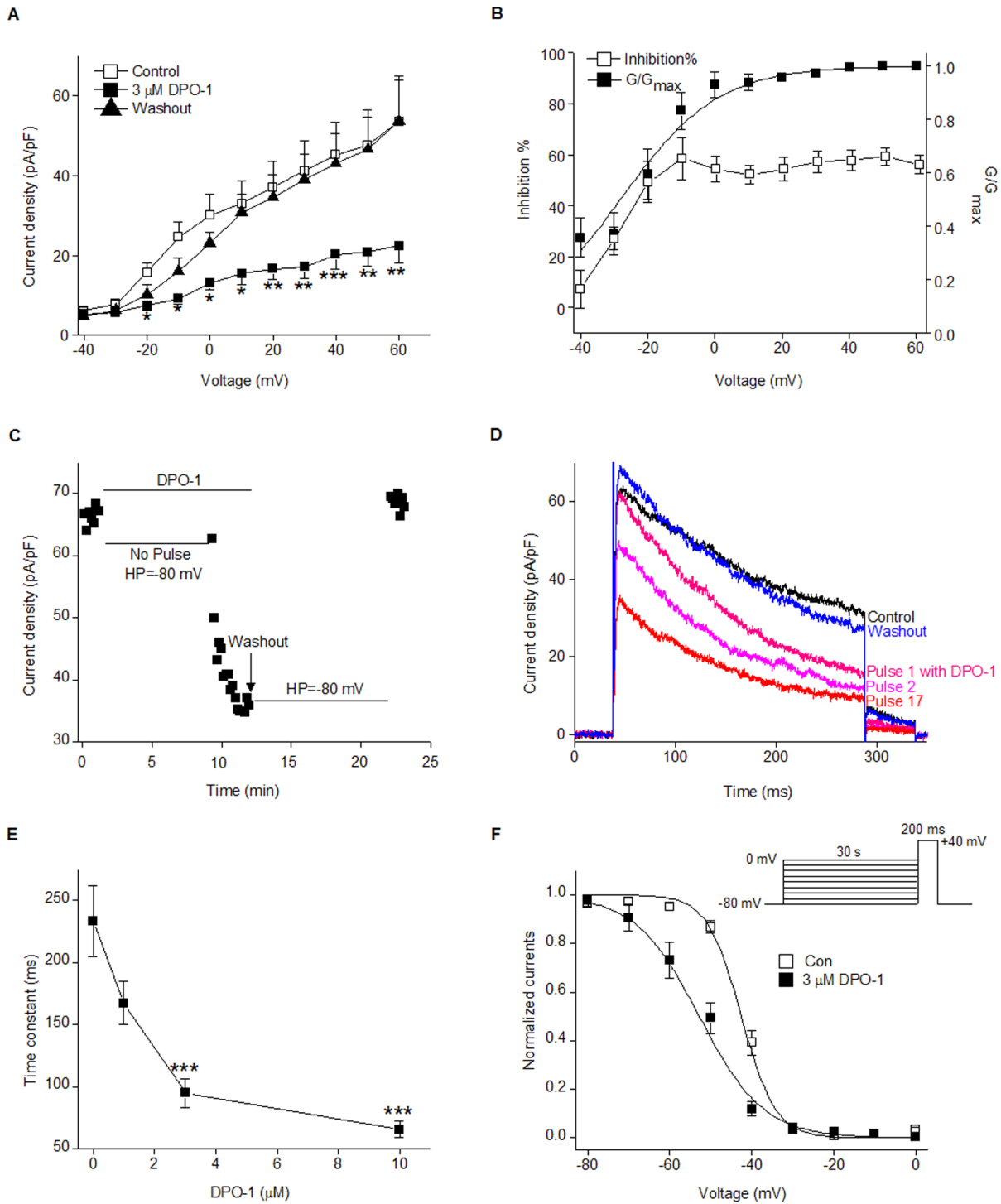
potential and maintaining  $\text{Ca}^{2+}$  influx along with the Kv1.3 channel [10], [25].  $\text{K}_{\text{Ca}2.2}$  is the main  $\text{K}_{\text{Ca}}$  channel involved in the  $\text{Ca}^{2+}$  signaling of Jurkat cells [26], whereas  $\text{K}_{\text{Ca}3.1}$  is the main  $\text{K}_{\text{Ca}}$  channel in peripheral T cells [25]. Therefore, we investigated whether DPO-1 could also block  $\text{K}_{\text{Ca}}$  currents in both these two kinds of human T cells. Figure 4A shows ramp currents in normal Ringer solution before and after application of DPO-1 in Jurkat cells. To avoid any confounding effects of the Kv currents, we measured the  $\text{K}_{\text{Ca}}$  slope conductance between  $-120$  mV and  $-60$  mV and fitted with the results as a linear function. DPO-1 at  $3$   $\mu\text{M}$  had no apparent effects on the slope factor for  $\text{K}_{\text{Ca}}$  ( $P=0.709$ ). To increase the slope conductance for  $\text{K}_{\text{Ca}}$  and make it more accurate to measure, we used the high  $\text{K}^+$  Ringer solution, which was also widely used in many studies [26], [27–29]. Application of  $1$   $\mu\text{M}$  TRAM-34, a selective  $\text{K}_{\text{Ca}}$  channel blocker [29], robustly decreased the  $\text{K}_{\text{Ca}}$  slope conductance (Figure 4A and 4B). In contrast, DPO-1 at  $3$   $\mu\text{M}$  had no effects on  $\text{K}_{\text{Ca}}$  in both Jurkat cells and human peripheral blood T cells (Figure 4C and 4D), which was also confirmed after the blockade Kv1.3 currents in the presence of MgTX (Figure S1). The slope conductance for Jurkat cells was  $1.39 \pm 0.07$  nS in control group, and  $1.39 \pm 0.09$  nS in the DPO-1 group ( $P=0.884$ ). Likewise, slope

conductance for human peripheral blood T cells was  $1.62 \pm 0.28$  nS in the control group and  $1.62 \pm 0.29$  nS in cells treated with DPO-1 ( $P=1.000$ ). The results for the control T cells were similar to previous reports [28], [30]. Therefore, our results suggest that DPO-1 had no effect on  $\text{K}_{\text{Ca}}$  either in physiological or high  $\text{K}^+$  external solution.

#### Kv1.3 channel current density and protein expression was inhibited by pre-treatment with DPO-1 in Jurkat cells

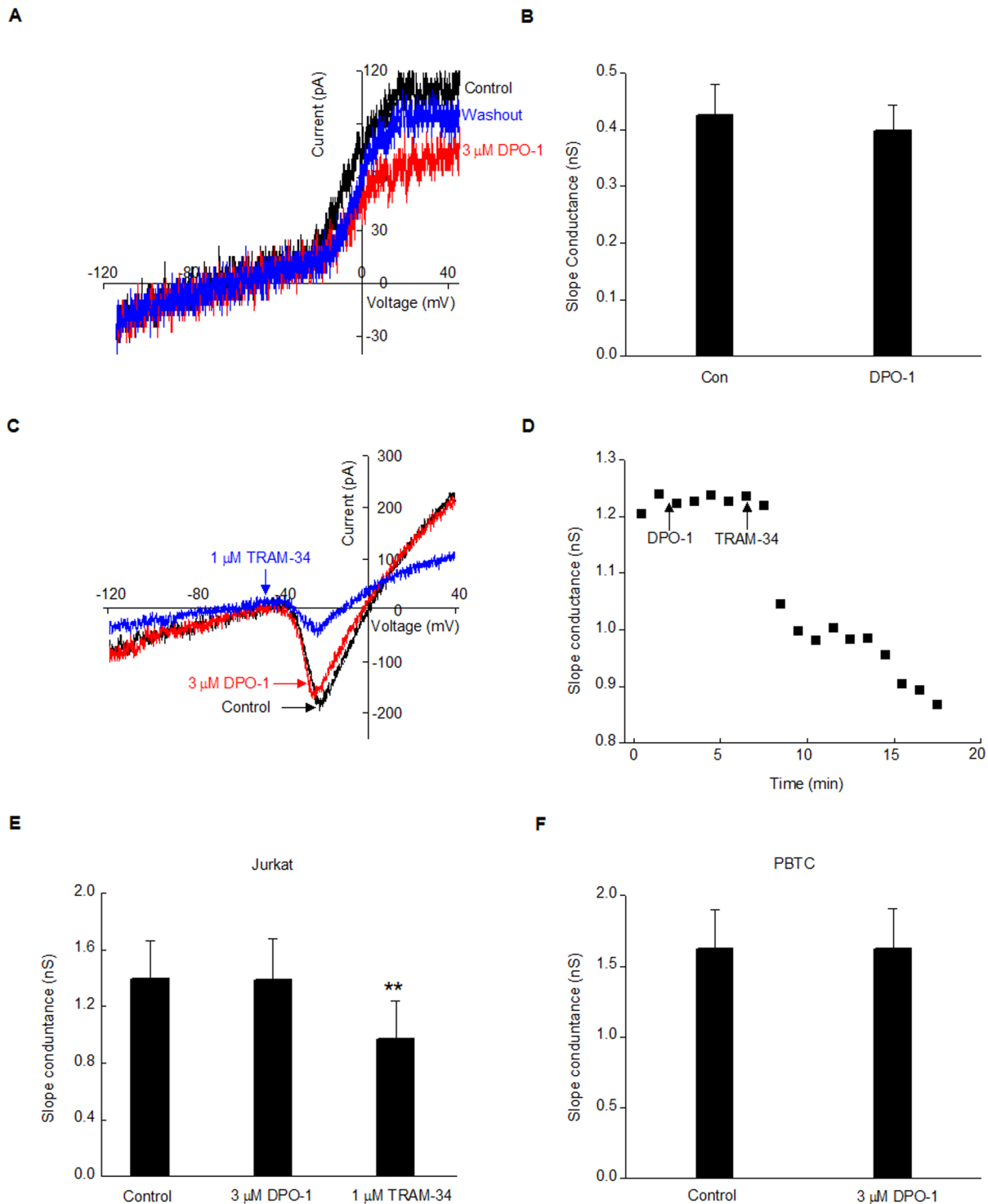
We further investigated the effects of 24 h DPO-1 pre-treatment on Kv1.3 channel currents and protein expression levels in Jurkat cells. Similar to acute administration, pre-incubation with DPO-1 also blocked the Kv1.3 channel current in a concentration-dependent manner. The current density at  $+40$  mV decreased from  $48.52 \pm 2.31$  pA/pF in control cells to  $24.37 \pm 2.21$ ,  $21.77 \pm 2.21$  and  $18.54 \pm 2.10$  pA/pF in 1, 3, and 10  $\mu\text{M}$  DPO-1 treated cells, respectively ( $P < 0.001$  vs. control for all concentrations) (Figure 5A).

Immunoblotting was used to analyze the protein expression of Kv1.3 channel (Figure 5B and Figure 5C). Pre-treatment with 3 and 10  $\mu\text{M}$  DPO-1 for 24 h decreased Kv1.3 channel protein expression by 47.9% and 60.0%, respectively, ( $P < 0.05$  vs. control



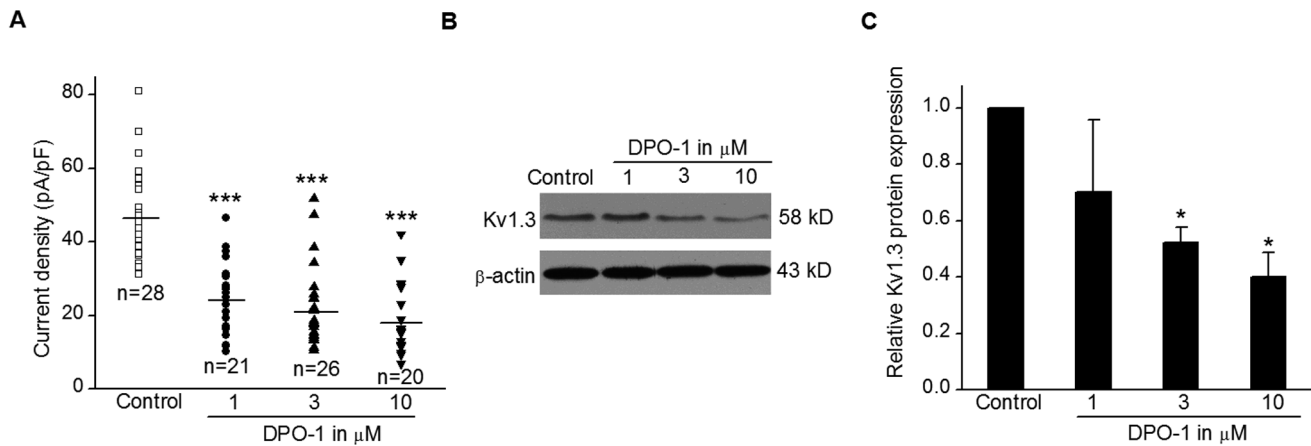
**Figure 3. DPO-1 blocked the Kv1.3 channel in Jurkat cells.** (A) An average current density-voltage relationship for peak Kv1.3 current in the absence (open squares), presence of 3  $\mu\text{M}$  DPO-1 (filled squares), and washout (filled triangles),  $**P < 0.01$  and  $***P < 0.001$  vs. control (n is cell number, n=6). (B) Inhibition (%) of peak Kv1.3 currents vs. test voltages obtained from panel A. The percent inhibition (open squares) are superimposed with the activation curve of Kv1.3 (filled squares) (n=6). (C) The time course of Kv1.3 inhibition by 3  $\mu\text{M}$  DPO-1. Currents were elicited by a 250 ms depolarizing to +40 mV. DPO-1 (3  $\mu\text{M}$ ) was applied to the bath while the membrane potential was held at -80 mV. After an interval of 8 min, consecutive 200 ms pulses were applied every 10 s. (D) Superimposed current traces recorded in the presence of DPO-1 (pink and red curves) or following washout (in blue) corresponding to panel C. The numbers 1 to 17 refers to pulses 1 to 17. (E) Kv1.3 current inactivation at +40 mV was fitted with a monoexponential equation, and the time constant has been plotted against DPO-1 concentration ( $***P < 0.001$  vs. control, n=6). (F) Steady-state inactivation curves in the absence (open squares) and presence (filled squares) of DPO-1. Data were obtained from normalized currents at +40 mV, which followed 30 s prepulses to potentials between -80 mV and 0 mV and were fitted with the Boltzman equation (n=6). Data were expressed as mean  $\pm$  SEM.

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**Figure 4. The effect of DPO-1 on  $K_{Ca}$  channel currents in human T cells.** (A) Representative currents-voltage relationships were superimposed before (in black) and after application (in red) with 3  $\mu$ M DPO-1 in Jurkat cells using normal Ringer's Solution. Currents were elicited by 200 ms voltage-ramps from  $-120$  mV to  $+40$  mV from the holding potential of  $-40$  mV. (B) Summarized data from 5 Jurkat cells. (C) Representative currents-voltage relationships were superimposed before (in black) and after perfusion with DPO-1 (in red) or TRAM-34 (in blue) in Jurkat cells. Currents were elicited by 200 ms voltage-ramps from  $-120$  mV to  $+40$  mV from the holding potential of  $-40$  mV. (D) Time course of the effect of DPO-1 and TRAM-34 on the slope conductance in Jurkat cells.  $K_{Ca}$  slope conductance was measured between  $-120$  mV and  $-40$  mV. (E) Summarized data from at least 9 Jurkat cell experiments. \*\*  $P < 0.01$  vs. control. (F) Summarized data from 7 human peripheral blood T cells. Data were expressed as mean  $\pm$  SEM.

doi:10.1371/journal.pone.0064629.g004



**Figure 5. Effects of DPO-1 pre-treatment on Kv1.3 current density and protein expression in Jurkat cells.** (A) Kv1.3 peak current density at +40 mV under control condition (open squares) and pretreated 24-h with 1 (filled circles), 3 (filled triangles) and 10 (filled inverted triangles)  $\mu\text{M}$  DPO-1. n is the cell number, \*\*\* $P < 0.001$  vs. control. (B) Representative western blot analysis of Kv1.3 protein under control condition and pretreated 24-h with 1, 3, and 10  $\mu\text{M}$  DPO-1. (C) Data from 3 experiments replicates were normalized to the amount of  $\beta$ -actin (\* $P < 0.05$  vs. control). Data were expressed in mean  $\pm$  SEM.

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for both concentrations). However, 30 min pre-treatment with DPO-1 and 24 h pre-treatment with MgTX had no significant effects on Kv1.3 channel protein expression (Figure S2 and S3).

#### The $\text{Ca}^{2+}$ influx to $\text{Ca}^{2+}$ -depletion Jurkat cells was blunted by DPO-1

The Kv1.3 channel controls  $\text{Ca}^{2+}$  homeostasis in T cells, and Kv1.3 channel inhibition significantly reduces  $\text{Ca}^{2+}$  influx [10]. To confirm that DPO-1 inhibited  $\text{Ca}^{2+}$  influx, we measured intracellular  $\text{Ca}^{2+}$  concentration by laser confocal microscopy. Depletion of  $\text{Ca}^{2+}$  from the endoplasmic reticulum was induced by 1  $\mu\text{M}$  TG in  $\text{Ca}^{2+}$ -free Ringer's solution. In the absence of extracellular  $\text{Ca}^{2+}$ , TG induced a very small, transient intracellular  $\text{Ca}^{2+}$  concentration rise in Jurkat T cells. When 2 mM  $\text{CaCl}_2$  was applied to the  $\text{Ca}^{2+}$ -free Ringer's solution,  $\text{Ca}^{2+}$  flowed into the cell through the  $\text{Ca}^{2+}$  release-activated  $\text{Ca}^{2+}$  channel (CRAC) channel to significantly increase intracellular  $\text{Ca}^{2+}$ , as illustrated in Figure 6A. Pre-treatment with DPO-1 for 24 h led to a concentration-dependent inhibition of  $\text{Ca}^{2+}$  influx through the CRAC channel in  $\text{Ca}^{2+}$ -depleted Jurkat cells, but did not alter TG-induced intracellular store  $\text{Ca}^{2+}$  release (Figure 6). The peak  $\Delta\text{F}/\text{F}_0$  value representing  $\text{Ca}^{2+}$  influx decreased from 4.17 in control to 2.4, 1.2, and 0.2, in 0.3, 1 and 3  $\mu\text{M}$  DPO-1, respectively ( $P < 0.01$  vs. control for 0.3 and  $P < 0.001$  for 1 and 3  $\mu\text{M}$ ). Similar results were also found in the pre-treatment of DPO-1 for 30 min (Figure S4).

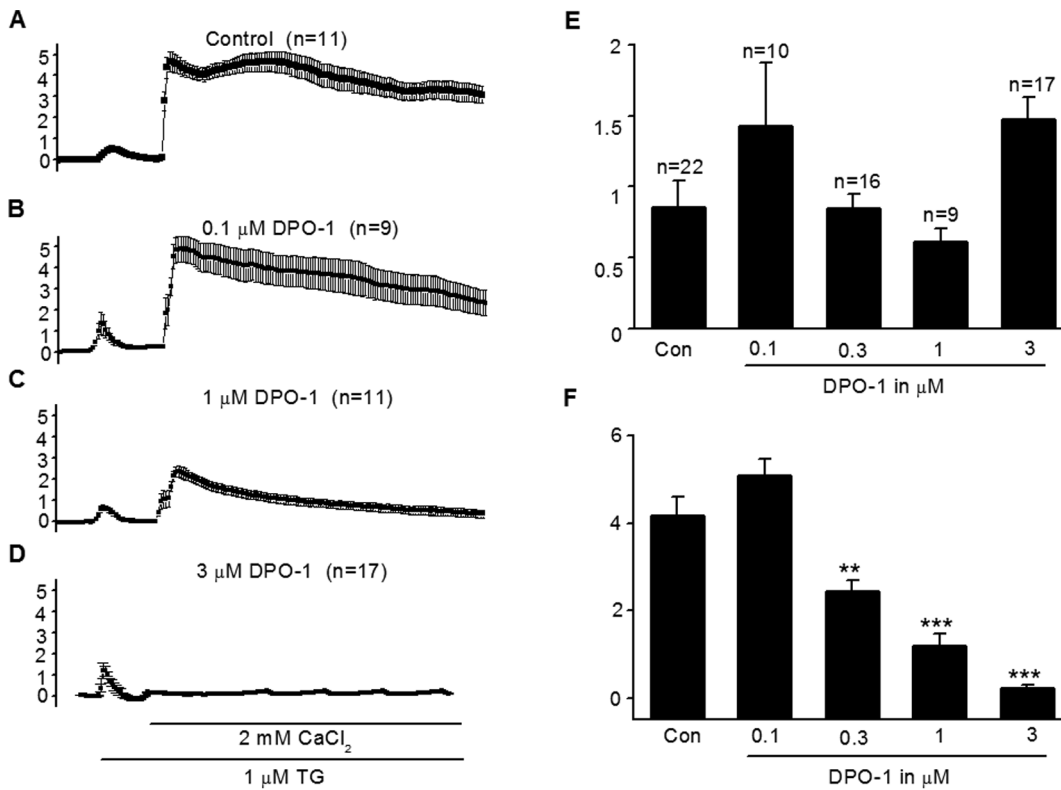
#### DPO-1 inhibited the secretion of IL-2 in activated Jurkat cells

To determine whether Kv1.3 channel and  $\text{Ca}^{2+}$  influx inhibition by DPO-1 could result in functional immunosuppression, we measured IL-2 secretion in the growth media by ELISA. As shown in Figure 7, IL-2 secretion was increased robustly upon stimulation with PHA+PMA ( $P < 0.001$ ), which served as the positive control. IL-2 levels were significantly reduced in the presence of 3 ( $P < 0.05$ ) and 10 ( $P < 0.01$ )  $\mu\text{M}$  DPO-1 pre-treatment for 24 h. IL-2 secretion was also reduced with 10 nM MgTX and 100 nM ChTX, two known Kv1.3 blockers [31].

## Discussion

This is the first study to investigate the effect of DPO-1, a novel Kv1.5 blocker, on potassium channels in human T cells. Our results demonstrated that DPO-1 blocked the Kv1.3 channel without any significant effect on the  $\text{K}_{\text{Ca}}$  channel. Similar to our previous observation on the Kv1.5 channel, DPO-1 blocked the Kv1.3 channel in a voltage- and concentration-dependent manner, preferentially blocking the open channel state [1]. Pre-treatment with DPO-1 for 24 h significantly reduced Kv1.3 current density and protein expression. Considering the importance of Kv1.3 in triggering  $\text{Ca}^{2+}$  influx and IL-2 synthesis, we further investigated the effects of DPO-1 on  $\text{Ca}^{2+}$  influx and IL-2 production. We found that DPO-1 inhibited  $\text{Ca}^{2+}$  influx as well as IL-2 secretion. Therefore, our results suggested that in addition to inhibiting Kv1.5 channels, DPO-1 has marked inhibitory effects on Kv1.3 currents and its expression,  $\text{Ca}^{2+}$  influx and IL-2 production.

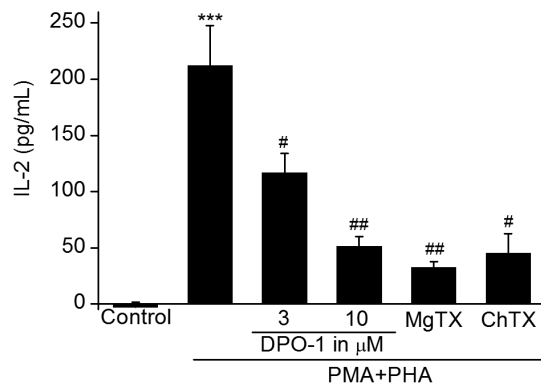
DPO-1 blocked the Kv1.3 channel in a voltage-dependent manner, and this inhibitory effect increased steeply from voltages of  $-40$  mV to 0 mV. Moreover, DPO-1 did not produce any obvious effect until the channel was first opened by a depolarization pulse, indicating that DPO-1 did not affect the Kv1.3 channel in the closed state. Combined, these effects suggest that DPO-1 interacts with the open state of Kv1.3 channels, which is the same mechanism previously observed for the Kv1.5 channel [1]. Of note, DPO-1 failed to cause a crossover phenomenon of tail current traces, which is in contrast with the properties of other open-channel blockers of the Kv1.3 channel [20], [32]. One possible explanation is that the functional dissociation of DPO-1 from the Kv1.3 channel at repolarization may not interfere with the transition of the channel from an open to a closed state. In addition, our data show that DPO-1 not only accelerated the time course of inactivation in a concentration-dependent manner but also shifted the voltage-dependent steady-state inactivation in the negative direction, indicating that DPO-1 also binds to the inactivated-state. Previous studies have shown that FK-506 [33] and verapamil [34], binding to both the open and inactivation states, caused significant negative shift of the voltage-dependence of steady-state inactivation. Unlike DPO-1, staurosporine [35] and fluoxetine [32], the pure Kv1.3 open channel blockers, had no



**Figure 6. Inhibitory effect of DPO-1 on calcium release and influx in Jurkat cells.** Ca<sup>2+</sup> release was induced by 1 μM thapsagargin (TG) in Ca<sup>2+</sup> free Ringer solution in Jurkat cells with or without 24 h pre-treatment of DPO-1. After 5 min, normal Ringer solution (2 mM CaCl<sub>2</sub>) was reintroduced, causing a rapid and sustained Ca<sup>2+</sup> influx. (A) Average Ca<sup>2+</sup> responses as the index ΔF/F<sub>0</sub> were shown in control, (B) 0.1 μM DPO-1, (C) 0.3 μM DPO-1, and (D) 3 μM DPO-1. (E) Summarized data of peak Ca<sup>2+</sup> release with TG. (F) Summarized data of the peak Ca<sup>2+</sup> influx with addition of 2 mM Ca<sup>2+</sup>, \*P<0.05 and \*\*P<0.01 vs. control. Data were expressed in mean±SEM. n is the cell number. doi:10.1371/journal.pone.0064629.g006

effects on the voltage-dependence of steady-state inactivation. However, the interactions between DPO-1 and the inactivation state require more sophisticated kinetic analysis.

In addition to the Kv1.3 channel, human T cells express K<sub>Ca</sub> channels that help set up the membrane potential to drive Ca<sup>2+</sup> influx necessary for IL-2 synthesis [10], [25]. Our electrophysio-



**Figure 7. Inhibitory effect of DPO-1 and potassium channel blockers on IL-2 production in activated Jurkat cells.** Jurkat cells were activated with PHA (5 μg/mL) and PMA (80 nM) for 24 h. DPO-1 (3 and 10 μM), MgTX (10 nM) and ChTX (100 nM) were added simultaneously. \*\*\* P<0.001 vs. control. # P<0.05 and ## P<0.01 vs. activated group. Data were expressed as mean±SEM. doi:10.1371/journal.pone.0064629.g007

logical data shows that acute application of 3 μM DPO-1 blocked the Kv1.3 channel, but had little effect on the K<sub>Ca</sub> channel. Meanwhile, the membrane potential was depolarized from  $-41.07 \pm 1.93$  mV to  $-25.99 \pm 2.91$  mV. In human T cells, a small fraction of Kv1.3 channel is active at rest to maintain the membrane potential [25], [36]. Therefore, we assumed that DPO-1 could block Kv1.3 channel even in the rest potential range. In addition, membrane depolarization by blocking Kv1.3 has been confirmed to be an effective method to prevent T-cell activation and therefore has applications in many autoimmune conditions [11]. DPO-1 also significantly decreased Ca<sup>2+</sup> influx and IL-2 secretion in activated Jurkat cells, leading to the attenuation of the immune response. Consistent with other reports, both MgTX and ChTX blocked Kv1.3 to inhibit IL-2 secretion [31]. Furthermore, we also demonstrated that pre-treatment with DPO-1 for 24 h but not 30 min significantly reduced the channel protein expression in T cells. Unlike DPO-1, pre-treatment with MgTX for 24 h had no effect on Kv1.3 protein expression. In addition, pre-treatment with DPO-1 for 24 h had no effects on Kv1.3 mRNA expression (Figure S5). These results suggested that direct and acute blockade of Kv1.3 channel by DPO-1 can lead to obvious inhibition of IL-2 secretion. Meanwhile, the inhibitory effect of DPO-1 on Kv1.3 protein expression may strengthen its immunosuppression potential. Overall, our data indicate that DPO-1, by blocking the Kv1.3 currents, down-regulating the Kv1.3 protein expression, and depolarizing membrane potential, blunts Ca<sup>2+</sup> influx and results in decreased production of IL-2.



Both Kv1.3 and  $K_{Ca}$  channels play crucial roles in T cell activation. However, the relative contribution varies in certain T cell subtypes. The activation of  $T_{EM}$  cells (the major pathogenic T cells in tissue sites of inflammation [37]) primarily depends on Kv1.3 channels, while  $K_{Ca}$  channels have a major role in the activation of naïve and central memory T cells [38]. Importantly, potent and selective Kv1.3 inhibition preferentially prevents  $T_{EM}$  function, whereas naïve and central memory T cells (necessary for physiological immune responses) escape inhibition by augmenting  $K_{Ca}$  expression [27]. In animal experiments (rats and monkeys), effective dose of DPO-1 as a Kv1.3 blocker showed no apparent cardiac side effects, except for prolonging atrial refractory period [39]. Therefore, the Kv1.3 channel may be considered an attractive target for immunomodulatory therapies [18], [40].

In summary, our study provides the first evidence that Kv1.5 blocker DPO-1 suppresses Kv1.3 channels and exerts immunomodulatory effects by the inhibition of calcium influx and stimulation induced IL-2 production. Our results suggest that DPO-1 may be effective in inflammatory autoimmune diseases and benefits for the development of new drug on immunoregulatory therapy.

## Supporting Information

**Figure S1 Block of  $K_{Ca}$  currents with DPO-1 in the presence of MgTX in high  $K^+$  solution.** First, 1 nM MgTX was applied to the external solution to inhibit Kv1.3 currents (in red), then, 3  $\mu$ M DPO-1 was added in the presence of MgTX (in blue). (TIF)

**Figure S2 30 min incubation with DPO-1 had no inhibitory effect on Kv1.3 protein expression.** 1, 3, 10  $\mu$ M DPO-1 was applied to incubate Jurkat cells for 30 min. Then the Kv1.3 protein expression level was determined by western blot. (TIF)

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**Figure S3 24 h incubation with MgTX had no inhibitory effect on Kv1.3 protein expression.** 10 nM MgTX was applied to incubate Jurkat cells for 24 h. Then the Kv1.3 protein expression level was determined by western blot. (TIF)

**Figure S4 30 min incubation with DPO-1 inhibited the  $Ca^{2+}$  influx in  $Ca^{2+}$ -depleted Jurkat cells.** Jurkat cells were loaded with 2  $\mu$ M fluo-3 AM for 30 min, washed twice and resuspended in  $Ca^{2+}$  free Ringer’s solution. Then the cells were incubated with 0 or 10  $\mu$ M DPO-1 for 30 min, with thapsargin in the solution to induce the  $Ca^{2+}$  depletion. The multifunctional enzyme mark instrument (PerkinElmer, USA) was used to measure the  $Ca^{2+}$  fluorescence intensity. The normal Ringer’s solution with 2 mM  $CaCl_2$  was applied to induce the  $Ca^{2+}$  influx. A, Average  $Ca^{2+}$  responses as the index  $\Delta F/F_0$  were shown in control and 10  $\mu$ M DPO-1; B, Summarized data of the peak  $Ca^{2+}$  influx with addition of 2 mM  $Ca^{2+}$ , \*\*\*  $P < 0.001$ . (TIF)

**Figure S5 DPO-1 had no inhibitory effect on Kv1.3 mRNA expression level.** Jurkat cells were incubated with 1, 3, 10  $\mu$ M DPO-1 for 24 h. Then the relative Kv1.3 mRNA expression level was determined by real-time PCR. (TIF)

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## Author Contributions

Conceived and designed the experiments: Y-MD Y-HL. Performed the experiments: NZ QD L-LD X-XF Y-MD. Analyzed the data: NZ Y-MD. Wrote the paper: NZ Y-MD Y-HL.

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