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## Lovastatin blocks Kv1.3 channel in human T cells: a new mechanism to explain its immunomodulatory properties

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Lovastatin is a member of Statins, which are beneficial in a lot of immunologic cardiovascular diseases and T cell-mediated autoimmune diseases. Kv1.3 channel plays important roles in the activation and proliferation of T cells, and have become attractive target for immune-related disorders. The present study was designed to examine the block effect of Lovastatin on Kv1.3 channel in human T cells, and to clarify its new immunomodulatory mechanism. We found that Lovastatin inhibited Kv1.3 currents in a concentration- and voltage-dependent manner, and the IC<sub>50</sub> for peak, end of the pulse was  $39.81 \pm 5.11$ ,  $6.92 \pm 0.95 \mu\text{M}$ , respectively. Lovastatin also accelerated the decay rate of current inactivation and negatively shifted the steady-state inactivation curves concentration-dependently, without affecting the activation curve. However,  $30 \mu\text{M}$  Lovastatin had no apparent effect on K<sub>Ca</sub> current in human T cells. Furthermore, Lovastatin inhibited Ca<sup>2+</sup> influx, T cell proliferation as well as IL-2 production. The activities of NFAT1 and NF- $\kappa$ B p65/50 were down-regulated by Lovastatin, too. At last, Mevalonate application only partially reversed the inhibition of Lovastatin on IL-2 secretion, and the siRNA against Kv1.3 also partially reduced this inhibitory effect of Lovastatin. In conclusion, Lovastatin can exert immunomodulatory properties through the new mechanism of blocking Kv1.3 channel.

Lovastatin belongs to the medication family called Statins. Statins are the oral competitive inhibitors of 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which catalyzes the conversion of HMG-CoA to L-Mevalonate, leading to the blockade of cholesterol biosynthesis. Meanwhile, L-Mevalonate is the precursor for many isoprenoid metabolites. Statins would also inhibit the biosynthesis of isoprenoid intermediates such as geranyl and farnesyl pyrophosphate, and then affect the posttranslational prenylation of several important cell-signaling proteins during immune responses<sup>1-3</sup>. Consequently, along with the lipid-lowering effects, Statins have been demonstrated to exert immunomodulatory properties which decrease the risk of cardiovascular events, such as ischemic heart disease<sup>4,5</sup> and atrial fibrillation<sup>6</sup>. In recent years, more research focus on the immunomodulatory effect of Statins in inflammatory autoimmune diseases<sup>7</sup>. Statins have been reported to be beneficial in a lot of T cell-mediated autoimmune diseases, including experimental autoimmune encephalomyelitis<sup>8</sup>, type 1 diabetes<sup>9</sup>, inflammatory arthritis<sup>10</sup>, autoimmune myocarditis<sup>11</sup>, and autoimmune thyroiditis<sup>12</sup>. Furthermore, many reports suggested that Statins inhibited T cell activation by blocking the Mevalonate pathway and reducing the isoprenoid metabolites<sup>9,13,14</sup>. Particularly, Lovastatin can inhibit the prenylation and posttranslational activation of

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Rho GTPase, which facilitates T cell migration, and then suppresses the autoimmune damage in encephalomyelitis and retinal inflammatory disease<sup>8,15</sup>. Nevertheless, the immunomodulatory mechanism of Lovastatin in the treatment of T cell-mediated inflammatory diseases which has not been clarified completely requires further study.

Recently, the Kv1.3 channel in T cells has been the novel target for the treatment of many T cell-mediated autoimmune diseases<sup>16</sup>. Kv1.3 channel belongs to the Shaker family, which is preferentially expresses in T cells. Along with  $K_{Ca}$  channels, Kv1.3 channel regulates the resting membrane potential and provides sustained driving force for the  $Ca^{2+}$  influx through  $Ca^{2+}$ -release activated  $Ca^{2+}$  (CRAC) channel in T cells.  $Ca^{2+}$  influx can activate the  $Ca^{2+}$ -dependent signal transcription pathway, and then induces the activation, proliferation and IL-2 secretion of T cells<sup>17,18</sup>. Blocking Kv1.3 channel can inhibit the  $Ca^{2+}$  influx and  $Ca^{2+}$ -mediated signal pathway, and then exert inhibitory effects on T cell activation<sup>19–22</sup>. In many autoimmune animal models such as multiple sclerosis, rheumatoid arthritis, Type I diabetes, the pathogenic  $T_{EM}$  (effector memory T) cells were reported to significantly up-regulate Kv1.3 channel expression after activation, therefore, Kv1.3 blockers can selectively inhibit  $T_{EM}$  cells and alleviate the immunologic damage<sup>16,23–26</sup>. Accordingly, Kv1.3 blockers were beneficial for T cell-mediated autoimmune diseases.

The present study was undertaken to determine if the immunomodulatory properties of Statins are mediated by blockade of Kv1.3 channel. In our study, we chose Lovastatin as a represent to validate the above hypothesis. Firstly, we found that Lovastatin concentration- and voltage-dependently blocked Kv1.3 channel in human T cells. However, Lovastatin had no effect on  $K_{Ca}$  current, Kv1.3 mRNA and protein expression. Furthermore, Lovastatin inhibited the  $Ca^{2+}$  influx,  $Ca^{2+}$ -activated transcription factors, T cell proliferation and IL-2 secretion. Finally, we applied Mevalonate and Kv1.3-siRNA to investigate the change of IL-2 production. Our research demonstrated for the first time that Lovastatin can block Kv1.3 channel, decrease  $Ca^{2+}$  influx and  $Ca^{2+}$ -activated transcriptional factors, and then inhibit the activation, proliferation of human T cells. We may conclude that Lovastatin exerts immunomodulatory effect through Kv1.3 channel.

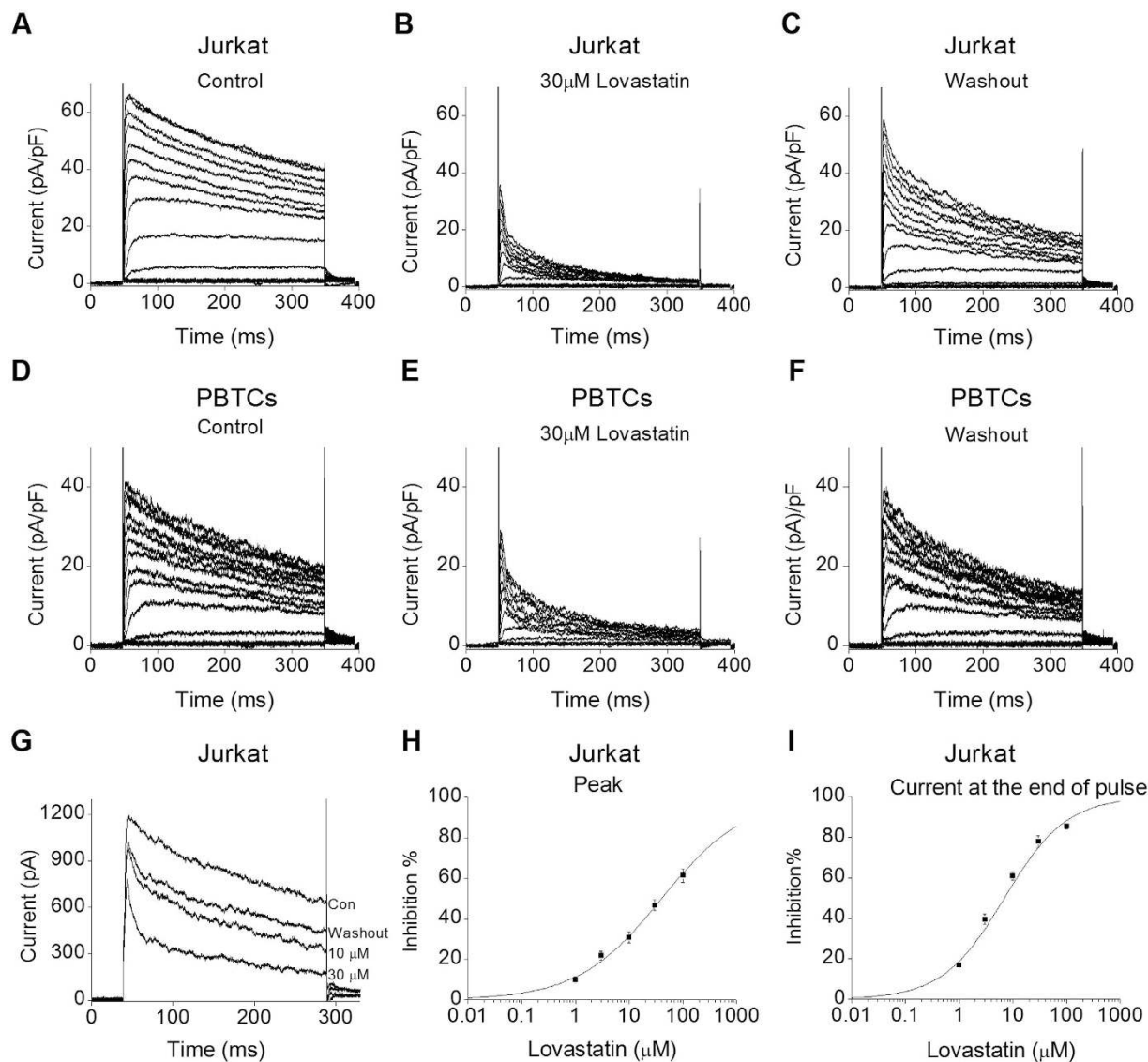
## Results

**Lovastatin blocked Kv1.3 channel currents in human T cells.** Jurkat cells were held at  $-80$  mV, and received the test potentials from  $-80$  to  $+80$  mV in  $10$  mV steps. As shown in Fig. 1A, at test potentials positive to  $-40$  mV, Kv1.3 channel currents rapidly activated and slowly inactivated. However, with  $30$   $\mu$ M Lovastatin application, Kv1.3 current was much smaller and inactivated faster (Fig. 1B). After washout, the peak current recovered nearly completely, while the current at the end of the pulse only reversed partially (Fig. 1C). We got the similar results from peripheral blood  $CD4^{+}$  T cells (PBTCs, Fig. 1D–F) and  $CD3^{+}$  T cells (data not shown). Next, different concentrations of Lovastatin ranging from  $1$  to  $100$   $\mu$ M were applied to block the Kv1.3 channel in Jurkat cells. The current traces in the absence and presence of  $10$ ,  $30$   $\mu$ M Lovastatin was shown in Fig. 1G. Whereas, the quantitative analysis of concentration-dependent block effect by Lovastatin on the peak and the current at the end of the pulse was shown in Fig. 1H,I, respectively. Using the Hill equation for non-linear fitting, the  $IC_{50}$  of Lovastatin on the peak current was calculated to be  $39.81 \pm 5.11$   $\mu$ M, with the Hill coefficient  $0.56 \pm 0.04$ ; and the  $IC_{50}$  on the current at the end of the pulse was  $6.92 \pm 0.95$   $\mu$ M, with the Hill coefficient  $0.75 \pm 0.06$ .

We further investigated the effects of Lovastatin on cloned human Kv1.3, Kv1.2 and Kv1.5 channels, which were transfected and expressed in HEK 293 cells. As shown in Fig. S1,  $30$   $\mu$ M Lovastatin was able to inhibit  $83.34 \pm 2.04\%$  of Kv1.3 channel peak currents and  $93.95 \pm 1.48\%$  of currents at the pulse end. Similar blockage effects were also observed in Kv1.2 ( $70.71 \pm 1.43\%$ ) and Kv1.5 ( $46.02 \pm 1.43\%$  at the peak,  $83.95 \pm 3.46\%$  at the end of pulse). These results suggested that Lovastatin was a non-selective Kv1.3 blocker.

**The blocking kinetics of Lovastatin on Kv1.3 channel.** Next, we observed the voltage-dependent block effect of Lovastatin on Kv1.3 channel. The current density-voltage relationship curves of the peak and the current end of the pulse, before and after  $30$   $\mu$ M Lovastatin application, were shown in Fig. 2A,B, respectively. Lovastatin at  $30$   $\mu$ M inhibited the peak and the pulse end current apparently above  $-40$  mV when Kv1.3 channel was open. Then, we calculated the inhibition% of  $30$   $\mu$ M Lovastatin on the peak and the pulse end current, which was plotted as a function of the test potentials. The activation curve was present in Fig. 3C, too. From  $-40$  mV to  $0$  mV, the inhibition percentage of Lovastatin on peak and pulse end current increased sharply from  $8.7\%$  and  $11.0\%$  to  $36.8\%$  and  $71.1\%$ , respectively. This voltage range was consistent with the voltage for Kv1.3 channel opening. The inhibition increased to a plateau between  $+10$  mV and  $+60$  mV when the channel was fully activated. This blocking character suggested that Lovastatin may bind to the open state of Kv1.3 channel.

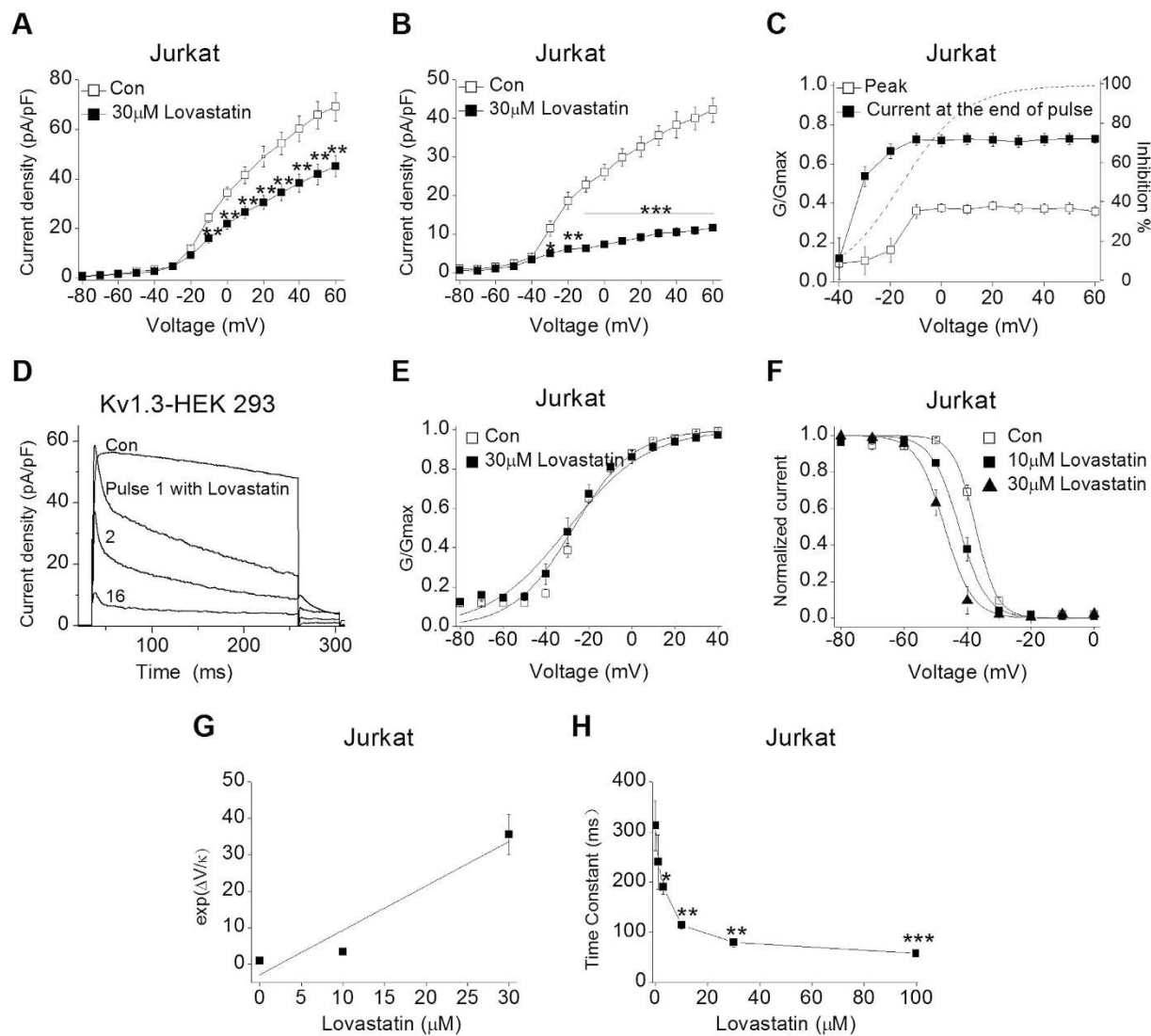
We further investigated whether Lovastatin blocked Kv1.3 channel in the closed state. Kv1.3 currents expressed in HEK 293 cells were recorded before and after a pulse-free period of incubation with Lovastatin. Membrane potential was held at  $-80$  mV and Lovastatin ( $30$   $\mu$ M) was applied to the bath without any depolarization pulses for  $8$  mins and then the consecutive  $250$  ms pulses (at  $+40$  mV) were given every  $10$  s. As shown in Fig. 2D, Lovastatin ( $30$   $\mu$ M) did not affect the peak currents at pulse 1 ( $59.27$  vs  $55.64$  pA/pF), suggesting that little Lovastatin block happened while the channel was closed. However, the onset of blockade occurred rapidly once the channel was opened (the amplitude peak



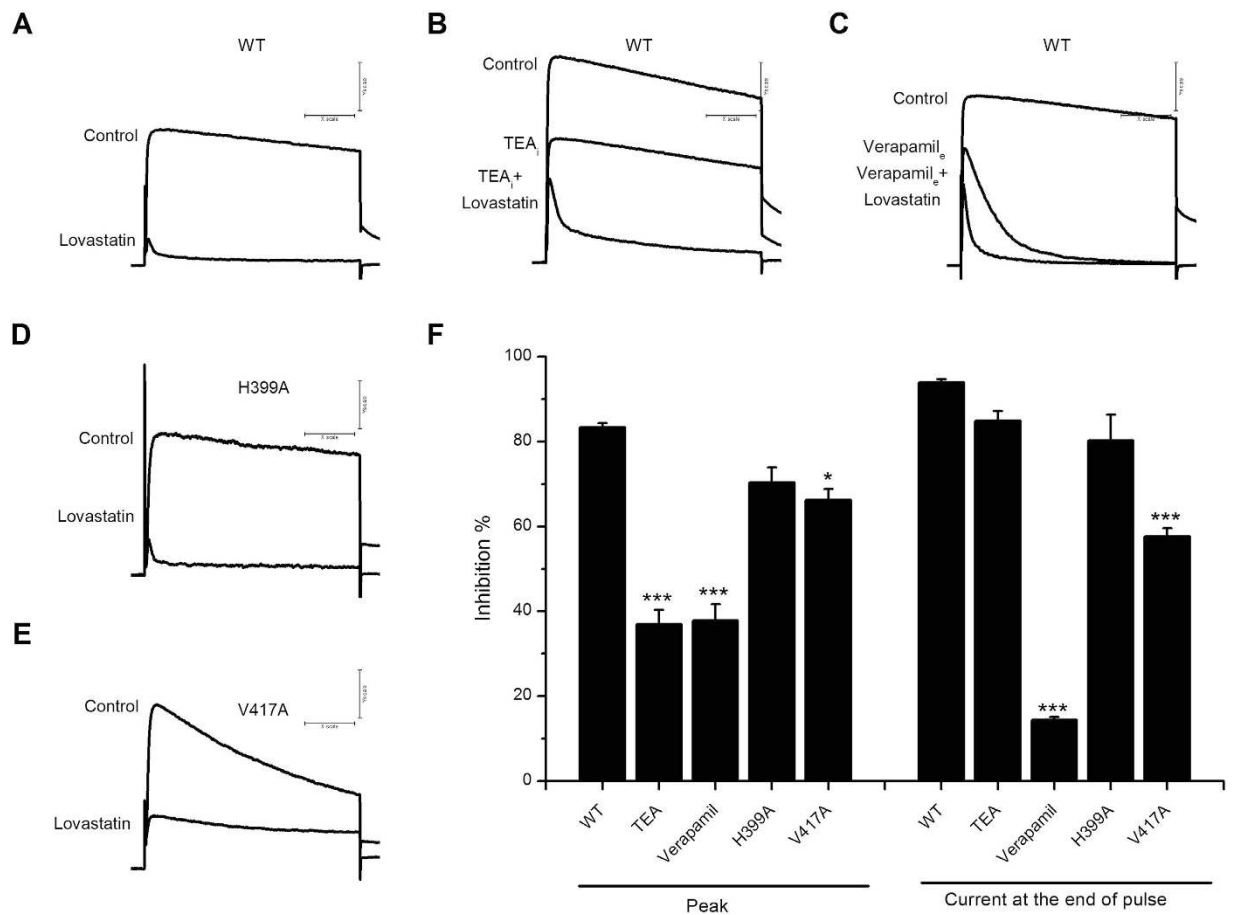
**Figure 1. Lovastatin blocked Kv1.3 channel currents in human T cells.** (A–C) The representative Kv1.3 current traces recorded in Jurkat cells in the absence (A), presence (B) and washout (C) of 30 µM Lovastatin, which were elicited by 300 ms pulses at the test potentials from  $-80$  to  $+60$  mV in 10 mV steps. (D–F) Representative Kv1.3 traces recorded in PBTCs in the absence (D), presence (E) and washout (F) of 30 µM Lovastatin. (G) Superimposed original Kv1.3 current traces at control, with 10, 30 µM Lovastatin applications, and washout in Jurkat cells. Kv1.3 currents were elicited by the depolarizing step pulses at  $+40$  mV with the holding potential at  $-80$  mV every 10 s. (H) The dose-response curve of the peak Kv1.3 currents fitted with the Hill equation in Jurkat cells. The summarized data (6 cells) for each concentration are expressed as mean  $\pm$  SEM. The  $IC_{50}$  and Hill coefficient were  $39.81 \pm 5.11$  µM and  $0.56 \pm 0.04$ . (I) The dose-response curve of Kv1.3 currents at the end of the pulse from 6 Jurkat cells were fitted with the Hill equation. The  $IC_{50}$  and Hill coefficient were  $6.92 \pm 0.95$  µM and  $0.75 \pm 0.06$ , respectively.

current at pulse 2 was 36.35 vs. 59.27 pA/pF at pulse 1). These results indicated that Lovastatin interacted with the binding sites as the Kv1.3 channel open, with no indication of drug binding in the closed state.

In Fig. 2E, the activation curves in the absence and presence of 30 µM Lovastatin were generated by plotting the normalized conductance with step potentials from  $-80$  mV to  $+40$  mV using Boltzman equation. Under control, the value of  $V_{1/2}$  for activation was  $-27.31 \pm 1.90$  mV and slope factor  $\kappa$  was  $14.01 \pm 1.10$ ; with Lovastatin application,  $V_{1/2}$  was calculated to be  $-29.57 \pm 2.30$  mV and  $\kappa$  was  $18.39 \pm 1.33$  ( $P > 0.05$ ). To investigate the effect of Lovastatin on Kv1.3 channel inactivation, in Fig. 2F, we use a standard double-pulse protocol<sup>19</sup> to generate the steady-state inactivation curves. The resulting normalized current-voltage relationships were fitted using Boltzman equation to acquire the half inactivation voltage  $V_{1/2}$  and  $\kappa$ , which were  $-37.40 \pm 0.65$  mV and  $3.36 \pm 0.18$  at control. Lovastatin



**Figure 2. Blocking kinetics of Lovastatin on Kv1.3 channel.** (A) The summarized current density-voltage relationship for the peak Kv1.3 currents in the absence (open square) and presence (filled square) of 30  $\mu$ M Lovastatin. Kv1.3 currents were elicited by the same protocol as in Fig. 1A–C. (B) The summarized current density-voltage relationship for the end of pulse Kv1.3 currents under control (open square) and in the presence (filled square) of 30  $\mu$ M Lovastatin. (C) The inhibition% of the peak Kv1.3 currents (open square) and currents at the end of pulse (filled square) were plotted with the test voltages. The dashed line showed the fitted activation curve of Kv1.3 currents at control. (D) Superimposed current traces recorded before and after a pulse-free period of incubation with Lovastatin. Currents through Kv1.3 channel expressed in HEK 293 were elicited by a 250 ms depolarizing to +40 mV. Lovastatin (30  $\mu$ M) was applied to the bath while the membrane potential was held at –80 mV. After an interval of 8 min, consecutive 250 ms pulses were applied every 10 s. The numbers 1 to 16 refers to pulses 1 to 16. (E) The activation curves in the absence and presence of 30  $\mu$ M Lovastatin were fitted with Boltzmann equation. G value was defined as  $I/(V-E_{rev})$ , where  $I$  was peak amplitude,  $V$  was corresponding test voltage, and  $E_{rev}$  was the reversal potential (–90 mV) of Kv1.3 channel. (F) The steady-state inactivation curves under control and in the presence of 10, 30  $\mu$ M Lovastatin. The data were obtained from the normalized currents at +40 mV, following a 30 s pre-pulse potentials from –80 mV to 0 mV, and fitted with the Boltzmann equation to acquire the  $V_{1/2}$  value and  $\kappa$ . (G) Plot of  $\text{Exp}(\Delta V/\kappa)$  value against the Lovastatin concentration. The potential corresponding to half-inactivation voltage  $V_{1/2}$  and slope factor  $\kappa$  were acquired from the curves in Fig. 2F. (H) The time constants of the Kv1.3 currents decay phase at +40 mV were acquired with mono-exponential equation and plotted against Lovastatin concentration. (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs. control).



**Figure 3. Interaction of Lovastatin with Kv1.3 Wild-Type and Kv1.3 Mutant Channels.** Currents were elicited by 200-ms depolarizing voltage steps from a holding potential of  $-80$  to  $+40$  mV every 10 s. (A) Representative currents through WT Kv1.3 channel expressed in HEK 293 cells before and after application of  $30 \mu$ M Lovastatin. (B) Effects of  $200 \mu$ M internally applied TEA alone and in combination with externally applied  $30 \mu$ M Lovastatin. (C) Effects of  $20 \mu$ M externally applied verapamil alone and in combination with externally applied  $30 \mu$ M Lovastatin. (D–E) Representative currents through H399A (D) and V417A (E) Kv1.3 mutant channels expressed in HEK 293 cells before and after application of  $30 \mu$ M Lovastatin. (F) Average inhibition% of Kv1.3 channel currents by  $30 \mu$ M Lovastatin. (\* $P < 0.05$ , \*\*\* $P < 0.001$  vs. WT).

caused a concentration-dependently negative shift of the steady-state inactivation curve. The  $V_{1/2}$  was  $-42.23 \pm 0.99$  ( $P < 0.05$  vs. control) with  $10 \mu$ M Lovastatin, and  $-47.91 \pm 1.55$  ( $P < 0.001$ ) with  $30 \mu$ M Lovastatin. However, Lovastatin did not alter the  $\kappa$  value, which was  $4.22 \pm 0.25$  at  $10$ ,  $3.04 \pm 0.14$  at  $30 \mu$ M Lovastatin ( $P > 0.05$ ). Then, the  $K_i$  value of Lovastatin on inactivated-state Kv1.3 channel was calculated to be  $0.95 \pm 0.21 \mu$ M in Fig. 2G. Accordingly, the effect of Lovastatin on channel inactivation was 40 times more potent than on the peak current ( $39.81 \pm 5.11 \mu$ M), and 7 times more potent than on the pulse end current ( $6.92 \pm 0.95 \mu$ M). Lovastatin may preferentially bind to the inactivated-state of Kv1.3 channel.

Furthermore, the decay phase of Kv1.3 channel current at  $+40$  mV was fitted using a mono-exponential equation, revealing a time constant of  $312.52 \pm 49.70$  ms under control, and  $240.26 \pm 54.11$  ( $P > 0.05$ ),  $190.67 \pm 14.95$  ( $P < 0.05$ ),  $114.29 \pm 7.65$  ( $P < 0.01$ ),  $79.79 \pm 8.45$  ( $P < 0.01$ ) and  $57.73 \pm 6.41$  ( $P < 0.001$ ) ms at 1, 3, 10, 30,  $100 \mu$ M Lovastatin (Fig. 2H). Lovastatin accelerated the channel decay rate in a concentration-dependent manner, similar to many other Kv1.3 open channel blockers<sup>27,28</sup>.

**Interaction of Lovastatin with Kv1.3 Wild-Type and Kv1.3 Mutant Channels.** To characterize and identify the binding site in Kv1.3 channel that is responsible for the blocking effect of Lovastatin, we firstly performed competition experiments to investigate the interaction of Lovastatin with the known inner pore blockers tetraethyl-ammonium (TEA) and verapamil. Lovastatin ( $30 \mu$ M) caused less blocking effects in the presence of internal TEA (Fig. 3B) or external verapamil (Fig. 3C) than expected for an independent action (Fig. 3A). We then examined the effects of Lovastatin on Kv1.3 H399A and V417A channels, which have been demonstrated to be involved in interactions with small molecular blockers

after channel opened. H399A located in the out pore region and V417A located in the S6 domain. As reported, the two mutants can produce substantial outward currents during depolarization<sup>29</sup>. The H399A channel was inhibited by Lovastatin (30  $\mu$ M) to an extent similar to WT (Fig. 3D), whereas, the inhibitory effect of Lovastatin (30  $\mu$ M) was significantly less on the V417A channel (Fig. 3E). Figure 3F summarized the inhibitory effect of Lovastatin (30  $\mu$ M), measured as percentage inhibition of peak and pulse end currents at +40 mV. These results suggested that Lovastatin interacted with the inner pore domain of Kv1.3 channel, and Val417 might be important for the block by Lovastatin.

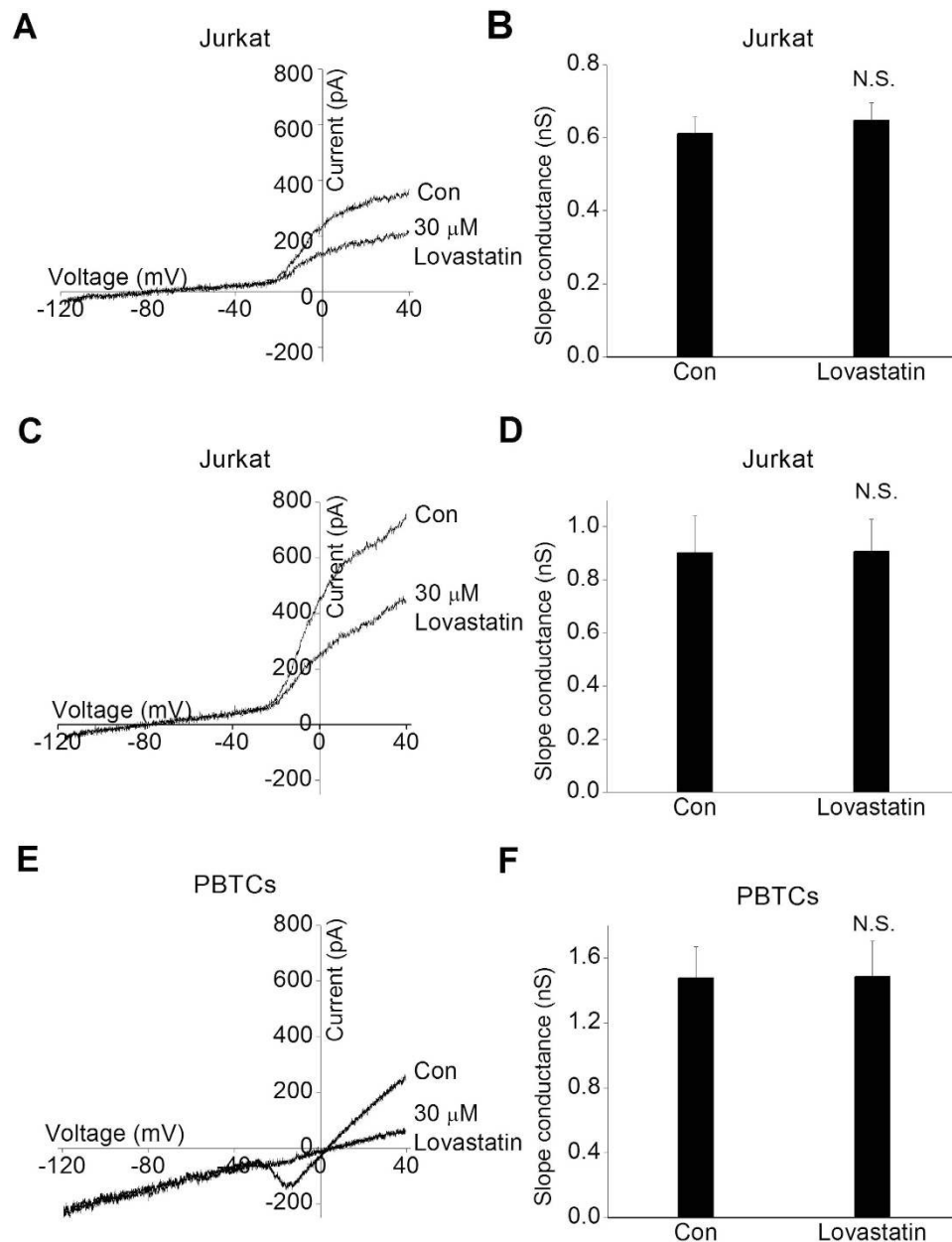
**Lovastatin did not block  $K_{Ca}$  currents in human T cells.** Next, we observed the block effect of Lovastatin on  $K_{Ca}$  channel both in Jurkat T cells and PBTCs, because  $K_{Ca2.2}$  is the main  $K_{Ca}$  channel expressed in Jurkat T cells<sup>30</sup>, whereas  $K_{Ca3.1}$  is the main  $K_{Ca}$  channel involved in PBTCs<sup>17</sup>.  $K_{Ca}$  current was elicited by a 200 ms ramp pulse ranging from  $-120$  mV to  $+40$  mV at the holding potential of  $-40$  mV. The representative current traces were shown in Fig. 4A,C,E. To avoid the mixture of outward Kv currents, the slope conductance between  $-120$  and  $-40$  mV was measured by fitting the curve with linear equation. As shown in Fig. 4B,D, 30  $\mu$ M Lovastatin did not block  $K_{Ca}$  currents in both Jurkat cells and PBTCs in normal Ringer's solution. In Jurkat cells, the slope conductance was  $0.61 \pm 0.05$  nS at control, and  $0.65 \pm 0.05$  nS with Lovastatin application ( $n = 5$ ,  $P > 0.05$ ). In PBTCs, the slope conductance was  $0.90 \pm 0.14$  nS at control, and  $0.91 \pm 0.12$  nS with Lovastatin application ( $n = 5$ ,  $P > 0.05$ ). In Fig. 4E,F, to increase their slope conductance, the  $K_{Ca}$  currents were recorded in  $K^+$  Ringer's solution, and similarly, 30  $\mu$ M Lovastatin exerted no block effect on  $K_{Ca}$  channel ( $1.48 \pm 0.19$  vs.  $1.48 \pm 0.22$  nS,  $P > 0.05$ ). Consequently, our results suggested that Lovastatin had no apparent block effect on  $K_{Ca}$  channel in human T cells.

**Effect of Lovastatin on the expression of Kv1.3 channel in Jurkat cells.** Besides the acute inhibition of functional Kv1.3 channel, Kv1.3 blockers may exert anti-inflammatory effect through the down-regulation of channel expression<sup>19,20,31,32</sup>. To evaluate the effect of Lovastatin on Kv1.3 expression, Jurkat cells were incubated with 10, 30 and 100  $\mu$ M Lovastatin for 24 h, and then collected to investigate the Kv1.3 mRNA and protein expression. In Fig. 5A, Lovastatin up to 100  $\mu$ M performed no apparent effect on Kv1.3 mRNA expression. Similarly, the Kv1.3 protein expression was not changed, either ( $P > 0.05$ , Fig. 5C).

**The  $Ca^{2+}$  influx to  $Ca^{2+}$ -depleted Jurkat cells was blunted by Lovastatin.** Many previous studies have shown that Kv1.3 channel plays a vital role in the  $Ca^{2+}$  homeostasis of T cells through maintaining the negative membrane potential, and blockade of Kv1.3 channel significantly reduces  $Ca^{2+}$  influx<sup>19,31,33</sup>. As shown in Fig. 6A, in  $Ca^{2+}$ -free Ringer's solution, 1  $\mu$ M TG caused a small increase of the intracellular  $Ca^{2+}$  concentration. After 2 mM  $CaCl_2$  application,  $Ca^{2+}$  flowed into Jurkat cells through CRAC channel and induced significant intracellular  $Ca^{2+}$  increase. Incubation with 10 and 30  $\mu$ M Lovastatin did not alter the  $Ca^{2+}$  release induced by TG, but 100  $\mu$ M Lovastatin slightly inhibited this reaction ( $P < 0.05$ , Fig. 6B). In Fig. 6C, 10, 30 and 100  $\mu$ M Lovastatin concentration-dependently inhibited the  $Ca^{2+}$  influx by 25.36% ( $P < 0.01$ ), 39.29% ( $P < 0.001$ ) and 64.29% ( $P < 0.001$ ), respectively.

**Lovastatin down-regulated the  $Ca^{2+}$ -related transcription factors NFAT1 and NF- $\kappa$ B p65/50 activities.** In T cells,  $Ca^{2+}$  influx and intracellular  $Ca^{2+}$  concentration elevation activate  $Ca^{2+}$ -dependent enzyme, and thereby  $Ca^{2+}$ -related transcription factors<sup>18,34</sup>, so we further investigated the modulation effect of Lovastatin on NFAT1 and NF- $\kappa$ B p65/50. In Fig. 7, the expression of NFAT1 and phospho-NF- $\kappa$ Bp65/50 was increased with stimulation by PHA + PMA ( $P < 0.01$  for NFAT1 and p50,  $P < 0.05$  for p65). Treatment with 10, 30 and 100  $\mu$ M Lovastatin apparently suppressed the expression level of all these three factors concentration-dependently.

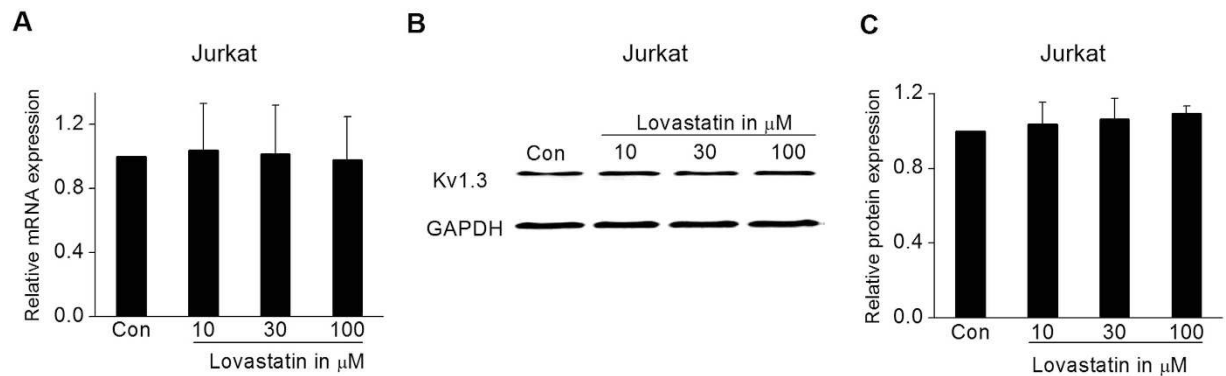
**Lovastatin inhibited the proliferation and IL-2 secretion of human T cells.** To investigate the functional immunosuppression effect performed by Lovastatin through inhibiting the Kv1.3 channel, we measured T cell proliferation and IL-2 production. CCK-8 kit was used to access the proliferation of anti CD3/CD28-stimulated human PBTCs, and IL-2 secretion from PHA + PMA activated Jurkat cell was measured by ELISA. In Fig. 8A,B, 3, 10, and 30  $\mu$ M Lovastatin inhibited the T cell proliferation and IL-2 production concentration-dependently. Since Statins can exert immunomodulatory effect by blocking the isoprenoid pathway<sup>1,3</sup>, we applied 1 mM Mevalonate (the intermediate of isoprenoid pathway) along with 30  $\mu$ M Lovastatin. We found that the blocking effect of Lovastatin on IL-2 secretion was partially neutralized, from 68.57% to 39.11%, which suggested that Lovastatin probably play immunomodulatory effect only partly through isoprenoid pathway. Furthermore, we knocked down Kv1.3 expression using specific Kv1.3-siRNA in Jurkat cells. The Kv1.3-siRNA transfection resulted in reduced inhibitory effect of 30  $\mu$ M Lovastatin on IL-2 production ( $34.27 \pm 4.74\%$  vs. NC-siRNA  $65.94 \pm 0.80\%$ ,  $P < 0.01$ ). Simultaneously, NC-siRNA transfection did not alter the block effect of Lovastatin on IL-2 secretion ( $65.94 \pm 0.80\%$  vs. Jurkat cell  $68.57 \pm 2.20\%$ ,  $P > 0.05$ ). As shown in Fig. 8E, Lovastatin also concentration-dependently reduced IL-2 secretion in Kv1.3 knockdown Jurkat cells. In conclusion, Lovastatin exerted immunomodulatory effect partly through Kv1.3 channel.



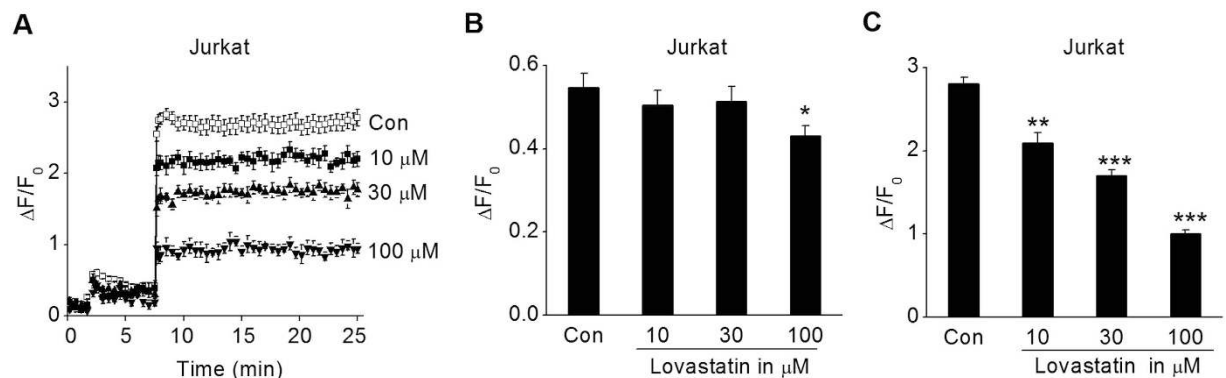
**Figure 4. Block effect of Lovastatin on  $K_{Ca}$  currents in human T cells.**  $K_{Ca}$  currents were elicited by 200 ms voltage-ramp from  $-120$  to  $+40$  mV at the holding potential of  $-40$  mV. The currents slope conductance was acquired by fitting the curves between  $-120$  and  $-40$  mV with linear equation. (A) Representative current-voltage relationship traces recorded in the absence or presence of  $30\mu\text{M}$  Lovastatin in Jurkat cells. (B) Summarized slope conductance data from 5 Jurkat cells. (C) Representative current-voltage traces of  $K_{Ca}$  channel obtained from PBTCs. (D) Summarized slope conductance from 5 PBTCs. (E) Representative superimposed current-voltage relationships traces from a Jurkat cell recorded in  $K^+$  Ringer solution with or without  $30\mu\text{M}$  Lovastatin. (F) Summarized data from 5 Jurkat cells. N.S. represented no statistical significance. Data are expressed as the mean  $\pm$  SEM.

## Discussion

This is the first study to investigate the block effect of Lovastatin on Kv1.3 channel in human T cells. Our research demonstrated that Lovastatin blocked Kv1.3 channel in a concentration- and voltage-dependent manner, accelerated the decay rate of current curve and negatively shifted the steady-state inactivation curve without significant effect on  $K_{Ca}$  channels. Different from DPO-1, Acacetin and  $18\beta$ -Glycyrrhetic acid<sup>19,20,31</sup>, Lovastatin incubation for 24h did not change the mRNA and protein expression of Kv1.3 channel, which suggested that Lovastatin may exert its effect by direct blockade. Similar with our previous studies on other Kv1.3 blockers<sup>19,20,31</sup>, Lovastatin inhibited the  $Ca^{2+}$  influx,  $Ca^{2+}$ -activated transcription factors NFAT1 and NF- $\kappa$ B p65/50, T cell proliferation and IL-2 production. The Mevalonate



**Figure 5. Effect of Lovastatin on Kv1.3 expression in Jurkat cells.** (A) Jurkat cells were incubated with 10, 30, and 100  $\mu\text{M}$  Lovastatin for 24 h. Then, the relative Kv1.3 mRNA expression level normalized to GAPDH was measured by real-time PCR. The summarized data from 3 replicates was shown. (B) Representative Western blotting analysis of Kv1.3 protein expression under control and after 24 h treatment with 10, 30, or 100  $\mu\text{M}$  Lovastatin. (C) The summarized data from 3 replicates which were normalized to the protein expression of GAPDH. All the data was expressed as the mean  $\pm$  SEM.

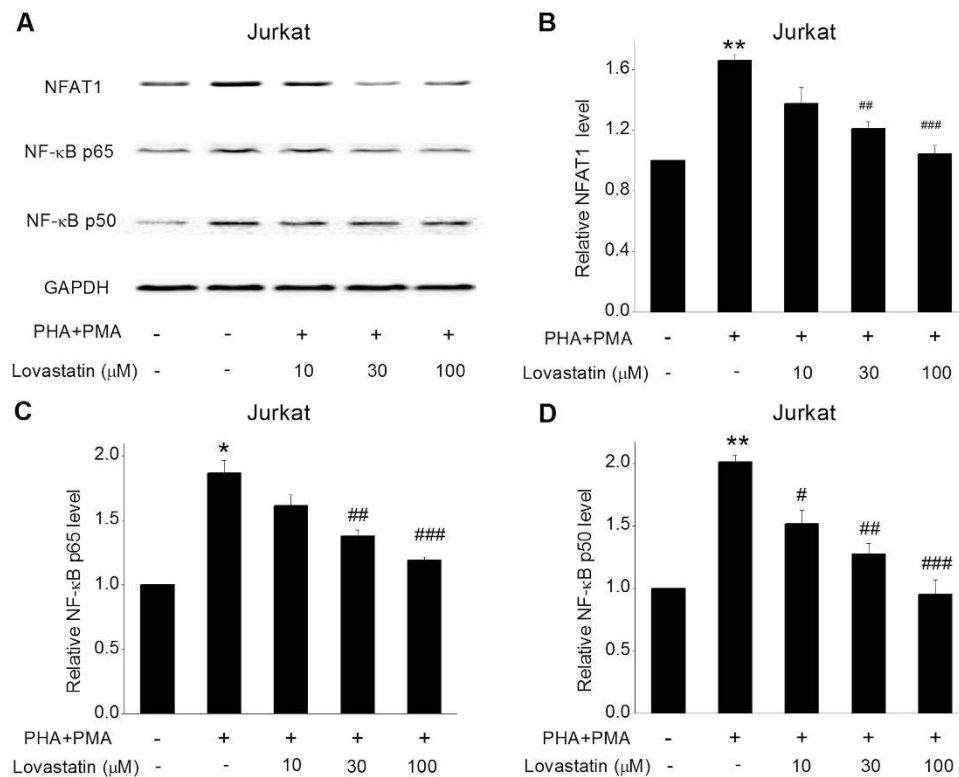


**Figure 6. Effect of Lovastatin on the  $\text{Ca}^{2+}$  influx to  $\text{Ca}^{2+}$ -depleted Jurkat cells.** Jurkat cells were loaded with fluo-4 AM and re-suspended in the  $\text{Ca}^{2+}$ -free Ringer solution. Then, 10, 30 or 100  $\mu\text{M}$  Lovastatin was applied into the extracellular solution. After 30 min incubation, intracellular  $\text{Ca}^{2+}$  release and  $\text{Ca}^{2+}$  influx were elicited by 1  $\mu\text{M}$  TG and 2 mM  $\text{CaCl}_2$ , respectively. (A) The summarized time course of  $\Delta\text{F}/\text{F}_0$  value was shown for the cells treated with 0 or 10, 30, 100  $\mu\text{M}$  Lovastatin. (B) The summarized  $\Delta\text{F}/\text{F}_0$  value of the peak intracellular  $\text{Ca}^{2+}$  release response induced by TG. \* $P < 0.05$  vs. control. (C) The summarized  $\Delta\text{F}/\text{F}_0$  value of the maximum  $\text{Ca}^{2+}$  influx response after 2 mM  $\text{CaCl}_2$  application. \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs. control. All the data are expressed as mean  $\pm$  SEM from at least 5 replicate experiments.

pathway is the important mechanism of Statins' immunomodulatory effect, but Mevalonate application only partially reversed the inhibition of Lovastatin on IL-2 secretion. Furthermore, siRNA against Kv1.3 channel reduced this inhibitory effect of Lovastatin on IL-2 production. Accordingly, Lovastatin can exert anti-inflammatory and immunomodulatory effect partly through Kv1.3 channel in human T cells.

Previous study has proved that Lovastatin blocked Kv1.3 channel in murine thymocytes<sup>35</sup> without clarifying the blocking characteristic. The  $\text{IC}_{50}$  of Lovastatin for Kv1.3 current end of the pulse was  $6.92 \pm 0.95 \mu\text{M}$ , and the peak plasma Lovastatin bioactivity could reach  $12.3 \mu\text{M}$ <sup>36</sup>. Accordingly, the effective concentration of Lovastatin for Kv1.3 blockade has good clinical relevance. We found that Lovastatin blocked Kv1.3 channel in a voltage-dependent manner, and this effect increased sharply between the voltage ranges from  $-40$  to  $0 \text{ mV}$  (the voltage of channel open). Moreover, Lovastatin did not produce any obvious effect until the channel was firstly opened by a depolarization pulse, which was similar to the effects of classical Kv channel pore blocker TEA<sup>37</sup>. But, unlike TEA, Lovastatin failed to cause the tail current crossover phenomenon, probably because Lovastatin is not a pure open-channel blocker. Furthermore, in contrast to TEA, Lovastatin concentration-dependently accelerated the decay phase of Kv1.3 channel, and negatively shifted the steady-state curves in a concentration-dependent manner, which indicated that Lovastatin exerted block effect by interacting with inactivated channel, because pure Kv1.3 open channel blockers staurosporine and fluoxetine have no effect on the steady-state inactivated



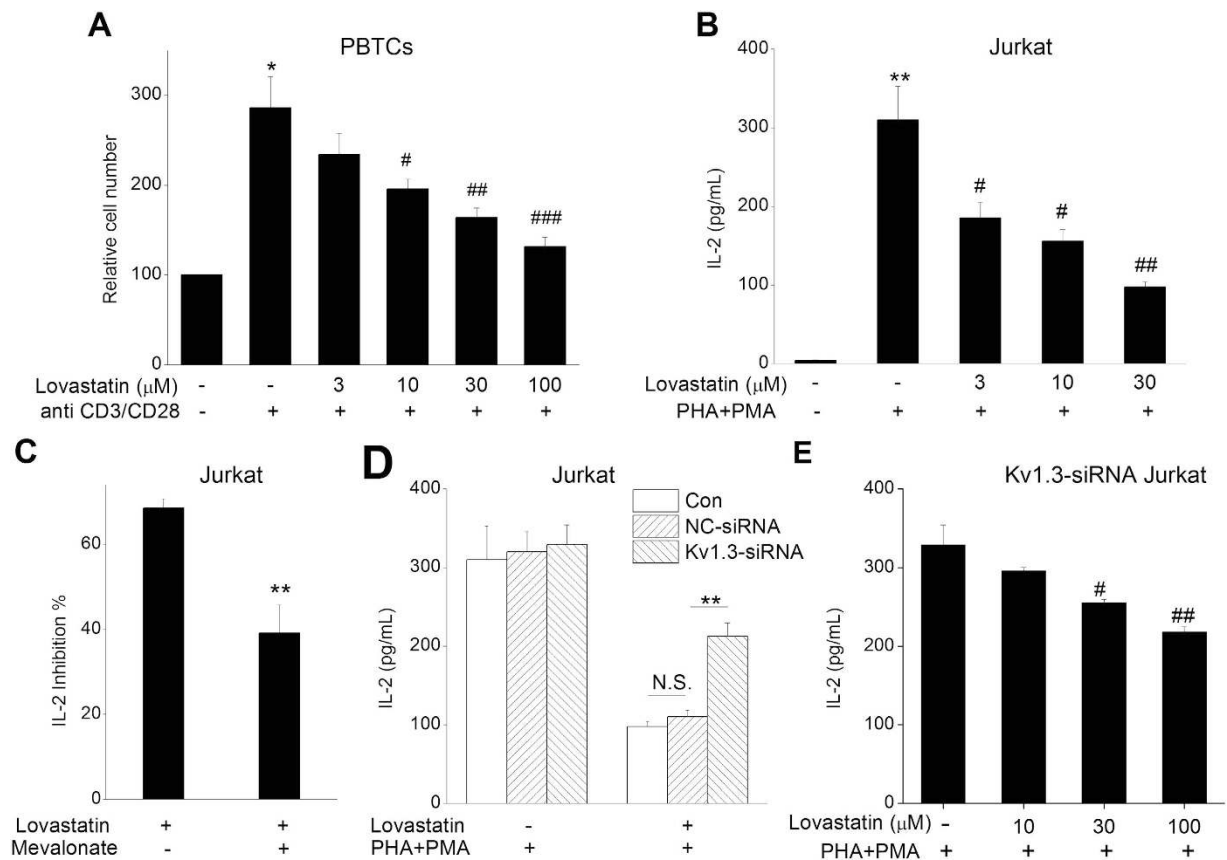


**Figure 7. Effect of Lovastatin on the NFAT1 and NF-κB p65/p50 activities.** Jurkat cells were pre-treated with 0, 10, 30, or 100 μM Lovastatin for 30 min, then stimulated with PHA + PMA for 4 h. The cells received equal volume of DMSO were defined as control. (A) Representative western blotting analysis of NFAT1 and phospho-NF-κB p65/p50 activities. The summarized relative NFAT1 and phospho-NF-κB p65/p50 expression analysis from at least 3 experiments was shown in (B) (NFAT1), (C) (p65), and (D) (p50). \* $P < 0.05$ , \*\* $P < 0.01$  vs. control, # $P < 0.05$ , ## $P < 0.01$  and ### $P < 0.001$  vs. PHA + PMA stimulated group without Lovastatin.

curves<sup>38,39</sup>. In conclusion, Lovastatin blocked both the open and inactivated states of Kv1.3 channel, similar with Kv1.3 blockers DPO-1<sup>19</sup>, 18β-Glycyrrhetic acid<sup>20</sup>, FK-506<sup>28</sup> and verapamil<sup>40</sup>. We calculated the  $K_i$  value for Lovastatin binding to the inactivated Kv1.3 channel, and compared this value with the  $IC_{50}$  of Lovastatin on peak or pulse end Kv1.3 current. The  $K_i$  value reduced by 40- and 7-fold relatively, which suggested that Lovastatin is much more potent on channel inactivation. This characteristic is very similar to FK-506<sup>28</sup>.

There are not many reports studying the binding sites of Statins and ion channels. Our results of the competitive experiments with well-defined inner-pore blockers TEA and verapamil suggested that Lovastatin interacted with the inner pore of Kv1.3. We further investigated the effects of Lovastatin on the mutant channels (H399A and V417A) and found that V417A significantly reduced the blockade effects of Lovastatin. This particular position has been described previously to be involved in the binding of verapamil to Kv1.3<sup>41</sup>. However, the precise binding sites of Lovastatin require further study.

Kv1.3 is the dominantly expressed  $K^+$  channel in human T cells, which provides driving force for  $Ca^{2+}$  influx by maintaining the resting membrane potential. Blocking Kv1.3 channel can depolarize the membrane potential<sup>42</sup>, decrease  $Ca^{2+}$  influx and then inhibit the T cell activation<sup>18</sup>. Along with Kv1.3 channel, T cells also express  $K_{Ca}$  channel, which assist to maintain the membrane potential<sup>17,43,44</sup>. In our study, acute application with Lovastatin significantly inhibited the  $Ca^{2+}$  influx into Jurkat cells. But Lovastatin at 30 μM apparently blocked Kv1.3 channel without affecting the  $K_{Ca}$  currents. Thus, the effect of Lovastatin on  $Ca^{2+}$  influx may attribute to the blocking of Kv1.3 instead of  $K_{Ca}$  channels. However, the direct blockade of Lovastatin on CRAC channels (the channel for  $Ca^{2+}$  entry) requires further investigation.  $Ca^{2+}$  influx can activate the  $Ca^{2+}$ -related transcription factors, and ultimately lead to cytokine release and T cell proliferation as well<sup>18</sup>. We also observed that Lovastatin down-regulated the  $Ca^{2+}$ -related NFAT1 and NF-κB p65/p50 activities. In a previous study, Lovastatin inhibited the IL-2 secretion of human PBTCs in a concentration-dependent manner<sup>45</sup>. Similarly, we found that Lovastatin apparently decreased the IL-2 production of Jurkat cells and PBTCs proliferation. The knockdown of Kv1.3 channel significantly attenuated the block effect of Lovastatin on IL-2 secretion, which indicated that Lovastatin exert anti-inflammatory action by blocking Kv1.3 channel.



**Figure 8. Effect of Lovastatin on T cell proliferation and IL-2 production.** (A) PBTCs were seeded and pre-incubated with 0, 3, 10, 30, or 100  $\mu\text{M}$  Lovastatin. After 30 min, anti-CD3/CD28 antibodies were added to induce PBTCs proliferation. After 3 days, the relative cell number was determined by CCK-8 kit. The summarized data from at least 5 duplicates was expressed as mean  $\pm$  SEM. \*\* $P < 0.01$  vs. control, # $P < 0.05$ , ## $P < 0.01$  vs. CD3/CD28 stimulated group without Lovastatin. (B) Jurkat cells were pre-treated with 0, 3, 10, 30, or 100  $\mu\text{M}$  Lovastatin for 30 min, then stimulated with PHA + PMA for 24 h. The supernatants were collected for IL-2 measurement. \*\* $P < 0.01$  vs. control, # $P < 0.05$ , ## $P < 0.01$  vs. PHA + PMA stimulated group. (C) Jurkat cells were treated using 100  $\mu\text{M}$  Lovastatin with or without 1 mM Mevalonate application. Then, the inhibition% of Lovastatin on IL-2 secretion was calculated and showed. (D) Con Jurkat cells and NC- or Kv1.3-siRNA-transfected cells were pre-treated with 0 or 100  $\mu\text{M}$  Lovastatin for 30 min, then stimulated with PHA + PMA for 24 h. The supernatants were collected for the measurement of IL-2. \*\* $P < 0.01$  vs. NC-siRNA group and N.S. represented no statistical significance. (E) Kv1.3-siRNA-transfected Jurkat cells were pre-treated with 0, 10, 30, or 100  $\mu\text{M}$  Lovastatin for 30 min, then stimulated with PHA + PMA for 24 h. The supernatants were collected for the measurement of IL-2. (# $P < 0.05$ , ## $P < 0.01$  vs. PHA + PMA stimulated group).

Many previous studies have proved that Lovastatin can down-regulate the serum inflammation mediators IL-6, TNF- $\alpha$ , and CRP<sup>46,47</sup>. Lovastatin also performs immunomodulatory function in T cell-mediated autoimmune diseases by inhibiting the isoprenoid and Mevalonate pathway<sup>3</sup>. For example, Lovastatin alleviated the autoimmune retinal disease by inhibiting the isoprenoid pyrophosphate synthesis and activation of GTPase<sup>15</sup>. In experimental autoimmune phalomyelitis model, Lovastatin exerted therapy action through altering the Th1/Th2 cytokines ratio<sup>8</sup>. In a rat model for Guillain-Barré syndrome, Lovastatin attenuated the nerve damage by decreasing the proliferation and migration of autoimmune lymphocytes, which can be neutralized by Mevalonate injection<sup>48</sup>. But in our study, we applied Mevalonate in the presence of Lovastatin, and found that the inhibitory action of Lovastatin on IL-2 was only partly neutralized. Therefore, besides the classical Mevalonate pathway, Lovastatin can perform immunomodulation function by blocking Kv1.3 channel. In T cell-mediated autoimmune diseases, Lovastatin may possess important therapeutic meanings by blocking Kv1.3 channel and inhibiting the pathogenic action of T<sub>EM</sub> cells without affecting the physiological immune reactions (which is mediated by the up-regulation of K<sub>Ca</sub> channels<sup>49</sup>).

In summary, our study proved that HMG-CoA inhibitor Lovastatin can block Kv1.3 channel, decrease  $\text{Ca}^{2+}$  influx and  $\text{Ca}^{2+}$ -activated transcriptional factors, and finally inhibit the proliferation, IL-2 secretion of T cells, which is probably a new mechanism for its immunomodulatory effect. Kv1.3 blocker Lovastatin might play important therapeutic roles in T cell-mediated immune diseases.

## Methods

**Ethics statement.** Our study protocol of the healthy volunteers' blood samples was conducted according to the Declaration of Helsinki and Tokyo for humans. The protocol was also approved by the Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology (Approval reference number: IORG0003571). Healthy volunteers were all provided with written informed consent for the collection of blood and the subsequent T cell isolation and analysis.

**Chemicals.** Lovastatin was a gift from Prof. Huang (Xiamen, China). Phytohemagglutinin (PHA) and phorbol ester (PMA) were obtained from Sigma-Aldrich (St Louis, MO, USA). Thapsigargin (TG) was obtained from Alomone Laboratories (Jerusalem, Israel), and fluo-4 AM was purchased from Invitrogen (Carlsbad, CA, USA). RPMI 1640, fetal bovine serum, and penicillin/streptomycin were all from Gibco (Grand Island, NY, USA). Lovastatin was dissolved in DMSO at 100 mM stock concentration. All aliquoted stock was kept light protected at  $-20^{\circ}\text{C}$ . During all experiments, the final concentration of DMSO in cell culture medium were no more than 0.1%, and the control with corresponding DMSO was used to discard the possible non-specific effects.

**Cell preparation.** The human Jurkat T cell-line was obtained from the China Center for Type Culture Collection (Wuhan, China). Mononuclear cells were separated using the density Ficoll gradients from the whole blood of healthy volunteers. Then,  $\text{CD4}^{+}$  T cells were purified using a  $\text{CD4}^{+}$  T Cell Isolation Kit (Miltenyi Biotec, Bergisch-Gladbach, Germany). All the cells mentioned above were cultured in RPMI1640 supplemented with 10% heat-inactivated FBS, 10 mM HEPES, 2 mM L-glutamate and 1% penicillin/streptomycin at  $37^{\circ}\text{C}$  in humidified atmosphere (5%  $\text{CO}_2$ ).

Human embryonic kidney 293 (HEK 293) cells were cultured in the same condition as described above. HEK 293 cells were transiently transfected with appropriate cDNA plasmids using Lipofectamine LTX and PLUS (Invitrogen) method as described previously<sup>50</sup>. The cDNA encoding human Kv1.2 (from Prof. Yingliang Wu, Wuhan University, Wuhan, China), human Kv1.3 (generously provided by Dr. Garcia Maria, Merck & Co. Inc., West Point, PA, USA) and human Kv1.5 (from Prof. Guirong Li, Hongkong University, Hongkong, China) were subcloned into the vector of pIRES2-EGFP (Clontech, USA). Point mutations were introduced into the Kv1.3 channel (H399A, V417A) and subcloned into the pRc/CMV plasmid expression vector, which were kindly given by Prof. Yingliang Wu, Wuhan University, Wuhan, China. All plasmids were verified by DNA sequencing before expression. Currents were recorded 48 h after transfection.

**Electric recordings and analysis.** The Jurkat T cell-line, human PBTCs and HEK 293 cells were used for the patch-clamp electrophysiological recordings. All studies were performed in the whole-cell recording mode at room temperature as described in our previous studies<sup>19,20,31</sup>. The external solution contained (in mM): 137 NaCl, 4 KCl, 1  $\text{MgCl}_2$ , 1.8  $\text{CaCl}_2$ , 10 Glucose and 10 HEPES, adjusted to pH 7.4 with NaOH. The pipette solution for Kv1.3 channel currents recording (in mM) was 130 KCl, 1  $\text{MgCl}_2$ , 5 EGTA, 5 Mg-ATP, and 10 HEPES, adjusted to pH 7.2 with KOH. For  $\text{K}_{\text{Ca}}$  recording, the pipette solution contained (in mM) 130 potassium aspartate, 10 EGTA, 8.55  $\text{CaCl}_2$ , 2.08  $\text{MgCl}_2$ , and 10 HEPES, adjusted to pH 7.2 with KOH. In some studies of  $\text{K}_{\text{Ca}}$ , the external solution was replaced by the  $\text{K}^{+}$  Ringer solution, in which all NaCl was replaced by KCl. The depolarization pulse protocol was described in the corresponding results and Figure Legends. The pClamp9.0 and Origin 8.5 software were used for the data acquisition and analysis. The equations used were the same as previously described<sup>19</sup> to obtain the  $\text{IC}_{50}$  (drug concentration required for the 50% inhibition of the channel currents), Hill coefficient, inhibition%, half-maximum voltage ( $V_{1/2}$ ) and slope factor ( $k$ ) for activation or inactivation, and the reciprocal of the slope ( $K_i$ ).

**Quantitative real-time PCR.** Jurkat T cells were seeded and incubated with 10, 30, 100  $\mu\text{M}$  Lovastatin for 24 h. The control group was treated with the corresponding amount of DMSO. Total RNA was isolated from Jurkat T cells using trizol reagent (Invitrogen, CA, USA). cDNA synthesis was performed with RT reagent Kit (Takara Bio., Shiga, Japan). The primers used for the Kv1.3 and GAPDH are as previously described<sup>31</sup>. The real-time PCR Kit (Takara) was used and the reactions were performed in Stepone ABI system. GAPDH was applied as an internal reference. In all experiments, negative controls were included in the absence of the reverse transcriptase. The relative expression quantity  $2^{-\Delta\Delta\text{Ct}}$  value was calculated to reflect the differences among the groups.

**Western blotting.** For the detection of Kv1.3 protein expression, Jurkat cells were treated with 10, 30, 100  $\mu\text{M}$  Lovastatin for 24 h. For the measurement of the NFAT1 and NF- $\kappa\text{B}$  activities, Jurkat cells were stimulated with 5  $\mu\text{g}/\text{mL}$  PHA and 80 nM PMA for 4 h with or without 10, 30, 100  $\mu\text{M}$  Lovastatin pretreatment for 30 min. Next, the Jurkat cells were collected, and then, the lysate was prepared and

analyzed as previously described<sup>19</sup>. The primary antibodies against Kv1.3 (1:500, Alomone), NFAT1 (1:500, Genetex, Irvine, CA, USA), phospho-NF- $\kappa$ B p65/p50 (1:1000, Cell Signaling Technology, MA, USA) and GAPDH (1:2000, Abcam, MA, USA) were applied in our experiments. GAPDH was used as the internal reference. The protein expression levels were measured by enhanced chemiluminescence and analyzed with Image Lab Software.

**Intracellular Ca<sup>2+</sup> measurement.** Jurkat T cells were loaded with 2  $\mu$ M Fluo-4 AM for 30 min at room temperature, washed twice, and then seeded into the 96-well plate. Lovastatin at 0, 10, 30, 100  $\mu$ M was applied for 30 min incubation. Thapsargin (TG, 1  $\mu$ M) was used to induce Ca<sup>2+</sup> release in Ca<sup>2+</sup> free Ringer's solution, then, 2 mM CaCl<sub>2</sub> was added for stimulating Ca<sup>2+</sup> influx. The Ca<sup>2+</sup> free Ringer's solution contained (in mM): 155 NaCl, 4.5 KCl, 3 MgCl<sub>2</sub>, 10 Glucose, 5 HEPES, 1 EGTA. With the Enspire Multimode Plate Reader, cells in 96-well plate were illuminated at 488 nm and the fluorescence emissions at 525 nm were captured at 10 s intervals. The index  $\Delta F/F_0$  was calculated to represent the average Ca<sup>2+</sup> response ( $\Delta F = F - F_0$ ,  $F_0$  is the mean value of the background fluorescence).

**Measurement of cell proliferation.** Cell proliferation was measured using the Cell Counting Kit-8 (CCK-8) according to the manufacturer's instructions. Briefly, PBTCs were seeded into 96-well plate in 200  $\mu$ L medium ( $5 \times 10^5$  per well). Every group contained 4-5 duplicates. PBTCs were pre-incubated with 0, 10, 30, 100  $\mu$ M Lovastatin for 30 min, then, 1  $\mu$ g/mL soluble purified anti-CD3 and anti-CD28 antibodies were applied to stimulate T cells for 3 days. 20  $\mu$ L CCK-8 solution was added to every well for the last 2 h. The optical density (OD) value of every well at 450 nm was measured. The relative cell number was calculated as the percentage of  $(OD_{test} - OD_{blank}) / (OD_{control} - OD_{blank})$ , in which  $OD_{test}$  is the OD value of wells exposed to Lovastatin or antiCD3/CD28 antibodies,  $OD_{control}$  is the OD of the control sample and  $OD_{blank}$  is the OD of wells without PBTCs.

**IL-2 production measurement.** Jurkat cells were pre-treated with 0, 10, 30 100  $\mu$ M Lovastatin in the absence or presence of 1 mM Mevalonate for 30 min. Then the cells were stimulated with 5  $\mu$ g/mL PHA and 80 nM PMA for 24 h. The supernatants were collected, and the IL-2 production was measured with an ELISA kit following the manufacturer's instructions. Each experiment was repeated at least 3 times in duplicate.

**Knockdown of Kv1.3 expression with small interfering RNA.** The Jurkat T cell-lines stably transfected with lentivirus-delivered negative control (NC)-siRNA or Kv1.3-siRNA were provided by Genepharma Company (Shanghai, China). Western blotting has been performed to confirm the efficiency of siRNA knockdown<sup>31</sup>. The cell-lines were treated as described above and the supernatants were collected for IL-2 production measurement.

**Statistical Analysis.** All results are delivered as mean  $\pm$  SEM. The significance of differences before and after drug application was evaluated using paired t-test. Comparisons between groups were accomplished by analysis of variance with Turkey's post-test. Significance was set at  $P < 0.05$ .

## References

- Bu, D. X., Griffin, G. & Lichtman, A. H. Mechanisms for the anti-inflammatory effects of statins. *Curr Opin Lipidol* **22**, 165–170 (2011).
- Chow, S. C. Immunomodulation by statins: mechanisms and potential impact on autoimmune diseases. *Arch Immunol Ther Exp (Warsz)* **57**, 243–251 (2009).
- Zeiser, R. *et al.* Regulation of different inflammatory diseases by impacting the mevalonate pathway. *Immunology* **127**, 18–25 (2009).
- Jain, M. K. & Ridker, P. M. Anti-inflammatory effects of statins: clinical evidence and basic mechanisms. *Nat Rev Drug Discov* **4**, 977–987 (2005).
- Pasterkamp, G. & van Lammeren, G. W. Pleiotropic effects of statins in atherosclerotic disease. *Expert Rev Cardiovasc Ther* **8**, 1235–1237 (2010).
- Siu, C. W., Lau, C. P. & Tse, H. F. Prevention of atrial fibrillation recurrence by statin therapy in patients with lone atrial fibrillation after successful cardioversion. *Am J Cardiol* **92**, 1343–1345 (2003).
- Khattri, S. & Zandman-Goddard, G. Statins and autoimmunity. *Immunol Res* **56**, 348–357 (2013).
- Stanislaus, R., Gilg, A. G., Singh, A. K. & Singh, I. Immunomodulation of experimental autoimmune encephalomyelitis in the Lewis rats by Lovastatin. *Neurosci Lett* **333**, 167–170 (2002).
- Brumeanu, T. D., Goldstein, R. & Casares, S. Down-regulation of autoreactive T-cells by HMG CoA reductase inhibitors. *Clin Immunol* **119**, 1–12 (2006).
- Leung, B. P. *et al.* A novel anti-inflammatory role for simvastatin in inflammatory arthritis. *J Immunol* **170**, 1524–1530 (2003).
- Azuma, R. W. *et al.* HMG-CoA reductase inhibitor attenuates experimental autoimmune myocarditis through inhibition of T cell activation. *Cardiovasc Res* **64**, 412–420 (2004).
- Gullu, S., Emral, R., Bastemir, M., Parkes, A. B. & Lazarus, J. H. *In vivo* and *in vitro* effects of statins on lymphocytes in patients with Hashimoto's thyroiditis. *Eur J Endocrinol* **153**, 41–48 (2005).
- Ghittoni, R. *et al.* Simvastatin inhibits T-cell activation by selectively impairing the function of Ras superfamily GTPases. *Faseb J* **19**, 605–607 (2005).
- Dunn, S. E. *et al.* Isoprenoids determine Th1/Th2 fate in pathogenic T cells, providing a mechanism of modulation of autoimmunity by atorvastatin. *J Exp Med* **203**, 401–412 (2006).
- Gegg, M. E. *et al.* Suppression of autoimmune retinal disease by lovastatin does not require Th2 cytokine induction. *J Immunol* **174**, 2327–2335 (2005).

16. Beeton, C. *et al.* Kv1.3 channels are a therapeutic target for T cell-mediated autoimmune diseases. *Proc Natl Acad Sci USA* **103**, 17414–17419 (2006).
17. Cahalan, M. D. & Chandy, K. G. The functional network of ion channels in T lymphocytes. *Immunol Rev* **231**, 59–87 (2009).
18. Feske, S. Calcium signalling in lymphocyte activation and disease. *Nat Rev Immunol* **7**, 690–702 (2007).
19. Zhao, N. *et al.* Potent Suppression of Kv1.3 Potassium Channel and IL-2 Secretion by Diphenyl Phosphine Oxide-1 in Human T Cells. *PLoS One* **8**, e64629 (2013).
20. Fu, X. X. *et al.* 18beta-Glycyrrhetic acid potently inhibits Kv1.3 potassium channels and T cell activation in human Jurkat T cells. *J Ethnopharmacol.* **148**, 647–654 (2013).
21. Price, M., Lee, S. C. & Deutsch, C. Charybdotoxin inhibits proliferation and interleukin 2 production in human peripheral blood lymphocytes. *Proc Natl Acad Sci USA* **86**, 10171–10175 (1989).
22. Leonard, R. J., Garcia, M. L., Slaughter, R. S. & Reuben, J. P. Selective blockers of voltage-gated K<sup>+</sup> channels depolarize human T lymphocytes: mechanism of the antiproliferative effect of charybdotoxin. *Proc Natl Acad Sci USA* **89**, 10094–10098 (1992).
23. Rangaraju, S., Chi, V., Pennington, M. W. & Chandy, K. G. Kv1.3 potassium channels as a therapeutic target in multiple sclerosis. *Expert Opin Ther Targets* **13**, 909–924 (2009).
24. Faeth, A. E., Cao, D., van Vollenhoven, R., Trollmo, C. & Malmstrom, V. CD28<sup>null</sup>CD4<sup>+</sup> T cells—characterization of an effector memory T-cell population in patients with rheumatoid arthritis. *Scand J Immunol* **60**, 199–208 (2004).
25. Viglietta, V., Kent, S. C., Orban, T. & Hafler, D. A. GAD65-reactive T cells are activated in patients with autoimmune type 1a diabetes. *J Clin Invest* **109**, 895–903 (2002).
26. Beeton, C. *et al.* Selective blockade of T lymphocyte K<sup>+</sup> channels ameliorates experimental autoimmune encephalomyelitis, a model for multiple sclerosis. *Proc Natl Acad Sci USA* **98**, 13942–13947 (2001).
27. Ahn, H. S. *et al.* Open channel block of Kv1.3 by rosiglitazone and troglitazone: Kv1.3 as the pharmacological target for rosiglitazone. *Naunyn Schmiedebergs Arch Pharmacol* **374**, 305–309 (2007).
28. Ahn, H. S. *et al.* Calcineurin-independent inhibition of Kv1.3 by FK-506 (tacrolimus): a novel pharmacological property. *Am J Physiol Cell Physiol* **2**, C1714–C1722 (2007).
29. Xie, Z. *et al.* Human alpha-defensins are immune-related Kv1.3 channel inhibitors: new support for their roles in adaptive immunity. *FASEB J* **29**, 4324–4333 (2015).
30. Grissmer, S., Lewis, R. S. & Cahalan, M. D. Ca<sup>2+</sup>-activated K<sup>+</sup> channels in human leukemic T cells. *J Gen Physiol* **99**, 63–84 (1992).
31. Zhao, N. *et al.* Acacetin blocks kv1.3 channels and inhibits human T cell activation. *Cell Physiol Biochem* **34**, 1359–1372 (2014).
32. Villalonga, N. *et al.* Immunomodulatory effects of diclofenac in leukocytes through the targeting of Kv1.3 voltage-dependent potassium channels. *Biochem Pharmacol* **80**, 858–866 (2010).
33. Li, Z. *et al.* Immunomodulatory synergy by combining atorvastatin and rapamycin in the treatment of experimental autoimmune encephalomyelitis (EAE). *J Neuroimmunol* **250**, 9–17 (2012).
34. Randriamampita, C. & Trautmann, A. Ca<sup>2+</sup> signals and T lymphocytes; “New mechanisms and functions in Ca<sup>2+</sup> signalling”. *Biol Cell* **96**, 69–78 (2004).
35. Kazama, I., Baba, A. & Maruyama, Y. HMG-CoA reductase inhibitors pravastatin, lovastatin and simvastatin suppress delayed rectifier K<sup>+</sup>-channel currents in murine thymocytes. *Pharmacol Rep* **66**, 712–717 (2014).
36. Holstein, S. A., Knapp, H. R., Clamon, G. H., Murry, D. J. & Hohl, R. J. Pharmacodynamic effects of high dose lovastatin in subjects with advanced malignancies. *Cancer Chemother Pharmacol* **57**, 155–164 (2006).
37. Yellen, G., Jurman, M. E., Abramson, T. & MacKinnon, R. Mutations affecting internal TEA blockade identify the probable pore-forming region of a K<sup>+</sup> channel. *Science* **251**, 939–942 (1991).
38. Choi, J. S. *et al.* Mechanism of fluoxetine block of cloned voltage-activated potassium channel Kv1.3. *J Pharmacol Exp Ther* **291**, 1–6 (1999).
39. Choi, J. S., Hahn, S. J., Rhie, D. J., Jo, Y. H. & Kim, M. S. Staurosporine directly blocks Kv1.3 channels expressed in Chinese hamster ovary cells. *Naunyn Schmiedebergs Arch Pharmacol* **359**, 256–261 (1999).
40. Robe, R. J. & Grissmer, S. Block of the lymphocyte K<sup>+</sup> channel mKv1.3 by the phenylalkylamine verapamil: kinetic aspects of block and disruption of accumulation of block by a single point mutation. *Br J Pharmacol* **131**, 1275–1284 (2000).
41. Schmid, S. I. & Grissmer, S. Effect of verapamil on the action of methanethiosulfonate reagents on human voltage-gated Kv1.3 channels: implications for the C-type inactivated state. *Br J Pharmacol* **163**, 662–674 (2011).
42. Leonard, R. J., Garcia, M. L., Slaughter, R. S. & Reuben, J. P. Selective blockers of voltage-gated K<sup>+</sup> channels depolarize human T lymphocytes: mechanism of the antiproliferative effect of charybdotoxin. *Proc Natl Acad Sci USA* **89**, 10094–10098 (1992).
43. Cahalan, M. D., Wulff, H. & Chandy, K. G. Molecular properties and physiological roles of ion channels in the immune system. *J Clin Immunol* **21**, 235–252 (2001).
44. Lewis, R. S. & Cahalan, M. D. Potassium and calcium channels in lymphocytes. *Annu Rev Immunol* **13**, 623–653 (1995).
45. Cheng, S. M. *et al.* Modulation of human T cells signaling transduction by lovastatin. *Int J Cardiol* **140**, 24–33 (2010).
46. Schonbeck, U. & Libby, P. Inflammation, immunity, and HMG-CoA reductase inhibitors: statins as antiinflammatory agents? *Circulation* **109**, I18–I26 (2004).
47. Kwak, B. R., Mulhaupt, F. & Mach, F. Atherosclerosis: anti-inflammatory and immunomodulatory activities of statins. *Autoimmun Rev* **2**, 332–338 (2003).
48. Sarkey, J. P., Richards, M. P. & Stubbs, E. J. Lovastatin attenuates nerve injury in an animal model of Guillain-Barre syndrome. *J Neurochem* **100**, 1265–1277 (2007).
49. Ghanshani, S. *et al.* Up-regulation of the IKCa1 potassium channel during T-cell activation. Molecular mechanism and functional consequences. *J Biol Chem* **275**, 37137–37149 (2000).
50. Dong, Q. *et al.* Blocking of the human ether-a-go-go-related gene channel by imatinib mesylate. *Biol Pharm Bull* **36**, 268–275 (2013).

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### Author Contributions

N.Z., Q.D. and C.Q. performed the experiments. N.Z., S.L., X.C. and Y.H.L. analyzed the data. Q.F.W., D.D., J.L., B.B.W., J.J.X. and K.F.G. helped with cell culture and materials. N.Z. and Y.M.D. designed the study and wrote the manuscript. All authors discussed the results and reviewed the manuscript.

### Additional Information

**Supplementary information** accompanies this paper at <http://www.nature.com/srep>

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# SCIENTIFIC REPORTS

## OPEN **Corrigendum: Lovastatin blocks Kv1.3 channel in human T cells: a new mechanism to explain its immunomodulatory properties**

Ning Zhao, Qian Dong, Cheng Qian, Sen Li, Qiong-Feng Wu, Dan Ding, Jing Li, Bin-Bin Wang, Ke-fang Guo, Jiang-jiao Xie, Xiang Cheng, Yu-Hua Liao & Yi-Mei Du

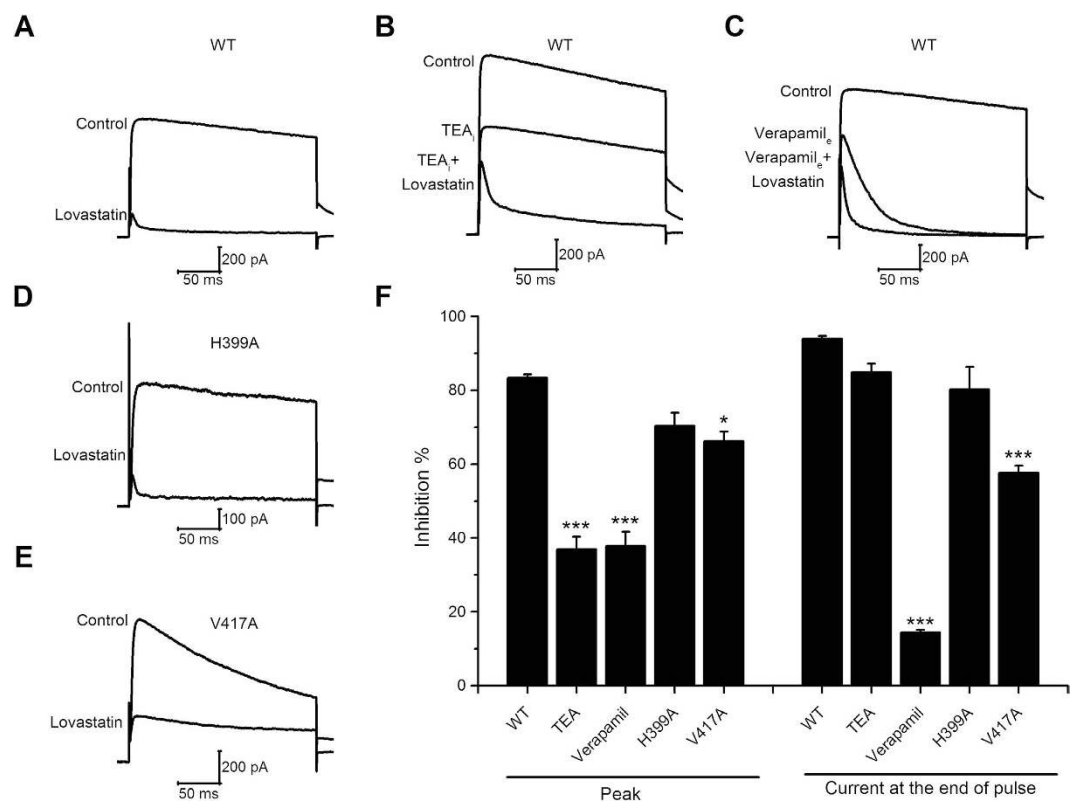
*Scientific Reports* 5:17381; doi: 10.1038/srep17381; published online 30 November 2015; updated on 10 March 2016

This Article contains errors in Figure 3 and Figure 7a.

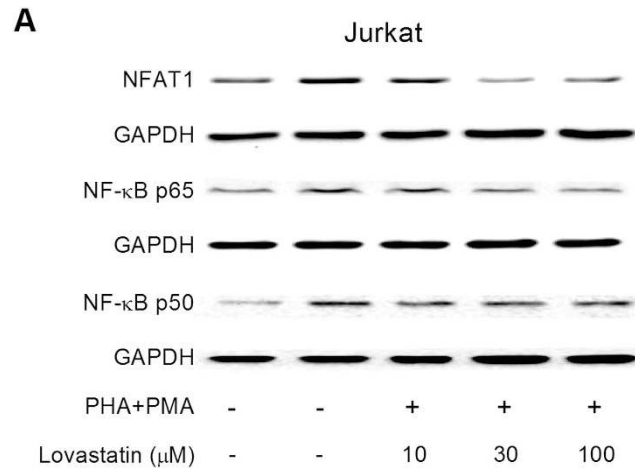
In Figure 3A, 3B, 3C, 3D and 3E the current labels were incorrectly provided.

In Figure 7a, the GAPDH Western blot bands were omitted from each corresponding target NFAT1 and NF- $\kappa$ B p65.


The correct Figures 3 and 7a appear below as Figs 1 and 2 respectively.



**Figure 1.**



**Figure 2.**

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