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TNF-α inhibits the CD3-mediated upregulation of voltage-gated K^+ channel ($K_v1.3$) in human T cells

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ABSTRACT

A long term treatment of T cells with tumor necrosis factor alpha (TNF-α) paradoxically inhibits the immunologic responses to TCR/CD3 stimulation. The voltage-gated K^+ channels (K_v) of T cells attracted attention as a pharmacological target for the treatment of autoimmune diseases. Here, the authors investigated the effects of TNF-α on the K_v current (I_{Kv}) and its upregulation by CD3 in human T cells. Acute treatment with TNF-α (10 min) temporarily decreased I_{Kv} in Jurkat-T cells (cells subsequently recovered after treatment >12 h), whereas CD3 stimulation for 24 h increased I_{Kv} amplitude more than two-fold. Furthermore, chronic pretreatment with TNF-α almost completely blocked the I_{Kv} increase induced by CD3 stimulation. An immunoblot study confirmed an increase in the protein level of K_v induced by CD3 stimulation, and its inhibition by TNF-α pretreatment. In addition, the facilitation of I_{Kv} by CD3 stimulation and its inhibition by pretreatment with TNF-α were confirmed in freshly isolated human peripheral CD4(+) T cells, in which the voltage-dependence of I_{Kv} was unaffected by TNF-α and/or CD3 stimulation. We conclude that the inhibition of CD3-induced K_v upregulation by TNF-α might be associated with the paradoxical suppression of T cell function by TNF-α under conditions of chronic inflammation.

Introduction

T cells play principal roles in cellular immune responses. Specific interaction between presented antigens and T cell receptor (TCR)/CD3 complex activate a variety of intracellular signaling such as PLCγ1/IP_3-induced Ca^{2+} release [1]. Furthermore, Ca^{2+} release from ER stores activates Ca^{2+} influx (store-operated Ca^{2+} entry, SOCE), which is critically required for T cell activation and proliferation. In terms of optimal Ca^{2+} signaling, the modulation of electrical driving force caused by K^+ channel activity has been suggested to play an important role. In T cells, subtypes of voltage-gated K^+ channel ($K_v1.3$) and Ca^{2+}-activated K^+ channel (IKCa1) are known to have such modulating effects [2–6]. In addition, the relative contributions made by $K_v1.3$ and IKCa1 to the control of membrane potentials are known to be dependent on T cell subtype and the activation states. Several experiments demonstrated that $K_v1.3$ blockers inhibit Ca^{2+}signaling and the mitogen-induced proliferation of resting T cells [7,8]. Furthermore, the expression of $K_v1.3$ is particularly strong in effector memory T (T_{EM}) cells, which immediate execute functions peripheral organs. For these reasons, selective blockers for $K_v1.3$ are being investigated in the context of regulating autoimmune diseases like multiple sclerosis [9–11].

Tumor necrosis factor alpha (TNF-α) is a pleiotropic cytokine and a key mediator of inflammation. However, in stark contrast to its acute proinflammatory and costimulatory effects, chronic exposure to TNF-α suppresses T cell proliferative and cytokine responses following TCR stimulation *in vitro* and *in vivo* [12,13]. Furthermore, it has been proposed that these paradoxical effects of TNF-α could explain the hyporesponsiveness of T cells to TCR stimulation under chronic inflammatory conditions [15]. Interestingly, this negative immunomodulation by chronic TNF-α has also been suggested in studies on autoimmunity in TNF-α and TNF receptor (TNFR)-deficient mice [16,17].

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In terms of the molecular mechanisms responsible for the inhibitory effects of TNF-α, it has been suggested that TNF-α reduces the phosphorylations of TCRζ chain and its associated protein ZAP 70. However, TCRζ reconstitution failed to restore T cell responses after long-term TNF-α treatment [18,19]. Of the above-mentioned TNF-α studies, studies on the ion channel-mediated signaling in T cells are very rare except some brief descriptions of decreased Ca^{2+} signaling by chronic TNF-α treatment [13,14]. A recent microarray study of BDC2.5 diabetogenic $CD4^+$ T cells demonstrated down-regulations of $Ca_V1.2$ and $Ca_V2.2$, which encode voltage-gated Ca^{2+} channels [20]. However, the actual functions of Ca_V in non-excitable T cells are unclear. Actually, no previous study has directly investigated the effects of chronic TNF-α treatment on ion channels in lymphocytes. Accordingly, to investigate the role of $K_v1.3$ channels in T cells, we examined the effects of TNF-α on voltage-gated K^+ currents (I_{Kv}) in human T cells under control and CD3-stimulated conditions. Jurkat-T cells (a human leukemic T cell line) and primary CD4+ T cells from human peripheral blood were utilized, and were investigated using a whole-cell voltage clamp technique.

Materials and methods

Cell culture and peripheral T cell isolation. Jurkat-T cells (clone E6-1) were purchased from ATCC (Manassas, VA, USA) and grown in RPMI 1640 media (Gibco, Grand Island, USA) supplemented with 10% (v/v) fetal bovine serum (Gibco), 1 mM sodium pyruvate and 1% penicillin/streptomycin (Gibco) at 37 °C in 20% O_2/5% CO_2.

The human primary CD4+ T cells were derived from the peripheral blood samples of three healthy male volunteers. Mononuclear cells were harvested from blood by Ficoll-Paque gradient centrifugation (Amersham Bioscience, Buckinghamshire, UK) and CD4+ T cells were purified using magnetic activated cell sorting (MACS®) with anti-CD4 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) as recommended by the manufacturer. The purities of collected CD4+ T cells (>92%) were determined by flow cytometry using anti-CD4 mAb.

Electrophysiological measurements. Membrane currents were measured using a patch clamp amplifier (Axopatch-1D, Axon instruments, Foster City, CA) at room temperature (22–25 °C). pCLAMP software v.10.2 and Digidata-1440A (Axon Instruments) were used to acquire data and apply command pulses. Cells were transferred into a bath (approximately 0.15 ml) mounted on an inverted microscope (IX-70, Olympus, Osaka, Japan) stage and superfused with HEPES-buffered normal Tyrode (NT) solution at 5 ml/min. Patch pipettes with a free-tip resistance of about 3 MΩ (Jurkat-T) or of about 4.5 MΩ (primary T cells) were used.

$[Ca^{2+}]_c$ measurements. Jurkat-T cells were loaded with fura-2 acetoxymethyl ester (5 μM, 30 min, 25 °C), and washed once with fresh NT solution. Fluorescence was monitored in a stirred quartz microcuvette (1 ml), in the thermostat controlled cell holder of a fluorescence spectrophotometer (Photon Technology Instrument, Birmingham, NJ) at wavelengths of 340 and 380 nm (excitation), and 510 nm (emission). The ratio of fluorescence ($F_{340/380}$) was normalized versus the maximum $F_{340/380}$ obtained by adding 5 μM ionomycin with 5 mM $CaCl_2$ to tested cells.

Immunoblot assay. Jurkat-T cells were pretreated with TNF-α (10 ng/ml for 2 or 10 days) and anti-CD3 antibodies (αCD3, 10 μg/ml) was added after 2 or 10 days, respectively. Membrane fractions of cell lysates were separated using 8% SDS–polyacrylamide gels, and then transferred to Immobilon-P membranes (Millipore, Billerica, MA). Membranes were blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20 (TTBS) at room temperature for 1 h, and then with anti-$K_v1.3$ (diluted 1:1000, Santa Cruz, CA) antibody (overnight at 4 °C in 5% nonfat milk in TTBS). After washing membranes with TTBS three times, they were treated with HRP-coupled goat anti-mouse IgG (Pierce Biotechnology, Rockford, IL) secondary antibodies. Antigen–antibody complexes were visualized by Enhanced Chemiluminescence Plus Kit (GE healthcare, NJ).

Solutions. The KCl pipette solution used for whole cell patch clamps was composed of 145 KCl, 1 $MgCl_2$, 10 HEPES, 10 EGTA, and 3 MgATP (in mM) and was of pH 7.2 (adjusted with KOH). Normal bath solution contained (in mM) 140 NaCl, 3.6 KCl, 1.3 $CaCl_2$, 1 $MgCl_2$, 5 glucose, and 10 HEPES (pH of 7.4 titrated with NaOH).

Chemicals and drugs. Anti-human CD3 antibody (OKT 3 clone), TNF-α, and fura-2 AM were purchased from eBioscience (San Diego, CA), R&D SYSTEMS (Minneapolis, MN) and Molecular Probes (Eugene, OR), respectively. $K_v1.3$-specific siRNA was purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA). TNF-α was reconstituted at 10 μg/ml in sterile PBS containing 0.1% bovine serum albumin. All other chemicals were purchased from Sigma (St. Louis, MO).

Data analysis and statistics. Data was managed and analyzed using Origin v.6.1 (Microcal Software Inc, Northampton, USA). Results are presented as means ± SEMs. The student's *t* test (unpaired) was used to test for significance, which was accepted for P values of <0.05. To investigate the voltage-dependence of I_{Kv}, membrane conductance (g) was defined as $I/(V - E_{rev})$ where I is the peak amplitude of the whole-cell current, V is the test voltage, and E_{rev} is the reversal potential (−90 mV) of the K^+ current. To obtain the voltage-dependent activation curve, values of g/g_{max} were plotted and fitted to the Boltzmann equation; $1/(1 + \exp(V - V_{1/2})/k$ where $V_{1/2}$ and k represent the half-activation voltage and slope factor, respectively.

Results

In whole-cell clamps using KCl pipette solution, ramp-like depolarization of Jurkat-T cells induced outwardly rectifying currents that reached a quasi-steady-state at 0 mV, which is consistent with the voltage-dependent activation and inactivation properties of K_v channel currents (I_{Kv}). Interestingly, repetitive application of a ramp-pulse (from −80 to 90 mV, holding voltage −60 mV) after making the whole-cell configuration induced an increase of I_{Kv} over 20–40 s (267.04 ± 34.31% of the initial amplitude, n = 16, Fig. 1A). After confirming I_{Kv} stability, various levels of step pulses were applied, and these revealed the time-dependent inactivation of outward currents (Fig. 1B). The peak current to voltage relation (I–V curve) of I_{Kv} was obtained from responses to step pulses in Jurkat-T cells; averaged results are displayed in Fig. 1C (control). Initially, we tested the acute effects of TNF-α (10 ng/ml) on I_{Kv}. When Jurkat-T cells were treated with TNF-α for 10–15 min I_{Kv} seemed to decrease, but then recovered after 30–60 min (Fig. 1C) and remained at this level at 2 days. However, at 10 days a significant reduction in I_{Kv} was observed (Fig. 1D).

We then investigated the effects of chronic CD3 stimulation on I_{Kv} in Jurkat-T cells. I_{Kv} increased by almost three-fold at 24 h after treating cells with αCD3 (10 μg/ml, Fig. 2A). However, after pretreating Jurkat-T cells with TNF-α for 24 h, additional treatment with CD3 for 24 h did not increase I_{Kv} (Fig. 2B, upper), and the same inhibitory effect was observed after 9 days of TNF-α pretreatment (Fig. 2B, lower). The spontaneous increase in I_{Kv} induced by repetitive ramp pulses was slightly attenuated in αCD3-stimulated cells, though this may have been due to higher initial I_{Kv} amplitude (Fig. 2C).

$K_v1.3$ is regarded as the primary voltage-gated K^+ channel in T lymphocytes [5,9], and thus, we investigated whether the protein level of $K_v1.3$ was affected by CD3 and TNF-α treatment. Immunoblot assays using human $K_v1.3$-specific Ab showed 51 and

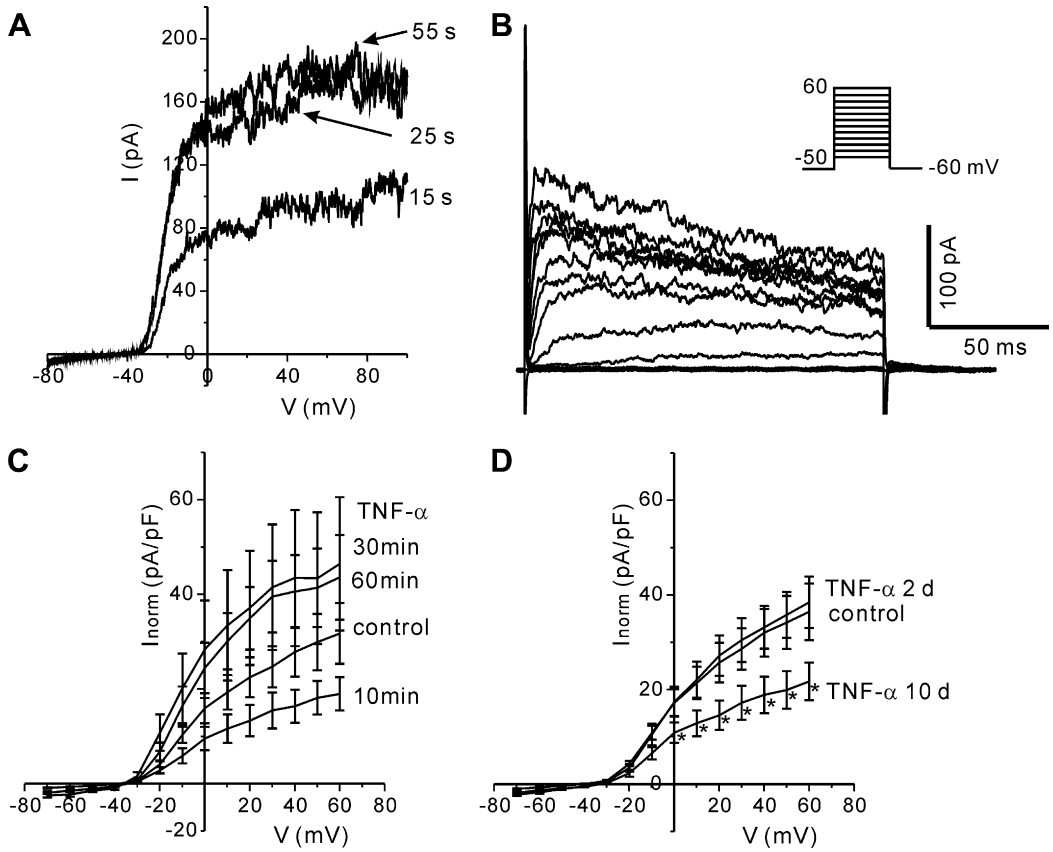

Fig. 1. Inhibition of I_{Kv} by TNF-α in Jurkat-T cells. (A) Representative current responses to ramp-like pulses from −80 to 100 mV at 15, 25 and 55 s after membrane break-in showing spontaneous increases in outward currents. (B) Representative currents obtained by step pulses from −50 to 60 mV in 10 mV steps. In (C) and (D), the same protocol was used to derive current–voltage relations (*I–V* curves). The figures summarize I_{Kv} responses to both acute (10 min, $n = 8$; 30 min, $n = 8$; 60 min, $n = 13$) (C) and chronic TNF-α 10 ng/ml treatment (2 days, $n = 12$; 10 days, $n = 10$) (D). The mean values of normalized currents (pA/pF) are plotted against voltage (mV). $^*P < 0.05$; 10 days TNF-α versus control.

60 kDa products, among which the 51 kDa band was almost completely abolished by $K_v1.3$-specific siRNA treatment (arrow in Fig. 2D). Both 51 and 60 kDa products were elevated after 24 h of CD3 treatment (Fig. 2D), and only the upregulation of 51 kDa signal was prevented by TNF-α treatment. The above results suggest that long-term TNF-α treatment inhibits the TCR/CD3-mediated signaling cascades that lead to $K_v1.3$ upregulation in Jurkat-T cells.

We then questioned whether TNF-α exerts an inhibitory effect on PLCγ-mediated Ca^{2+} signaling mechanisms in T cells. To investigate this possibility, we compared αCD3-induced increases in cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_c$) in control and TNF-α-pretreated T cells. In the absence of extracellular Ca^{2+}, αCD3 treatment induced a transient increase in $[Ca^{2+}]_c$, reflecting PLCγ-dependent IP_3 receptor-mediated Ca^{2+} release from ER stores. Then, an addition of $CaCl_2$ (5 mM) induced a large transient increased in $[Ca^{2+}]_c$ followed by a plateau-like phase, that was believed to reflect SOCE. Both initial Ca^{2+} release and presumed SOCE signals were slightly reduced by treatment with TNF-α for 1 day, but no difference was observed between TNF-α treated cells and controls after 9 days of TNF-α treatment (Fig. 3).

Finally, we investigated the effects of αCD3 and/or TNF-α on freshly isolated human peripheral T cells. CD4+ T cells were cultured for 27–32 h with CD3 or with TNF-α for 3 h followed by αCD3 plus TNF-α for 24–29 h. The TNF-α pretreatment time and the total culture time were shorter than those used in experiments on Jurkat-T cells, because we were concerned that prolonged culture might alter the properties of primary T cells.

The average capacitances of primary T cells were generally lower than those of Jurkat-T cells (3.2 ± 0.06 pF ($n = 32$) versus 5.4 ± 0.17 pF ($n = 64$), respectively). Nevertheless, the I_{Kv} amplitudes of primary T cells were comparable to those of Jurkat-T cells (Fig. 4A and B). After 24 h of CD3 stimulation, I_{Kv} amplitudes in the primary T cells were increased, and this was prevented by 3 h of TNF-α pretreatment (Fig. 4B). However, TNF-α alone did not affect the amplitude of I_{Kv} in primary T cells (Fig. 4B). The voltage-dependence of K_v activation was estimated by fitting normalized conductance against clamp voltages using the Boltzmann distribution equation. However, neither TNF-α alone nor combined treatment with αCD3 changed the voltage-dependence of I_{Kv} (Fig. 4D).

Discussion

This study demonstrates for the first time the effects of long-term TNF-α treatment on ion channels in lymphocytes. Our results reveal that pretreatment with TNF-α effectively suppresses the upregulation of $K_v1.3$ by TCR stimulation in human T cells. Although we did not investigate the signaling pathways responsible for $K_v1.3$ upregulation by long-term αCD3 treatment, an earlier electrophysiological study demonstrated the upregulation of $K_v1.3$ by combined treatment with ionomycin (a Ca^{2+} ionophore) and phorbol myristate (a PKC activator) [21]. Thus, we suppose that TNF-α may attenuate CD3-induced Ca^{2+} signaling and/or PKC activation. The changes in I_{Kv} amplitude observed in the present study are consistent with our immunoblotting results. However, the similar protein levels of $K_v1.3$ observed after 10 days of TNF-α treat-

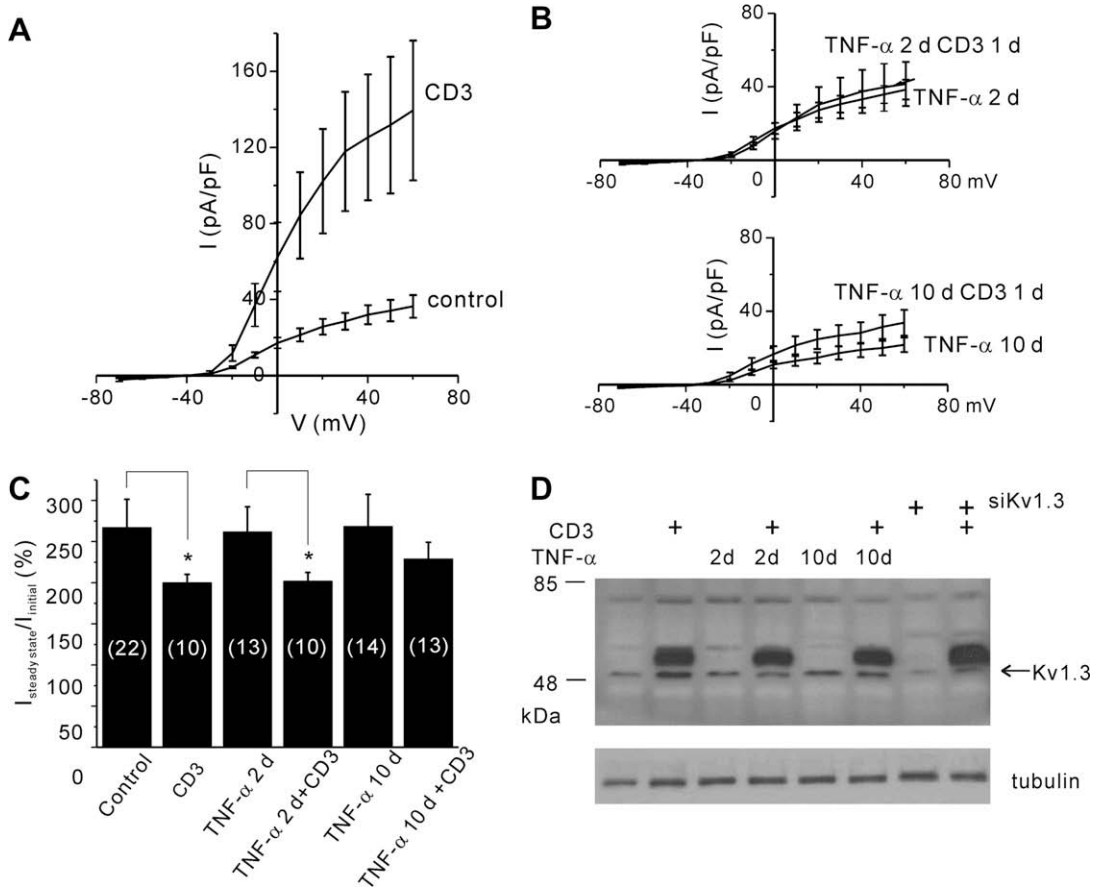

Fig. 2. Upregulation of $K_v1.3$ by chronic αCD3 treatment and the inhibition of this upregulation by TNF-α in Jurkat-T cells. (A) *I–V* curves obtained using the step-pulse protocol described in the legend of Fig. 1, showing an increase in I_{Kv} density after αCD3 treatment (5 μg/ml, 24 h). (B) Summary of $K_v1.3$ response to long-term TNF-α treatment (10 ng/ml for 2 or 10 days) with or without CD3 stimulation (24 h). Upper panel; *I–V* curves obtained after 2 days of TNF-α treatment ($n = 12$) and after 1 day of TNF-α treatment with CD3 stimulation ($n = 10$). Lower panel; after treatment with TNF-α for 10 days ($n = 10$) and after treatment with TNF-α for 10 days and with αCD3 on day 10 ($n = 13$). (C) Summary of the spontaneous increase observed in I_{Kv} after membrane break-in. Outward current amplitudes at 0 mV were measured and averaged steady-state to initial amplitude ratios are shown. $^*P < 0.05$ (The numbers of tested cells are shown in the bar graph). (D) Western blot analysis using $K_v1.3$-specific antibodies. The expected size of $K_v1.3$ was about 51 kDa (arrow), which was weakened by $K_v1.3$-specific siRNA transfection. Furthermore, this 51 kDa signal was increased by CD3 stimulation (24 h), and this increase was prevented by TNF-α pretreatment for 2 or 10 days. A representative example of three similar results is shown.

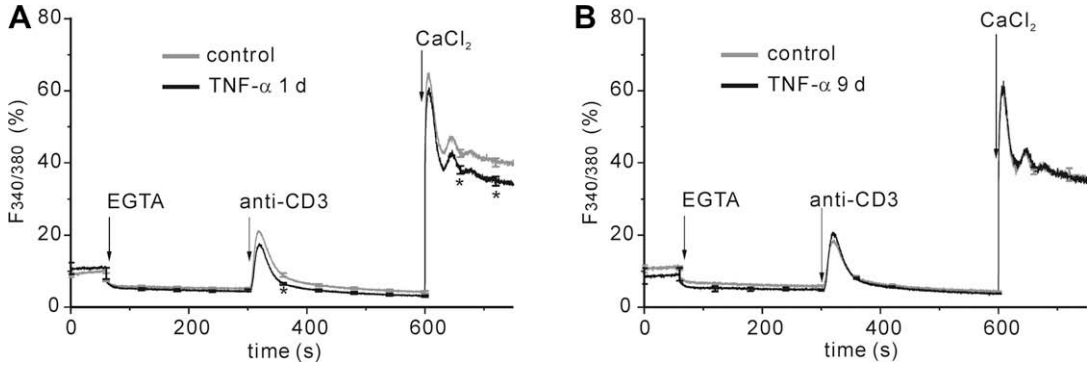

Fig. 3. Effects of chronic TNF-α on anti-CD3-induced calcium signaling and on SOCE in Jurkat-T cells. (A) In the absence of extracellular Ca^{2+} (achieved by adding 2 mM of EGTA), anti-CD3 Ab (5 μg/ml) treatment induced a transient increase in $[Ca^{2+}]_c$. The addition of Ca^{2+} (5 mM $CaCl_2$) to the bath solution induced a sharp increase in $[Ca^{2+}]_c$. Pretreatment of Jurkat-T cells with TNF-α for 1 day slightly suppressed Ca^{2+} signaling (A), whereas longer term TNF-α treatment (10 days) had no effect. $^*P < 0.05$ ($n = 3$, respectively).

ment and at baseline are not consistent with the reduced current amplitudes observed after 10 days of TNF-α treatment.

It has been reported that long-term TNF-α treatment suppresses TCR/CD3-mediated intracellular signaling cascades, and $[Ca^{2+}]_c$ increases [13,19]. However, in the present study, αCD3-induced $[Ca^{2+}]_c$ increases were only marginally reduced by TNF-α (Fig. 3), which we find difficult to explain. Nonetheless, this lack of an effect suggests that the inhibitory effects of TNF-α on TCR-dependent signaling are not wholly due to its targeting of PLCγ-associated pathways, at least in Jurkat-T cells, and that the inhibitory effects of long-term TNF-α are not mediated by the 'uncoupling' of signaling steps from TCR complex. The results of

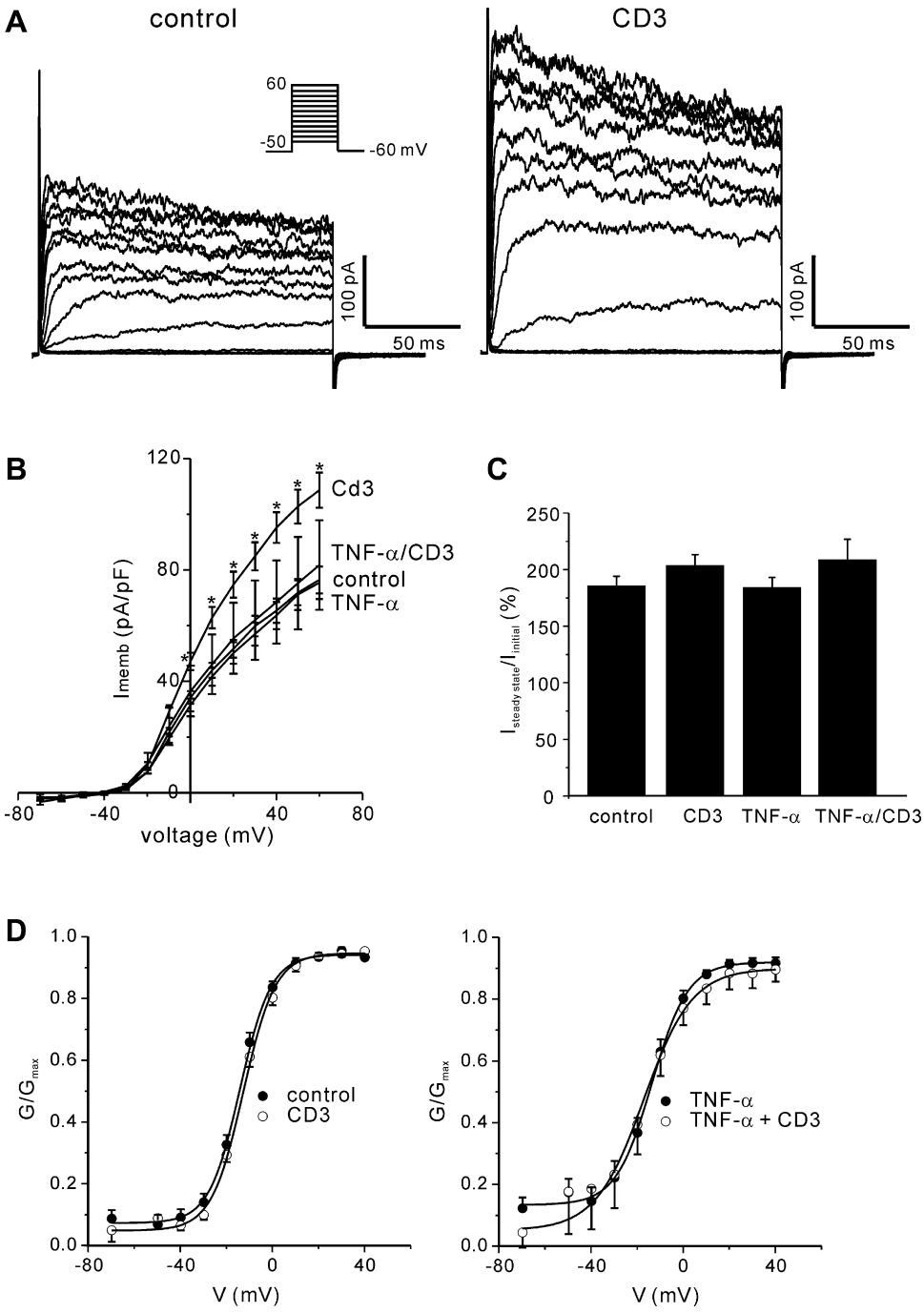

Fig. 4. Effects of CD3 and TNF-α on I_{Kv} in primary T cells. (A) Representative current traces elicited by step pulses (see *inset*) in human peripheral CD4(+) T cells cultured for 1 day with (right panel) or without αCD3 (right panel). (B) Summary of current–voltage relationship of I_{Kv} in human T cells. Mean values of normalized membrane current (pA/pF) after pretreating cells for 24 h with αCD3 (n = 24), for 27 h with TNF-α (n = 28), or with TNF-α for 3 h and with TNF-α plus anti-CD3 Ab for 24 h (n = 12). (C) Summary of the spontaneously increase of amplitude of $K_v1.3$ at 0 mV on human T cells. (D) Analyses of the voltage-dependence of outward currents derived from *I–V* relation in (B) (refer to Methods for details of analysis). Half-activation voltage ($V_{1/2}$) values were −14.2 ± 0.62 mV for the non-treated control cells, −12.6 ± 0.62 mV for αCD3 treated cells (1 day), −13.6 ± 0.54 mV for TNF-α treated cells (1 day), and −17.9 ± 1.29 for TNF-α + αCD3 treated cells (1 day).

previous studies are also contradictory in terms of the level of TCR-dependent signaling where the chronic TNF-α treatment dominantly affects [18,19].

In the present study, we applied voltage clamp pulses as soon as the membrane break-in and electrical capacitance measurement because changes in the cytosolic environment induced by TNF-α or αCD3 might be dialyzed in terms of the $K_v1.3$ regulation. Both CD4(+) T cells and Jurkat-T cells consistently showed spontaneous increase in I_{Kv} after membrane break-in. However, the initial spontaneous increase of I_{Kv} was generally observed throughout the experimental conditions. Previous studies have suggested that the voltage-dependence of K_v is modulated by intracellular ATP in a PKC-dependent manner [22]. However, in the present study, we found that the spontaneous increase in I_{Kv} was consistently observed without ATP in the pipette solution, but that it was abolished by sustained dialysis at a holding voltage of −60 mV before applying the voltage clamp pulse (data not shown), which suggests that the spontaneous increase might reflect the equilibrium pro-

cess of ion activity by dialyzing through patch pipettes or the recovery of activated $K_v1.3$ from a partially inactivated state.

Based on the accepted roles of K^+ channels in non-excitable cells, $K_v1.3$ channels are thought to maintain a partially hyperpolarized membrane potential, and thus, to provide the driving force for Ca^{2+} influx. This role of $K_v1.3$ is believed to provide a mechanism for the effects of $K_v1.3$ inhibitors in autoimmune diseases [9]. However, it has also been suggested that $K_v1.3$ functions in a different manner in immunocytes. In particular, the recruitment of $K_v1.3$ to the so-called 'lipid raft domain' or immunological synapse region suggests that $K_v1.3$ provides a platform for immunological signaling pathways [23–25]. Actually, it has been reported that $K_v1.3$ activation affects integrin-dependent signaling in a physically associated manner [26].

Conclusion

In the presence of chronic infections or inflammatory disease, the immune system is continuously exposed to a multitude of antigens and cytokines like TNF-α. The inhibition of TCR/CD3-triggered signaling by TNF-α might offer a means of suppressing T cell autoreactivity under these conditions. Furthermore, although chronic TNF-α may have several intracellular targets, the present study suggests that it suppresses $K_v1.3$ upregulation by CD3 stimulation.

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