IMMUNOLOGY

Inhibition of two-pore channels in antigen-presenting cells promotes the expansion of TNFR2-expressing CD4^{+}Foxp3^{+} regulatory T cells

Tianzhen He^{1}, De Yang^{2}, Xiao-Qing Li^{2,3}, Mengmeng Jiang^{1}, Md Sahidul Islam^{1}, Shaokui Chen^{1}, Yibo Chen^{1}, Yang Yang^{1}, Chon-Kit Chou^{1}, Anna L. Trivett^{2}, Joost J. Oppenheim^{2*}, Xin Chen^{1*}

CD4^{+}Foxp3^{+} regulatory T cells (T_{regs}) are pivotal for the inhibition of autoimmune inflammatory responses. One way to therapeutically harness the immunosuppressive actions of T_{regs} is to stimulate the proliferative expansion of TNFR2-expressing CD4^{+}Foxp3^{+} T_{regs} via transmembrane TNF (tmTNF). Here, we report that two-pore channel (TPC) inhibitors markedly enhance tmTNF expression on antigen-presenting cells. Furthermore, injection of TPC inhibitors including tetrandrine, or TPC-specific siRNAs in mice, increases the number of T_{regs} in a tmTNF/TNFR2-dependent manner. In a mouse colitis model, inhibition of TPCs by tetrandrine markedly attenuates colon inflammation by expansion of T_{regs}. Mechanistically, we show that TPC inhibitors enhance tmTNF levels by disrupting surface expression of TNF-α–converting enzyme by regulating vesicle trafficking. These results suggest that the therapeutic potential of TPC inhibitors is mediated by expansion of TNFR2-expressing T_{regs} and elucidate the basis of clinical use in the treatment of autoimmune and other inflammatory diseases.

INTRODUCTION

CD4^{+}Foxp3^{+} regulatory T cells (T_{regs}) are indispensable for the maintenance of immune homeostasis and for the prevention of allergy, asthma, and autoimmune diseases (1). There is compelling evidence that tumor necrosis factor (TNF), a pleiotropic cytokine, has both proinflammatory and immunosuppressive effects (2), preferentially activates and expands T_{regs} through stimulation of TNF receptor type II (TNFR2) (3–5). The biological function of TNF is transduced by two receptors, TNFR1 and TNFR2 (6). TNF is initially expressed as a membrane-bound form or transmembrane TNF (tmTNF) that can be cleaved into soluble TNF (sTNF) by TNF-α–converting enzyme (TACE) (7, 8). It is known that sTNF has much higher affinity for TNFR1 than TNFR2 (9). In contrast, tmTNF preferentially binds to and fully activates TNFR2 (10), which is mostly expressed by lymphoid cells, especially T_{regs} (11). There is increasing evidence that TNFR2 mediates the anti-inflammatory effect of TNF and the activation of the tmTNF-TNFR2 axis favors immune tolerance (12, 13), which is presumably mainly based on the activation and expansion of T_{regs} (12, 14).

It has been reported that some pharmacological agents can stimulate the expansion of T_{regs} through up-regulation of tmTNF expression and consequently enhance the interaction between TNF and TNFR2. For example, it was reported that vitamin D3 up-regulates the expression of tmTNF by dendritic cells (DCs) and consequently promoted the induction of T_{regs} through TNFR2 (15). Adalimumab, a humanized anti-TNF antibody used for the treatment of rheumatoid arthritis (RA) and other autoimmune diseases, antagonizes TNF stimulation of TNFR1, but can also concomitantly up-regulate the expression of tmTNF on monocytes and consequently stimulate the activation and expansion of T_{regs} through TNFR2 in RA patients (12). Methotrexate, a disease-modifying anti-rheumatic drug, was shown to enhance tmTNF expression on peripheral blood mononuclear cells (PBMCs) from RA patients and thus could further promote the effect of adalimumab in the expansion of T_{regs} (14). Therefore, up-regulation of tmTNF expression and subsequent expansion of TNFR2^{+} T_{regs} may represent a previously unidentified mechanism underlying the anti-inflammatory action of some pharmacological agents.

In this study, we examined a number of anti-inflammatory agents including some used in conventional medicine and traditional Chinese medicine on tmTNF expression by antigen-presenting cells (APCs). Our results found that tetrandrine, a traditional Chinese herb–derived immunosuppressive compound used in the treatment of autoimmune diseases (16), markedly up-regulated tmTNF expression on APCs and consequently induced proliferative expansion of T_{reg} via TNFR2 signaling. Because tetrandrine is a well-characterized inhibitor of calcium channel, we further determined the effect of various types of calcium channel inhibitors on tmTNF expression by APCs and found that two-pore channel (TPC) inhibitors and TPC small interfering RNAs (siRNAs) had the same effect as tetrandrine. Furthermore, our studies showed that TPC inhibition was able to inhibit the cell surface expression of TACE. Therefore, our results indicate that this capacity of TPC inhibitors may be therapeutically harnessed in the treatment of inflammatory diseases.

RESULTS

In vitro treatment with TPC inhibitors up-regulates tmTNF expression on APCs

To identify compound(s) with the capacity to up-regulate tmTNF expression on APCs, we examined the effect of a number of clinical anti-inflammatory agents on the surface expression of TNF by Raw264.7 cells, including conventional medicines [steroidal and nonsteroidal anti-inflammatory drugs (SAID and NSAID, respectively)] and Chinese herbal extracts manufactured for the administration to patients in China (table S1). The result showed that a Chinese
herbal product containing tetrandrine markedly up-regulated tmTNF expression on Raw264.7 cells, a macrophage-like cell line (fig. S1, A and B). We then treated Raw264.7 cells with purified tetrandrine compound from commercial source and found that the treatment with 1 μM tetrandrine resulted in a about twofold increase of tmTNF expression (Fig. 1, A to C). Furthermore, treatment with tetrandrine also markedly up-regulated tmTNF expression on mouse DC2.4 cell line, mouse bone marrow–derived DCs (BMDCs) and human monocyte-derived DCs (MoDCs) (P < 0.001; Fig. 1, A to C). Moreover, we also observed that tetrandrine treatment up-regulated tmTNF expression on human monocyte-derived macrophages and monocytes (fig. S2A). In contrast, tetrandrine treatment inhibited the production of sTNF by mouse BMDCs (P < 0.001; Fig. 1D), which is consistent with the results of previous report (17). Treatment with tetrandrine did not up- or down-regulate the TNF mRNA level in mouse Raw264.7 cells, mouse DC2.4 cells, mouse BMDCs, or human MoDCs (fig. S2, C to F) and did not change the total TNF protein level in mouse Raw264.7 cells (Fig. 1E). In contrast, lipo-polysaccharide (LPS) treatment enhanced the expression of both tmTNF and sTNF, as well as TNF mRNA expression (figs. S1, B to D, and S2, C to F). Therefore, tetrandrine appears to selectively up-regulate tmTNF expression and this effect is unlikely due to the contamination with LPS.

Tetrandrine is a well-characterized natural Ca\(^{2+}\) channel blocker that has the capacity to inhibit L-type calcium channel (18) and TPCs (19). Therefore, it is possible that the effect of tetrandrine on TNF expression was based on its inhibitory effect on calcium signaling. To test this, the effect of some common calcium signaling inhibitors on TNF expression was investigated. The results showed that three other structurally distinct L-type channel inhibitors, namely, diltiazem, nimodipine and verapamil, rather than \(\omega\)-agatoxin (P/Q type channel inhibitor; fig. S3A) and \(\omega\)-conotoxin (N-type channel inhibitor; fig. S3B), also have the same effects as tetrandrine on the expression of tmTNF (P < 0.001; Fig. 1, F and G) by DCs. In contrast, gabapentin, a representative of another class of L-type calcium channel antagonist, had no effect on tmTNF expression (fig. S3C).

It is known that tetrandrine, diltiazem, nimodipine, and verapamil each also inhibited calcium signaling triggered by nicotinic acid adenine dinucleotide phosphate (NAADP). We thus examined the effect of a NAADP inhibitor on tmTNF expression. As shown in Fig. 1 (F and G), NAADP-specific inhibitor Ned 19 also has the capacity to up-regulate tmTNF expression and inhibited the production of sTNF (P < 0.001; Fig. 1H) by BMDCs. To gain further insight into the connection between NAADP-mediated signaling, tmTNF expression, and \(T_{\text{reg}}\) proliferation, we examined the effect of TPCs, the major calcium channels activated by NAADP (20), on tmTNF expression. TPCs are also activated by the phosphoinositide PI(3,5,5)\(_2\) (phosphatidylinositol 3,5-bisphosphate) with highly conserved proteins TPCN1 and TPCN2 (21). We thus further used TPC siRNAs to specifically disrupt their functions (knockdown deficiency >70%; fig. S3, D and E), and this also resulted in the up-regulation of tmTNF expression by DCs (fig. 1, I to K), while sTNF levels were decreased and TNF mRNA remained unchanged (fig. S3, F and G).

Tetrandrine-treated DCs develop the capacity to selectively induce \(T_{\text{reg}}\) expansion in vitro

To determine if tetrandrine-treated DCs (TET-DCs) had the capacity to selectively induce proliferative expansion of \(T_{\text{reg}}\) autologous mouse BMDCs were generated and treated with or without tetrandrine for 48 hours. After washing, these BMDCs were cocultured with carboxyfluorescein diacetate succinimidyl ester (CFSE)–labeled CD4 T cells from either wild-type (WT) or TNFR2 knockout (KO) mice. As shown in Fig. 2 (A to C), DCs pretreated with tetrandrine acquired the capacity to selectively stimulate the proliferation of \(T_{\text{reg}}\) but not effector T cells (T$_{\text{eff}}$), in the cocultured CD4 T cells. Coculture of CD4 T cells with DC–TET resulted in the proliferation of 39.8% of \(T_{\text{reg}}\), which was approximately twofold greater than that in coculture with control DCs (DC–Ctrl) (P < 0.001). As expected, the addition of interleukin-2 (IL-2) markedly enhanced the proliferation of \(T_{\text{reg}}\) in all coculture conditions. The \(T_{\text{reg}}\)-stimulatory effect of DC–TET was dependent on TNF because the anti-TNF antibody completely blocked the proliferation of \(T_{\text{reg}}\) induced by DC–TET, while IL-2–induced proliferation of \(T_{\text{reg}}\) was not affected (P < 0.001; Fig. 2, A and B). Furthermore, TET-DCs failed to stimulate the proliferation of \(T_{\text{reg}}\) deficient in TNFR2 (Fig. 2, D and E). Tetrandrine did not up-regulate tmTNF expression on human CD4 cells or purified mouse T(reg) (fig. S2, A and B) and failed to stimulate the proliferation of mouse T(reg) (fig. S4A). Therefore, the interplay between APCs and T(reg) through the TNF-TNFRII pathway played a crucial role in the proliferation of T(reg) in the cocultures.

In vivo treatment with tetrandrine increases the number of T(reg) in WT mice, but not in mice deficient in TNFR2 and TNF

We next examined if tetrandrine treatment could also up-regulate the expression of tmTNF on APCs and consequently stimulate proliferative expansion of \(T_{\text{reg}}\) in vivo. To this end, WT C57BL/6j mice were injected intraperitoneally with tetrandrine for 3 days. On day 4, mice were sacrificed and the number, phenotype, and function of T(reg) present in the peripheral lymphoid tissues were analyzed (gating strategy was shown in fig. S4B). As shown in Fig. 3A, the treatment with tetrandrine markedly up-regulated tmTNF expression on CD11c$^+$ DCs in WT mice (P < 0.05). The proportion of Foxp3$^+$ cells in splenic CD4$^+$ T cells was increased in a dose-dependent manner (9.6, 23, and 29.6% increase in mice treated with tetrandrine at 50, 100, and 150 mg/kg per day, respectively; Fig. 3B; P < 0.01). The absolute number of T(regs) in the spleen was also increased by ~1.5-fold after tetrandrine treatment (100 mg/kg per day) (P < 0.01 to 0.001; Fig. 3C). Similarly, the proportion of Foxp3$^+$ cells in CD4$^+$ T cells present in lymph nodes (axillary, inguinal, and mesenteric regions) was also markedly increased after tetrandrine injection (P < 0.05 to 0.001; fig. S4C). The expression of Ki-67 in T(regs) was markedly increased after tetrandrine treatment (P < 0.05; Fig. 3D).

Ki-67 is a proliferating marker coexpressed with TNFR2 in highly suppressive T(regs) (22). This result thus suggested that the increased number of T(regs) in mice treated with tetrandrine was due to expansion of preexisting naturally occurring T(regs) (nT(regs)), rather than from the conversion of naïve CD4 T cells into induced T(regs) (iT(regs)). This is supported by our recent report that tetrandrine did not increase the conversion of iT(regs) in both in vitro and in vivo studies (23). TNFR2 expression by T(reg) from tetrandrine-treated WT mice was also markedly increased (P < 0.05; Fig. 3E), indicative of highly suppressive phenotype (11). Because proliferative expansion of T(regs) could potentially reduce their suppressive function (24), we also carefully examined the activity of T(reg) isolated from tetrandrine-treated mice. As shown in fig. S5 (A and B), T(regs) from tetrandrine-treated mice actually were markedly more suppressive, as compared with their counterparts from vehicle control mice (P < 0.001).
Fig. 1. In vitro treatment with TPC inhibitors up-regulates tmTNF expression on APCs. (A to E) Raw264.7 cells, DC2.4 cells, mouse BMDCs, or human MoDCs were treated with 1 µM tetrandrine. After 48 hours, tmTNF expression was analyzed by FCM (A and B) or by Western blot (C) [total TNF in (E), Raw264.7 cell lysate]. Supernatant TNF levels were determined by enzyme-linked immunosorbent assay (ELISA) (D). MFI, mean fluorescence intensity. (F to H) BMDCs were treated with 100 µM diltiazem (DIL), nimodipine (NIM), verapamil (VER), or Ned 19 (NED) for 48 hours. tmTNF expression was analyzed by FCM. (I to K) Mouse DC2.4 cells were transfected with TPCN1-specific (I and J) and TPCN2-specific (I and K) siRNA. After 48 hours, tmTNF was analyzed by FCM. Data were presented as means ± SEM (n = 3 to 6 independent experiments). By comparison with vehicle control group, *P < 0.05, **P < 0.01, and ***P < 0.001; n.s., no significant differences.
Furthermore, T\(_{\text{reg}}\) in tetrandrine-treated mice appeared to be more activated, as evidenced by the up-regulation of CD152, CD69, and CD44 expression (fig. S5C, \(P < 0.05\)). Therefore, in addition to increasing the number of T\(_{\text{reg}}\), tetrandrine treatment could also enhance the immunosuppressive function of T\(_{\text{reg}}\).

To further verify the crucial role of the TNF-TNFR2 interaction in the expansion of T\(_{\text{reg}}\) in tetrandrine-treated mice, we examined the effect of tetrandrine on mice deficient in TNFR1 (TNFR1 KO), TNFR2 (TNFR2 KO), or TNF (TNF KO). Results showed that tetrandrine treatment retained the capacity to up-regulate tmTNF expression on DCs in TNFR1 KO mice (\(P < 0.05\); Fig. 3F) and TNFR2 KO mice (\(P < 0.001\); Fig. 3G). Furthermore, treatment with tetrandrine also increased the proportion and number of T\(_{\text{reg}}\) in TNFR1 KO mice (\(P < 0.01\); Fig. 3H). This was accompanied by the up-regulation of Ki-67 expression (\(P < 0.05\); fig. S5D) and increased TNFR2 expression (\(P < 0.05\); fig. S5E) by T\(_{\text{reg}}\). In sharp contrast, tetrandrine treatment failed to induce the expansion of T\(_{\text{reg}}\) in TNFR2 KO mice (\(P > 0.05\); Fig. 3I) and in TNF KO mice (\(P > 0.05\); Fig. 3J). These data indicate that the TNF-TNFR2 interaction is required for the expansion of T\(_{\text{reg}}\) in tetrandrine-treated mice.

TPCs regulate the in vivo proliferation of T\(_{\text{reg}}\) through TNF-TNFR2 pathway

To determine the role of TPC inhibition in T\(_{\text{reg}}\) expansion in vivo, WT mice or mice deficient in TNFR1 or TNFR2 were treated with TPCN1 or TPCN2 siRNAs. The treatment effectively knocked down Tpcn1 or Tpcn2 mRNA expression (fig. S6, A to F). The expression of tmTNF on CD11c\(^+\) DCs was markedly increased in all three mouse strains with knockdown of Tpcn1 or Tpcn2 (\(P < 0.01\); Fig. 4, A, F, and I). The proportion of Foxp3\(^+\) cells in CD4 T cells and the number of T\(_{\text{reg}}\) in spleen were increased by 1.5- to 2-fold in WT (\(P < 0.05\) to 0.001; Fig. 4, B and C) and TNFR1 KO mice (\(P < 0.05\) to 0.01; Fig. 4, G and H). Ki-67 and TNFR2 expression was
Fig. 3. In vivo treatment with tetrandrine expands T_reg in a TNF/TNFFR2-dependent manner. (A to E) WT mice were injected with tetrandrine (TET; intraperitoneally) for 3 days. Spleen and lymph nodes were harvested 24 hours after the last injection. tmTNF expression on CD11c+ cells (A), proportion of T_reg in splenic CD4 cells (B), number of T_reg (C), and expression of Ki-67 (D) and TNFR2 (E) by splenic T_reg were analyzed by FCM. (F to J) Three types of gene KO mice, including TNFR1 KO (F and H), TNFR2 KO (G and I), or TNF KO (J) mice, were injected with tetrandrine (intraperitoneally) for 3 days. tmTNF expression on splenic CD11c+ cells (F and G), proportion of T_reg in splenic CD4 cells, and number of T_reg in spleen (H to J) were analyzed by FCM. For typical FCM plots, the number indicated proportion of gated cells. Data (means ± SEM) were representative of three separate experiments with similar results (B and C, n = 6; G and I, n = 4; others, n = 3 mice). Compared with vehicle control, *P < 0.05, **P < 0.01, and ***P < 0.001; n.s., no significant differences.
Fig. 4. Knockdown of TPCs induces $T_{reg}$ proliferation. (A to E) WT mice were intraperitoneally injected with TPC siRNAs or nontargeting control for 2 days, and 24 hours later, tmTNF expression on splenic CD11c$^+$ cells (A), proportion and number of splenic $T_{reg}$ (B and C), and expression of Ki-67 (D) or TNFR2 (E) by $T_{reg}$ were analyzed by FCM. (F to K) Two types of gene KO mice, including TNFR1 (F to H) or TNFR2 KO mice (I to K), were intraperitoneally injected with TPC siRNAs for 2 days. tmTNF expression on splenic CD11c$^+$ cells (F and I) and proportion and number of $T_{reg}$ in the spleen (G, H, J, and K) were analyzed by FCM. (L and M) WT mice were intraperitoneally injected with lentivirus encoding $Tpcn1$ or $Tpcn2$ or control. Twenty-four hours later, mice were treated with tetrandrine for 3 days. The proportion of $T_{reg}$ in splenic CD4 cells (L) and the number of splenic $T_{reg}$ (M) were analyzed by FCM. For typical FCM plots, the number indicated proportion of gated cells. Data (means ± SEM, $n = 3$ mice) were representative of three separate experiments. Compared with the indicated group, *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$; n.s., no significant differences.
also up-regulated by treatment with TPC siRNAs in WT mice (Fig. 4, D and E). However, the proportion and number of $T_{reg}$ were not changed by the treatment with TPC siRNAs in TNFR2 KO mice ($P > 0.05$; Fig. 4, J and K).

We further determined the role of TPCs in the regulation of $T_{reg}$ proliferation by overexpressing $Tpcn1$ and $Tpcn2$ in mice. WT mice were infected with lentivirus encoding $Tpcn1$ or $Tpcn2$ or with control lentivirus. After 5 days, the mRNA expression of $Tpcn1$ and $Tpcn2$ was increased by more than twofold (Fig. S6, G and H). The treatment of tetrandrine resulted in 17.5% increase in the proportion of $T_{reg}$ in CD4 cells and in 39% increase in the number of $T_{reg}$ in the spleen of mice treated with control lentivirus ($P < 0.001$; Fig. 4L). In control, the capacity of tetrandrine in promoting $T_{reg}$ expansion was abolished in mice infected with lentivirus encoding $Tpcn1$ or $Tpcn2$ (Fig. 4, L and M). These data further support the notion that TPCs negatively regulate $T_{reg}$ proliferation induced by tetrandrine treatment.

**Expansion of $T_{reg}$ contributes to the anti-inflammatory effect of tetrandrine in mouse colitis model**

Tetrandrine is known to have in vivo anti-inflammatory effects (16), including ameliorating colitis in mouse model (25). We next examined if the anti-inflammatory effect of tetrandrine was dependent on its capacity to induce $T_{reg}$ expansion. To this end, a mouse colitis model induced by transfer of naïve CD4 T cells into lymphopenic Rag1 KO mice (CD45.2+) was established. Naïve CD4 T cells (flow-sorted CD4+ CD25+ CD45RBhi T cells, $T_{eff}$) from Ly5.1 B6 mice (CD45.1+) were transferred alone or cotransfected with CD4+ Foxp3/GFP+ (green fluorescent protein–positive) cells ($T_{regs}$) from Foxp3/GFP knock-in (KI) mice (CD45.2+) (experimental procedure was schematically shown in Fig. 5A). To show the effect of tetrandrine, a low ratio of $T_{regs}$ versus naïve CD4 T cells was cotransferred (2.97% of $T_{reg}$ in total transferred cells; Fig. 5B, left). The mice were treated with tetrandrine or vehicle control starting from 1 day after cell injection for 5 weeks. As shown in Fig. 5B and fig. S7A, 8 weeks after cell cotransfer, the proportion of $T_{reg}$ (CD4+ CD45.2+) in total colonic CD4 T cells (18.3%) in Rag1 KO mice treated with tetrandrine was 6.6-fold higher as compared with that in the recipient mice without tetrandrine treatment (2.76%, $P < 0.01$ to 0.001). This was unlikely to be caused by the alteration of trafficking or homing pattern of $T_{regs}$, because the proportion of $T_{reg}$ in CD4 cells in the spleen and mesenteric lymph nodes was also markedly increased ($P < 0.01$; fig. S7B). Furthermore, the number of $T_{reg}$ in the spleen of mice treated with tetrandrine (5.46 × 10^6 cells) was also markedly higher than that in mice without tetrandrine treatment (1.74 × 10^6 cells; $P < 0.01$ to 0.001; Fig. 5C). Tetrandrine treatment did not increase Foxp3-expressing cells in CD4+ CD45.1+ population and proportion of CD45.2+ cells in CD4 cells in mice transferred with naïve CD4 T cells only ($P > 0.05$; fig. S7, C to E), providing further evidence that an increased number of $T_{reg}$ were caused by proliferative expansion of preexisting n$T_{reg}$, rather than from conversion of iT$T_{reg}$ (Fig. 3D and our previous report (23)). Moreover, the expression of TNFR2 on $T_{reg}$ was accordingly up-regulated by the treatment with tetrandrine ($P < 0.01$ to 0.001; Fig. 5D), which is a critical marker of the highly suppressive $T_{reg}$ in the inflammatory environment as we previously reported (11). The expression of tmTNF on CD11c+ cells in Rag1 KO mice was up-regulated by the treatment with tetrandrine ($P < 0.01$ to 0.001; Fig. 5E). Furthermore, the development of colitis was inhibited by tetrandrine treatment, which was attributable to the expansion of $T_{reg}$ induced by tetrandrine treatment, because both $T_{reg}$ transfer alone or tetrandrine treatment alone did not markedly inhibit colitis in this model (Fig. 5, F to I, and fig. S7, F and G).

**Tetrandrine down-regulates membrane-bound TACE expression on DCs through inhibition of TPCs**

The cleavage of pro-TNF to generate sTNF is mediated by TACE (also named ADAM17). Among known substrates, pro-TNF is preferentially catalyzed by TACE (26). We thus hypothesized that up-regulation of tmTNF by tetrandrine was due to the inhibition of expression or activity of TACE. Treatment of BMDCs with tetrandrine markedly inhibited the expression of membrane-bound TACE (mTACE) on BMDCs ($P < 0.05$; Fig. 6, A and B) and on Raw264.7 cells (Fig. 6D, left). Therefore, the capacity of TACE to cleave consequent TNF was decreased by tetrandrine, which resulted in the increase of tmTNF expression on cell surface and concomitant decreased secretion of sTNF. Because there was no effect of tetrandrine on TACE mRNA expression (Fig. 6C) and the expression of TACE total protein (Fig. 6D, right), the inhibitory effect of tetrandrine on TACE was presumably due to induction of cytosolic retention during protein translocation at the posttranslational level. To further determine the role of TACE in $T_{reg}$ expansion in mice treated with tetrandrine, we injected nilotinib, a small–molecule compound that had the capacity to increase TACE activity (27), into the mice. The result showed that the treatment with nilotinib completely abrogated tetrandrine-induced expansion of $T_{reg}$ ($P < 0.01$; Fig. 6, E and F). Nilotinib also reduced the number of $T_{reg}$ in mice without tetrandrine treatment ($P < 0.05$; Fig. 6, E and F), suggesting that TACE may negatively regulate homeostasis of $T_{reg}$ pool in the steady state. Together, these data establish that the TPC-TACE-tmTNF regulatory pathway is responsible for $T_{reg}$ expansion after tetrandrine treatment.

**TPCs control the intracellular translocation and surface expression of TACE**

We then examined the effect of tetrandrine on TACE activity. As shown in fig. S8A, treatment with tetrandrine did not inhibit the enzymatic activity. TPCs are endolysosomal ion channels that play a critical role in protein trafficking and exocytosis (28). Thus, inhibition of TPC may interrupt the TACE intracellular trafficking and surface expression. To test this, the effect of TPC inhibitors on TACE expression was investigated. The results showed that all examined TPC inhibitors including diltiazem, nimodipine, verapamil, and Ned 19 had the capacity to down-regulate surface expression of TACE on DCs ($P < 0.001$; Fig. 6, G and H). We further used TPC siRNA to specifically disrupt their function. This also resulted in the marked down-regulation of surface TACE expression on DCs (Fig. 6, I to K); however, the levels of mRNA expression (fig. S8B) and total TACE protein remained unchanged (fig. S8C). Our data suggested that down-regulation of TACE expression on plasma membrane by the treatment of TPC inhibitors was presumably due to a defect in TACE trafficking from cytoplasm to plasma membrane. We also found that TPCN1 and TPCN2 were colocalized with TACE in DCs by confocal microscopy, and TPCN1 and TPCN2 were associated with TACE in DCs by immunoprecipitation (fig. S8, D and E). All of these data support the notion that TPCs have the capacity to regulate the translocation of TACE from cytoplasm to plasma membrane and consequently expanded $T_{reg}$ through the enhanced tmTNF-TNFR2 interaction.
Fig. 5. Tetrandrine inhibits colitis by promoting expansion of Tregs. (A) Schematic diagram of experimental procedure. In brief, naïve CD4 T cells (Teffs, 4 × 10⁵ cells per mouse) from Ly5.1 B6 mice (CD45.1⁺) alone or together with CD4⁺Foxp3/GFP⁺ cells (Tregs, CD45.2⁺, 2 × 10⁴ cells per mouse) were injected intraperitoneally into Rag1⁻/⁻ mice. The mice were treated with tetrandrine (100 mg/kg per day, intraperitoneally) or vehicle control twice a week, starting from the second day after cell injection, for 5 weeks. The mouse colon and spleen were harvested on week 8 after cell transfer. (B to E) Proportion of Tregs (CD45.2⁺) in colonic CD4 cells before (B, left) or 8 weeks after transfer (B, middle and right), number of splenic Tregs (C), TNFR2 expression on colonic Tregs (D), and tmTNF expression on colonic CD11c⁺ cells (E) were analyzed by FCM. (F) Hematoxylin and eosin staining of colon. (G) Histological score. (H) Length of colon. (I) Body weight change (% of initial). Arrows indicated inflammatory cell infiltrates. Data (means ± SEM) shown (n = 4 to 5 mice) were representative of three separate experiments. Compared with the indicated group, *P < 0.05, **P < 0.01, and ***P < 0.001; n.s., no significant differences.
**DISCUSSION**

It has been reported that TPC inhibitors such as tetrandrine (16), diltiazem (29), nimodipine (30), verapamil (31), and NAADP inhibitors (32) have the anti-inflammatory activity. However, the effect of these TPC inhibitors on Tregs remains largely unknown. Although diltiazem was reported to promote the generation of iTregs from naïve CD4 cells (33), its effect on preexisting nTregs is not clear. In this study, we identified a previously unrecognized activity of tetrandrine and other TPC inhibitors in the up-regulation of tmTNF expression on APCs, and consequently induce the proliferative expansion of highly suppressive Tregs, in both in vitro and in vivo settings, in a TNF/TNFR2-dependent manner. Interruption of intracellular trafficking and surface expression of TACE is an underlying mechanism of the Treg-boosting effect of TPC inhibitors.

Although TNF was originally described as a proinflammatory cytokine, it also has an anti-inflammatory function (34). It has become clear that the binding of sTNF to TNFR1 transduces the signaling responsible for the proinflammatory effect of TNF, while the interaction between tmTNF and TNFR2 exerts an anti-inflammatory effect (12, 13, 35), which is presumably mainly based on the activation and expansion of immunosuppressive Tregs (12, 14). TPC inhibitors inhibited the release of sTNF, resulting in the up-regulation of tmTNF expression, which stimulates the activation and expansion of Tregs. By doing so, TPC inhibitors convert TNF from a potent...
proinflammatory mediator to a key driver of immunosuppression. Our study thus clarified the basis of anti-inflammatory action of TPC inhibitors, and this property of TPC inhibitors may be therapeutically harnessed in the treatment of inflammatory disorders. Because TPCs are widely expressed, the systemic effect of TPC inhibitors on the expansion of Tregs, as well as on the function of other immune cells, should be further investigated.

Certain types of DCs, such tolerogenic DCs, have the capacity to induce Tregs by expressing immunosuppressive molecules such as IL-10, transforming growth factor β (TGFβ), PD-L1 (programmed death-ligand 1), or IDO (indoleamine 2,3-dioxygenase) (36). Although it is tempting to use such DCs to induce or reestablish immune tolerance for therapeutic purpose, the plasticity of their tolerogenic phenotype in the inflammatory environment is a major concern (36). As shown in our study, DCs treated with TPC inhibitors acquired the capacity to stimulate the expansion of Tregs through up-regulation of tmTNF. High expression of tmTNF by such DCs is likely to be maintained or even further enhanced in an inflammatory environment. Therefore, the Treg–boosting capacity of DCs treated with TPC inhibitors should be more stable than previously described tolerogenic DCs, and thus, they may be more useful in DC-based cellular therapy for inflammatory diseases.

TACE has the highest affinity for TNF among all known substrates (26). It was reported that the TACE inhibitor TAPI (TNF-α protease inhibitor) increased tmTNF expression on monocytes and enhanced the effect of anti-CD3 antibody in the induction of TNFR2-expressing CD8+ Foxp3+ Treg, in PBMCs of RA patients (37). Furthermore, conditional deletion of TACE in myeloid cells protected mice from septic shock due to reduction in sTNF production (38). Therefore, inhibition of TACE directly using pharmacological agent(s) may also represent a sounding strategy to promote the expansion of Tregs. TPC inhibition is likely to have a broader effect on APCs, as compared to TACE inhibition. It was shown that the TPC inhibitor diltiazem could inhibit the production of IL-12 and enhance the production of IL-10 from LPS-stimulated DCs (33). Therefore, inhibition of TPCs may have an additional beneficial effect on APCs in the induction of immune tolerance. This possibility merits further investigation. It is also likely that undesirable side effects may be associated with the systemic administration of TPC inhibitor because of the expression of TPCs in different tissues. This off-target effect may be mitigated by an APC-targeted delivery system, if the purpose is to generate immune tolerance by expansion of Tregs through the TNF-TNFFR2 interaction.

Together, our data indicate that TPC inhibitors had the capacity to inhibit sTNF production while enhancing tmTNF expression on APCs, and to promote the proliferative expansion of highly immunosuppressive Tregs through the TNF-TNFFR2 interaction. Therefore, our study elucidates the cellular and molecular basis of the anti-inflammatory effect of TPC inhibitors and support further investigation to repurpose the approved TPC inhibitors or develop novel TPC inhibitors for the treatment of major inflammatory human diseases such as autoimmune reactions, allergy, allograft rejection, and graft-versus-host disease (GvHD).

**METHODS**

**Reagents**

Antibodies purchased from BD Pharmingen (San Diego, CA) consisted of perdinin chlorophyll protein (PerCP)–Cy5.5 anti-mouse CD3 (145-2C11), phycoerythrin (PE) anti-mouse CD4 (GK1.5), PE anti-mouse CD120b/TNFFR2 (TR75-89), PerCP-Cy5.5 anti-mouse CD25 (PC61), PerCP-Cy5.5 anti-mouse T cell receptor (TCR) β chain (H57-597), and PE anti-mouse TNF (MP6-XT22). Antibodies purchased from eBioscience included PE-Cy7 anti-mouse CD4 (GK1.5), APC anti-mouse/rat Foxp3 staining set (FIJK-16s), fluorescein isothiocyanate (FITC) anti-mouse CD11c (N418), FITC anti-human Ki-67 monoclonal antibody (20Raj1), FITC anti-human TNF (MAB11), and FITC anti-mouse CD45RB (C363.16A). Antibodies purchased from BioLegend included anti-mouse CD45.1 antibody (A20). Antibodies purchased from Tonbo Biosciences included APC anti-human CD11c (3.9) and PerCP-Cyanine5.5 anti-mouse CD45.2 (104). Human TACE/ADAM17 ectodomain PE-conjugated antibody (111633) was purchased from R&D Systems. Recombinant mouse IL-2, mouse GM-CSF (granulocyte-macrophage colony-stimulating factor), human IL-4, and human GM-CSF were obtained from PeproTech (Rocky Hill, NJ). TACE activity assay kit was purchased from Anaspec Inc. Pharmacological inhibitors were purchased from Calbiochem (trans-19, Cay17527, San Diego, CA) or from Sigma-Aldrich (diltiazem, D2521; nimodipine, N149; verapamil, V4629; gabapentin, G154; ω-agonotoxin, A6719; ω-conotoxin, C9915; St. Louis, MO). Diltiazem, nimodipine, verapamil, and trans-19 were dissolved by dimethyl sulfoxide (DMSO) initially and diluted with culture medium (culture medium that contained the same concentration of DMSO was used as vehicle control). Gabapentin, ω-agonotoxin, and ω-conotoxin were dissolved by distilled water. Nilotinib (S1033) was ordered from Selleck Chemicals.

**In vivo administration of tetrandrine**

WT, TNFR1 KO, TNFR2 KO, TNF KO, and Foxp3/GFP KI mice were injected intraperitoneally with tetrandrine solution (WT mice: 50, 100, and 150 mg/kg per day; TNFR1 KO and TNFR2 KO and TNF KO and Foxp3/GFP KI mice: 100 mg/kg per day) or control solution for 3 days. Tetrandrine powder (Sigma-Aldrich) was dissolved in 0.1 N HCl and then the pH was adjusted to 7.0 with 1 N NaOH. In some experiments, WT mice were intraperitoneally injected with nilotinib [24 mg/kg per day, dissolved in 4% DMSO + 30% PEG300 (polyethylene glycol, molecular weight 300) + 5% Tween 80 + ddH2O2]. On day 4, mice were sacrificed and lymphoid tissues (spleen and axillary lymph nodes, inguinal lymph nodes, and mesenteric lymph nodes) were harvested to measure the number and phenotype of Tregs. For Treg function assay, CFSE-labeled responder CD4+Foxp3+ cells (5 × 10^4 cells per well, sorted from tetrandrine- or control-treated Foxp3/GFP KI mice) were cultured alone or cocultured with flow-sorted CD4+Foxp3/GFP Treg at ratios of 10:0, 10:2, and 10:5. The cells were stimulated with 3000-rad irradiated APCs (CD4-depleted splenocytes, 2 × 10^5 cells per well) and soluble anti-CD3 antibody (BD, clone 145-2C11, 1 μg/ml). After 72-hour incubation, the proliferation of responder cells was analyzed by FCM (flow cytometry), based on the dilution of CFSE.

**T cell transfer model of colitis**

Naive CD4+ CD25− CD45RB− T cells were flow-sorted from WT congenic B6 (CD45.1^−, 4 × 10^6 cells per mouse) mice and injected intraperitoneally into Rag1^−/− recipients (CD45.2^−) alone or cotransferred with CD4+Foxp3/GFP Treg (CD45.2^+, 2 × 10^4 cells per mouse). In T eff and Treg cotransfer experiments, cells were flow-sorted from WT congenic B6 mice (CD45.1^−) and CD4+Foxp3/GFP mice (CD45.2^+) were mixed at a 20:1 ratio and intraperitoneally injected.
into Rag1 KO mice. Mice were monitored weekly for the clinical symptoms of colitis such as rectal bleeding, loose feces/diarrhea, rough/hunched posture, and body weight by animal facility staff. Any mice losing >20% of its starting body weight or showing severe signs of disease were euthanized. All mice were euthanized on week 8 after cell transfer. The colon and lymphoid tissues were harvested for FC analysis. For histopathological analysis, mouse colon was fixed in 10% neutral buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin. Histopathological score was assessed according to grading scales described previously (39).

**Generation of human MoDCs and mouse BMDCs**

Human MoDCs were generated by culturing magnetic-activated cell sorting (MACS)–purified CD14+ monocytes (5 × 10^5 cells/ml) with complete RPMI 1640 medium (Mediatek Inc.) supplemented with 10% fetal bovine serum (GemCell), 2 mM glutamine (Lonza), 25 mM Heps (Quality Biological), penicillin (100 U/ml) (Lonza), streptomycin (100 μg/ml) (Lonza), and 50 μM 2-mercaptoethanol (Sigma-Aldrich) containing recombinant human GM-CSF (50 ng/ml) (PeproTech) and recombinant human IL-4 (PeproTech) at 37°C in a humidified CO2 (5%) incubator for 5 days with 50% of the culture medium, which was replaced with prewarmed fresh medium after 3 days of culture. After 5 days, cells in suspension were harvested and used as immature MoDCs (purity almost 99.99% observed under microscopy) for subsequent experiments. Mouse BMDCs were generated in complete RPMI 1640 containing recombinant mouse GM-CSF (20 ng/ml) (PeproTech) for 6 days, which were isolated from the femurs and tibias of C57BL/6J mice. After 6 days, mouse immature BMDCs were harvested and used for subsequent experiments.

**Cell purification and in vitro cell culture**

BMDCs were generated from WT C57BL/6J mice as mentioned above and plated in a 24-well plate with or without tetrads (1 μM) for 48 hours. Mouse lymphocytes were harvested from spleens, axillary lymph nodes, inguinal lymph nodes, and mesenteric lymph nodes of WT or TNFR2 KO mice. CD4 T cells were purified from lymphocytes by using CD4 (L3T4) microbeads (Milteny Biotec, 130-097-145) and MS column (Milteny Biotec). MACS-purified CD4 cells were labeled with CFSE, and cells (5 × 10^5 cells per well) were cocultured with BMDCs (5 × 10^5 cells per well) in a 96-well plate and then stimulated with/without IL-2 for 3 days. Anti-TNF antibody (BioLegend, clone MP6-XT22, 100 μg/ml) was added to some wells. Proliferation of T regs or T effs was assessed by FCM analysis. For histopathological analysis, mouse colon was stained with hematoxylin and eosin. Histopathological score was assessed according to grading scales described previously (39).

**TCG knockdown and overexpression**

To knock down the expression of TPCs, siRNAs specific for TPCN1 or TPCN2 were purchased from Qiagen (in vitro study) and Life Technologies (in vivo study). TPC1 target sequences are as follows: CAGGATGACATTCAATTAGA and CTCAAGATTCTCATAGTCTAGTA. TPC2 target sequences are as follows: CAGGCTGTGGTACTCTGCTGA and CAGGCTGTGGTCCGCCAAACA. As nontargeting controls, AllStars Negative Control siRNA and Negative Control siRNA were used (Qiagen). DC2.4 cells were transfected with siRNA using RNAiMAX (Life Technologies) following the manufacturer’s protocol in 24-well plates. For in vivo delivery experiment, TPC siRNAs were mixed with in vivo-jetPEI (Polyplus, New York, NY) according to the manufacturer’s instructions. These mixtures were injected intraperitoneally into mice for 2 days (0.2 ml per mouse per day).

For overexpression experiments, mice were infected by intraperitoneal injection with 100-μl lentivirus encoding Tpcn1 or Tpcn2 or with control lentivirus [LV5-EGFP, 1 × 10^6 TU (transduction units) ml^-1]. The gene overexpression lentivirus particles (LV5-TPCN1-EGFP, LV5-TPCN2-EGFP, and LV5–negative control–EGFP) were generated by Shanghai GenePharma Co. Ltd. (Shanghai, China). After 24-hour infection with lentivirus, these mice were injected with tetrandrine or vehicle control (100 mg/kg per day), once a day for 3 days. On day 5, mice were sacrificed and spleen and lymph nodes were harvested for FC analysis.

**Quantitative reverse transcription polymerase chain reaction**

To determine the fold change in the TNF, TACE, Tpcn1, and Tpcn2 mRNA expression by DCS, total RNA was extracted and purified using Qiagen RNeasy Mini Kit following the manufacturer’s protocol. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using a Qiagen RT-PCR kit with SYBR Green with specific primers (Qiagen). β-Actin–specific primer (Qiagen) sets served as internal controls.

**Flow cytometry**

After blocking FcR, cells were incubated with appropriately diluted antibodies and finally suspended in FC buffer for cytometric analysis. Acquisition was performed using a BD LSRII flow cytometer or BD LSRFortessa. Data were analyzed with FlowJo software (Tree Star Inc., Ashland, OR).

**Measurement of sTNF levels**

The supernatants were collected from the same cultures in which the cells were used to determine tmTNF expression. The sTNF levels in the supernatants were measured using ultrasensitive plate assays (Mesos Scale Discovery). The plates were analyzed using the SECTOR Imager 2400 (Mesos Scale Discovery) according to the manufacturer’s instruction. The detection limit for the mouse sTNF is 0.0327 pg/ml.

**Immunofluorescence microscopy**

DC2.4 cells plated on coverslips were fixed with 4% paraformaldehyde for 10 min, washed three times with phosphate-buffered saline (PBS) for 10 min, permeabilized with 0.1% Triton X-100 in PBS for 5 min, blocked by 10% donkey serum (Abcam, ab7475) for 1 hour, and then incubated with anti-TPCN1 (1:100, Abcam, ab94731), anti-TPCN2 (1:100, Abcam, ab119915), or anti-TACE (1:50, Abcam, ab13535) antibody for 1 hour at room temperature, followed by incubation with donkey anti-goat immunoglobulin G (IgG) H&L (TRITC, Abcam, ab66828) or donkey anti-rabbit IgG H&L (Alexa Fluor 488, Abcam, ab150073) secondary antibodies (1:2000) for 1 hour. After washing three times with PBS, cells were finally mounted on glass slides using ProLong Gold antifade reagent with 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen). Confocal images were acquired with a laser scanning confocal microscope (Zeiss LSM).

**Coimmunoprecipitation and Western blotting**

Coimmunoprecipitation analysis was performed using the Pierce Co-Immunoprecipitation Kit (Thermo Fisher Scientific) by following the manufacturer’s instruction. Antibodies (anti-TACE antibody, Abcam, ab75609; anti-IgG antibody, Abcam, ab6709) were

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immobilized by amino link plus coupling resin. Cell lysates were precleared by using the control agarose resin, and then the cell lysate was added to the spin column containing antibody-coupled resin and incubated overnight at 4°C. Then, proteins were eluted by elution buffer. The samples were subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE) gradient gel (4 to 12% bis–tris protein gel; Thermo Fisher Scientific), and the separated proteins were transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% nonfat milk or 5% bovine serum albumin, probed with primary antibodies (anti-TACE antibody, Abcam, ab75609; anti-TPCN1 antibody, Abcam, ab94731; anti-TPCN2 antibody, Abcam, ab119915) overnight at 4°C, and then incubated with horseradish peroxidase (HRP)–conjugated secondary antibodies (1:5000) at room temperature for 1 hour. The protein bands were detected with a G-box imager.

To determine the effect of tetrandrine on the expression of tmTNF or mTACE, the Membrane Protein Extraction Kit (Beyotime Biotechnology, P0033) was used to extract membrane proteins from cells. For total TNF or TACE expression, the whole-cell lysates were prepared by using a standard whole-cell lysis protocol (pH 6.8; 62.5 mM tris–HCl, 2% SDS, 10% glycerol, 50 mM dithiothreitol, 0.01% bromophenol blue). The above samples were subjected to SDS-PAGE gel, and the separated proteins were transferred onto PVDF membranes. After blocking by 5% nonfat milk, the membranes were probed with primary antibodies (anti-TNF antibody, Abcam, ab215188; anti-TACE antibody, Abcam, ab57484; Na+/K⁺ ATPase, Cell Signaling Technology, #3010; β-actin, Abcam, ab8227) for overnight at 4°C and then incubated with HRP-conjugated secondary antibodies (1:5000) at room temperature for 1 hour. The protein bands were examined by Tanon-5200.

**Study approval**
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**Statistical analysis**

Comparisons of two groups of data were analyzed by two-tailed Student’s t test by using GraphPad Prism 7.0 (GraphPad, San Diego, CA), and comparisons of more than two groups of data were analyzed by one-way analysis of variance (ANOVA) by using GraphPad Prism 7.0 (GraphPad, San Diego, CA). All P values less than 0.05 were considered significant. In the in vitro studies, n stands for the individual mice used in the experiments. In the in vitro cell culture studies, n (the dots shown in the bar chart) stands for independent experiments.
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