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Short communication

Tetrandrine inhibits differentiation of proinflammatory subsets of T helper cells but spares *de novo* differentiation of iTreg cells

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ABSTRACT

Tetrandrine (TET) is an anti-inflammatory compound isolated from Chinese herb *Stephania tetrandra* S. Moore. It was reported recently that the differentiation of Th17 cells was inhibited, while the generation of induced Treg cells (iTregs) was promoted, by TET treatment. We therefore carefully examined the effect of TET on the differentiation of four major subsets of T helper cells. The results showed that *in vitro* treatment with TET potently inhibited the differentiation of Th1, Th2 and Th17 cells. Administration of LPS resulted in a mixed Th1, Th2 and Th17 responses in normal mice, and such effect of LPS was inhibited by *in vivo* TET treatment as well. In contrast, TET did not promote or inhibit the *in vitro* generation of iTregs from naïve $CD4^+CD25^-Foxp3/gfp^-$ T cells. Furthermore, spontaneous and rapamycin-induced conversion of naïve $CD4^+CD25^-Foxp3/gfp^-$ T cells into Foxp3-expressing iTregs in congenic mice was not affected by TET treatment. Thus, TET had the capacity to inhibit the differentiation of proinflammatory Th1, Th2 and Th17 cells, while sparing the generation of Tregs. As a Treg-friendly and broad spectrum anti-inflammatory agent, the molecular mechanism and the therapeutic potential of TET in various human inflammatory diseases should be further studied.

1. Introduction

Tetrandrine (TET) is an anti-inflammatory bisbenzylisoquinoline alkaloid isolated from *Stephania tetrandra* S. Moore, a traditional Chinese herb which has been used in the treatment of patients with rheumatoid arthritis for centuries [1]. In China, TET has been used for the treatment of autoimmune diseases, hypertension and silicosis [1]. Over the past decades, the pharmacological basis of the therapeutic effect of TET has been extensively studied. Its effect on reactive oxygen species (ROS), autophagic flux, multi-drug resistance, caspase pathway, cell cycle and calcium channels has been reported [2]. Among many other pharmacological activities, there is compelling *in vitro* and *in vivo* evidence that TET has immunosuppressive effects. For example, it was shown that proinflammatory mediators such as tumor necrosis factor-alpha (TNF) and nitric oxide (NO) produced by activated human monocytes or macrophages were inhibited by TET [3,4]. More recently, studies indicate that the inhibition of differentiation of proinflammatory T helper cells may also contribute to the anti-inflammatory effect of TET. For example, it was reported that TET, by activating aryl hydrocarbon receptor (AHR), inhibited the generation of Th17 cells and promoted the conversion of naïve CD4 T cells into induced $CD4^+Foxp3^+$ regulatory T cells (iTreg), and consequently suppressed the inflammatory responses in mouse collagen-induced arthritis [5,6].

By responding to specific pathogens or autoantigens, T helper cells play critical roles in mediating adaptive immune responses and inflammatory responses such as in the autoimmunity, asthma and allergy. Upon TCR stimulation in a particular cytokine environment, naïve CD4 T cells can differentiate into functional distinct effector subsets, such as Th1, Th2, Th17, and iTreg [7]. To date, the effect of TET on the differentiation of four major subsets of T helper cells have not been examined and compared side by side. In this study, we carefully evaluated the effect of TET on the differentiation of Th1, Th2, Th17 and iTregs in the standards *in vitro* differentiation conditions. Our results clearly show that TET markedly inhibited the differentiation of proinflammatory Th1, Th2 and Th17 cells. Intriguingly, TET did not inhibit or promote the conversion of naïve CD4 T cells into iTregs. Furthermore, the results of our *in vitro* experiment were able to be verified by *in vivo* studies. Therefore, inhibition of differentiation of proinflammatory Th subsets while sparing the generation of iTreg may be a basis of therapeutic effect of TET on Th1-, Th2- and Th17-mediated diseases.

2. Materials and methods

2.1. Mice and reagents

Wild-type (WT) C57BL/6 mice (Ly5.2), Ly5.1 C57BL/6 mice

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(B6.SJL-Ptprca Pepcb/BoyJ) and Foxp3/gfp KI (knock in) mice (8–12 weeks old, male and female) were purchased from The Jackson Laboratory (JAX) and maintained in the Animal Facility of University of Macau. The animal study protocol was approved by Animal Research Ethics Committee of University of Macau. Anti-mouse antibodies (Abs) were purchased from BD Biosciences (San Diego, CA) consisted of anti-mouse CD3 (145-2C11), TCRβ (H57-597), CD45.2 (104), IL-13 (JES10-5A2) and IL-17A (TC11-18H10). Anti-mouse CD4 (GK1.5) and IFN-γ (XMG1.2) Abs, and Foxp3 Staining Set (FJK-16s) were purchased from eBioscience (San Diego, CA). Functional grade purified hamster anti-mouse CD3e (145-2C11) and CD28 (37.51) Abs were obtained from BD Biosciences. Murine IL-2, IL-4, IL-6 were purchased from BD Biosciences and murine IL-12 was from eBioscience. Human rTGFβ1 was from R&D Systems (Minneapolis, MN). Rapamycin (Cat#: R-5000) was from LC Laboratories. Tetrandrine (TET, Cat#: T2695) and lipopolysaccharides (rough strains) from Salmonella (LPS) (Cat#: L9764) were from Sigma-Aldrich. TET was dissolved in 0.1 N HCl, with adjustment of PH to 7.0 with 1 N NaOH, as previously reported [11,12]. Stock concentration of TET was 25 mg/ml, stored in −80 °C in small aliquot.

2.2. Cell proliferation assay

Cell proliferation was determined using a colorimetric 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT) assay. Naïve $CD4^+CD25^-Foxp3/gfp^-$ T cells were stimulated with anti-CD3 and anti-CD28 Abs for 72 h, in the presence of medium or 0.05–100 μM of TET, in 96-well plates. MTT (5 mg/ml) was then added. After 3 h, the optical density was measured at 570 nm.

2.3. Purification of cells and in vitro cell activation and differentiation

Mouse lymphocytes were harvested from spleens, axillary lymph nodes, inguinal lymph nodes, and mesenteric lymph nodes of Foxp3/gfp KI mice. Naïve $CD4^+CD25^-Foxp3/gfp^-$ T cells were sorted with BD FACSAria™ Fusion flow cytometer. The cells (5×10^4 cells/well) were cultured in a 96-well plate, stimulated with plate-bound anti-CD3e Ab (5 μg/ml) and soluble anti-CD28 Ab (2 μg/ml) for 3 days. Th1 cells were generated by addition of IL-2 (20 ng/ml) and IL-12 (20 ng/ml) into the culture. Th2 cells were generated *via* addition of IL-2 (20 ng/ml) and IL-4 (10 ng/ml). For the generation of Th17 cells or Tregs, naïve T cells were cultured in the presence of TGFβ (2 ng/ml), with or without IL-6 (20 ng/ml) respectively. TET was added to the wells at the desired concentrations.

2.4. In vivo administration of LPS and tetrandrine

C57BL/6 mice were injected intraperitoneally (i.p.) with 200 μg of LPS in 0.2 mL PBS. Some mice were treated with TET at dose of 50 mg/kg through intraperitoneal injection (i.p., in 0.2 mL PBS) for three consecutive days, starting immediately after LPS treatment. After 24 h of last treatment, mice were sacrificed. The spleens, and lymph nodes at axillary, inguinal and mesenteric regions were harvested for FACS analysis.

2.5. In vivo iTreg conversion study

The *in vivo* conversion of iTregs from naïve CD4 T cells was performed according to report published previously [8], FACS-sorted $CD4^+CD25^-Foxp3/gfp^-$ cells (1×10^6, $CD4^+Foxp3^+$ cells < 0.1%) from Foxp3/gfp KI mice ($CD45.2^+$) were intravenously (i.v.) injected into C57BL/6 recipient mice (Ly5.1, $CD45.1^+$) on day 0. After cell transfer, the recipient mice were treated on days 1, 2 and 3 with HBSS or rapamycin (RPM, 3 mg/kg/day, i.p.). Some mice were then injected with TET (50 mg/kg/day, i.p.) for three consecutive days immediately after rapamycin treatment. On day 6, spleen and lymph node cells from recipient mice were harvested. The expression of Foxp3 by initially transferred cells was analyzed with FACS, by gating on $CD45.2^+CD4^+$ cells.

2.6. Flow cytometry and intracellular cytokine staining

After blocking FcR, cells were incubated with appropriately diluted antibodies. For intracellular cytokines staining, cells were re-stimulated with phorbol myristate acetate (PMA, 20 ng/ml; Sigma-Aldrich St. Louis, MO) and ionomycin (1 μM; Sigma-Aldrich) in the presence of GolgiPlug (BD Biosciences) for 5 h. The cells were then fixed and permeabilized with Cytofix/Cytoperm (BD Pharmingen) and then stained with anti-IFNγ, or anti-IL-13 or anti-IL-17A antibodies. For detection of Foxp3, cells were fixed and permeabilized using the anti-mouse Foxp3 staining kit (FJk-16S, eBioscience). Acquisition was performed by BD FACSCanto II. Data analysis was conducted by using FlowJo software (Tree Star Inc., Ashland, OR, USA).

2.7. Statistical analysis

Comparisons of two groups of data were analyzed by *t*-test using GraphPad Prism 6.0. Comparisons of more than two groups of data were analyzed by one-way ANOVA by using GraphPad Prism 6.0 (GraphPad, San Diego, CA, USA).

3. Results

3.1. Effect of tetrandrine on the differentiation of T helper subsets in vitro

We firstly determined the effect of TET on the activation of naïve CD4 T cells. To this end, naïve $CD4^+CD25^-Foxp3/gfp^-$ T cells were stimulated with anti-CD3 and anti-CD28 Abs for 72 h, in the presence of medium or 0.05–100 μM of TET. As shown in Fig. 1A, TET at a concentration range of 0.05–1 μM did not markedly inhibit the proliferation of activated naïve T cells ($p > 0.05$), which was consistent with a previously report [5]. Therefore, 0.1–1 μM of TET was used in subsequent *in vitro* studies.

To investigate the effect of TET on the differentiation of T helper subsets *in vitro*, naïve $CD4^+CD25^-Foxp3/gfp^-$ T cells were flow-sorted and stimulated under the standard Th1-, Th2-, Th17- or iTreg-polarizing culture conditions as described previously [9]. The differentiation of T helper subsets was assessed by the expression of IFNγ, IL-13, IL-17A and Foxp3, respectively. As shown in Fig. 1B, TET markedly inhibited the differentiation of Th1, Th2 and Th17 cells from naïve CD4 cells in a dose dependent manner ($p < 0.05$–0.001). The percent inhibition of Th1, Th2 and Th17 differentiation by 1 μM of TET was 67.24%, 42.01% and 70.68%, respectively. Interestingly, TET did not inhibit or promote the differentiation of Foxp3-expressing Tregs ($p > 0.05$). The mRNA of Foxp3 expression by iTregs was not affected by TET treatment as well (Supplementary Fig. 1). In the "Th0" culture condition, *e.g.*, without cytokines required for the differentiation of Th subsets, TET did not induce the expression of IFNγ, or IL-13, or IL-17A or Foxp3 (Supplementary Fig. 2). Therefore, TET had the capacity to inhibit the *in vitro* differentiation of proinflammatory Th1, Th2 and Th17 cells, while sparing the differentiation of iTreg cells.

3.2. Effect of tetrandrine on Th1, Th2 or Th17 responses in LPS-treated mice

LPS through binding to the Toll-like receptor 4 (TLR4), mainly expressing on the cell surface of monocytes and macrophages, stimulates a mixed Th1, Th2 and Th17 responses [10,11]. Therefore, LPS-treated mice were used to examine if TET has *in vivo* inhibitory activity on Th1, Th2 and Th17 responses. To this end, the mice were treated with single dose of LPS (200 μg, i.p.) alone, or treated with TET (50 mg/kg, i.p.) for three consecutive days starting immediately after LPS treatment. After

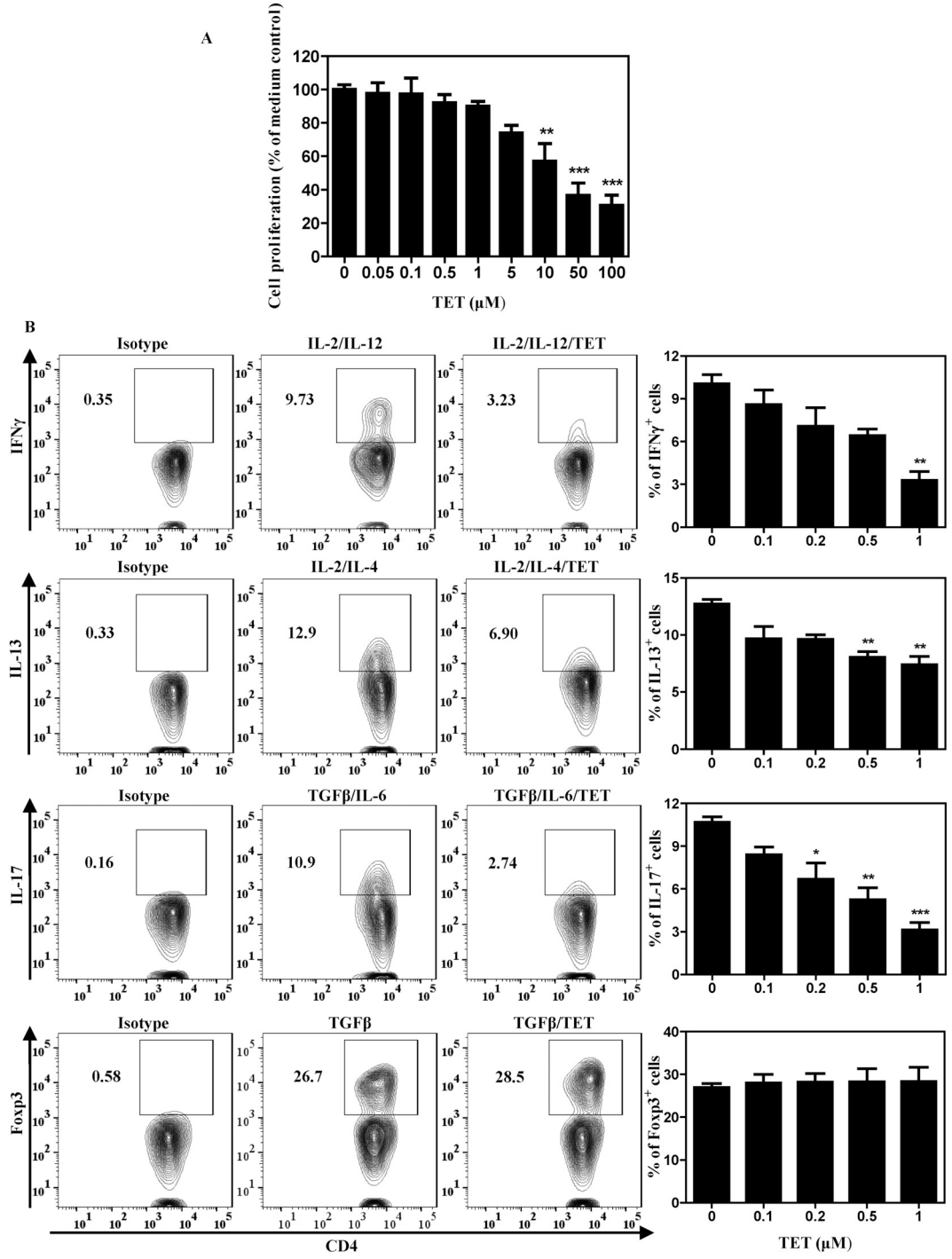

Fig. 1. Effect of tetrandrine on the differentiation of Th subsets *in vitro*. (A) Effect of TET on the proliferation of naïve CD4 T cells. Flow-sorted naïve $CD4^+CD25^-Foxp3/gfp^-$ T cells were stimulated with anti-CD3 and anti-CD28 Abs for 72 h, in the presence of medium or 0.05–100 μM of TET. The proliferation of cells was assessed with MTT assay. The percent proliferation was calculated, based on the medium culture alone. The data shown are pooled from three experiments with similar results (means ± SEM, $N = 9$). $^{**}p < 0.01$, $^{***}p < 0.001$, as compared with medium control (without TET). (B) FACS-sorted naïve $CD4^+CD25^-Foxp3/gfp^-$ T cells were cultured under Th1-, Th2-, Th17- and iTreg-polarizing conditions in the presence of TET (0, 0.1, 0.2, 0.5, and 1 μM) for 3 days. The phenotype of Th subsets was analyzed by FACS. Representative FACS data from at least three separate experiments with similar results are shown on left panel. Number in the FACS data indicates the proportion of gated cells. The right panel show summarized data (means ± SEM), pooled from 3 to 4 separate experiments ($N = 9$–12). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, as compared with medium control (without TET).

24 h of last treatment, the proportion of IFNγ-, IL-13- and IL-17-producing cells in splenic or LN $CD4^+$ T cells was analyzed by FACS. The study protocol was schematically shown in Fig. 2A. As can be seen in Fig. 2B, we were able to confirm that LPS treatment resulted in a mixed Th1, Th2 and Th17 responses, as evidenced by the markedly increase of IFNγ-, or IL-13- or IL-17-producing cells in splenic or LN $CD4^+$ T cells ($p < 0.01$–0.001). Treatment with TET markedly inhibited the proportion of LPS-induced IFNγ-, or IL-13-, or IL-17-producing cells by 47.31%, 51.07% and 52.25% in the spleens and 32.81%, 41.47% and 52.72% in the LNs (Fig. 2B, $p < 0.05$–0.01). Furthermore, the master transcription factors of Th1, Th2 and Th17 cells, namely T-bet, Gata3 and RORγt, and absolute number of Th1, Th2 and Th17 cells in the

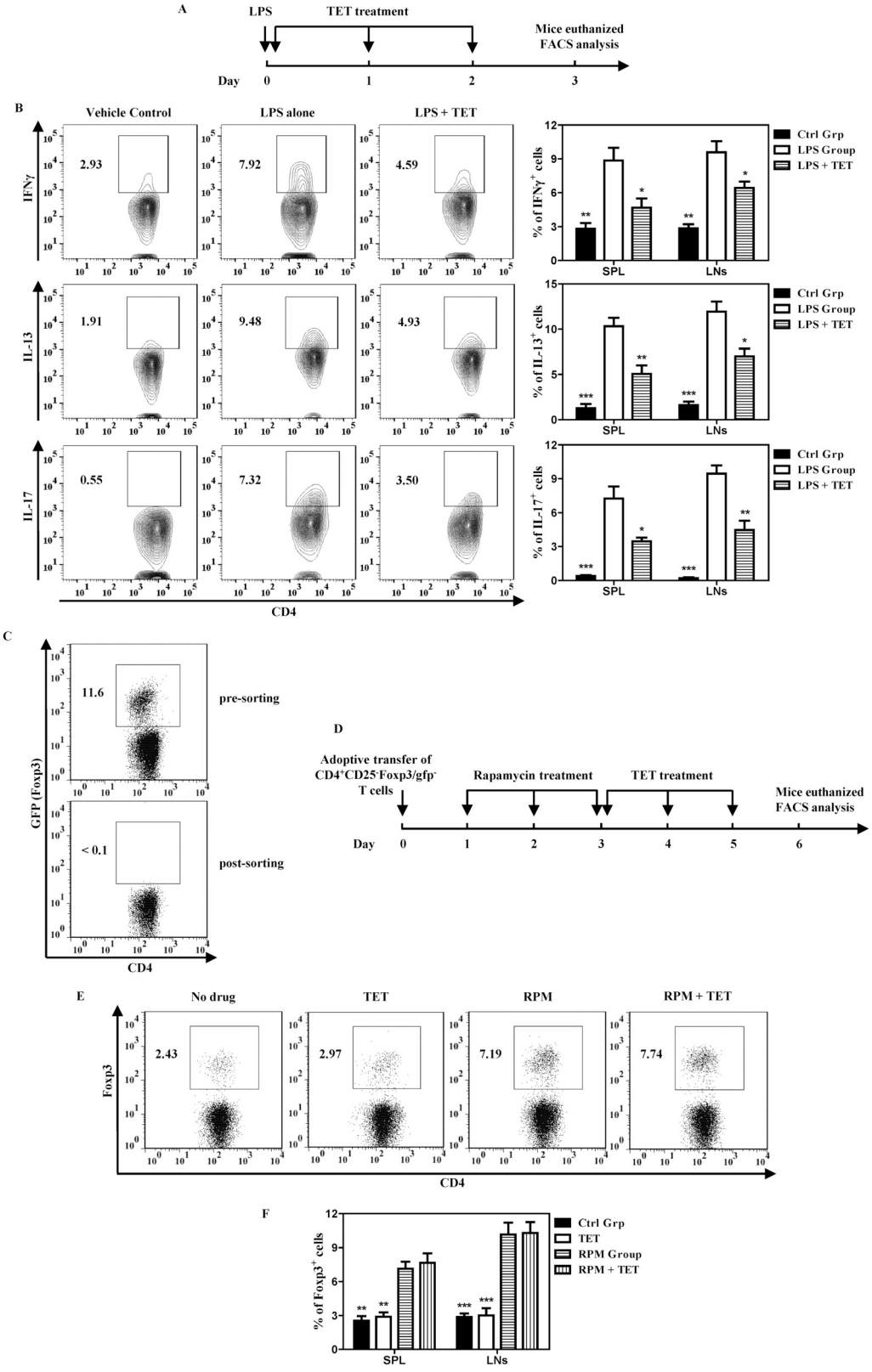

Fig. 2. Effect of tetrandrine on the responses of Th subsets *in vivo*. (A-B) Effect of tetrandrine on Th1, Th2 or Th17 responses in LPS-treated mice. (A) The schematical diagram of experimental procedure. C57BL/6 mice were injected with 200 μg of LPS (i.p.) or PBS, and treated with or without TET (50 mg/kg/day, i.p.) immediately after LPS challenge for three days. All mice were sacrificed 24 h after last treatment. Spleens (SPLs) and lymph nodes (LNs) were harvested. The cells were re-stimulated with PMA and ionomycin in the presence of GolgiPlug for 5 h. The proportion of Th subsets in $CD4^+$ T cells was then analyzed by FACS. (B) Representative FACS data are shown on the left panel. Number in the FACS data indicates the proportion of gated cells. The right panel of (B) show summarized data ($N = 9$, means ± SEM), pooled from three separate experiments with similar results. By comparison with LPS treatment group, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (C-F) Effect of tetrandrine on *de novo* generation of Tregs *in vivo*. (C) Naïve $CD4^+CD25^-Foxp3/gfp^-$ T cells from Foxp3/gfp KI mice ($CD45.2^+$) were sorted by FACS. FACS plots show GFP (Foxp3)-expressing cells in the pre- (*upper*) and post-sorting population (*lower*). (D) The schematic diagram of experimental procedure. Ly5.1 recipient mice ($CD45.1^+$) were injected (i.v.) with a million of sorted $CD4^+CD25^-Foxp3/gfp^-$ T cells on day 0. The mice were treated on days 1, 2 and 3 with HBSS or rapamycin (RPM, 3 mg/kg/day, i.p.). Some mice were then injected with TET (50 mg/kg/day, i.p.) for three consecutive days immediately after rapamycin treatment. (E) Foxp3 expression by $CD45.2^+CD4^+$ T cells present in the spleens and lymph nodes of recipient mice were analyzed on day 6 with FACS. The percentages of induced $Foxp3^+$ cells are indicated. Typical FACS plots are shown. (F) Summary of the proportion of iTregs in $CD45.2^+CD4^+$ T cells in the spleens (SPLs) and lymph nodes (LNs). Data are pooled from three separate experiments with similar results ($N = 9$, means ± SEM). By comparison with RPM alone group, $^{**}p < 0.01$, $^{***}p < 0.001$.

spleen of LPS-treated mice were also potently inhibited by TET treatment (Supplementary Fig. 3, Supplementary Fig. 4). Therefore, TET has both *in vitro* and *in vivo* activity in the inhibition of the Th1, Th2 and Th17 responses.

3.3. Effect of tetrandrine on de novo generation of Tregs in vivo

To examine the effect of TET on *de novo* differentiation of iTregs, $CD4^+CD25^-Foxp3/gfp^-$ cells from Foxp3/gfp KI mice (C57BL/6 background, $CD45.2^+$) were flow-sorted. The resultant naïve CD4 T cells contained < 0.1% of $Foxp3^+$ cells (Fig. 2C). Naïve CD4 T cells

(1×10^6) were i.v. injected into Ly5.1 C57BL/6 mice ($CD45.1^+$), as schematically shown in Fig. 2D. After 6 days, the conversion of Foxp3/gfp^- naïve CD4 T cells into $Foxp3^+$ Tregs in the spleens and LNs in the recipient mice was analyzed with FACS, by gating on $CD45.2^+CD4^+$ cells, *e.g.*, the initially transferred naïve CD4 T cells. As shown in Fig. 2E, after 6 days of transfer, a minor but a clear fraction (~2.4%) of initially Foxp3/gfp^- cells were converted into Foxp3-expressing cells. Treatment with TET (50 mg/kg/day, i.p.) for three consecutive days had no effect on this spontaneous conversion of naïve CD4 T cells into iTregs (Fig. 2E). It was reported that the treatment with rapamycin could promote the conversion of naïve CD4 T cells into iTregs [8,9]. We therefore further examined if TET had the capacity to inhibit or enhance rapmycin-induced iTregs. To this end, Foxp3/gfp^- CD4 cell-recipient mice were treated with rapamycin (3 mg/kg, i.p.) for three days. Some mice were further treated with TET (50 mg/kg/day, i.p.) for three consecutive days immediately after rapamycin treatment (Fig. 2D). The result showed that rapamycin treatment resulted in the conversion of 7–10% of naïve CD4 T cells into iTregs in the spleens or in the LNs (Fig. 2E-F). The treatment with TET did not increase or inhibit rapamycin-induced iTregs (Fig. 2E-F, Supplementary Fig. 5, Supplementary Fig. 6). Therefore, TET has no effect on the conversion of iTregs in both *in vitro* and *in vivo* settings.

4. Discussion

Previously, it has been reported that TET could inhibit the development of a broad spectrum of inflammatory diseases. For example, streptozotocin-induced rat type 1 diabetes, a known Th1 response [12], could be inhibited by intravenous injection of TET [13]. TET was also purportedly able to inhibit the Th2 response-mediated inflammation, and consequently effective in the treatment of patients with asthma [14], dermatitis in rats [15], and experimental allergic conjunctivitis in mice [16]. Furthermore, it was shown that the treatment with TET in Lewis rats could reduce the incidence of relapsing experimental allergic encephalitis mediated by Th17 cells [17]. More recently, TET was shown to inhibit Th17 response-mediated CIA (collagen-induced arthritis) in mice [5,6]. The results of these studies can at least partially be explained by our observation that TET potently inhibits the differentiation of proinflammatory Th1, Th2 and Th17 cells, while sparing the differentiation of immunosuppressive iTreg cells, in both *in vitro* and *in vivo* experimental settings.

Although we could confirm the observation by Dai and colleagues that TET had the capacity to inhibit the differentiation of Th17 cells, however, we were not able to reproduce their observation that TET was able to promote *de novo* differentiation of Foxp3-expressing iTregs from naïve CD4 T cells [5,6]. As shown in Fig. 1B, TET has no effect on the induction of iTregs in both standard *in vitro* iTreg differentiation assay (TGFβ, anti-CD3/CD28 Abs), and *in vivo* spontaneous as well as rapamycin-induced iTreg conversion studies (Fig. 2E-F). In our experiments, flow-sorted $CD4^+CD25^-$Foxp3/gfp^- cells were used to unambiguously identify non-Tregs in the starting population. In Dai and colleagues study, “naïve CD4 T cells” were used in the iTreg convention study. It is known that the Treg pool contains a substantial proportion of cells with naïve phenotype [18] which could be sorted into “naïve CD4 T cells” population. Therefore, it is possible that in Dai and colleague's study, the increased number of Tregs after TET treatment was actually resulted from the expansion of Foxp3-experssing Tregs contained in “naïve CD4 T cells”. In fact, we have observed that TET had the capacity to expand pre-existing naturally occurring Tregs (nTregs) in both *in vitro* and *in vivo* studies (our unpublished data).

Previously it was shown that TET at a higher concentration (5 or 10 μM) inhibited Th1 or Th2 cytokine production from activated T cells, and this effect was attributable to the inhibition of CD28-costimulatory signaling pathway [19]. In our study, lower concentrations (0.1–1 μM) of TET were used in the *in vitro* study. In this concentration range, TET did not inhibit anti-CD3/CD28-induced the proliferation of naïve CD4 T cells (Fig. 1A) and did not inhibit the generation of iTregs (Fig. 1B). This mitigates the role of blockade of CD28-costimulatory signaling pathway in the inhibition of Th1, Th2 and Th17 differentiation. It was also proposed that agonistic effect of TET on aryl hydrocarbon receptor was responsible for the inhibition of differentiation of Th17 cells while promoting the generation of iTregs [5,6]. However, since iTreg generation was not changed by TET in our study. Therefore, in addition to the activation of aryl hydrocarbon receptor, other mechanism underlying the effect of TET in the inhibition of Th1, Th2 and Th17 responses should be further studied.

Some most frequently used immunosuppressive agents, such as calcineurin inhibitors (cyclosporine and tacrolimus, CNIs), can impair the function and reduce the number of Tregs [20]. This property may cause the break of immune tolerance and induction of unwanted inflammatory responses in a clinical setting. Thus, it is highly desirable to develop Treg-friendly immunosuppressive drugs [20]. The capacity of TET in the inhibition of differentiation of proinflammatory Th1, Th2 and Th17 cells, while permitting *de novo* iTreg differentiation, make this Chinese herb-derived compound a promising immunosuppressive agent for future research and development.

Ethics statement

This study was carried out in accordance with the recommendations of approved guidelines of Animal Research Ethics Committee, University of Macau. The protocol was approved by the Animal Research Ethics Committee of University of Macau.

Author contributions

HZ and TH performed the experiments. HZ and XC designed the experiments and wrote the manuscript. All authors agree to the submission of the manuscript.

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.01.040.

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