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Research Article

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p53 promotes the expansion of regulatory T cells via DNMT3a- and TET2- mediated Foxp3 expression in sepsis

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Abstract

Background: Immunosuppression is an important characteristic of sepsis and is closely related to poor outcomes. Regulatory T cells (Tregs) contribute to immune suppression by inhibiting effector T cell (Teff) proliferation and differentiation. We aimed to investigate the role of p53 in Treg expansion after sepsis.

Methods: We constructed a sepsis model in wild-type (WT) and $p53^{f/f}$/CD4-Cre^{+} mice by cecal ligation and puncture (CLP) and evaluated the proportions of $CD4^{+}CD25^{+}$ $Foxp3^{+}$ Tregs by flow cytometry. The expression levels of forkhead/winged helix transcription factor p3 (Foxp3), DNA methyltransferase enzyme (DMNT)3a and ten–eleven translocation (TET)2 were examined using quantitative real-time PCR and Western blot analysis. Treg-specific demethylation region (TSDR) methylation sites in cells were analyzed by bisulfite-sequencing PCR. Furthermore, the direct binding of p53 to the Dnmt3a and TET2 promoters was illustrated using a luciferase assay. The suppressive ability of Tregs was indicated by enzyme-linked immunosorbent assay analysis of cytokine levels and the proliferation of cocultured Teffs. Finally, mortality rates after CLP were compared among WT and $p53^{f/f}$/CD4-Cre^{+} mice.

Results: The proportion of $CD4^{+}CD25^{+}$ $Foxp3^{+}$ Tregs was significantly reduced in $p53^{f/f}$/CD4-Cre^{+} mice compared to WT mice after CLP. The enhanced expression of Foxp3 in WT mice was downregulated in the $p53^{f/f}$/CD4-Cre^{+} group. We found decreased DMNT3a and increased TET2 levels after CLP. However, the dysregulation of DNMT3a and TET2 was significantly reversed in $p53^{f/f}$/CD4-Cre^{+} mice. TSDR underwent increased demethylation in $p53^{f/f}$/CD4-Cre^{+} mice. Luciferase activity indicated direct binding of p53 to the promoter regions of DNMT3a and TET2 to regulate their transcription. Consequently, Tregs from $p53^{f/f}$/CD4-Cre^{+} CLP mice exhibited limited suppressive ability, as indicated by the reduced production of transforming growth factor-β and interleukin 10 (IL-10). In the coculture system, Teffs showed preserved production of IL-2, differentiation into Th1 cells and proliferation in the presence of Tregs isolated from $p53^{f/f}$/CD4-Cre^{+} CLP mice. Finally, the mortality rate of the $p53^{f/f}$/CD4-Cre^{+} group after CLP was significantly reduced in comparison to that of the WT group.

Conclusion: p53 appears to be critical for Foxp3 expression and consequent Treg expansion by regulating the induction of DNMT3a and TET2, thereby resulting in Foxp3-TSDR demethylation in the context of sepsis.

Key words: Sepsis, Immunosuppression, p53, Regulatory T cells, Foxp3, DNMT3a, TET2

Highlights

- Sepsis-induced p53 upregulation promoted Tregs expansion.
- p53 was responsible for enhanced Foxp3 expression during sepsis mediated by DNMT3a and TET2.
- p53 regulated DNMT3a and TET2 transcription by direct binding to their promoter regions.

Background

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection [1]. Sepsis has become a severe health-care problem leading to 11 million deaths annually [2]. Although clinical advances in antibiotic treatment and organ support have decreased in-hospital mortality, sepsis survivors still suffer a high risk of secondary infection [3].

In recent decades, accumulating evidence has demonstrated that immunosuppression drives sepsis to poor outcomes [4,5]. A significant loss of $CD4^+$ T cells has been revealed in septic patients, which is the leading cause of an impaired immune response. Moreover, $CD4^+$ T cells are prone to differentiate into suppressive helper T-cell (Th)2 cells that aggravate the immunosuppressive state [6,7].

Although regulatory T cells (Tregs) are a small proportion of $CD4^+$ T lymphocytes in the adaptive immune system, they exert potent suppressive effects on effector T cell (Teff) activation by secreting inhibitory cytokines, including interleukin 10 (IL-10), transforming growth factor-β (TGF-β) and IL-35, which make them pivotal immunosuppressive agents during sepsis [8]. A clinical study described an increased percentage of peripheral Tregs in patients with septic shock. The expansion of Tregs occurs from the early phase of sepsis and persisted at high levels in patients suffering from poor outcomes [9,10].

During oncogenesis, p53 acts as a surveillance factor and can lose its normal ability to eliminate hyperproliferative tumor cells. The relationship between p53 and Tregs is controversial in cancer studies. A marked increase in Tregs was reported in p53-null mice and correlated with accelerated tumor growth [11]. In breast carcinomas, positive p53 expression was significantly related to the infiltration of Tregs [12]. In rheumatoid arthritis, p53 skewed T-cell differentiation to Tregs and suppressed Th17 differentiation [13].

In our previous report, we revealed p53 to be an immunosuppressive molecule. In p53-deficient mice, splenic $CD4^+$ T cells exhibited more effective proliferation and were more prone to Th1 differentiation than those from wild-type (WT) mice after cecal ligation and puncture (CLP)-induced sepsis [14]. Following this evidence, we investigated the potential role of p53 in the expansion of Tregs and its regulatory pathway under septic challenge in this study.

Methods

Animals

C57BL/6 mice (male, 6–8 weeks, 18–22 g) were purchased from the Laboratory Animal Institute, Chinese Academy of Medical Sciences (Beijing, China) and housed in specific pathogen-free conditions. C57BL/6 mice containing floxed Trp53 alleles ($Trp53^{fl/fl}$) and Cre in CD4-positive cells (CD4-Cre) were purchased from Jackson Laboratories (Bar Harbor, ME, USA). The $p53^{f/f}$/CD4-Cre^+ mice were produced by crossbreeding $Trp53^{fl/fl}$ mice with heterozygous CD4-Cre mice. All animal experimental manipulations were performed in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of the Chinese PLA General Hospital (No. SYXK2016–0014), Beijing, China.

CLP

Mice were subjected to laparotomy under anesthesia via inhalation of 2% isoflurane. Afterwards the cecum was isolated, ligated and punctured through using a 21-gauge needle. Sham operation was performed without ligation and puncture. After operation, the peritoneum and skin were closed and pre-warmed normal saline was applied to all animals for resuscitation through subcutaneous injection.

Isolation of splenic cells

$CD4^+$ T cells were isolated using a mouse $CD4^+$ T cell isolation kit (MiltenyiBiotec, BergischGladbach, Germany) and then were further labeled with CD25-PE and anti-PE microbeads. $CD4^+CD25^-$ effector T cells were collected by passing through the LS column and the $CD4^+CD25^+$ Tregs were collected through a subsequent positive selection.

Cell culture and treatment

Isolated splenic $CD4^+$ T cells were resuspended in RPMI 1640 (containing glutamine, penicillin, streptomycin and HEPES) with 10% fetal bovine serum and seeded into

anti-mouse CD3 (1 μg/ml) pre-coated 96- or 6-well plates. Soluble anti-mouse CD28 antibodies (1 μg/ml) were supplied as well. After a 12-h incubation, cells were stimulated with lipopolysaccharide (LPS) (1 μg/ml), pifithrin-α (PFT) (20 μmol/l) or nutlin-3a (10 μmol/l).

Flow cytometry analysis

More than 10,000 events from each sample were analyzed by passing through a FACScan flow cytometer (BD Biosciences, CA). Results were analyzed using CellQuest software.

Western blotting

The collected cells were treated with RIPA lysis buffer to isolate the whole cell protein. Protein samples were separated by electrophoresis in a 10–12% SDS-PAGE system and transferred onto PVDF membranes. Afterwards, membranes were blotted as previous described [14]. The protein bands were quantified by densitometry using National Institutes of Health ImageJ software.

Real-time PCR

RNA was extracted from cells using an RNeasy Mini Kit (Qiagen) and reverse transcribed into cDNA using a Super Script III First-Strand Synthesis System (Takara Bio, Inc., Shiga, Japan) according to the manufacturer's instructions. A BioRad Real-Time PCR system was used for real-time PCR amplification. Primers are provided in the online Supplementary data Table 1.

Bisulfite-sequencing PCR

Genomic DNA was isolated and purified from $CD4^+$ T cells using an AllPrep DNA/RNA Micro kit (Qiagen GmbH, Hilden, Germany). Sodium bisulfite conversion bisulfite-sequencing PCR was performed using an EpiTech Bisulfite kit (Qiagen GmbH, Hilden, Germany). PCR products were subcloned into a pTZ57R/T vector (InsTAcloneTM PCR Cloning kit, Fermentas Inc., Ontario, Canada). Ligated vectors were then transferred into the Escherichia coli (E. coli) strains DH5α and ten positive colonies were extracted by Qiaprep® Spin Miniprep kit (Qiagen GmbH, Hilden, Germany) and sequenced. The sequences were analyzed with bisulfite sequencing DNA methylation analysis (BISMA) online software.

Transfection

Lentivirus vectors expressing the DNA fragments encoding GFP-tagged full-length DNA methyltransferase enzyme (DNMT) 3a, DNMT3a mRNA-targeted small interfering ribonucleic acid (siRNA), ten–eleven translocation 2 (TET2) and TET2 mRNA-targeted siRNA were designed, constructed, packed and purified by Obio Technology Co. Ltd (Shanghai, China). Jurkat T cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. Lentivirus was added to the culture medium and incubated for a further 8 h. Then cells were washed and subjected to further culture until stable expression of GFP was detected.

Luciferase assay

Trp53 expression full-length plasmid (pcDNA3.1-*p53*) was obtained by inserting the full length of *p53* into pcDNA3.1 vector. The binding site of p53 on the Dnmt3a or TET2 promoter region was predicted on the PROMO online tool. Mutant sequences were constructed by Cyngentech Co. Ltd. Luciferase reporter plasmids pGL4.1 were reconstructed by inserting the DNMT3a promoter sequence, TET2 promoter sequence or their mutant sequences and transfected into HEK293T cells along with pcDNA3.1-*p53* and cultured for 3 days. Luciferase activity was assessed using the dual-luciferase reporter kit according to the manufacturer's instructions (Promega, Madison, WI, USA).

Enzyme-linked immunosorbent assay for cytokine concentrations

TGF-β, IL-10, IL-2, IL-4 and interferon (IFN)-γ concentrations in culture supernatants were quantitated by enzyme-linked immunosorbent assay (ELISA) kits for mice following the protocols provided by the manufacturer. Plates were read in a microplate reader (Spectra MR, Dynex, Richfield, MN, USA) at 450 nm.

Cell-viability assessment

Cell viability was evaluated using the cell counting kit-8 from Dojindo Laboratories (Kumamoto, Japan) according to the manufacturer's instructions.

Carboxfluorescein succinimidyl ester

$CD4^+CD25^-$ T cells were stained with carboxfluorescein succinimidyl ester (CFSE; BD Biosciences, Mountain View, CA, USA) and then cocultured with $CD4^+CD25^+$ Tregs isolated from WT or CLP $p53^{f/f}$/CD4-Cre^+ mice in 24-well plates (ratio 4 : 1). After stimulation with anti-CD3/CD8 antibodies for 72 h, CSFE dilution in cells was analyzed by FACS (BD Biosciences, Mountain View, CA, USA).

Statistical analysis

The results were analyzed with a one-way ANOVA and shown as means ± SD. The significant differences between groups were measured by Fisher's least significant difference. Survival of experimental animals was assessed by the Kaplan–Meier method and the log-rank test was performed to analyze significant differences. SPSS 17.0 was used for statistically significant analysis and a difference was set at $p < 0.05$.

Results

p53 is upregulated in expanded Tregs in sepsis

We isolated $CD4^+CD25^+$ Tregs from $CD4^+$ T cells and determined the expression of p53 in $CD4^+CD25^-$ Teffs and Tregs. The expression of p53 increased rapidly in Tregs and peaked after 24 h. In comparison, p53 activation in Teffs was delayed and peaked at 72 h after CLP (Figure 1a). The majority of $CD4^+CD25^+$ Tregs expressed forkhead/winged helix transcription factor p3 (Foxp3), which is critical for their suppressive function. Consistent with the upregulation

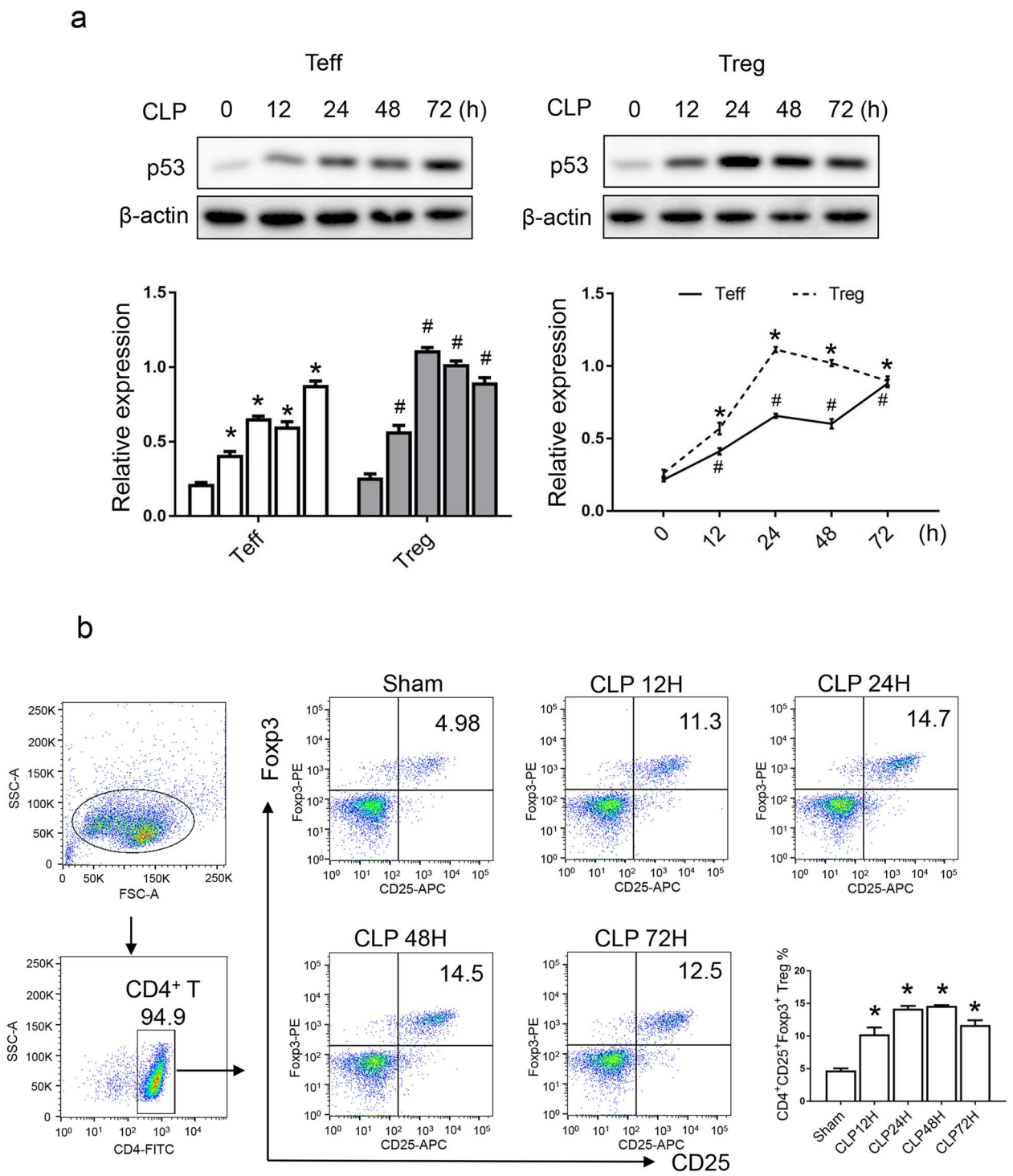

Figure 1. Sepsis activates p53 expression in expanded Tregs. (**a**) C57BL/6 mice (n = 15) were subjected to CLP operation. Splenocytes were isolated and separated into $CD4^+CD25^-$ Teffs and $CD4^+CD25^+$ Tregs, and cell lysate was extracted and used for western blot analysis of p53 and β-actin. The relative expression of p53 was quantified using ImageJ and normalized to that of the loading control. $^*p < 0.01$ *vs* Teffs sham group; $^\#p < 0.01$ *vs* Tregs sham group. (**b**) C57BL/6 mice were subjected to sham (n = 3) or CLP (n = 5) operations and sacrificed after 12 to 48 h. Percentages of $CD4^+CD25^+Foxp3^+$ Tregs in splenic $CD4^+$ T cells were analyzed using flow cytometry. $^*p < 0.01$ *vs* sham group. *CLP* cecal ligation and puncture

of p53, Foxp3 expression in $CD4^+$ T cells was induced within 24 h (supplementary Figure 1a, see online supplementary material). The proportion of $CD4^+CD25^+$ $Foxp3^+$ Tregs was evaluated as shown in Figure 1b, and these cells were 4.98% of $CD4^+$ T cells in sham mice, increased to 14.7% at 24 h and were sustained at high levels after CLP. Although the total numbers of splenic lymphocytes and $CD4^+$ T cells decreased, the absolute number of $CD4^+CD25^+$ $Foxp3^+$ Tregs increased

(supplementary Figure 1b–d). Thus, p53 expression was upregulated along with the expansion of Tregs in the early phase of sepsis.

Expansion of Tregs is restricted in p53-deficient mice

To investigate the impact of upregulated p53 on the differentiation of Tregs, we injected the p53 inhibitor PFT prior to CLP and observed decreased Treg proportions in comparison to CLP alone (Figure 2a). To exclude the influence of other immune cells, we generated CD4-specific p53 knockout mice ($p53^{f/f}$/CD4-Cre^{+}). The expression of p53 was completely depleted in $CD4^{+}$ T cells (supplementary Figure 2, see online supplementary material). As shown in Figure 2b, the percentage of Tregs in WT mice increased from 4.62% in the sham group to 13.7% in the CLP group. However, in $p53^{f/f}$/CD4-Cre^{+} mice, the increase in the Tregs proportion after CLP (5.05 to 10.3%) was greatly decreased. *In vitro* experiments were performed on isolated splenic $CD4^{+}$ T cells and presented a similar result (Figure 2c). *In vitro* stimulation with LPS led to an expanded proportion of Tregs in $CD4^{+}$ T cells from WT mice (5.1 to 10.5%); however, this expansion was limited in p53-deficient $CD4^{+}$ T cells (5.3 to 8.2%). *In vivo* and *in vitro* results suggested that p53 was important for the expansion of Tregs in sepsis.

Foxp3 expression is decreased in the absence of p53

Foxp3 has been defined as a pivotal regulator that controls the differentiation and function of Tregs. Foxp3 mRNA transcription was activated after CLP in $CD4^{+}$ T cells from WT mice but was significantly downregulated in those from $p53^{f/f}$/CD4-Cre^{+} mice (Figure 3a). *In vitro* assessment further confirmed an increase in Foxp3 mRNA expression in LPS-exposed $CD4^{+}$ T cells that was inhibited by PFT. In contrast, treatment with nutlin-3a, a specific p53 activator, activated Foxp3 mRNA transcription (Figure 3b). Consistently, the protein level of Foxp3 was increased in $CD4^{+}$ T cells from WT CLP mice but was significantly lower in $p53^{f/f}$/CD4-Cre^{+} CLP mice (Figure 3c). Moreover, cells expressing Foxp3 were greatly increased after CLP in WT mice. In the $p53^{f/f}$/CD4-Cre^{+} mice, however, the percentages of $Foxp3^{+}$ cells were significantly lower in both the sham and CLP groups (Figure 3d). Accumulating evidence has clarified that demethylation of the cytosine–guanine dinucleotide (CpG) motif in the Treg-specific demethylation region (TSDR) is dispensable for Foxp3 expression [15]. Herein, we compared the methylation status of TSDR in WT and $p53^{f/f}$/CD4-Cre^{+} mice. TSDR in $CD4^{+}$ $CD25^{+}$ Tregs underwent high demethylation (supplementary Figure 3, see online supplementary material). However, demethylation sites in $CD4^{+}$ $CD25^{-}$ T cells were increased after CLP in both types of mice, which might indicate a partial conversion from Teffs into Tregs during sepsis. Compared to sham CLP mice, the demethylation of TSDR in $CD4^{+}$ $CD25^{-}$ T cells from $p53^{f/f}$/CD4-Cre^{+} CLP mice was inhibited, as indicated by a higher percentage of methylated CpG motifs (Figure 3e). Collectively, demethylation-mediated Foxp3 expression was largely dependent on p53 in the development of sepsis.

Foxp3 expression is related to DNMT3a and TET2

DMNTs methylate the 5 positions of the C residue in CpG to form 5-methylcytosine, which is reversely catalyzed by the TET family to yield 5-hydroxymethylcytosine and consequent demethylation [16]. Among DNMTs, DNMT3a mRNA levels were obviously downregulated after CLP, and TET2 was the only affected member of the TET family that was upregulated at the mRNA level (Figure 4a, b). Western blot results are shown in Figure 4c and confirmed the alterations in protein levels. *In vitro* results indicated consistent dysregulation of DNMT3a and TET2 after LPS treatment (supplementary Figure 4a, see online supplementary material). We further interfered with the expression of DNMT3a or TET2 with transfecting mRNA- or siRNA-carrying plasmids in Jurkat T cells (supplementary Figure 4b). As shown in Figure 4d, Foxp3 expression was elevated in LPS-treated vector cells, and this effect was abolished in DNMT3a-overexpressing cells but not affected in DNMT3a-knockdown cells. In TET2-overexpressing cells, the basal level of Foxp3 was increased but was not further enhanced after LPS stimulation. In contrast, in TET2-knockdown cells, the basal level of Foxp3 was largely decreased, and although it was slightly activated by LPS treatment, it was significantly lower than that in vector cells (Figure 4e). Therefore, the Dnmt3a and TET2 imbalance might contribute to the induction of Foxp3 expression after sepsis.

p53 regulates DNMT3a and TET2 expression

Considering the involvement of p53 in Foxp3 expression, we further investigated its role in regulating DNMT3a and TET2 expression. The results in Figure 5a show that DNMT3a mRNA transcription was downregulated after CLP in WT mice; in contrast, it was maintained at a higher level in $p53^{f/f}$/CD4-Cre^{+} mice. TET2 mRNA showed the opposite change in expression after CLP. Western blot analysis indicated similar results at the protein level (Figure 5b). To verify the hypothesis that p53 interferes with DNMT3a or TET2 gene transcription, we used the PROMO 3.0.2 online tool to analyze the DNMT3a and TET2 promoter regions and found numerous possible binding sites, which are indicated by blue arrows in Figure 5c, d. We cotransfected p53-expressing pcDNA3.1 with the WT DNMT3a/TET2 promoter or the binding site deletion mutant sequences expressing the luciferase reporting plasmid into HEK293T cells. As shown in the left panels, the WT DNMT3a promoter had decreased transcriptional activity upon p53 expression, whereas this inhibition was relieved when the predicted binding sites were all deleted. In contrast, p53 enhanced WT TET2 promoter transcriptional activity, which was significantly abolished in its mutant promoter. The direct binding sites of p53 are present in human DNMT3a and TET2 promoters (supplementary Figure 5, see online supplementary material). We further cut the promoter sequences into three segments according to the distribution of the predicted binding sites to investigate the possible binding regions (right panels in Figure 5c, d). Among the three mutant DNMT3a promoters, mutant 3 ($-259 \sim -1$)

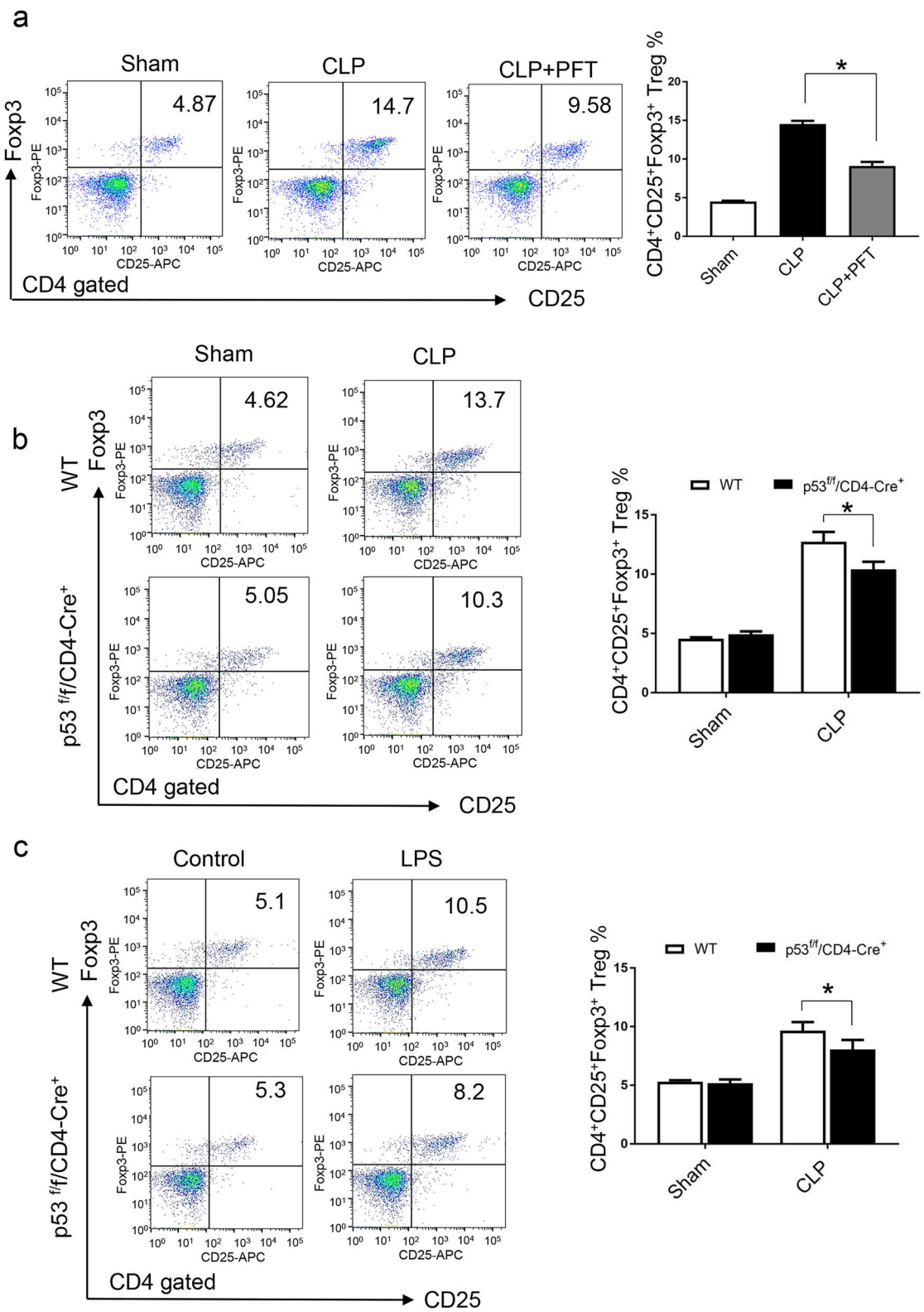

Figure 2. p53 is involved in the expansion of Tregs in sepsis. (**a**) C57BL/6 mice were subjected to sham (n = 3) or CLP (n = 10) operations. PFT was peritoneally injected prior to CLP. After 24 h mice were sacrificed; (**b**) WT and $p53^{f/f}$/CD4-Cre^{+} mice were subjected to sham (n = 3) or CLP (n = 5) procedures and sacrificed 24 h after operation. The percentages of $CD4^{+}CD25^{+}Foxp3^{+}$ Tregs in splenic $CD4^{+}$ T cells were analyzed using flow cytometry (*$p < 0.01$). (**c**) Splenic $CD4^{+}$ T cells were isolated from both WT and $p53^{f/f}$/CD4-Cre^{+} mice and exposed to PBS (control) or LPS (1 μg/ml) for 24 h. Afterwards, the percentages of $CD4^{+}CD25^{+}Foxp3^{+}$ Tregs were analyzed by flow cytometry (*$p < 0.01$). *CLP* cecal ligation and puncture, *PFT* pifithrin-α, *WT* wild type, *PBS* phosphate-buffered saline, *LPS* lipopolysaccharide, *Foxp3* forkhead/winged helix transcription factor p3

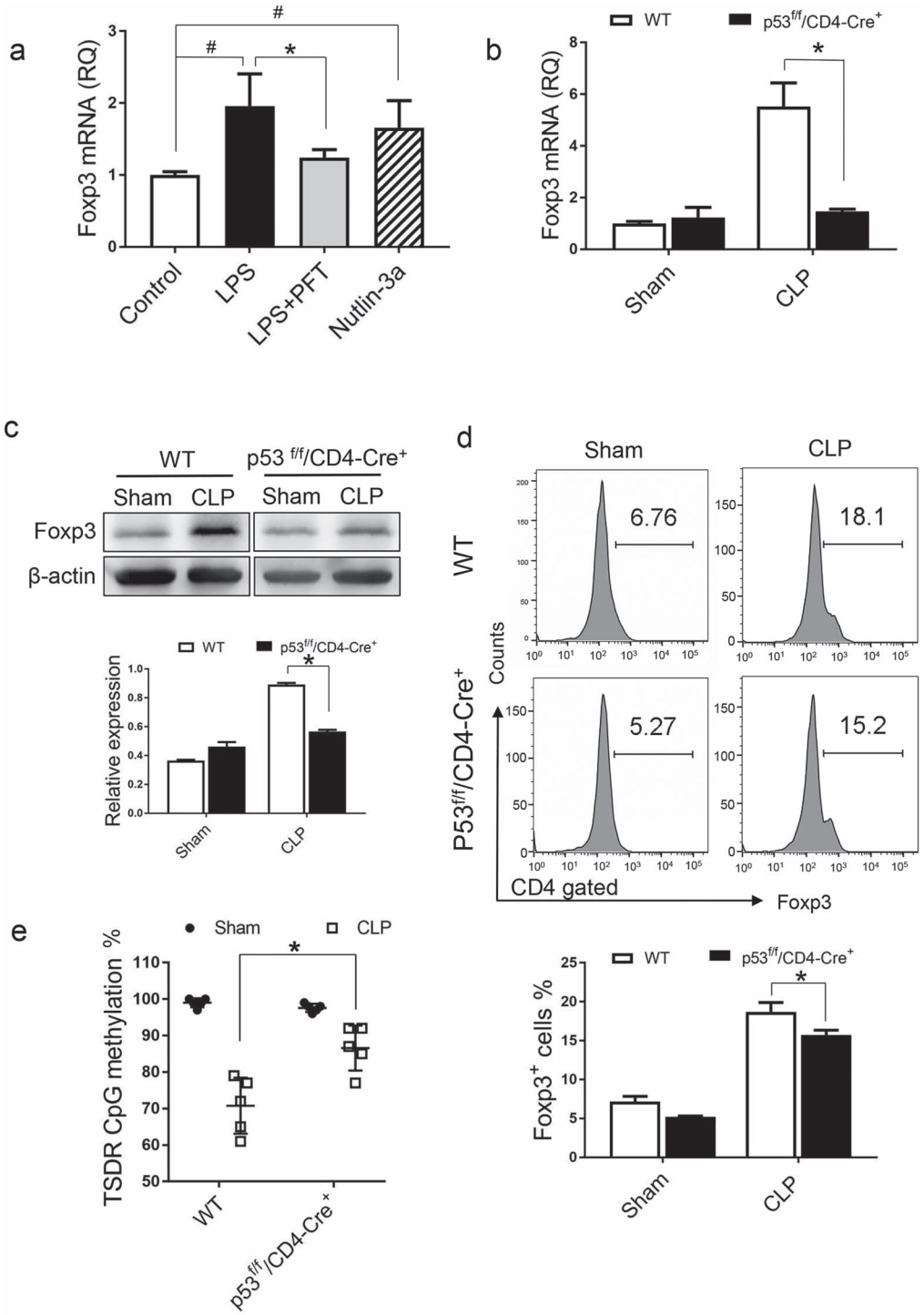

Figure 3. Sepsis induces Foxp3 expression required for p53. (**a**) Splenic CD4$^+$ T cells were isolated from C57BL/6 mice and exposed to PBS (control), LPS (1 μg/ml), LPS + PFT (20 μM) or Nutlin-3a (10 μM) for 24 h. Then, mRNAs were extracted from cells and subjected to qPCR analysis of Foxp3 and β-actin mRNAs. The relative expressions were normalized to the loading control ($^*p < 0.01$ *vs* LPS; $^\#p < 0.01$ *vs* control group). (**b–d**) WT and p53$^{f/f}$/CD4-Cre$^+$ mice were subjected to sham ($n = 10$) or CLP ($n = 20$) operations and sacrificed after 24 h. (b) The total RNAs were extracted from splenic CD4$^+$ T cells and subjected to qPCR analysis of Foxp3 mRNA. The relative expression levels were normalized to sham of WT or p53$^{f/f}$/CD4-Cre$^+$ group, respectively ($^*p < 0.01$). (c) Splenic CD4$^+$ T were isolated and used for Western blot analysis of Foxp3 and β-actin. The relative expression of Foxp3 was quantified using ImageJ and normalized to that of β-actin ($^*p < 0.01$). (d) The percentages of Foxp3$^+$ cells were analyzed by flow cytometry ($^*p < 0.01$). (**e**) Total DNA was extracted from CD4$^+$CD25$^-$ T cells and subjected to BSP. The percentages of methylated CpG motifs in TSDR are indicated ($^*p < 0.01$). *PBS* phosphate-buffered saline, *LPS* lipopolysaccharide, *PFT* pifithrin-α, m*RNA* messenger ribonucleic acid, *qPCR* quantitative real-time polymerase chain reaction, *Foxp3* forkhead/winged helix transcription factor p3, *WT* wild type, *CLP* cecal ligation and puncture, *DNA* deoxyribonucleic acid, *CpG* cytosine–guanine dinucleotides, *TSDR* Treg specific demethylation region, *BSP* bisulfite-sequencing polymerase chain reaction

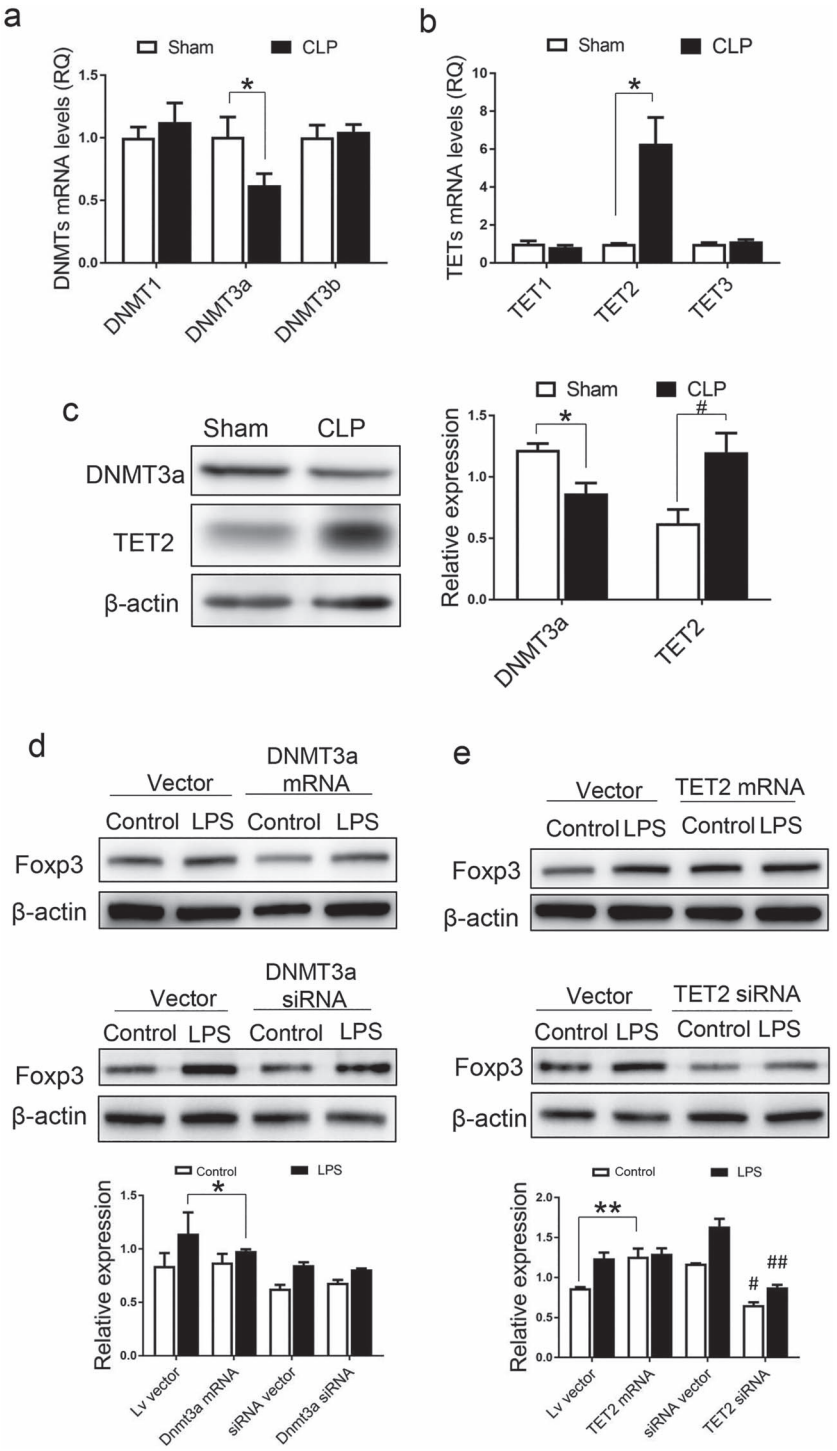

Figure 4. Foxp3 expression is regulated by DNMT3a and TET2. (**a**, **b**) C57BL/6 mice were subjected to sham (n = 5) or CLP (n = 10) procedure and sacrificed after 24 h. Total mRNAs were extracted from splenetic $CD4^+$ T cells and subjected to qPCR analysis of DNMT1, DNMT3a, DNMT3b, TET1, TET2 and TET. β-Actin was used as the loading control. The mRNA levels were normalized to the sham group ($^*p < 0.01$). (**c**) Total proteins were extracted from cells and used for western blotting of DNMT3a, TET2 and β-actin. The relative expressions were quantified using ImageJ and normalized to that of the loading control ($^*p < 0.01$, $^\#p < 0.01$). (**d**) Jurkat T cells were transfected with lentivirus containing DNMT3a mRNA or siRNA expressing plasmids or blank vector. (**e**) Jurkat T cells were transfected with lentivirus containing TET2 mRNA or siRNA expressing plasmids or blank vector. After stable expression, cells were stimulated with PBS (control) or LPS (1 μg/ml) for 24 h. Then total proteins were extracted from cells and used for western blotting analysis of Foxp3 and β-actin. The relative expression of Foxp3 was quantified using ImageJ and normalized to that of β-actin ($^*p < 0.05$, $^{**}p < 0.01$, $^\#p < 0.01$ *vs* control group in siRNA vector, $^{\#\#}p < 0.01$ *vs* LPS group in siRNA vector). *CLP* cecal ligation and puncture, m*RNA* messenger ribonucleic acid, *siRNA* small interfering ribonucleic acid, *qPCR* quantitative real-time polymerase chain reaction, *DNMT* DNA-buffered saline, *LPS* lipopolysaccharide, *Foxp3* forkhead/winged helix transcription factor p3

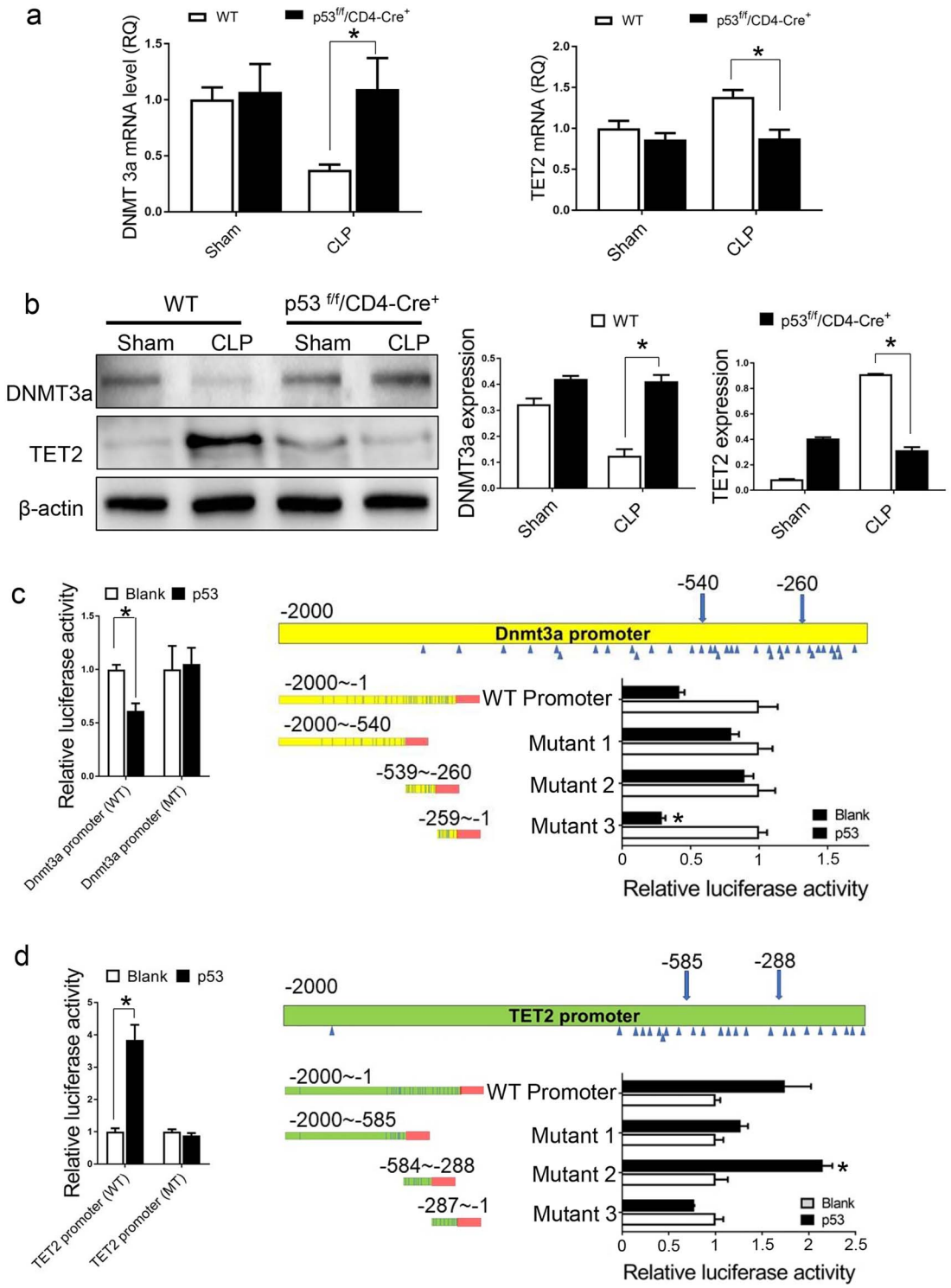

Figure 5. p53 directly regulates DNMT3a and TET2 expression. (**a**, **b**) WT and p53$^{f/f}$/CD4-Cre^{+} mice were subjected to sham (n = 5) or CLP (n = 10) operation. After 24 h mice were sacrificed and CD4^{+} T cells were isolated from spleens. (**a**) Cells were subjected to mRNA extraction followed by qPCR analysis of DNMT3a and TET2 mRNAs. β-Actin was used as the loading control. The relative mRNA levels were normalized to that in the sham group ($^{*}p < 0.01$). (**b**) Cells were subjected to protein extraction and western blot analysis of DNMT3a and TET2. β-Actin was used as the loading control. The relative expression levels were quantified using ImageJ and normalized to that of the loading control ($^{*}p < 0.01$). (**c**, **d**) Predicted binding sites of p53 on DNMT3a or TET2 promoter regions are indicated with blue arrows or vertical lines. pcDNA3.1-*p53* and pGL4.1 carrying WT promoter or mutant promoter with binding sites deletion (left panels) or segment of promoter sequence (right panels) were co-transfected into HEK293T cells. After 3 days, cells were lysed and subjected to luciferase activity detection ($^{*}p < 0.01$ *vs* blank group). *CLP* cecal ligation and puncture, m*RNA* messenger ribonucleic acid, *qPCR* quantitative real-time polymerase chain reaction, *DNMT* DNA methyltransferase enzyme, *TET*, ten–eleven translocation, *HEK293T* human embryonic kidney 293 T cells, *WT* wild type

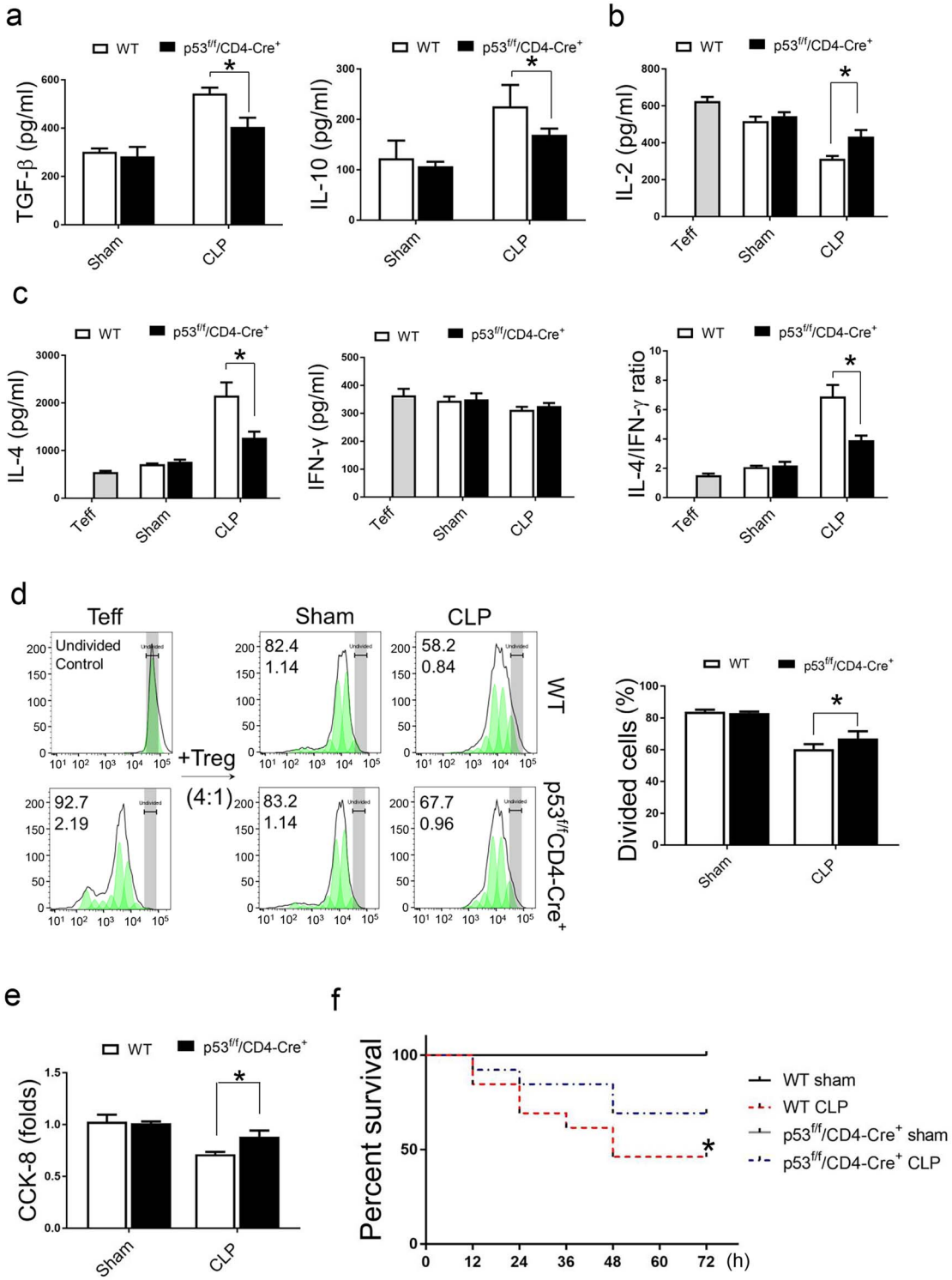

Figure 6. p53 is associated with the suppressive ability of Tregs in sepsis. WT and p53$^{f/f}$/CD4-Cre^{+} mice were subjected to sham (n = 5) or CLP (n = 10) operations and sacrificed after 24 h. (**a**) CD4^{+}CD25^{+} Tregs were isolated and cultured for 24 h, and the secretion of TGF-β and IL-10 was measured by ELISA (*$p < 0.01$). (**b**, **c**) CD4^{+}CD25^{+} Tregs were isolated from sham and CLP mice and co-cultured with WT CD4^{+}CD25^{-} Teffs in a ratio of 1 : 4 for 3 days. Then the supernatants were collected and subjected to ELISA analysis of IL-2, IL-4 and IFN-γ levels. The ratio of IL-4 and IFN-γ was analyzed. Teffs alone were used as a negative control (*$p < 0.01$). (**d**) CSFE-labeled WT CD4^{+}CD25^{-} Teffs were mixed with CD4^{+}CD25^{+} Tregs in a ratio of 4 : 1 and cultured for 3 days. Cell proliferation was detected by CFSE dilution using flow cytometry and indicated as a percentage of divided cells (*$p < 0.01$) and divided index. (**e**) The CD4^{+} T cells were isolated from mice and cultured for 3 days, and CCK-8 was added into the culture medium. The absorbance was detected and normalized with the sham group (*$p < 0.01$). (**f**) WT and p53$^{f/f}$/CD4-Cre^{+} mice were subjected to sham (n = 10) or CLP (n = 10) operations. The survival rates were recorded up to 72 h after CLP (*$p < 0.01$ *vs.* WT CLP group). *WT* wild type, *CLP* cecal ligation and puncture, *TGF* transforming growth factor, *IL* interleukin, *ELISA* enzyme-linked immunosorbent assay, *IFN* interferon, *CSFE* carboxfluorescein succinimidyl ester, *CCK-8* cell counting kit-8

presented significantly decreased transcriptional activity in response to p53, while the other two mutants showed a slight decrease that was not significant. Among the mutant TET2 promoters, only mutant 2 ($-584 \sim -288$) showed robust induction of transcription activity. Therefore, p53 modulated the expression of DNMT3a and TET2 through direct binding to their promoter regions and regulating transcription, thereby leading to enhanced Foxp3 expression.

Impaired function of $CD4^+CD25^+$ Tregs in p53-deficient mice

Tregs isolated from $p53^{f/f}$/CD4-Cre^+ mice produced limited suppressive cytokines, including TGF-β and IL-10, in comparison to those of WT mice after CLP (Figure 6a). To assess the suppressive effects of Tregs, we mixed resting WT Teffs with WT or $p53^{f/f}$/CD4-Cre^+ Tregs from sham or CLP mice (in a ratio of 4 : 1). Afterward, IL-2 production was obviously reduced in the presence of WT Tregs isolated from CLP mice, but it was markedly elevated in cocultures of Tregs from $p53^{f/f}$/CD4-Cre^+ CLP mice (Figure 6b). Moreover, Tregs from WT CLP mice led to Teff differentiation toward Th2 cells, as indicated by an increased ratio of IL-4/IFN-γ, which was relatively preserved by $p53^{f/f}$/CD4-Cre^+ Tregs (Figure 6c).

Furthermore, Tregs from $p53^{f/f}$/CD4-Cre^+ CLP mice showed limited suppressive effects, as evidenced by reduced CSFE dilution (Figure 6d). As a result, the viability of all $CD4^+$ T cells was higher in $p53^{f/f}$/CD4-Cre^+ mice than in WT mice after CLP (Figure 6e). Consequently, we noted reduced mortality in $p53^{f/f}$/CD4-Cre^+ mice compared with that in WT mice secondary to septic challenge (Figure 6e). Accordingly, p53 might mediate the functional expansion of Tregs, which possess the ability to suppress Teffs and are related to poor outcomes in sepsis.

Discussion

Sepsis is a syndrome with life-threatening organ dysfunction caused by a dysregulated host response to infection. Immunosuppression is now identified as a major cause of poor outcomes [17]. Although Tregs are a small proportion of $CD4^+$ T cells, they exert potent immunosuppressive effects on Teffs by inhibiting proliferation and functional differentiation [18]. Following the evidence from our previous study that upregulated p53 in $CD4^+$ T cells mediated the loss of proliferation and function, we further investigated the potential role of p53 in regulating Treg expansion and function in sepsis [14].

In the current study, we showed the early accumulation of p53 in $CD4^+$ $CD25^+$ Tregs, and the Treg proportion in $CD4^+$ T cells increased in the early phase of sepsis. Other studies in mice showed similar increases in Tregs after CLP, not only within 48 h but lasting up to 7 days [19,20]. Consistently, in clinical observations, Gupta *et al*. [21] reported a significantly higher Treg population in trauma patients who developed sepsis than in those without sepsis. However, in our study, we induced severe sepsis in mice that led to 50% mortality within 2 days and did not observe long-term outcomes. Some studies have noted that the increased proportion of Tregs is due to preferential loss of other non-Treg subsets [22,23]. In the current study, we observed a reduction in total splenic lymphocytes and $CD4^+$ T-cell numbers after CLP. However, the absolute number of Tregs was increased, indicating an expansion of peripheral Tregs (supplementary Figure 1).

Generally, Tregs develop in the thymus and are essential for the maintenance of immune tolerance. Some studies have used Foxp3-Cre mice to investigate the dispensable roles of target genes in Foxp3-expressing Tregs, including their impacts on the functions and maintenance of the Treg lineage [24,25]. In addition to natural development in the thymus, some postthymic events, such as sepsis, can also generate Tregs from conventional Foxp3(−) T cells [26]. To assess the role of p53 in sepsis-induced Treg expansion that was primarily derived from the conversion of $CD4^+foxp3^-$ cells, we generated $p53^{f/f}$/CD4-Cre^+ mice and found relatively lower promotion of Tregs after CLP than in WT mice. Kawashima *et al*. also reported a lower level of Tregs in $p53^{f/f}$/CD4-Cre^+ mice. However, the mice gradually developed autoimmune disease at 6 months due to fewer Tregs, which reduced body weight and induced inflammatory lesions in organs [27]. In this study, we used $p53^{f/f}$/CD4-Cre^+ mice aged 6–8 weeks, and we did not observe any dysplasia or abnormities prior to the CLP operation. Following sepsis challenge, p53 deficiency prevented Treg expansion, which might protect mice from fatal immunosuppression-associated outcomes.

In our results, we indicated an expansion of Tregs, which might be secondary to a conversion from peripheral non-Tregs in the spleen but not thymus-developed Tregs. Foxp3 is a dominant regulator of the development and immunosuppressive effects of Tregs [28,29]. Transcription of the Foxp3 gene largely depends on the demethylation of the CpG motif in noncoding sequence 2, which is also known as TSDR [15]. TSDR is almost fully methylated in effector $CD4^+$ T cells or in *in vitro* TGF-β-induced Tregs in which Foxp3 expression is transient and undergoes full demethylation to induce stable expression of Foxp3 in Tregs [30,31]. In our study, TSDR presented almost complete methylation in $CD4^+CD25^-$ T cells from sham mice. Following the onset of sepsis, some sites became demethylated, which might promote Foxp3 expression. After full demethylation, the cells might convert into Tregs by expressing stable Foxp3. Consistently, in human T cells, Kressler *et al*. established targeted demethylation of TSDR that could induce $Foxp3^+$ cells from Th1 cells [32]. These results demonstrated that demethylation of TSDR could overcome the predetermined lineage and promote the expansion of Tregs from non-Tregs.

DNA methylation balance is modulated by DNMTs (DNMT1, DNMT3a and DNMT3b) and TET proteins (TET1, TET2 and TET3) [33,34]. Among DNMTs, only DNMT3a was significantly decreased in CLP mice. Overexpression of DNMT3a in Jurkat T cells inhibited Foxp3 expression. However, a slight increase in DNMT1 in CLP mice cannot be ignored. DNMT1 is responsible for

maintaining existing methylation, while DNMT3a and DNMT3b are responsible for *de novo* methylation [35]. Wang *et al*. revealed that DNMT1 did not affect DNA methylation of Foxp3 but was required for Treg development and function [36]. Accordingly, different DNMTs might be affected in this context. As shown in our results, DNMT3a but not DNMT1 or DNMT3b expression was downregulated in the early phase of sepsis.

Sepsis greatly induced TET2 expression. TET2 deficiency led to significant suppression of Foxp3 expression. In TET2-overexpressing cells, Foxp3 expression was activated in sham mice and could not be further activated after LPS exposure. Similarly, in TGF-β-induced Tregs, vitamin C was found to augment Foxp3 expression and consequent Treg efficacy by potentiating TET2 activity [37]. In LPS-exposed macrophages, TET2 activity was inhibited, which dampened the inflammatory response [38]. Although the response of TET2 to infectious challenge varied depending on cell type, dysfunctional macrophages or expanded Tregs led to suppression of the immune response.

We further revealed p53 as a central regulator of the downregulation of DNMT3a and upregulation of TET2 in sepsis via direct binding to their promoter regions. Consistently, observations in human and mouse embryos showed that p53 restricted the expression of DNMT3a and DNMT3b but upregulated TET1 and TET2 levels [39]. In addition to regulating DNA methylation via DNMT3a and TET2, p53 has been implicated in inhibiting Foxp3 transcription directly by binding to its promoter [27]. Park *et al*. [13] noticed that p53 directly interacted with STAT5, which in turn activated Foxp3 expression. Regardless of DNA methylation or transcriptional activation, p53 is critically involved in Foxp3 expression secondary to septic challenge.

However, there are several notable limitations to the present study. First, we did not determine the dynamic changes in the expression of DNMTs and TETs or the demethylation of TSDR and Foxp3 expression in the development of sepsis, which requires further examination and might provide insights for the clinical prediction of sepsis outcomes. Second, the imbalance between DNMTs and TETs in p53-deficient embryos resulted in global DNA methylation and clonal homogeneity [39]. In this study, we used $p53^{f/f}$/CD4-Cre^{+} mice to avoid the impact on embryo development and other systems. However, the methylation status of other genes expressed in $CD4^{+}$ T cells was not examined. Third, we investigated the possible binding between p53 and DNMT3a or TET2 by detecting luciferase activity *in vitro*, while direct binding was not evidenced in the septic model *in vivo*.

Conclusions

In summary, we suggest an indirect effect of p53 on Foxp3 expression by regulating DNMT3a and TET2 expression, which contributes to the demethylation of the TSDR region in the Foxp3 locus, thereby upregulating its expression and leading to the functional expansion of Tregs. The current data provide new evidence suggesting that p53 exerts an immunosuppressive effect on the development of sepsis by promoting functional Treg expansion.

Abbreviations

BSP: Bisulfite-sequencing PCR; CCK-8: Cell counting kit-8; CLP: Cecal ligation and puncture; CpG: Cytosine–guanine dinucleotides; CSFE: Carboxfluorescein succinimidyl ester; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electropheresis; GFP: green fluorescent protein; DMNT: DNA methyltransferase enzyme; ELISA: Enzyme-linked immunosorbent assay; ANOVA: analysis of variance; Foxp3: Forkhead/winged helix transcription factor p3; IFN: Interferon; IL: Interleukin; siRNA: LPS: Lipopolysaccharide; PFT: Pifithrin-α; siRNA: Small interfering ribonucleic acid; Teffs: Effector T cells; TET: Ten–eleven translocation; TGF: Transforming growth factor; Th: Helper T cell; Tregs: Regulatory T cells; TSDR: Treg-specific demethylation region; WT: Wild-type.

Supplementary data

Supplementary material is available at *Burns & Trauma Journal* online.

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Authors' contributions

HZ and TW performed the experiments, analyzed the data and drafted the manuscript. CR and YW were responsible for animal-model construction and cell culture. ND was responsible for flow cytometry analysis. YY designed the study and revised the manuscript. All the authors read, critically revised and agreed to be accountable for the content of the work.

Conflict of interests

None declared.

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