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Sirtuin4 suppresses the anti-neuroinflammatory activity of infiltrating regulatory T cells in the traumatically injured spinal cord

Wenping Lin,[1,2] Wenkai Chen,[2] Weifeng Liu,[3] Zhengquan Xu[4] and Liqun Zhang[4]

[1]*Department of Spine Surgery, Shenzhen Pingle Orthopedic Hospital, Shenzhen, Guangdong,* [2]*Department of Orthopedic Surgery, the Second Affiliated Hospital of Fujian Medical University, Quanzhou, Fujian,* [3]*Department of Anesthesiology, the Second Affiliated Hospital of Fujian Medical University, Quanzhou, Fujian, and* [4]*Department of Spine Surgery, the First Affiliated Hospital of Fujian Medical University, Fuzhou, Fujian, China*

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Correspondence: Liqun Zhang, Department of Spine Surgery, the First Affiliated Hospital of Fujian Medical University, 20 Chazhong Road, Fuzhou, Fujian, China.
Email: zhangliqun@fjmu.edu.cn
Senior author: Wenping Lin.

Summary

The neuroinflammation following traumatic spinal cord injury (SCI) is a critical process that impacts both the injury and the recovery of spinal cord parenchyma. Infiltrating regulatory T (Treg) cells are potent anti-inflammatory cells that restrain post-SCI neuroinflammation. To understand the molecular mechanisms underlying the activity of infiltrating Treg cells, we used a mouse spinal cord compression injury model to analyze the role of Sirtuins (SIRTs) in the modulation of infiltrating Treg cell functions. We found that the expressions of SIRT4 and SIRT6 were upregulated in infiltrating Treg cells. Using lentivirus-mediated gene expression or RNA interference, we revealed that SIRT4 substantially inhibited the expression of Foxp3, interleukin-10, and transforming growth factor-β in Treg cells, whereas SIRT6 had little effect on Treg cells. Consistently, SIRT4 overexpression weakened the suppressive effect of Treg cells on lipopolysaccharide-stimulated spinal cord CD11b$^+$ myeloid cells. Knockdown of SIRT4 enhanced the anti-inflammatory activity of infiltrating Treg cells in the parenchyma of injured spinal cords. Additionally, SIRT4 overexpression blocked *in vitro* Treg cell generation from conventional T cells. Furthermore, SIRT4 down-regulated 5′ AMP-activated protein kinase (AMPK) signaling in Treg cells, whereas the AMPK agonist AICAR restored the expression of Foxp3 and interleukin-10 in SIRT4-overexpressing Treg cells. In conclusion, our research unveils a new mechanism by which the post-SCI neuroinflammation is regulated.

Keywords: flow cytometry; neuroinflammation; T cell.

Introduction

Spinal cord injury (SCI) is a refractory neurological disorder comprising the primary mechanical injury and secondary inflammatory response-mediated injury. Broken bones, distorted disk tissues, and ligaments bruise or tear into the spinal cord tissue, initiating the primary damage immediately after the traumatic injury has taken place. The secondary neuroinflammatory reactions, which mediate additional and extensive neurological injury, start within minutes and continue for several weeks following the primary injury. After that, the injury enters the chronic phase for days to years after the acute injury, causing long-term neurological damage in both orthograde and retrograde directions. Alleviating the harmful acute neuroinflammatory reactions could be a therapeutic strategy to limit the injury and promote functional recovery.

Abbreviations: ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; APC, allophycocyanin; FITC, fluorescein isothiocyanate; IL-10, interleukin-10; iTreg cells, inducible Treg cells; LE, control lentivirus; LS4, lentivirus encoding SIRT4; LS6, lentivirus encoding SIRT6; mAb, monoclonal antibody; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor-κB; nTreg cells, natural Treg cells; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PE, phycoerythrin; PerCP, peridinin chlorophyll protein; PI, propidium iodide; rm, recombinant murine; SCI, spinal cord injury; SIRT, Sirtuin; TCR, T-cell receptor; TGF-, β, transforming growth factor-β; TNF-, α, tumor necrosis factor-α ; Treg cells, regulatory T cells

T cells, together with other blood-borne leukocytes, take part in the progression of post-SCI neuroinflammation. It is believed that T cells undergo autoimmunization following SCI and play both protective and destructive roles during the recovery process. SCI-induced leakage of central nervous system antigens into the periphery leads to the activation and expansion of autoreactive T cells that transmigrate to the injured spinal cord parenchyma, reaching peak accumulation 1 week after SCI.[1–4] Helper T cells are involved in recruitment and activation of macrophages, which cause focal axonal injury and demyelination.[4] Meanwhile, autoreactive cytotoxic T cells can mediate remarkable white matter pathology.[5,6] However, there is also evidence that helper T cells are necessary for neurological recovery following SCI.[7] Furthermore, $\gamma\delta$ T cells are found at the lesion sites within 24 hr after SCI and secrete the pro-inflammatory cytokine interferon-γ.[8]

Regulatory T (Treg) cells, which are critical for the maintenance of immune homeostasis, also play a significant role in some neurological disorders. The Treg cells can be divided into natural Treg (nTreg) cells and inducible Treg (iTreg) cells, which originate from different T-cell subsets under distinctive physiological or pathological circumstances.[9] It has been reported that nTreg cells are the predominant infiltrating Treg cells in the parenchyma of traumatically injured central nervous system.[7] Treg cell apoptosis increases neuronal survival after SCI.[10] Depletion of Treg cells at the subacute/chronic phase interferes with tissue remodeling.[7] The repairing effect of Treg cells possibly lies in their strong anti-inflammatory activity.[11,12] However, the molecular mechanisms underlying the modulation of Treg cell activity in the spinal cord parenchyma have not been thoroughly understood.

The Sirtuin (SIRT) family consists of seven members (SIRT1–7), which act as NAD^+-dependent protein deacetylases and/or ADP-ribosylases.[13] They participate in the regulation of cellular processes, including metabolism, stress, and genome stability. The roles of SIRTs, especially SIRT1, in the immune system have been recently disclosed. SIRT1-deficient mice show enhanced T-cell activation, a compromise of $CD4^+$ T-cell tolerance, and the onset of T helper type 17-associated autoimmune diseases.[14] Activation of SIRT1 inhibits T-cell activation.[15] Interestingly, SIRT1 targeting up-regulates Foxp3 expression and promotes Treg cell immunosuppressive function.[16] Consistently, SIRT1 results in Foxp3 proteasomal degradation, while SIRT1 inhibition increases Foxp3 transcriptional activity.[17,18] However, the significance of other SIRTs to Treg cells has not been studied.

In the current study, we tested the expression of SIRTs in the infiltrating Treg cells in the spinal cord parenchyma in a mouse SCI model. We found that the expressions of SIRT4 and SIRT6 were up-regulated in infiltrating Treg cells. To determine the role of these SIRTs in Treg cell activity, we transduced splenic nTreg cells with SIRT4- or SIRT6-encoding lentiviruses. Interestingly, exogenous SIRT4 overexpression substantially alleviated the expression of Foxp3, interleukin-10 (IL-10) and transforming growth factor-β (TGF-β) in nTreg cells, while SIRT6 overexpression had no significant effect on nTreg cells. Consistently, SIRT4 overexpression weakened the inhibitory effect of nTreg cells on lipopolysaccharide (LPS) -stimulated spinal cord $CD11b^+$ myeloid cells. Knock-down of SIRT4 increased the anti-inflammatory activity of infiltrating nTreg cells in the parenchyma of injured spinal cords. Additionally, SIRT4 overexpression decreased iTreg cell generation *in vitro*. Furthermore, we found that SIRT4 down-regulated 5′-AMP-activated protein kinase (AMPK) signaling in nTreg cells. The AMPK agonist AICAR restored the expression of Foxp3 and IL-10 in nTreg cells. Therefore, we concluded that SIRT4 inhibits the anti-neuroinflammatory activity of Treg cells by blocking AMPK signaling.

Materials and methods

Mice

This study was carried out in accordance with the recommendations of the Animal Care and Use Committee of Fujian Medical University. The protocol was approved by the Animal Care and Use Committee of Fujian Medical University. Eight-week-old wild-type C57BL/6 mice and Foxp3-GFP knock-in mice (initially generated in JAX Laboratory, Bar Harbor, ME) were purchased from the Animal Center of Renmin Hospital of Wuhan University. The mice were raised under the specific pathogen-free conditions before and after surgery.

Spinal cord compression injury model

This model was established following the previously reported protocols with minor modifications.[19,20] Briefly, mice were anesthetized using inhalation of 3% isoflurane. A longitudinal skin incision was made to expose the spine between T2–T12 vertebral body levels. The subcutaneous tissues were carefully separated to expose the muscles using a Swiss Tweezer Forceps #5. The muscles were slowly separated with cotton pellets to expose T9–T12 vertebral bodies. The spinous process, lamina, and pedicles of T10 lamina were removed with eye scissors to expose the underlying intact dura and spinal cord. The T9 spinous process and the paraspinal tissues were immobilized using two pairs of forceps to keep the spine straight. The T10 spinal cord was bilaterally compressed for 2 min using forceps that were 1 mm wide with a 1-mm spacer. After that, the subcutaneous tissues and skin were closed. Mice were returned to their cages after they regained the righting reflex. The urinary retention was relieved by twice-daily bladder expressions. The sham-

operated mice received every surgical step except for the spinal cord compression.

Enrichment of immune cells from spinal cords and peripheral blood

At day 14 after SCI, mice were anesthetized by inhalation of 3% isoflurane, the peripheral blood was collected from the tail vein, and red blood cells were lyzed using Tris–NH_4Cl buffer. Blood leukocytes were resuspended in phosphate-buffered saline (PBS) for further tests. Each mouse then received transcardial perfusion with 200 ml of PBS. The T10 spinal cord was taken, minced into approximately 1-mm^3 pieces, and treated with RPMI-1640 supplemented with 2 mg/ml collagenase IV (ThermoFisher Scientific, Waltham, MA), 200 U/ml DNase I (Sigma-Aldrich, St Louis, MO), 10% fetal bovine serum and 2·0 mM $CaCl_2$ for 30 min on an orbital shaker while shaking at 150 r.p.m. at 37° in an incubator. Digested spinal cord tissues were then filtered through 70-μm cell strainers and overlaid on 20% Percoll (GE Healthcare, Chalfont St Giles, UK), followed by centrifugation at 250 *g* for 10 min. The cell pellet was resuspended in PBS or culture medium before further experiments. In some experiments, the immune cells were pooled from spinal cords of 5–10 mice.

Flow cytometry

The following fluorophore-conjugated antibodies were purchased from eBioscience (San Diego, CA): peridinin chlorophyll protein-conjugated (PerCP-) anti-T-cell receptor-β (TCR-β) (H57-597), allophycocyanin-conjugated (APC-) anti-CD4 (GK1.5), APC-anti-Gr-1 (1A8-Ly6g), phycoerythrin-conjugated (PE-) anti-IL-10 (JES5-16E3), PE-Cy7-anti-IL-17A (TC11-18H10.1), APC-Cy7-anti-IL-6 (MP5-20F3), fluorescein isothiocyanate-conjugated (FITC-) anti-tumor necrosis factor-α (TNF-α) (MP6-XT22), and PE-Cy7-anti-CD11b (M1/70). PE-anti-IL-1β (166931) and PE-anti-IL-23 p19 (320244) were purchased from R&D Systems (Minneapolis, MN). For cell surface marker staining, cells were stained with 2 μg/ml each antibody on ice for 15 min. Dead cells were excluded by staining with 1 μg/ml propidium iodide (PI; eBioscience). For intracellular staining, cells were fixed with 4% paraformaldehyde and permeabilized with 90% ice-cold methanol for 30 min, followed by antibody incubation (5 μg/ml each antibody) for 1 hr at room temperature. Cells were then washed with PBS twice and loaded either onto a BD LSR-II flow cytometer (BD Biosciences, San Jose, CA) for analysis or a BD FACSAria II sorter (BD Biosciences) for sorting.

Real-time RT-PCR

Total cellular RNAs were purified using the ARCTURUS PicoPure RNA Isolation Kit (ThermoFisher Scientific). A SuperScript® III First-Strand Synthesis Kit (ThermoFisher Scientific) was used to prepare cDNA. Using a 7300 q-PCR thermocycler (Invitrogen, Carlsbad, CA), a real-time polymerase chain reaction (PCR) was conducted with Fast SYBR®Green Master Mix (ThermoFisher Scientific) at the following steps: pre-warming at 50° for 2 min, 94° for 10 min, and then 40 cycles of 30 seconds at 94° and 1 min at 60°. The data were analyzed using the $2^{-\Delta\Delta Ct}$ method. The primers are listed in the Supplementary material (Table S1).

Immunoblotting

Cellular proteins were extracted using RIPA buffer (ThermoFisher Scientific, 89900) with protease inhibitor cocktail (Sigma-Aldrich, S8820). Protein concentrations were quantified using a Pierce BCA Protein Assay Kit (ThermoFisher Scientific, 23225). Twenty micrograms of total protein was loaded onto the 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel. The following antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX): anti-GAPDH (sc-47778, 1:500), anti-Foxp3 (sc-53876, 1:500), anti-LKB1 (sc-374300, 1:1000), anti-SIRT2 (sc-28298, 1:1000) and anti-SIRT6 (sc-517556, 1:500). Anti-SIRT4 antibody was purchased from Abcam (Cambridge, UK; ab124521, 1 μg/ml). AMPK phosphorylation was detected using the antibodies in the AMPK and ACC Antibody Sampler Kit (Cell Signaling Technology, Danvers, MA; #9957, 1:1000 each). Optical density was analyzed on a ChemiDoc XRS^+ system (Bio-Rad, Hercules, CA).

iTreg induction in vitro

Ninety-six-well flat-bottom plates were coated with attachment factor plus 5 μg/ml anti-CD3e monoclonal antibody (mAb) (eBioscience, 16-0031) overnight at 4°. Then, 1×10^5 splenic $CD4^+$ $CD25^-$ conventional T cells were seeded into each well with 2 μg/ml soluble anti-CD28 mAb (eBioscience, 16-0281) in the presence of 10 ng/ml recombinant murine IL-2 (rmIL-2; eBioscience, 14-8021-64) and 2 ng/ml rmTGF-β (R&D Systems, 7666-MB). Seventy-two hours after seeding, cells were stained with APC-anti-CD4 antibody (GK1.5) and FITC-anti-Foxp3 antibody (eBioscience, 11-5773-82) using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience, 00-5523-00), according to the manufacturer's instructions.

Lentiviral preparation

The mouse SIRT4 lentiviral vector (LV416705), mouse SIRT6 lentiviral vector (LV466463), SIRT4 siRNA/shRNA/RNAi Lentivector (i047573c), and SIRT6 siRNA/shRNA/RNAi Lentivector (i047965a) were purchased from Applied

Biological Materials Inc. (Richmond, BC, Canada). These vectors express the genes of interest and green fluorescent protein (GFP), so GFP^+ cells were regarded as successfully transduced cells in later lentiviral transduction. To produce the lentiviruses, 5×10^5 HEK293T cells per 10-cm culture dish were cultured for 16–18 hr till 80% confluency was reached. Cells were then treated with 8 μM chloroquine diphosphate (Abcam) for 2·5 hr, and 0·80 pmol pRSV-Rev (Addgene, Cambridge, MA), 1·5 pmol pMDLg/pRRE (Addgene), 2·0 pmol each lentiviral vector, and 60 μg polyethylenimine (Sigma-Aldrich) were mixed in 1 ml of Opti-MEM (ThermoFisher Scientific, 51985091) before adding to HEK293T cell culture. The culture medium was refreshed 18 hr later. On day 2 after transfection, the supernatants were harvested, centrifuged at 300 *g* for 10 min, and passed through 0·45-μm filters. The lentiviruses were purified with Lentivirus Purification Kit (ABM Inc., G173) and quantified with qPCR Lentivirus Titration Kit (ABM Inc., LV900). The lentivirus encoding SIRT4 was named LS4, the lentivirus encoding SIRT6 was named LS6, and the control lentivirus was named LE.

Lentiviral transduction

To transduce nTreg cells, splenic $CD4^+$ $CD25^+$ T cells were isolated from normal C57BL/6 mice using a MagCellect Mouse $CD4^+$ $CD25^+$ Regulatory T Cell Isolation Kit (R&D Systems, MAGM208) following the vendor's manual. $CD4^+$ $CD25^+$ T cells (1×10^6/ml) were stimulated *in vitro* for 24 hr with 5 μg/ml plate-bound CD3e mAb, 2 μg/ml soluble CD28 mAb, and 10 ng/ml rmIL-2. The medium was then replaced by fresh medium containing 5 μg/ml polybrene (Sigma-Aldrich, H9268), CD28 mAb and rmIL-2. The lentiviral particles were then added at a multiplicity of infection of 10 to incubate cells overnight. Cells were maintained for an additional 2 days in the presence of plate-bound anti-CD3 antibody, soluble anti-CD28 antibody plus rmIL-2. GFP^+ cells were evaluated by flow cytometry to analyze the transduction efficiency. In some experiments, 10 μg/ml Brefeldin A (Sigma-Aldrich, B6542) was added 4 hr before the termination of culture to block the cytokine secretion. In another experiment, 250 μM AICAR (an AMPK agonist) was added into nTreg cell culture immediately after the addition of lentiviral particles.

To transduce iTreg cells, iTreg cells were induced from $CD4^+$ $CD25^-$ conventional T cells as described above. At day 1 after induction, the lentiviral particles were added at a multiplicity of infection of 10 together with 5 μg/ml polybrene into the iTreg cell culture. Cells were maintained for an additional 2 days before further analysis.

nTreg cell–$CD11b^+$ myeloid cell co-culture

At day 3 after SCI, immune cells in the spinal cords were isolated as described above. These cells were then stained with APC-anti-Gr-1 antibody and PE-Cy7-anti-CD11b antibody. $CD11b^+$ $Gr\text{-}1^-$ myeloid cells, i.e. macrophages and microglia, were sorted from these immune cells by flow cytometry. Lentivirus-transduced nTreg cells ($2{\cdot}5 \times 10^5$/ml) were stimulated for 24 hr in each well of 96-well half-area plates in the presence of plate-bound CD3e mAb, soluble CD28 mAb, and rmIL-2. Then, 1×10^5/ml myeloid cells and 50 ng/ml LPS (Sigma-Aldrich) were added to co-culture with nTreg cells for 6 hr. Four hours before the end of co-culture, 10 μg/ml Brefeldin A was added. After co-culture, cells were treated with Trypsin-EDTA Solution (Sigma-Aldrich) for 5 min at room temperature. Total cells were then stained with PerCP-anti-TCR-β and the indicated intracellular cytokine antibodies for flow cytometry analysis.

Adoptive transfer

At day 5 after SCI, mice were anesthetized by inhalation of 3% isoflurane. Then, 2×10^6 lentivirus-transduced splenic $CD4^+$ $CD25^+$ T cells (in 100 μl of sterile PBS) were infused into each mouse via retro-orbital injection. Two days later, the mice were killed and immune cells in the spinal cords were isolated as described above. GFP^+ cells, i.e. transferred nTreg cells, were sorted from these immune cells by flow cytometry.

Immunofluorescence staining

Spinal cords were fixed with 4% paraformaldehyde followed by paraffin embedding. Five-micron thick cross-sections were cut. The sections were stained with mouse anti-GFP antibody (Santa Cruz Biotechnology, Santa Cruz, CA, sc-9996, 1:200) and rabbit anti-GFAP antibody (Abcam, ab7260, 1:500) at 4° overnight. After three PBS washes, the sections were stained with FITC-conjugated goat anti-mouse IgG and PE-conjugated goat anti-rabbit IgG (Abcam, ab6785 and ab72465, 1:500). Images were taken on a Leica DMIRE2 fluorescence microscope.

Statistical analysis

Experiments were independently conducted two or three times unless specified, with three to eight different samples in each group. Data were shown as mean ± standard deviation and were analyzed by GraphPad Prism 7.0 (GraphPad, San Diego, CA). Student's *t*-test or one-way analysis of variance was used to compare the mean values among the groups. A P-value $< 0{\cdot}05$ was considered significant.

Results

SIRT4 and SIRT6 are up-regulated in infiltrating Treg cells after SCI

To study Treg activities after SCI, we used Foxp3-GFP transgenic mice in the SCI model. At day 14 after SCI,

mononuclear cells were enriched from the peripheral blood and injured spinal cords. As indicated in the Supplementary material (Fig. S1), dead cells were excluded by PI staining, and mononuclear cells were distinguished based on forward- and side-scatter parameters. TCR-β^+ T cells were then recognized among mononuclear cells (Fig. 1a). $CD4^+$ GFP^+ cells, i.e. Treg cells, were further discerned from T cells and sorted by flow cytometry (Fig. 1a). The expression of SIRTs in sorted Treg cells was then analyzed via real-time RT-PCR. As demonstrated in Fig. 1(b–f), the mRNA levels of SIRT2 and SIRT4 were elevated in both blood Treg cells and infiltrating Treg cells, whereas SIRT6 mRNA was increased only in infiltrating Treg cells. No change was observed in the mRNA levels of SIRT1 and SIRT3. No amplification of SIRT5 and SIRT7 was achieved in all three groups (data not shown). Notably, in infiltrating Treg cells, the up-regulation of SIRT4 (~6-fold) and SIRT6 (~2·75-fold) was more robust than the up-regulation of SIRT2 (~1·5-fold). So we focused on SIRT4 and SIRT6 in the following experiments. The weak expression of SIRT4 and SIRT6 in blood Treg cells in sham-operated mice, as well as their up-regulation in post-SCI infiltrating Treg cells, were further illustrated by immunoblotting assay (Fig. 1g).

SIRT4 overexpression reduces the production of IL-10 and TGF-β in nTreg cells

To determine the roles of SIRT4 and SIRT6 in nTreg cell activity, we used lentiviral particles to introduce exogenous SIRT4 or SIRT6 into wild-type splenic $CD4^+$ $CD25^+$ T cells. The lentiviral transduction efficiency reached approximately 65–75% based on GFP

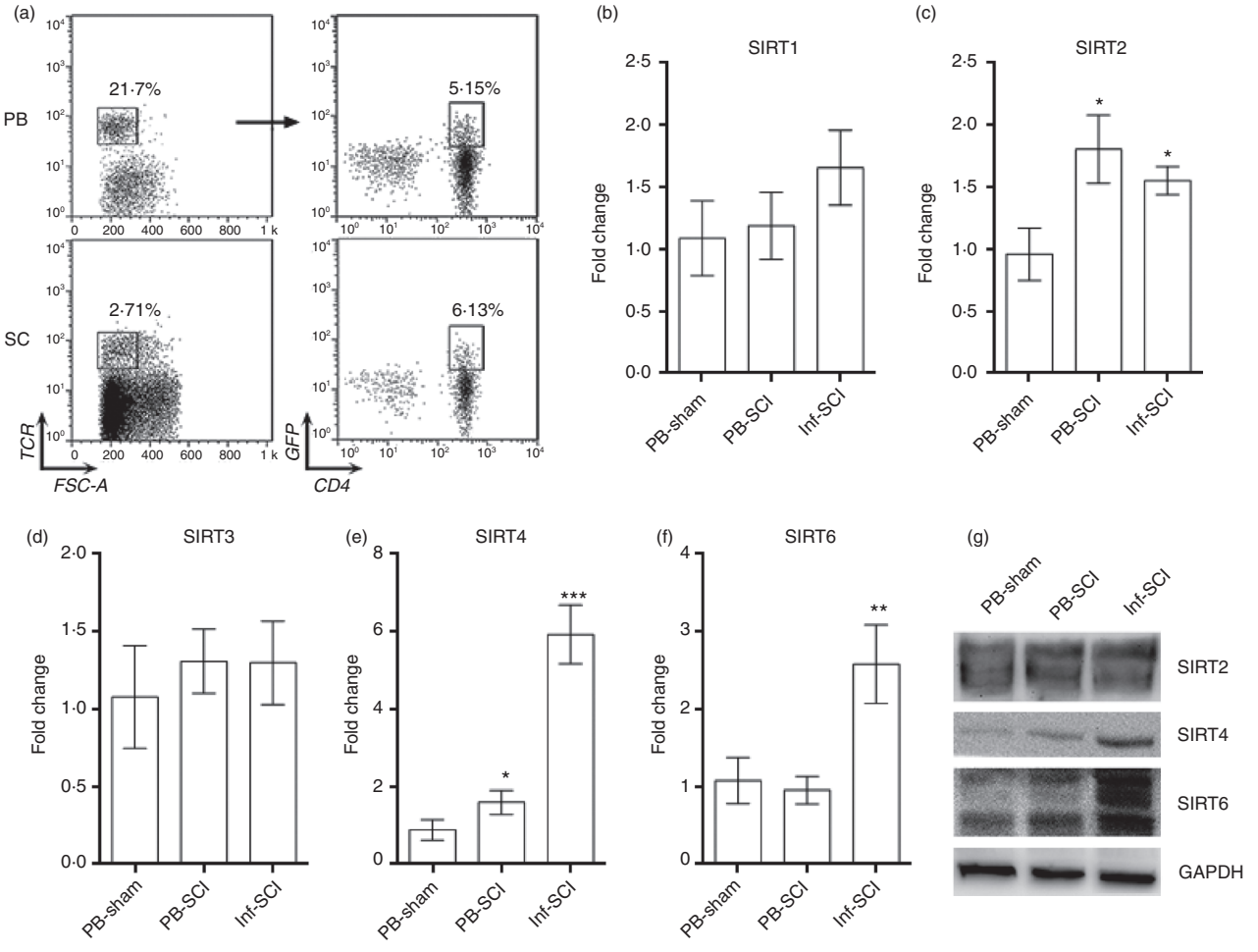

Figure 1. The expression of sirtuins (SIRTs) in blood and infiltrating regulatory T (Treg) cells. (a) Gating strategy for the Treg cells in blood and spinal cord mononuclear cells. PB: peripheral blood. SC: spinal cord. (b–f) The mRNA abundances of indicated SIRTs in Treg cells at day 14 after spinal cord injury (SCI). PB-sham: blood Treg cells in sham-operated mice. PB-SCI: blood Treg cells in SCI mice. Inf-SCI: infiltrating Treg cells in the spinal cords of SCI-operated mice. $*P < 0{\cdot}05$; $**P < 0{\cdot}01$; $***P < 0{\cdot}001$ in comparison to PB-sham group; $n = 4$ mice per group. (g) Protein levels of SIRT2, SIRT4 and SIRT6 in Treg cells at day 14 after SCI. The images are representative of two independent experiments.

expression after transduction (Fig. 2a). The expression of exogenous SIRT4 and SIRT6 was confirmed by immunoblotting assay (Fig. 2b). Moreover, the Foxp3 protein level was decreased in LS4-transduced nTreg cells compared with LE-transduced nTreg cells (Fig. 2b,c). The Foxp3 expression in LS6-transduced nTreg cells and LE-transduced Treg cells was comparable (Fig. 2b,c). We then tested the expression of immunoregulatory molecules in these nTreg cells. As indicated in Fig. 2(d), the mRNA abundances of IL-10 and TGF-β were both down-regulated in LS4-transduced nTreg cells compared with LE-transduced nTreg cells. The ICOS expression was not altered. Since some nuclear factors such as T-bet, GATA3 and RORγ also impact nTreg cell activity,[21–

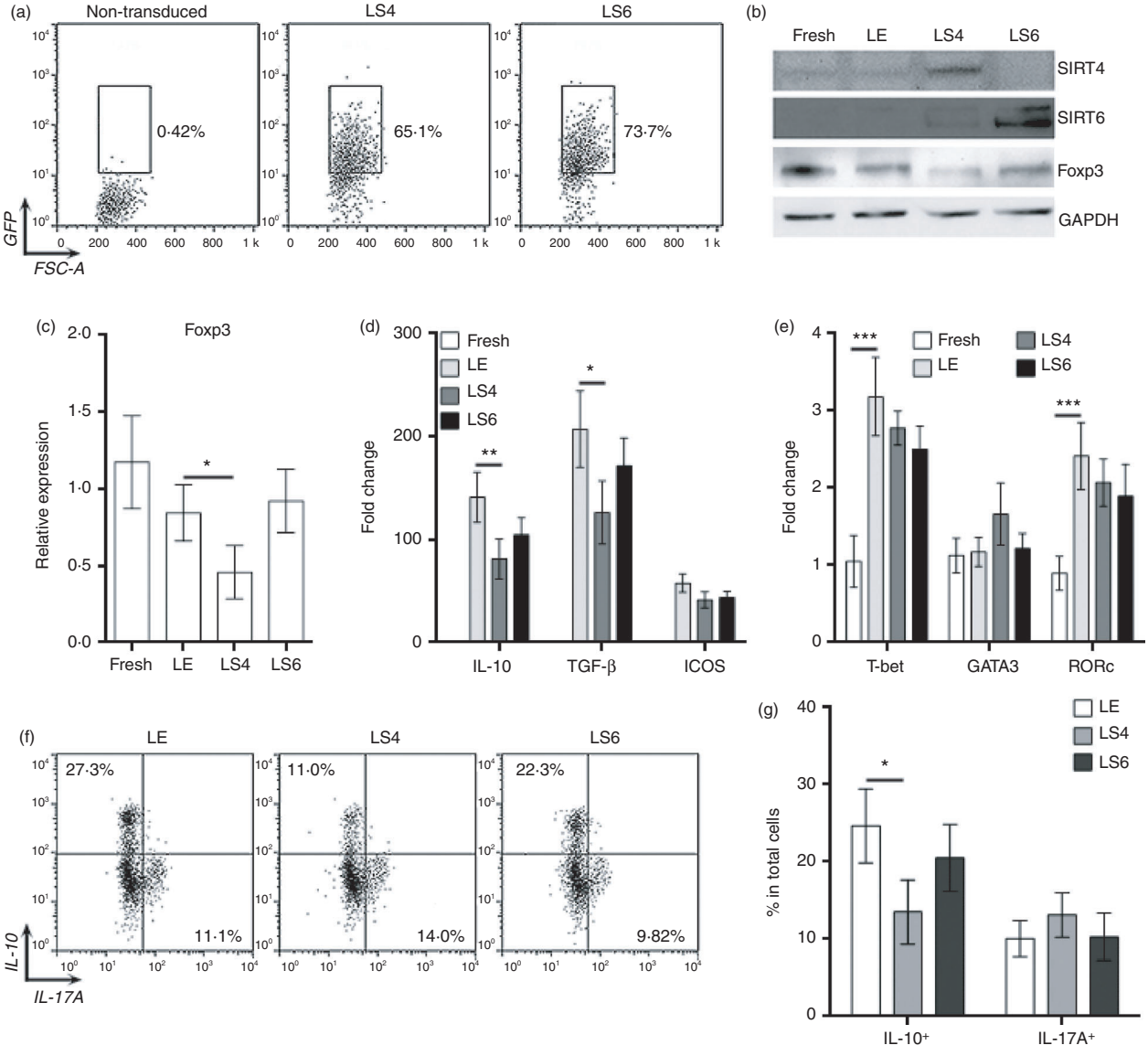

Figure 2. The effect of lentivirus-mediated sirtuin 4 (SIRT4) overexpression on the production of interleukin-10 (IL-10) and transforming growth factor-β (TGF-β) in natural regulatory T (nTreg) cells. (a) Efficiency of lentiviral transduction is shown by the GFP expression in nTreg cells. Non-transduced, no transduction; LS4, nTreg cells transduced by the lentivirus encoding SIRT4; LS6, nTreg cells transduced by the lentivirus encoding SIRT6. Data are representative of two independent experiments. (b) Protein levels of SIRT4, SIRT6 and Foxp3 in splenic nTreg cells after lentiviral transduction. Data are representative of three independent experiments. Fresh, freshly sorted nTreg cells; LE, lentivirus-transduced nTreg cells; LS4, LS4-transduced nTreg cells; LS6, LS6-transduced nTreg cells. (c) Statistics of Foxp3 expression in indicated groups, $n = 3$ samples per group. (d, e) The mRNA abundances of indicated molecules in lentivirus-transduced nTreg cells, $n = 6$ samples per group. (f, g) Intracellular staining of IL-10 and IL-17A in lentivirus-transduced nTreg cells. Representative flow cytometry dot plots are shown in (f), and statistics is shown in (g), $n = 4$ samples from two batches of transduced cells. $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$.

[23] we evaluated their expression and found that SIRT4 and SIRT6 did not change their mRNA abundances (Fig. 2e). The intracellular cytokine staining indicated that LS4-transduced nTreg cells produced less IL-10 in comparison with the other two groups, whereas IL-17A production was not altered by either SIRT4 or SIRT6 (Fig. 2f,g).

SIRT4 overexpression impairs the anti-inflammatory function of nTreg cells

To analyze the roles of SIRT4 and SIRT6 in the modulation of the anti-inflammatory activity of nTreg cells, we sorted $CD11b^+$ myeloid cells (including microglia and macrophages, see Supplementary material, Fig. S2) from spinal cords at day 7 post-SCI and stimulated them with

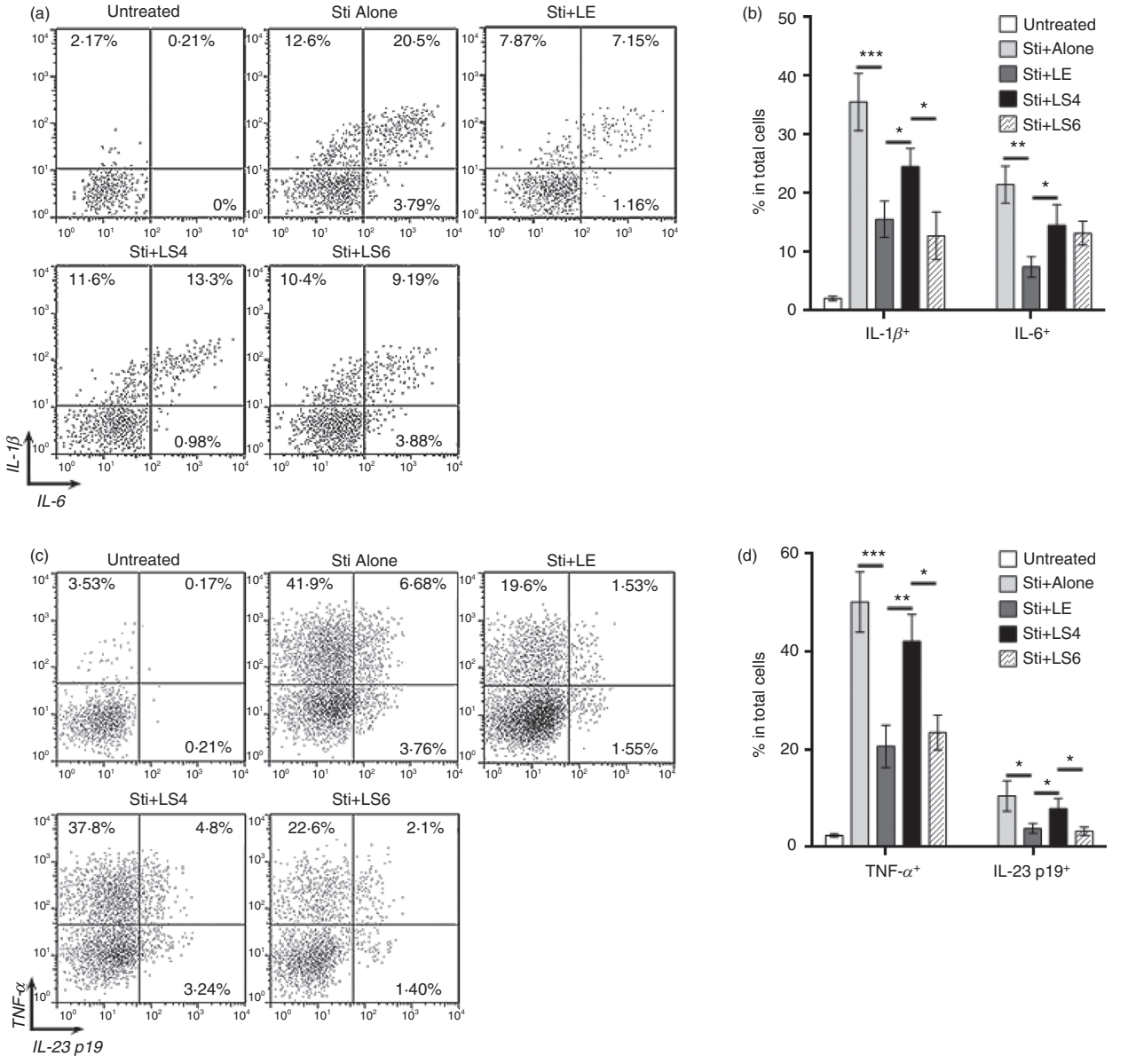

Figure 3. Sirtuin 4 (SIRT4) impairs the anti-inflammatory activity of natural regulatory T (nTreg) cells *in vitro*. (a, b). Intracellular staining of interleukin-1β (IL-1β) and IL-6 in lipopolysaccharide (LPS) -stimulated spinal cord myeloid cells after co-culture with lentivirus-transduced nTreg cells. Representative flow cytometry dot plots are shown in (a), and the statistics for IL-1β^+ or IL-6$^+$ myeloid cells are shown in (b). (c, d) Intracellular staining of tumor necrosis factor-α (TNF-α) and IL-23 p19 in LPS-stimulated spinal cord myeloid cells after co-culture with lentivirus-transduced nTreg cells. Representative flow cytometry dot plots are shown in (c), and the statistics for TNF-α^+ or IL-23 p19$^+$ myeloid cells are shown in (d). Untreated, untreated myeloid cells; Sti alone, LPS-stimulated myeloid cells cultured alone; Sti + E, LPS-stimulated myeloid cells co-cultured with lentivirus-transduced nTreg cells; Sti + LS4, LPS-stimulated myeloid cells co-cultured with nTreg cells transduced by the lentivirus encoding SIRT4; Sti + LS6, LPS-stimulated myeloid cells co-cultured with nTreg cells transduced by the lentivirus encoding SIRT6; $n = 5$ samples. *$P < 0{\cdot}05$; **$P < 0{\cdot}01$; ***$P < 0{\cdot}001$.

LPS. We then co-cultured lentivirus-transduced nTreg cells with these myeloid cells and evaluated pro-inflammatory cytokine production in these myeloid cells (see Supplementary material, Fig. S3). As shown in Fig. 3, LE-transduced Treg cells considerably reduced the production of IL-1β, IL-6, TNF-α, and IL-23 p19 in the myeloid cells. Meanwhile, LS4-transduced nTreg cells were less potent to inhibit the production of these cytokines, compared with LE-transduced nTreg cells. LS6-transduced nTreg cells inhibited the expression of IL-1β, TNF-α, and IL-23 p19, but were unable to reduce IL-6 expression to the same extent as LE-transduced nTreg cells did. In conclusion, our data suggest that SIRT4 is an inhibitory factor for nTreg cell-mediated anti-inflammatory responses.

SIRT4 knockdown promotes the anti-inflammatory activity of nTreg cells in SCI

To check the effect of SIRT4 *in vivo*, nTreg cells were enriched from normal mouse spleens, transduced with lentivirus encoding SIRT4 shRNA or SIRT6 shRNA (Fig. 4a), and then adoptively transferred into recipient SCI mice at day 12 after SCI. Knockdown of SIRT4 or SIRT6 did not alter the expression of IL-10, TGF-β, and Foxp3 *in vitro* (Fig. 4b,c), possibly due to the very low expression of SIRT4 and SIRT6 in cultured nTreg cells. Two days later (day 14 after SCI), transferred GFP^+ nTreg cells were retrieved from the injured spinal cords. As shown in Fig. 4(d), similar proportions of SIRT4-knockdown nTreg cells and SIRT6-knockdown nTreg cells were found in spinal cord mononuclear cells, suggesting that SIRT4 and SIRT6 did not influence the transmigration of nTreg cells into injured spinal cords. In comparison with control nTreg cells and SIRT6-knockdown nTreg cells, SIRT4-knockdown nTreg cells expressed higher IL-10 and TGF-β (Fig. 4e). To our surprise, the Foxp3 expression in SIRT4-knockdown nTreg cells was not remarkably increased *in vivo*, although there was a trend of increase (Fig. 4e,f).

To examine the location of infiltrating nTreg cells, we stained GFP and GFAP in injured spinal cords. In the periphery of the injury-caused cavity, we found GFP^+ nTreg cells in close proximity to the soma or processes of $GFAP^+$ astroglia (Fig. 4g), suggesting that these two cell types may interact through cell-to-cell contact.

SIRT4 overexpression blocks *in vitro* iTreg generation

Although it is highly likely that nTreg cells are predominant in post-SCI infiltrating Treg cells, to completely understand the role of SIRT4, we still checked the effect of SIRT4 on iTreg cells. Lentiviral particles were added into the $CD4^+$ $CD25^-$ conventional T-cell culture under the Treg cell induction condition, followed by analysis of iTreg cell generation. Compared with control conventional T cells and LE-transduced conventional T cells, LS4-transduced conventional T cells gave birth to significantly fewer $Foxp3^+$ iTreg cells (Fig. 5a,b). SIRT4 overexpression decreased Foxp3 expression (Fig. 5c). The exogenous SIRT4 expression was confirmed in Fig. 5d.

SIRT4 down-regulates AMPK signaling in nTreg cells

It has been reported that SIRT4 blocks AMPK signaling.[24] AMPK signaling is closely related to Treg cell generation and function.[25,26] To determine whether SIRT4 blocks AMPK signaling to impair Treg cell function, we first tested the phosphorylation of acetyl-CoA carboxylase (ACC) and AMPKα in lentiviral-transduced nTreg cells. SIRT4 overexpression decreased phospho-ACC and phospho-AMPKα in nTreg cells, but SIRT6 overexpression had no effect (Fig. 6a–c). To further substantiate the role of AMPK signaling in SIRT4-mediated functional changes of nTreg cells, we treated LS4-transduced nTreg cells with AMPK activator AICAR before analyzing the expression of IL-10 and IL-17A. As illustrated in Fig. 6(d,e), AICAR restored IL-10 expression and decreased IL-17A expression in SIRT4-overexpressing nTreg cells. AICAR also partially restored Foxp3 expression in LS4-transduced nTreg cells (Fig. 6f,g).

Discussion

In the present study, we evaluated the change in the expression of each SIRT family member in both blood Treg cells and infiltrating Treg cells after SCI. Interestingly, SIRT2 and SIRT4 were moderately increased in blood Treg cells, suggesting that some systemic factor(s) induces their expression. Additionally, SIRT4 and SIRT6 were remarkably up-regulated in infiltrating Treg cells, suggesting that the microenvironment in the injured spinal cord strongly promotes the transcription of SIRT4 and SIRT6 in infiltrating Treg cells. The *in vitro* lentivirus-mediated overexpression of exogenous SIRT4 or SIRT6 revealed a significant role of SIRT4 rather than SIRT6 in the inhibition of Treg cell anti-inflammatory activity. We focused on the neuroinflammation rather than adaptive immunity in the present research, so we did not check the immunosuppressive activity of lentivirus-transduced Treg cells. However, because SIRT4 overexpression down-regulated Foxp3 and immunoregulatory molecules such as IL-10 and TGF-β, it is plausible to deduce that the immunosuppressive activity of SIRT4-overexpressing Treg cells would be impaired.

SIRT4 is localized within mitochondria, where it exerts effects on multiple metabolic pathways, including fatty acid metabolism, the tricarboxylic acid cycle, glycolysis, reactive oxygen species, oxidative phosphorylation, protein metabolism, and the urea cycle.[27] Based on our knowledge, there is no evidence showing the direct effect of SIRT4 on the transcription of Foxp3 and cytokines. We identified the significance of AMPK signaling for SIRT4-mediated changes in Treg cell function. Consistent

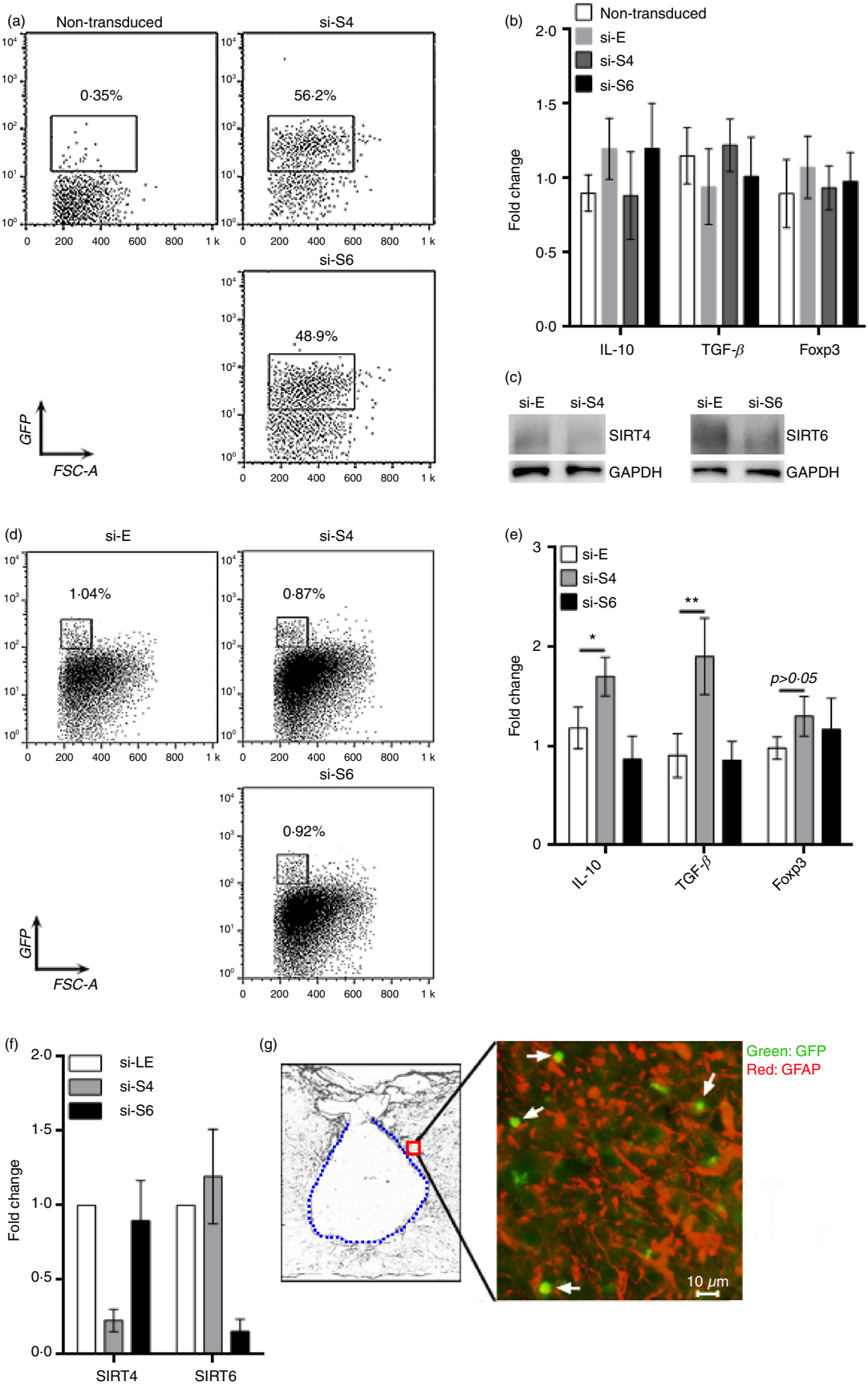

Figure 4. Sirtuin 4 (SIRT4) knockdown promotes the anti-inflammatory activity of infiltrating natrual regulatory T (nTreg) cells *in vivo*. (a) Representative flow cytometry dot plots showing the GFP$^+$ lentivirus-transduced nTreg cells *in vitro*. Si-S4, nTreg cells transduced with lentivirus encoding SIRT4 shRNA; Si-S6, nTreg cells transduced with lentivirus encoding SIRT46 shRNA. (b) The mRNA abundances of indicated molecules in GFP$^+$ lentivirus-transduced nTreg cells *in vitro*. Si-E, nTreg cells transduced with lentivirus expressing scramble shRNA; Si-S4, nTreg cells transduced with lentivirus encoding SIRT4 shRNA; Si-S6, nTreg cells transduced with lentivirus encoding SIRT46 shRNA; $n = 3$ per group. (c) Protein levels of SIRT4 and SIRT6 in lentivirus-transduced nTreg cells *in vitro*. Data are representative of two independent experiments. (d) Representative flow cytometry dot plots showing GFP$^+$ lentivirus-transduced nTreg cells retrieved from injured spinal cords at day 2 after adoptive transfer. (e) The mRNA abundances of indicated molecules in donor-derived nTreg cells retrieved from injured spinal cords; $n = 5$ mice per group. (f) The mRNA abundances of SIRT4 and SIRT6 in retrieved GFP$^+$ nTreg cells; $n = 2$ mice per group. (g) Representative immunofluorescence image showing the proximity between GFP$^+$ nTreg cells and GFAP$^+$ astroglia in injured spinal cords at day 2 after adoptive transfer. In the left panel, the dotted line outlines the cavity caused by spinal cord injury (SCI). The red square is the area where the nTreg cells and astroglia were shown in the right panel (magnification = 200×). This image is a section of SCI in a mouse which received LS4-transduced nTreg cells. $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$.

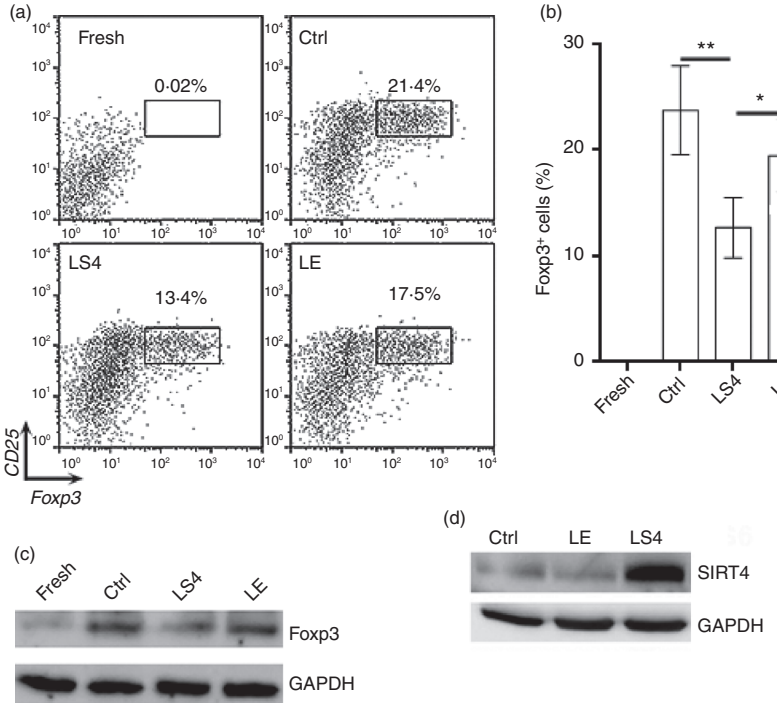

Figure 5. Sirtuin 4 (SIRT4) overexpression inhibits induced regulatory T (iTreg) cell generation *in vitro*. (a, b) Expression of CD25 and Foxp3 in conventional T cells after iTreg cell induction. Representative flow cytometry dot plots are shown in (a), and statistics of the proportion of Foxp$^+$ cells is shown in (b), $n = 6$ samples per group. $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$. (c, d) Protein levels of Foxp3 (c) and SIRT4 (d) in cultured T cells. Data are representative of two independent experiments. Fresh, freshly sorted conventional T cells; Ctrl, iTreg cell induction without lentiviral transduction; LE, iTreg cell induction with lentivirus transduction. LS4, iTreg cell induction with transduction by the lentivirus encoding SIRT4.

with previous research,[24] SIRT4 decreased the phosphorylation of AMPKα and its downstream target ACC. AMPK is an intracellular energy sensor that is activated by low energy levels such as a low ATP/ADP ratio. Once activated, AMPK up-regulates a set of genes related to energy generation such as glucose oxidation and, importantly, lipid oxidation.[25] It is assumed that AMPK could favor Treg cell generation through either promoting fatty acid oxidation or inhibiting mammalian target of rapamycin (mTOR).[25] Indeed, activated AMPK can drive naive T cells to differentiate toward Treg cells both *in vitro* and *in vivo*, and AMPK is highly expressed and active in Treg cells.[26] In our study, the AMPK agonist AICAR counteracted the effect of SIRT4 and restored the expression of Foxp3 and IL-10, indicating that it is highly possible that SIRT4 suppresses Treg cell function by inhibiting AMPK signaling. It is highly likely that as an ADP-ribosyl-transferase in the mitochondria, SIRT4 leads to increased cellular ATP levels, which subsequently inhibit AMPK signaling.

AMPK signaling is an inhibitory pathway for Th17 differentiation. However, we did not observe any increase in IL-17A or RORγt in SIRT4-overexpressing Treg cells. Meanwhile, SIRT4 is involved in other metabolic

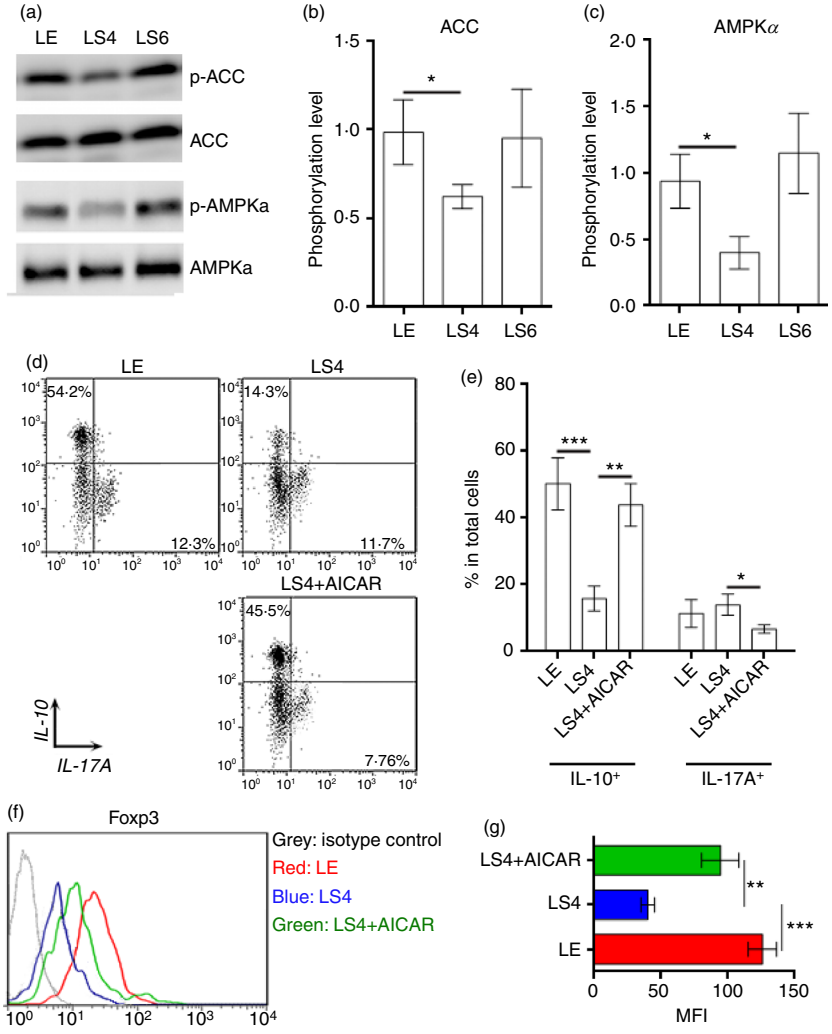

Figure 6. Sirtuin 4 (SIRT4) inhibits AMP-activated protein kinase (AMPK) signaling in natural regulatory T (nTreg) cells. (a–c) Phosphorylation of acetyl-CoA carboxylase (ACC) and AMPKα in lentivirus-transduced nTreg cells. The representative immunoblotting images are shown in (a), and the statistics for phospho-ACC and phospho-AMPKα are shown in (b and c). LE, lentivirus-transduced nTreg cells; LS4, nTreg cells transduced by the lentivirus encoding SIRT4; LS6, nTreg cells transduced by the lentivirus encoding SIRT6; $n = 5$ samples per group. (d, e) Intracellular staining of interleukin-10 (IL-10) and IL-17A in lentivirus-transduced nTreg cells with or without AICAR treatment. Representative flow cytometry dot plots are shown in (d), and statistics is shown in (e). LE, LE-transduced nTreg cells; LS4, LS4-transduced nTreg cells treated with vehicle; LS4 + AICAR, LS4-transduced nTreg cells treated with AICAR; $n = 4$ samples per group. $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$. (f, g) Foxp3 expression in lentivirus-transduced nTreg cells with or without AICAR treatment. Representative flow cytometry histograms were shown in (f) and statistics of mean fluorescent intensity (MFI) was shown in (g); $n = 4$ samples per group. $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$.

pathways as described above. Whether these pathways are also (or partially) responsible for suppressing Treg cell activity needs further investigation.

Interestingly, unlike SIRT4, SIRT6 was unable to induce similar changes in Treg cells. SIRT6 is localized in the nucleus whereas SIRT4 is in mitochondria.[28,29] This might be the reason for their different effects. Perhaps it is easier for SIRT4 than SIRT6 to contact AMPK signaling molecules. However, it has been confirmed that AMPK is present in the nucleus and cytoplasm in distinct circumstances. Under normal growth conditions, AMPK shuttles between the nucleus and the cytoplasm under the control of the mitogen-activated protein kinase kinase–extracellular signal-regulated kinase 1/2 cascade, whereas environmental factors such as stress regulate the intracellular localization of AMPK.[30] It is therefore necessary to check the localization of AMPK and determine whether SIRT4 is co-localized with AMPK in infiltrating Treg cells. Our ongoing study is testing this possibility.

The exact factor(s) that induces the expression of SIRT4 remains unclear. It is now known that mTOR complex 1 (mTORC1) represses SIRT4 by promoting the

proteasome-mediated destabilization of cAMP-response element binding-2.[31] Interestingly, mTORC1 is required for the generation of effector Treg cells and follicular Treg cells.[32,33] It is therefore possible that mTORC1 activity becomes lower when Treg cells enter the parenchyma of injured spinal cords, and SIRT4 expression is turned on. Our data also indicate that infiltrating Treg cells were spatially close to astroglia. Astroglia are the most abundant cells in the central nervous system, and they can exert various effects on T cells.[34–37] As one of the initial pro-inflammatory cell types found immediately after SCI, astroglia would impair the anti-inflammatory activity of infiltrating Treg cells by releasing unidentified mediators. In the next study, our team will test whether astroglia induce SIRT4 expression in Treg cells by diminishing mTORC1 activation.

Although we did not pay much attention to SIRT2 because its expression was not changed as significantly as that of SIRT4 and SIRT6, it is still necessary to check its role in the modulation of Treg cell function in future. There is not enough evidence to indicate the effect of SIRT2 on T cells. SIRT2 was found to activate Akt and downstream targets in mammalian cells.[38] Meanwhile, SIRT2 deacetylates p65 to inhibit nuclear factor-κB (NF-κB) activity.[39] Because Akt and NF-κB are both important for Treg cell activity,[40–43] it is likely that up-regulation of SIRT2 influences Treg cell immunosuppressive function. However, because the exact role of Akt and NF-κB in Treg cell activity is still under debate, it is difficult to deduce the effect of SIRT2. Further investigations would give the answer.

Taken together, we found a novel mechanism by which the anti-inflammatory activity of Treg cells is suppressed in the parenchyma of the injured spinal cord. Our results provide a clue to understand the process of post-SCI neuroinflammation.

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Authorship

WPL and WKC participated in the design of the study, performed the research, and did the analysis and interpretation of data and the writing of the manuscript. WFL, YTZ, and BYL participated in the design of the study, and analysis and interpretation of data. GYQ was responsible for study design, data interpretation, and manuscript writing.

Disclosures

The authors have no financial conflicts of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Specific primer for real-time PCR.

Figure S1. Gating of mononuclear cells isolated from injured spinal cords.

Figure S2. Gating strategy for enriching CD11b+ myeloid cells from injured spinal cords.

Figure S3. Distinguishing regulatory T cells and myeloid cells after co-culture.

