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RESEARCH ARTICLE

Immunodeficiencies and Autoimmunity

Oleic Acid Promotes Treg Cell Differentiation via Autophagy Induction and Ameliorates DSS-Induced Colitis

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ABSTRACT

Oleic acid (OA), a monounsaturated fatty acid (FA) prevalent in olive oil and key component of Mediterranean diet, exhibits anti-inflammatory effects in ulcerative colitis (UC), but its immunomodulatory mechanisms remain largely elusive. Here, we found that OA promotes T regulatory cell (Treg) differentiation in a dose-dependent manner. OA inhibited the activation of the mechanistic target of rapamycin complex 1 (mTORC1) while simultaneously promoting AMP-activated protein kinase (AMPK) activity, both of which are essential for the autophagy pathway. Notably, blockade of autophagy abolished OA-induced Treg differentiation, indicating that autophagy was essential for the immunomodulatory effects of OA. In a dextran sulfate sodium (DSS)-induced colitis model, OA administration significantly increased colonic $CD4^+Foxp3^+$ Treg populations and ameliorated disease severity, including reduced weight loss, bleeding, and histological damage. In summary, our findings revealed that OA promoted Treg differentiation both in vitro and in vivo, suggesting that OA or diets rich in this FA could be promising supplementary therapies for UC.

1 | Introduction

The mammalian immune system is held in check through mechanisms of thymic and peripheral tolerance [1]. T regulatory (Treg) cells that inhibit effector T cells when activated play a critical role in peripheral tolerance [2]. Tregs, primarily characterized by the expression of the transcription factor FOXP3, are essential for maintaining immune tolerance and suppressing excessive inflammation in the gut [3, 4]. Emerging evidence suggests that restoring Treg-mediated immune tolerance represents a promising strategy for the treatment of inflammatory bowel diseases such as ulcerative colitis (UC) [5, 6]. Approaches such as adoptive transfer of ex vivo-expanded Tregs [7], small-molecule agonists targeting Treg survival pathways, and microbiota-derived metabolites (e.g., short-chain fatty acids [FAs] and polyamines) have shown efficacy in preclinical models

Minghui Xia, Yang Liu, Ming Zeng, and Xiang-Ping Yang contributed equally

[8–10]. However, challenges remain, including maintaining Treg stability in the inflamed gut microenvironment and overcoming immune suppression resistance.

In contrast with activated effector T cells, activated Treg cells rely on aerobic metabolism of glucose and FAs compared with the anaerobic metabolism of effector T cells [11, 12]. Conversely, the presence and access to nutrients may also influence the development of regulatory and effector T cells [13, 14]. Starved cells are maintained by a process of autophagy, which is controlled by the competing actions of AMP-activated protein kinase (AMPK) and mechanistic target of rapamycin complex 1 (mTORC1) [15]. AMPK is an important energy sensor, and studies have shown that it can induce autophagy in cells under various stresses such as glucose starvation [16]. AMPK can positively regulate autophagy via ULK phosphorylation and the inhibition of mTORC1(15). By contrast, mTORC1 is a central component of the mTOR that drives protein translation and cell growth [17].

Oleic acid (OA) is a ω-9 monounsaturated FA containing one double bond (C18:1 *n*-9) that is present naturally in the human body. It is the most abundant dietary and plasma FA, representing 31% of total plasma free FAs [18, 19]. OA represents 70%–80% of olive oil composition, which is a key component of Mediterranean diet [20, 21]. Beyond its well-established cardioprotective effects [22, 23], OA demonstrates anti-inflammatory properties in a variety of inflammatory conditions such as inflammatory bowel disease [24], rheumatoid arthritis [25], central nervous system (CNS) inflammation [26], and host versus graft response [27]. The European Prospective Investigation into Cancer and Nutrition (EPIC) cohort study (n = 25,639), with a 7–11 years follow-up period, revealed a significant inverse association between OA intake and UC incidence, establishing dietary OA as a significant protective factor against UC pathogenesis [28]. Despite these associations, the mechanistic basis for OA's immunomodulation remains poorly defined, particularly its interplay with Treg biology.

In this study, we demonstrate that OA potentiates Treg cell differentiation in vitro in a dose-dependent manner, without affecting the proliferation ability of these cells. Second, we found that the enhancement of Treg cell differentiation by OA is IL-2/STAT5-independent. Furthermore, our data indicate that OA induces autophagy in Treg cells, and blockade of autophagy abolished the enhancing effect of OA on Treg cell differentiation. We found that OA administration ameliorated gut pathology in a murine dextran sulfate sodium (DSS)-induced colitis model, with increased frequency of lamina propria Tregs and elevated expression of the Treg functional markers GITR, CTLA-4, and ICOS. These findings uncover a novel mechanism by which OA promotes Treg differentiation and immune homeostasis, highlighting its potential as a therapeutic agent in autoimmune and inflammatory disorders.

2 | Result

2.1 | OA Potentiates Ex Vivo Treg Cell Differentiation

The anti-inflammatory effect of OA has been reported in several inflammatory or autoimmune experimental models [29]. Given the essential role of Tregs in maintaining immune homeostasis and preventing autoimmunity, we investigated whether OA could directly modulate Treg cell differentiation. To test this, we performed an ex vivo Treg cell differentiation assay using OA conjugated with bovine serum albumin (BSA) at different concentrations (25, 50, and 100 μM). Compared to the BSA controls, OA treatment significantly increased the frequencies of $CD4^+Foxp3^+$ Tregs (Figure 1A,B), as well as the mean fluorescence intensity (MFI) of Foxp3 (Figure 1B) in Tregs in a dose-dependent manner. Similarly, the mRNA levels of *Foxp3* were elevated upon OA treatment (Figure 1C).

As T cell differentiation is intricately linked with proliferation, we next investigated whether the positive effect of OA on Treg cell differentiation was due to altered proliferation ability. We labeled naïve T cells with CFSE and differentiated them into Treg cells with or without OA (50 and 100 μM) treatment. There was no difference in CFSE dilution between the tested groups, indicating that OA does not affect T cell proliferation (Figure 1D,E). Further analysis of the percentages of $Foxp3^+$ Tregs between OA-treated group and untreated group revealed enhanced Foxp3 expression in each individual cell cycle (Figure 1F). Taken together, these data demonstrate that OA enhanced Treg cell differentiation without affecting Treg proliferation.

2.2 | OA Enhances Treg Cell Differentiation Not Through STAT5 Activation

IL-2 signaling through STAT5 phosphorylation is a well-established pathway for Treg cell generation [30–32]. We wondered whether IL-2/STAT5 pathway contributes to OA-driven Treg cell differentiation. A 3-day differentiation assay revealed that OA treatment enhanced STAT5 phosphorylation (Figure 2A). However, acute stimulation (1.5 h) of TCR-activated $CD4^+$ T cells with OA (100 μM) failed to induce STAT5 activation, unlike recombinant IL-2 (Figure 2B), indicating that OA does not directly activate STAT5. To address whether OA enhances STAT5 phosphorylation in a per-cell level, we performed intracellular phospho-flow cytometry analyzing pSTAT5 in gated $Foxp3^+$ cells. The pSTAT5 MFI within the $Foxp3^+$ T cells was not significantly altered by OA treatment (Figure S1), indicating that IL-2/STAT5 pathway is not involved in OA-mediated Treg differentiation.

To definitively explore IL-2 dependency, we performed Treg differentiation under IL-2-neutralizing conditions (anti-mIL-2 antibodies) or exogenous IL-2 deprivation. We found that neither blockade of autocrine IL-2 production nor withdrawal exogenous rIL-2 impaired the ability of OA to potentiate Treg cell differentiation (Figure 2C,D), indicating OA promotes Treg cell differentiation in an IL-2/STAT5-independent fashion.

2.3 | OA Induces Autophagy in Treg Cells

Autophagy plays a critical role in the function of Treg cells [33]. OA has been reported to induce autophagy in several mammalian cell types, including hepatocytes and vascular smooth muscle cells [34, 35]. We wondered whether OA could affect autophagy flux in Treg cells. During autophagy,

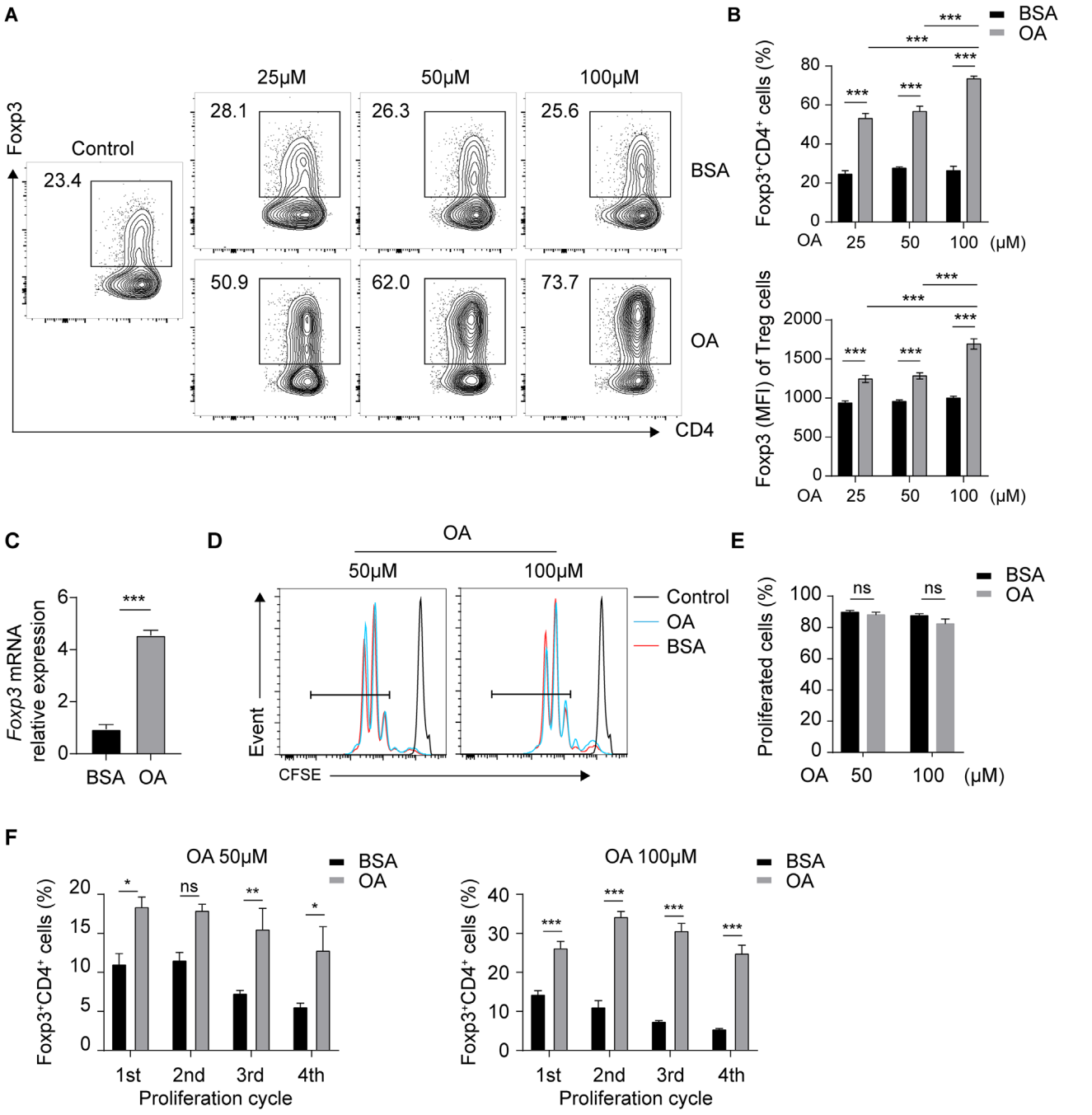

FIGURE 1 | Oleic acid (OA) potentiates ex vivo Treg cell differentiation. (A and B) Naïve CD4$^+$ T (CD44^{low}CD62L^{high}) cells enriched from spleens of C57BL/6 WT mice were cultured under Treg cell differentiation condition with increasing concentrations of OA and corresponding BSA. After 3 days of culture, cells were stained for intracellular Foxp3. Representative flow cytometry plots (A), frequencies of CD4$^+$Foxp3$^+$ T cells, and the mean fluorescence intensity of Foxp3 in CD4$^+$Foxp3$^+$ T cells (B) were determined. (C) Expression of *Foxp3* mRNA relative to β-actin was determined in Treg cells differentiated with OA (100 μM) or BSA control for 60 h. (D–F) CD4$^+$ T cells were labeled with CFSE dye and cultured for 72 h under Treg cell-polarizing conditions with OA (50 and 100 μM). Representative histogram graph (D) and the percentage of proliferating cells were determined (E). Foxp3$^+$ cells were gated on each proliferation cycle after incorporation of the CFSE proliferation dye (F). $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. (A and B) Data are representative of three experiments. (C) Data are pooled from three independent experiments. (D–F) Data are representative of two experiments. Data are means ± SEM and were analyzed by two-way ANOVA (B and F), unpaired Student's *t*-test (C and E). BSA, bovine serum albumin; MFI, mean fluorescence intensity; ns, no significance. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

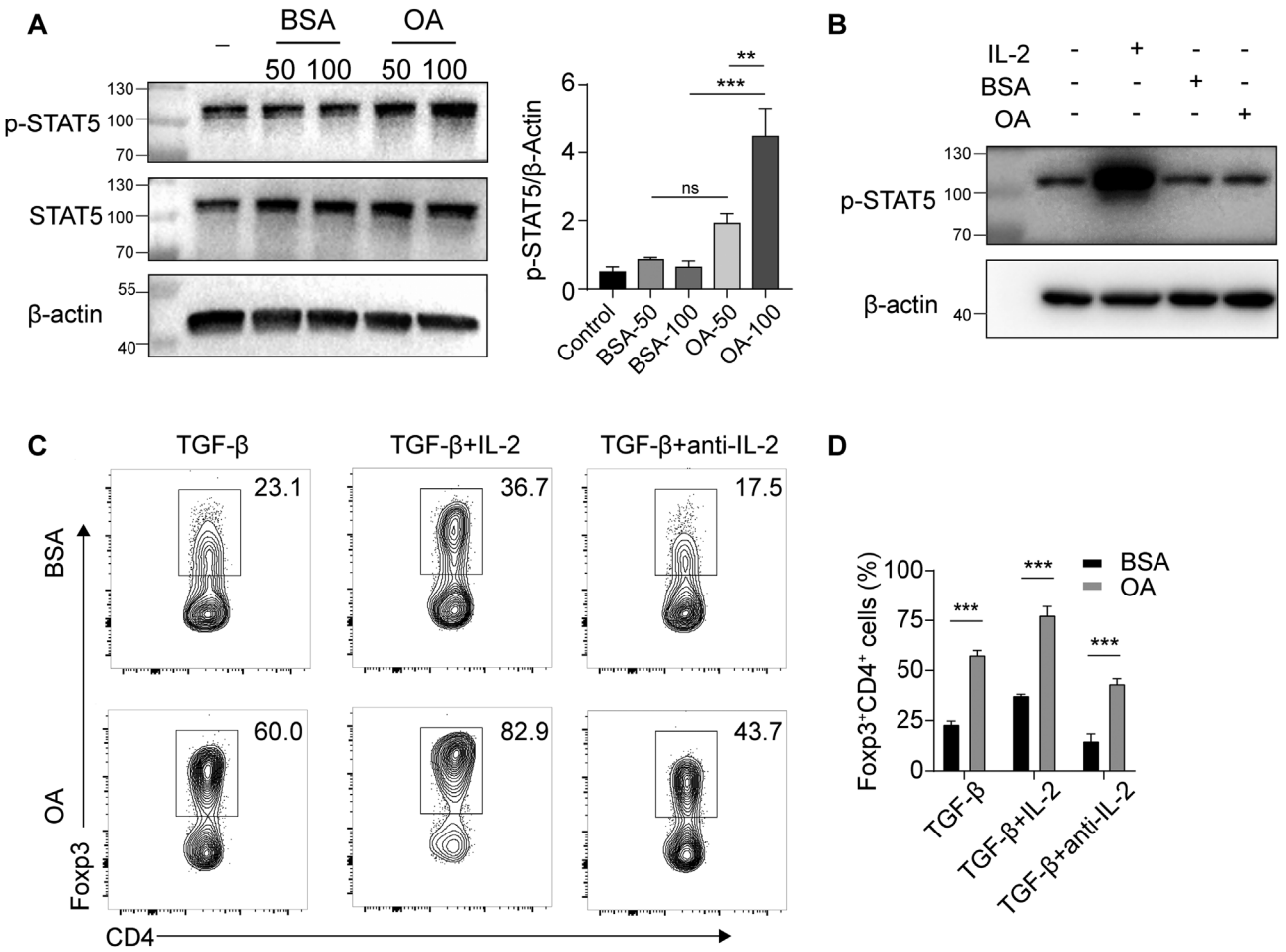

FIGURE 2 | OA enhances Treg differentiation not through STAT5 activation. (A) $CD4^+$ T cells were cultured with or without OA (50 and 100 μM) compared with BSA control under Treg cell differentiation condition for 3 days. Phosphorylated and total STAT5 proteins were determined by immunoblot in whole cell lysates. β-Actin was used as loading control for normalization of p-STAT5. (B) Isolated naïve $CD4^+$ T cells were stimulated with plate-bound anti-CD3/CD28 antibodies for 3 days. Cells were then treated with IL-2, OA (100 μM), or BSA control for 1.5 h to assess phosphorylated STAT5 proteins along with loading control β-actin detected by immunoblotting. (C and D) $CD4^+$ T cells were cultured in the presence of IL-2 and anti-IL-2 or absence of IL-2 for 3 days. Representative flow cytometry plots (C) and frequencies of $CD4^+Foxp3^+$ Treg cells were determined (D). $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. (A) Data are representative of four experiments. Data from immunoblot quantification were pooled from four independent experiments. (D) Data are representative of two experiments. Data are means ± SEM and were analyzed by one-way ANOVA (A), two-way ANOVA (D). BSA, bovine serum albumin; OA, oleic acid; ns, not significant.

cytosolic LC3-I is conjugated to phosphatidylethanolamine to form LC3-phosphatidylethanolamine conjugate (LC3-II), which is recruited to autophagosomal membranes [36]. We measured the levels of LC3 forms under Treg cell differentiation condition with or without OA treatment. We found OA treatment resulted in elevated LC3-II level (Figure 3A) and a significant decline of the autophagic substrate p62 (Figure 3A). These findings were associated with enhanced LC3 puncta observed in OA-treated Treg cells by immunofluorescence staining (Figure 3B).

Immunoblotting showed that OA-treated cells displayed a significant decrease in p-S6 levels (Figure 3C,D), indicating reduced mTORC1 activity. In contrast, we found that the phosphorylation of AMPK was elevated (Figure 3C,E). Together, these findings suggest that OA promotes autophagy by modulating AMPK and mTORC1 activity.

2.4 | The Regulatory Effect of OA on Treg Differentiation Is Autophagy Dependent

Next, we asked whether OA potentiates Treg differentiation via the induction of autophagy. To this end, we used 3-methyladenine (3-MA), an inhibitor of class III PI3K to block autophagy during Treg cell differentiation. Blockade of autophagy via 3-MA addition significantly impaired the ability of OA to promote Treg cell differentiation (Figure 4A,B). Similarly, the addition of chloroquine, which blocks the fusion of autophagosomes to lysosomes, partially attenuated OA-induced Treg differentiation (Figure 4C,D). Furthermore, we employed CRISPR-Cas9-based technology to delete *Atg7*, a core autophagy gene in Treg cells [33]. The ablation of *Atg7* markedly reduced the enhancement of Treg differentiation by OA compared to control cells (Figure 4E,F). Taken together, these data demonstrate that OA enhances Treg differentiation requiring a functional autophagy process.

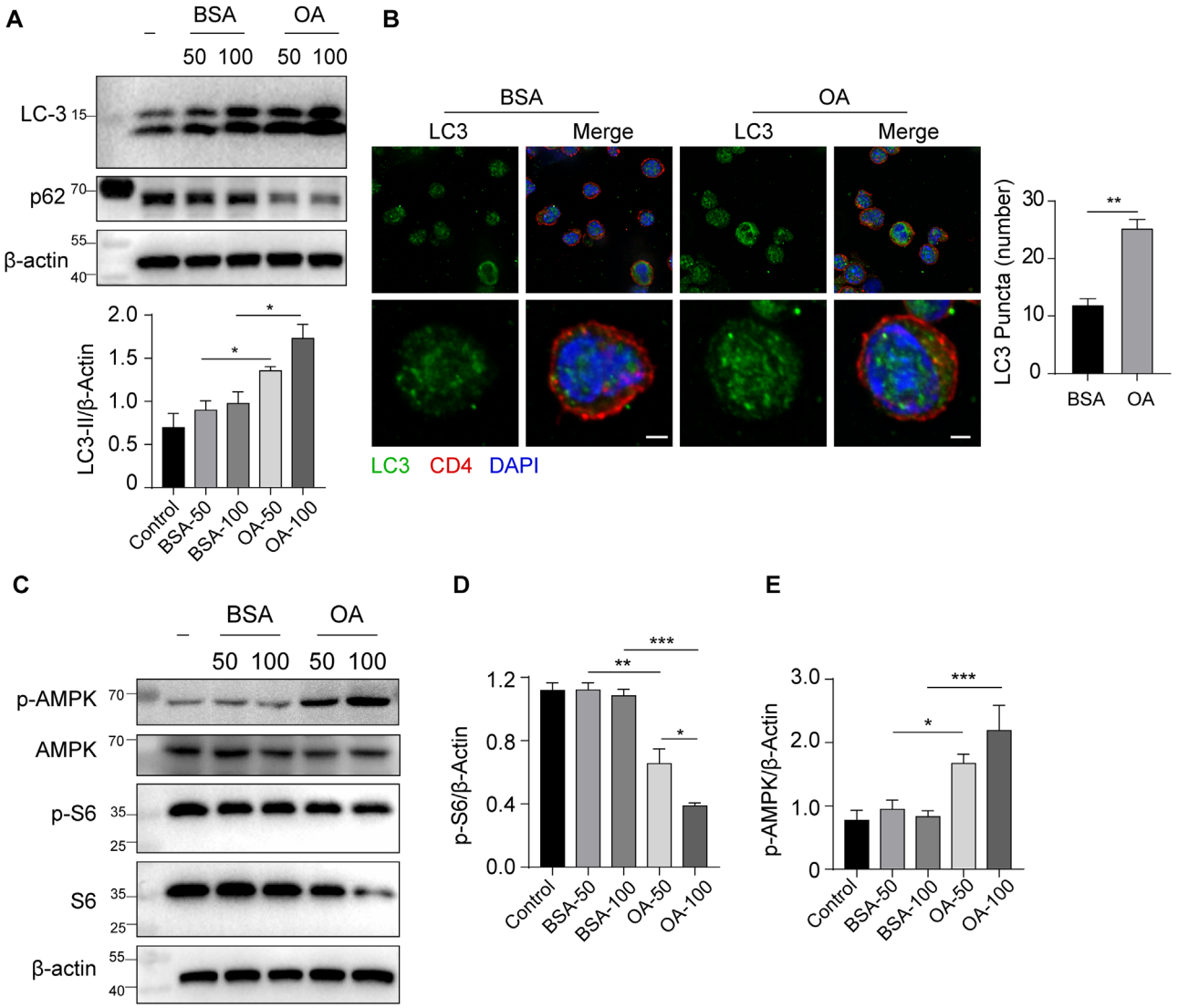

FIGURE 3 | Oleic acid (OA) induces autophagy in Treg cells. (A) Immunoblot analysis of LC3 and p62 in differentiated Treg cells upon OA (50 and 100 μM) stimulation. β-Actin was used as loading control for normalization of LC3. (B) Confocal microscopy analysis of LC3 (green) expression in Treg cells stimulated with OA (100 μM) compared with BSA control. Scale bars, 5 μm. LC3 puncta were measured by immunofluorescence in the presence or absence of OA (100 μM). (C) Naïve $CD4^+$ T cells from spleens were stimulated for 72 h with or without OA (50 and 100 μM) compared with BSA control under Treg differentiation conditions. Immunoblot analysis of p-AMPK, AMPK, p-S6, and S6 proteins in differentiated Treg cells upon OA (50 and 100 μM) stimulation. (A and C) Data are representative of four (A and C) experiments. Data from immunoblot quantification were pooled from four independent experiments. Data are means ± SEM and were analyzed by one-way ANOVA (A, D, E) and two-tailed, unpaired Student's *t*-test (B). BSA, bovine serum albumin; OA, oleic acid; ns, no significance. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

2.5 | OA Ameliorates Gut Pathology in DSS-Induced Colitis

Next, we asked whether OA can ameliorate inflammation in vivo. To this end, we used a DSS-induced colitis model in which Treg cells are critical in alleviating inflammation. In this model, OA was administrated continuously by gavage for 6 days prior to colitis induction, as depicted in the schematic representation (Figure 5A). Mice were then fed with 2% DSS-contained drinking water for 5 days. DSS-treated mice displayed continuous body weight loss, but the weight loss in mice treated with OA was significantly attenuated (Figure 5B). The bleeding scores (Figure 5C), diarrhea score (Figure 5D), and shortening of colon lengths were all ameliorated by OA treatment (Figure 5E,F). Next, we evaluated the histological changes by hematoxylin and eosin (HE) staining of the colon sections. DSS treatment resulted in massive infiltration of inflammatory cells and disruption of mucosal structures (Figure 5G). By contrast, OA administration was able to preserve the mucosal structures, accompanied with reduced infiltration of inflammatory cells (Figure 5G). Moreover, histological scores were substantially improved by OA treatment (Figure 5H). Together, these data demonstrate that OA could inhibit DSS-induced inflammation and pathology.

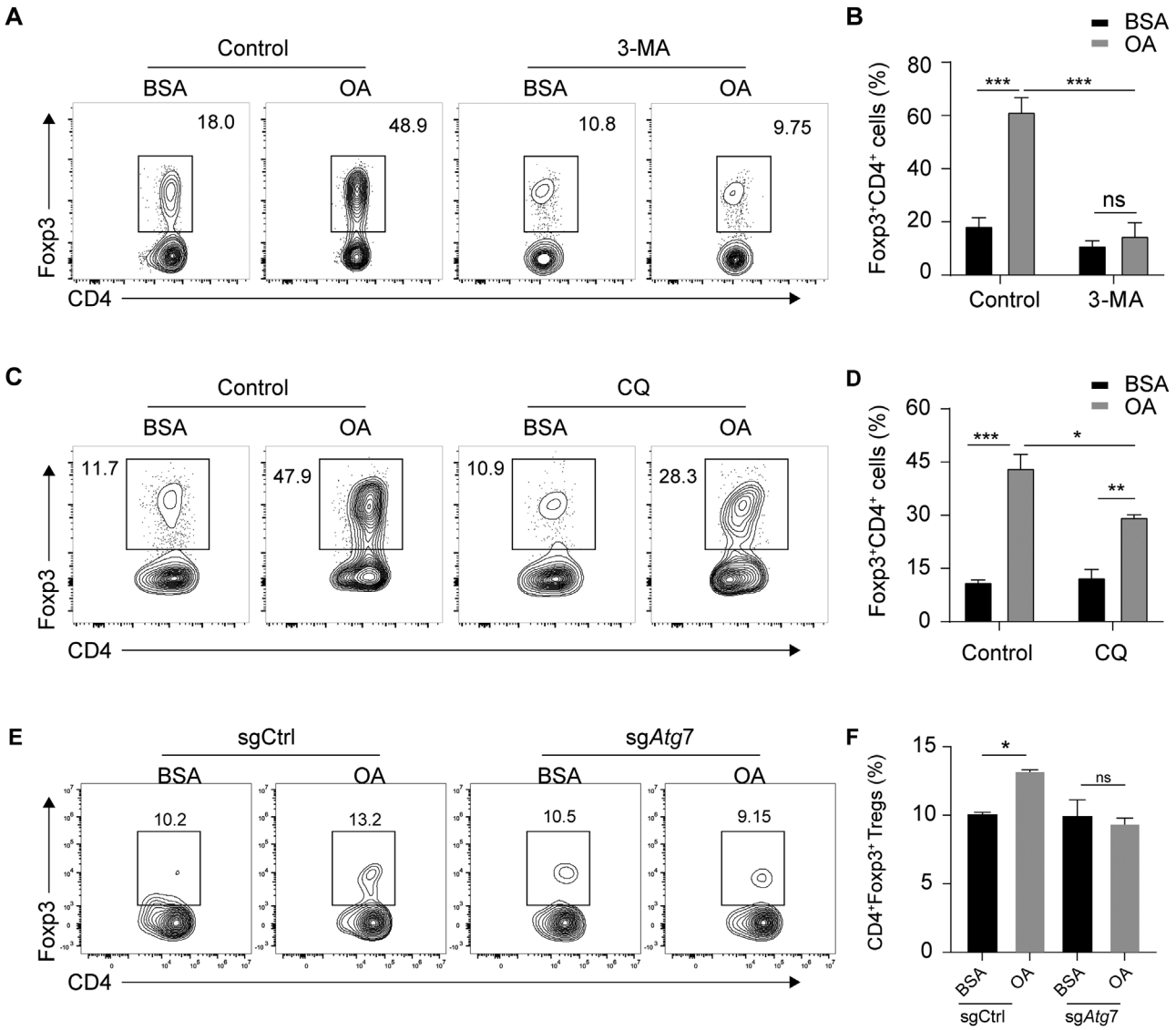

FIGURE 4 | The regulatory effect of oleic acid on Treg differentiation is autophagy dependent. (A–D) Naive $CD4^+$ T cells isolated from C57BL/6 WT mice were cultured with or without autophagy inhibitor (3-MA or CQ) in the presence or absence of OA (100 μM) under Treg cell conditions. On Day 3, the frequency of $CD4^+Foxp3^+$ T cells was quantified by flow cytometry. Representative flow cytometry plots (A and C) and frequencies of $CD4^+Foxp3^+$ T cells (B and D) were shown. (E and F) $CD4^+$ naïve T cells were transduced with sgRNA targeting a control locus (sgCtrl) or *Atg7* (sg*Atg7*) following culture under Treg-polarizing conditions with BSA (control) or OA. Representative flow cytometry plots (E) and frequencies of $CD4^+Foxp3^+$ T cells (F) were shown. (A and C) Data are representative of and pooled from three independent experiments. Data are means ± SEM and were analyzed by two-way ANOVA (B and D), one-way ANOVA (F). 3-MA, 3-methyladenine; BSA, bovine serum albumin; OA, oleic acid; ns, no significance. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

2.6 | OA Potentiates Treg Cell Differentiation In Vivo and Prevents DSS-Induced Colonic Inflammation

Next, we asked whether the protective effect of OA is associated with enhanced Treg differentiation in the DSS-induced colitis model. To test this, we analyzed the percentages of $CD4^+Foxp3^+$ Treg cells in the lamina propria using the gating strategy outlined in Figure S2. The proportions of Treg cells were not affected by DSS treatment, compared to the control group (Figure 6A,B). However, OA significantly enhanced the percentages of $CD4^+Foxp3^+$ Treg cells in DSS group (Figure 6A,B). Phenotype analysis of Tregs showed OA supplementation in DSS-treated mice (DSS + OA) significantly enhanced the expression of Treg functional markers GITR, CTLA-4, and ICOS (Figure 6C,D). Next, we detected the protein levels of TNF-α, monocyte chemotactic protein-1 (MCP-1), and IFN-γ, which are pro-inflammatory cytokines associated with colitis pathology in colons. Although DSS treatment resulted in significantly increased TNF-α, MCP-1, and IFN-γ, OA administration in DSS-treated mice significantly inhibited the levels of those pro-inflammatory cytokines (Figure 6E-G). In addition, the mRNA levels of *Tnfα*, *Ifnγ*, *Il1β*, and *Il17f* were also significantly inhibited by OA administration (Figure 6H-K). Together, these data demonstrate that OA pro-

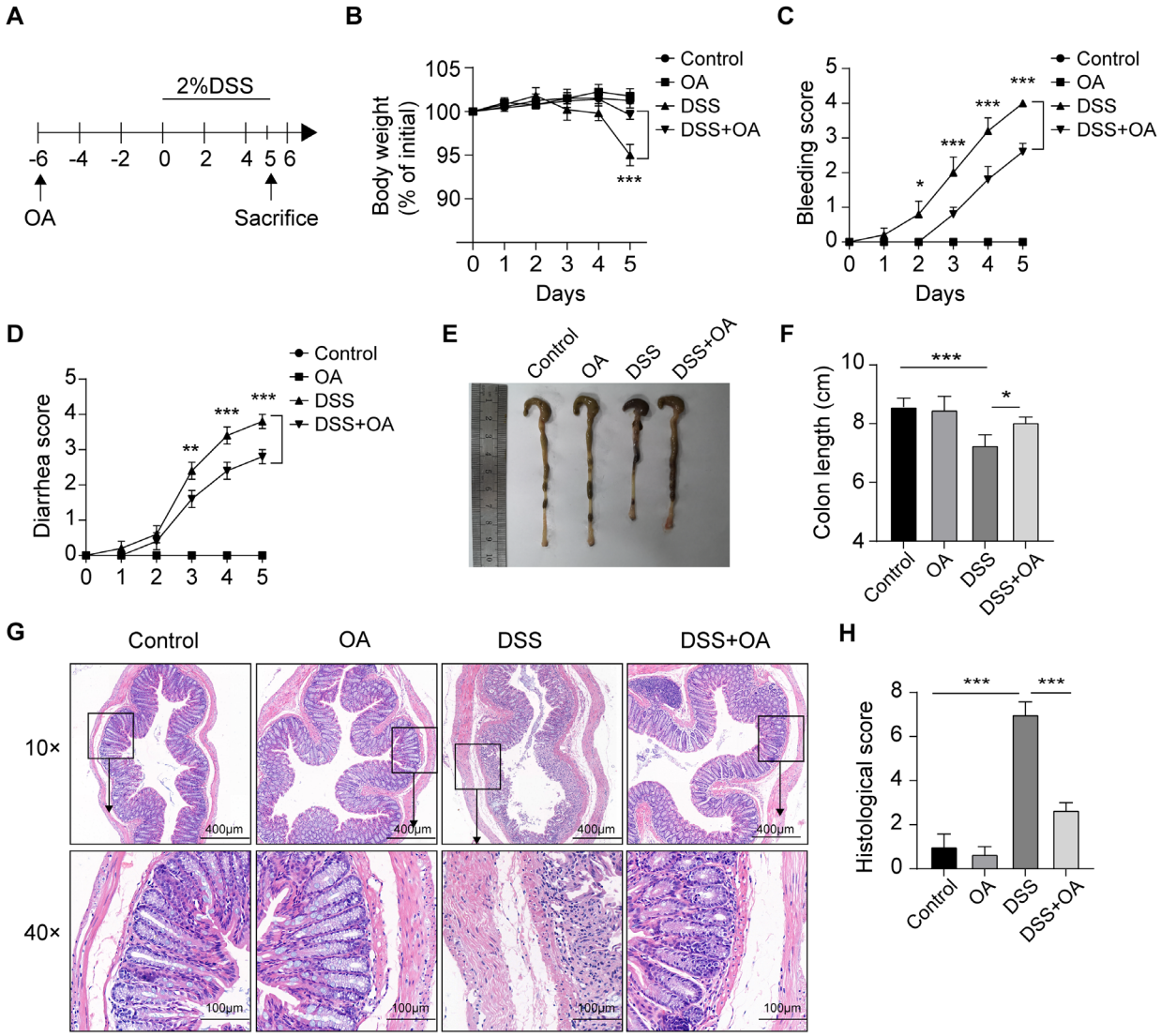

FIGURE 5 | Oleic acid ameliorates gut pathology in DSS-induced colitis. (A) Colitis was induced by oral administration of 2% w/v DSS in drinking water in C57BL/6J mice. OA (180 mg per mouse) was administrated by daily gavage for 6 days prior to DSS treatment ($n = 4, 5$). (B) Body weight was measured daily and is shown as percentage relative to initial body weight. (C) Bleeding scores of each group were measured. (D) Diarrhea scores during 5-day DSS treatment. (E) Representative photo images of the colon of each group. (F) Colon lengths by group. (G) H&E staining of colon sections. The upper row is 10× (scale bar 400 μm), and the lower row is enlarged to 40× (scale bar 100 μm). (H) Quantification of histological score for each group ($n = 3$). Data are means ± SEM and were analyzed by two-way ANOVA (B–D), one-way ANOVA (F and H). DSS, dextran sulfate sodium; OA, oleic acid. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

motes Treg cell generation, which is associated with reduced inflammation in DSS-induced colitis.

3 | Discussion

Regulatory T cells play a critical role in the establishment of intestinal immune homeostasis. The differentiation and maintenance of Treg cells are tightly regulated by homeostatic stimuli and environmental cues. We found that OA enhances the differentiation of Treg cells and alleviates DSS-induced colitis in mice.

Our findings align with the growing evidence supporting OA as a critical immuno-modulator in intestinal inflammation. Previous studies have found that impaired endogenous OA biosynthesis due to the deficiency of SCD1 exacerbates colitis [24], whereas exogenous OA supplementation exerts protective effects [37, 38]. Complementing with these experimental observations, the EPIC cohort study ($n = 25{,}639$) also identified a negative association between dietary OA intake and the incidence of UC. OA supplementation has been linked with Treg biology by several groups [39, 40]. OA availability during thymic development has been shown to program the epigenetics of $CD4^{+}$ T cells predisposing them toward Treg differentiation [39], as evidenced by its function as a source of acetyl and methyl residues for histone and DNA modification. It has also been reported that OA rescues Treg functional defects in multiple sclerosis via FAO-mediated metabolic support [40].

OA has been found to induce autophagy in various cell types [34, 35, 41, 42]. Our data demonstrate that OA promotes autophagy

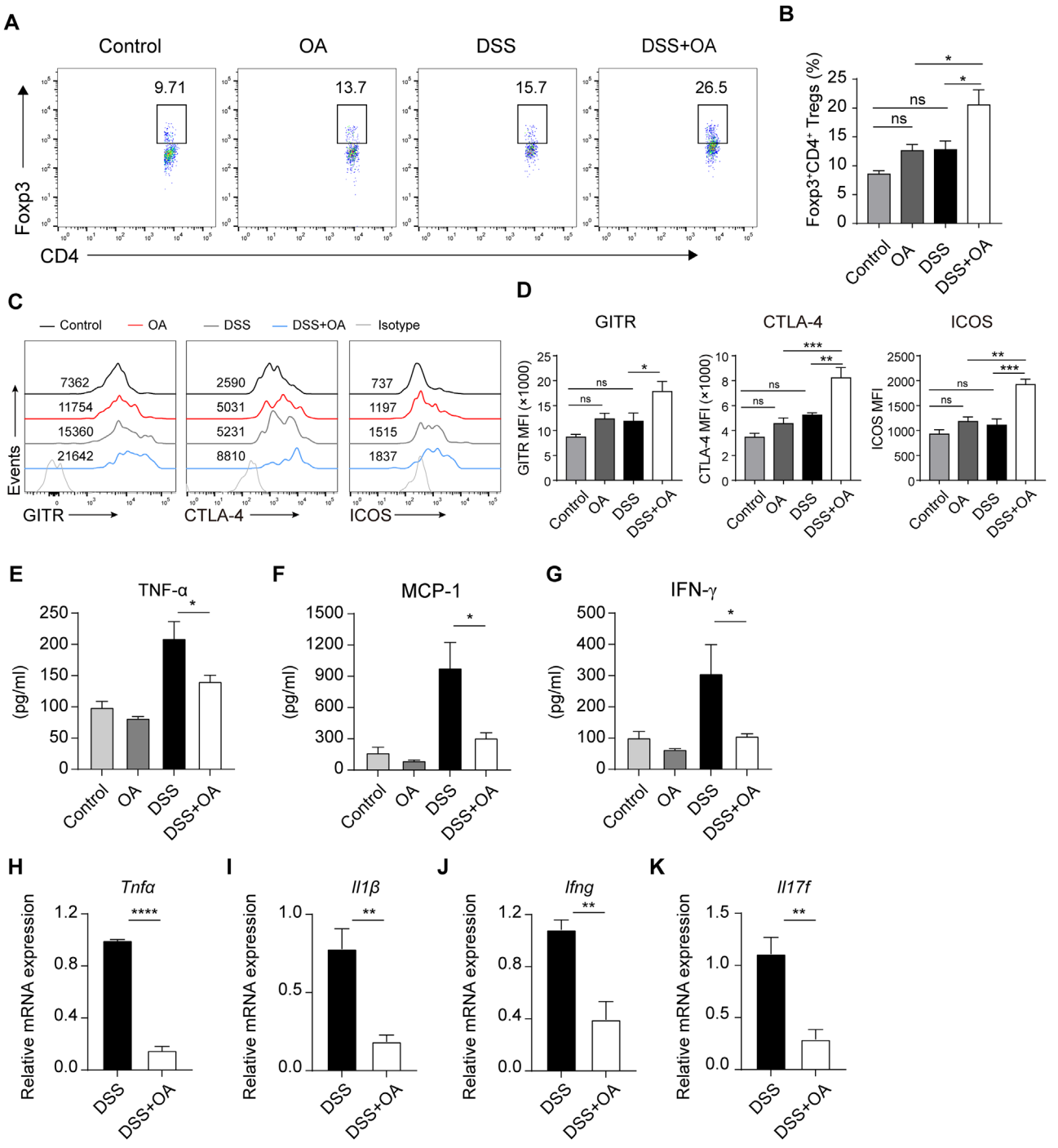

FIGURE 6 | OA potentiates Treg cells differentiation in vivo and prevents DSS-induced colonic inflammation. (A and B) Percentages of Treg were determined in lamina propria using flow cytometry. Representative flow cytometry plots (A) and the frequency of $CD4^+Foxp3^+$ T cells in lamina propria (B) were shown. (C and D) Representative histogram (C) and quantification (D) of the mean fluorescence intensity (MFI) for GITR, CTLA-4, and ICOS within gated $Foxp3^+$ cells from the colonic lamina propria ($n = 4, 5$). (E–G) The protein levels of inflammatory cytokines were determined by cytometric bead array (CBA) in the colon section ($n = 4, 5$). (H–K) Real-time PCR measurement of *Tnfα*, *Il1β*, *Ifnγ*, *Il17f* mRNA expression levels of inflammatory cytokines in colon tissues ($n = 3$). Data are means ± SEM and were analyzed by one-way ANOVA (B and D), two-tailed, unpaired Student's *t*-test (E–K). DSS, dextran sulfate sodium; OA, oleic acid.

induction in Treg cells by simultaneously inhibiting mTORC1 activity and activating AMPK signaling. This dual modulation creates a cellular environment favorable for autophagic flux, consistent with known regulatory roles of mTORC1 and AMPK in autophagy control [43]. Recent studies employing genetic ablation of core autophagy genes, including *Vps34* [44], *Atg16l1* [45], and *Atg5/7* [33], collectively underscore the indispensable role of autophagic processes in Treg cell maintenance and function. Treg-specific deletion of these proteins results in a profound reduction of Treg abundance, impaired Treg lineage stability, and

immune dysregulation. In the above research, *Atg7* knockout enhances glycolysis via mTORC1 activation, whereas *Atg16l1* loss impairs FAO; both result in compromised Treg generation and suppressive capacity. These findings establish that autophagy plays a regulatory role in the energy metabolism of Treg cells [33, 45]. In line with reports in multiple sclerosis, where OA rescues Treg function by augmenting FAO [40], autophagy licenses Treg metabolic adaptability by suppressing glycolysis [33]. Future research should investigate how autophagy interacts with Treg metabolism, particularly through metabolomic profiling and genetic mouse models.

In contrast to reports of autophagy inhibition in effector T cells at high OA concentrations (500–750 μM) [46], our study employed lower doses (25–100 μM) that specifically enhance autophagic flux in Tregs. This key distinction underscores that OA's pro-autophagic effects exhibit both cell type- and dose-dependent specificity, which may explain differential immunomodulatory outcomes in various contexts. It should be noted that, in our in vitro assays, the naïve $CD4^+$T cells were isolated on the basis of the $CD4^+CD44^{low}CD62L^{high}$ expression without CD25-negative selection, resulting in the inclusion of a small population of nTreg cells (data not shown). However, under Th0 conditions, OA did not expand $CD4^+CD44^{low}CD62L^{high}CD25^+$ population, further supporting the notion that OA primarily drives de novo Treg differentiation.

Our study has several limitations. First, in the CRISPR-Cas9 knockout experiments, the potentiating effect of OA on Treg differentiation was less pronounced than that in the standard assay. This attenuation is likely attributable to the prolonged culture and transfection stress inherent to the technical procedure, which may alter cellular responsiveness. Second, the upstream mechanism by which OA modulates the mTORC1/AMPK axis remains unclear and should be investigated using Treg-specific deletion of *Ampk* or *Raptor* mouse strains. To definitely validate the involvement of autophagy and further elucidate the downstream mechanism, Treg-specific conditional knockout models (e.g., *Atg7*- or *Atg16l1*-KO mice) should be employed.

In conclusion, our study demonstrates that OA promotes Treg differentiation through induction of autophagy and enhances Treg differentiation while suppressing DSS-induced colitis in mice. These findings suggest that OA or diets rich in OA could serve as a supplementary therapeutic approach for clinical management of autoimmune diseases or inflammatory conditions.

4 | Materials and Methods

4.1 | Mice

C57BL/6 (B6) mice of 6–8 weeks were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd. Mice were housed and bred in a specific pathogen-free facility at the Tongji Medical College, HUST, and all animal experiments were conducted in accordance with guidelines approved by the Institutional Animal Care and Use Committee of Tongji Medical College, HUST.

4.2 | Isolation and Culture of $CD4^+$ T Cells

For isolation of naive $CD4^+$ T lymphocytes ($CD4^+CD44^{low}CD62L^{high}$ T cells), cells were collected from spleens of mice and enriched by naïve $CD4^+$ T cell negative isolation kit (Cat: 130-104-454, Miltenyi Biotec). Cells were cultured in RPMI 1640 complete medium supplemented with 10% (v/v) heat-inactivated FBS (Gibco), 1% penicillin-streptomycin, 1 mM sodium pyruvate (Thermo Fisher Scientific), 1× non-essential amino acids (NEAA, Thermo Fisher Scientific), and 50 μM β-mercaptoethanol (Millipore Sigma). For Th0 conditions, naïve $CD4^+$ T cells were stimulated with plate-bound anti-CD3 (5 μg/mL, Bio X Cell) and anti-CD28 (5 μg/mL, Bio X Cell) in the presence of anti-IL-4 (10 μg/mL) and anti-IFN-γ (10 μg/mL). For Treg differentiation, cells were treated with TGF-β (4 ng/mL, R&D Systems), human IL-2 (10 ng/mL, PeproTech), anti-IL-4 (10 μg/mL), and anti-IFN-γ (10 μg/mL) for 3 days. In vitro differentiation was assessed by flow cytometry. For OA conjugation to BSA, sodium oleate (≥99%, Sigma) in RPMI 1640 medium was mixed with FA-free BSA (Sigma) at a final molar ratio of 6:1 (OA:BSA). Genetic ablation of *Atg7* was performed in primary murine naïve $CD4^+$ T cells using CRISPR-Cas9 technology. Briefly, a CRISPR-Cas9 ribonucleoprotein (RNP) complex targeting *Atg7* (target sequence: AACTCCAACGTCAAGCGGGTGGG) was assembled and delivered into cells via the ProteanFect transfection system (Cat no. PT06, Suzhou) following the manufacturer's instructions.

4.3 | DSS-Induced Colitis

Male C57BL/6 mice aged 6–8 weeks were used. Colitis was induced by administering 2% (w/v) DSS (MW 36–50 kDa, MP Biomedicals) in drinking water for 5 days. Mice were pretreated with 200 μL (180 mg) of OA (Sigma, O364525) or PBS by oral gavage for 6 days prior to DSS exposure. Body weight and clinical signs of colitis, including rectal bleeding and diarrhea, were recorded daily. Disease activity index (DAI) was scored from 0 to 4 based on weight loss, stool consistency, and bleeding.

4.4 | Isolation of Colonic Lamina Propria Lymphocytes

Colons were collected and washed with HBSS containing 1 M HEPES to remove fecal content. Epithelial cells were removed by incubation in HBSS containing 0.5 M EDTA and 1 M HEPES at 37°C for 20 min with gentle shaking. Tissues were then minced and digested enzymatically in 4.5 mL RPMI 1640 containing 0.28 mg/mL Liberase TL (Roche) and 0.2 mg/mL DNase I (Biosharp) for 15 min at 37°C with shaking. Digested tissues were passed through a 70 μm cell strainer on ice. Cells were isolated using a 40% Percoll gradient and centrifuged at 550 × *g* for 35 min at 4°C. The cell pellet was collected, washed, and resuspended for flow cytometry analysis.

4.5 | Colon Histological Analysis

Histological scores were evaluated as described [47] and were assigned by experimenters blinded to sample identity. Epithelial

damage was scored as 0 = normal; 1 = hyperproliferation, irregular crypts, and goblet cell loss; 2 = mild-to-moderate crypt loss (10%–50%); 3 = severe crypt loss (50%–90%); 4 = complete crypt loss with intact surface epithelium; 5 = small to medium ulcer (<10 crypt widths); 6 = large ulcer (≥10 crypt widths). Inflammatory cell infiltration was scored separately for mucosa (0–3), submucosa (0–2), and muscle/serosa (0–1). Total histology scores ranged from 0 to 12.

4.6 | Inflammatory Cytokine Assay

Cytokines in the colon tissue homogenates were analyzed by a mouse inflammation CBA kit following the manufacturer's instructions. This kit allows detecting six cytokines (IL-6, IL-10, MCP-1, IFN-γ, TNF, and IL-12p70) simultaneously. All assay tubes were added 25 μL of the PE detection reagent and incubated in the dark at room temperature for 2 h. After the incubation, each assay tube was added 1 mL of wash buffer and centrifuged at 200 × *g* for 5 min, followed by discarding the supernatant. The bead pellet was resuspended with 200 μL of wash buffer for the flow cytometry analysis.

4.7 | Flow Cytometry

Cells were stained in PBS containing 0.5% BSA with indicated fluorochrome-conjugated antibodies for surface marker analysis. For Foxp3 staining, cells were fixed and permeabilized using fixation and permeabilization buffers (Thermo Fisher Scientific) followed by staining with APC-conjugated anti-Foxp3 (FJK-16s; eBioscience) according to the manufacturer's instructions.

4.8 | Immunoblot and Immunofluorescence Analyses

Cells were lysed in RIPA buffer containing protease and phosphatase inhibitors (Servicebio) for 30 min on ice, followed by centrifugation at 12,000 × *g* for 10 min at 4°C. Protein concentration was determined by BCA assay. Proteins were separated by SDS-PAGE and transferred to membranes, which were probed with primary antibodies overnight at 4°C: anti-p-STAT5 (Tyr694, CST #C71E5), anti-STAT5 (CST #D206Y), anti-p-S6 (Ser235/236, CST #D57.2.2E), anti-S6 (CST #54D2), anti-LC3B (CST #D3U4C), anti-p62 (MBL #PM045), and anti-β-actin (CST #13E5). Blots were incubated with HRP-conjugated secondary antibodies and developed using enhanced chemiluminescence (ECL, GE Healthcare). Band intensities were quantified using ImageJ and normalized to β-actin. For immunofluorescence, naïve $CD4^+$ T cells were stimulated under Treg-polarizing conditions for 60 h, fixed with 4% PFA, permeabilized with 0.5% Triton X-100, blocked with 5% BSA, and incubated with anti-LC3 (MBL #PM036) and anti-CD4 (Thermo Fisher #RM4-5), followed by Cy3- and FITC-conjugated secondary antibodies. Nuclei were stained with DAPI.

4.9 | Quantitative Real-Time PCR

Total RNA was extracted using TRIzol (Invitrogen), reverse transcribed using a cDNA synthesis kit (TOYOBO, FSQ-201), and analyzed using a Bio-Rad SYBR Green intercalating fluorophore system (Applied Biosystems). Data were normalized to the expression of β-actin using ΔΔCT. The following primers were used in the present study: mouse Foxp3 forward: 5′-CCAGTACTCAGGGCAGTGTG-3′, mouse Foxp3 reverse: 5′-ACTATTGCCATGGCTTCCCC-3′.

4.10 | Statistical Analysis

Data were analyzed using GraphPad Prism and are presented as mean ± SEM. Differences between groups were assessed by Student's *t*-test or ANOVA, as appropriate. A p value <0.05 was considered statistically significant.

Author Contributions

Yang Liu, Minghui Xia, and Xiang-Ping Yang designed the experiment and wrote the manuscript. Yang Liu, Minghui Xia, Xiujuan Zhao, Qiuyang Du, Xinxin Xiong, and Zijie Chen performed the experiments and analyzed the data. Kamran Ghoreschi helped analyze the data and assisted with the experimental design. Junyan Zhang, Ming Zeng, and Jing Wang provided essential reagents and assisted with experimental design and data analysis. Yang Liu, Minghui Xia, Xiang-Ping Yang, and Arian Laurence wrote the article. Xiang-Ping Yang supervised the project.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Peer Review

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Supporting Information

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