Interleukin-17A promotes proliferation and osteogenic differentiation of human ligamentum flavum cells through regulation of β-catenin nuclear translocation

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Research Article

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Abstract

Thoracic ossification of the ligamentum flavum (TOLF) is the leading cause of thoracic spinal stenosis with no specifc treatment available. IL-17A has received widespread attention for its key contribution in the regulation of bone metabolism and heterotopic ossification. However, it is unclear whether IL-17A is involved in TOLF. Our results showed that IL-17A expression was elevated in ossified ligamentum flavum tissue compared to normal ligamentum flavum. In *in vitro* experiments, IL-17A stimulation promoted the proliferation and osteogenic differentiation of ligamentum flavum cells (LFCs) derived from TOLF patients. Mechanistically, we found that IL-17A stimulation promoted nuclear translocation of β-catenin, whereas silencing of β-catenin attenuated IL-17A-induced proliferation and osteogenic differentiation of LFCs. Furthermore, co-culture of T helper 17 (Th17) cells with LFCs showed that IL-17A secreted by Th17 cells significantly enhanced the accumulation of β-catenin in the nucleus, as well as the proliferation and osteogenic differentiation of LFCs. However, these effects were markedly attenuated after the neutralization of IL-17A. Our study demonstrates that IL-17A secreted by Th17 cells in the lesion microenvironment may be involved in TOLF by regulating nuclear translocation of β-catenin to promote proliferation and osteogenic differentiation of LFCs. This study helps to elucidate the pathological mechanism of TOLF and suggests the possibility of IL-17A as a potential target for the prevention and treatment of TOLF.

Introduction

Ossification of the ligamentum flavum (OLF) is a spinal disorder characterized by the presence of ectopic bone within ligamentum flavum. Notably, thoracic ossification of the ligamentum flavum (TOLF) is the leading cause of thoracic spinal stenosis[1], which is mainly found in East Asia, such as China[2] and Japan[3]. Since the blood supply to the thoracic spinal cord is relatively weak, once the ossified mass forms compression on the spinal cord, it may cause severe neurological dysfunction and impose a tremendous physical and psychological burden on the patients. The current view is that the development of OLF is associated with genetic[4, 5], local stress[6], and metabolic factors[7], but the exact pathogenesis is unclear.

Multiple lines of evidence suggest that the inflammatory response is closely associated with heterotopic ossification[8–10]. Undoubtedly, the process is extremely complex and the inflammatory state regulated by the involvement of various inflammatory factors will directly affect the osteogenic process[11, 12]. Endochondral ossification is considered to be the predominant pathological process of OLF[13, 14]. Likewise, inflammatory response plays an important role in endochondral ossification[15]. However, the role of various inflammatory factors in the OLF remains to be elucidated.

IL-17 is an early initiator of T cell-induced inflammatory responses and mediates the amplification of inflammatory responses by promoting the release of pro-inflammatory cytokines[16]; it also serves as a key mediator of new bone formation[17]. Several pieces of experimental evidence indicate that IL-17A plays an significant role in promoting heterotopic ossification[18]. In addition, IL-17 could promote

osteogenic differentiation of human mesenchymal stem cells[19]. However, its effect on osteogenic differentiation of ligamentum flavum cells (LFCs) remains poorly understood.

In the present study, we first analyzed the levels of IL-17A in plasma and ligamentum flavum tissues of TOLF patients *versus* non-TOLF patients. Next, we obtained TOLF patient-derived LFCs to examine the effect of IL-17A on LFC proliferation and osteogenic differentiation. Finally, we elucidated the possible mechanism of IL-17A involvement in OLF by regulating the nuclear translocation of β-catenin. The aim of this study was to elucidate the role and mechanism of IL-17A in TOLF.

Materials And Methods

Clinical specimen collection

We collected whole blood specimens from a total of 30 patients. 15 specimens were obtained from patients diagnosed with TOLF as the TOLF group and the other 15 were from patients without any spinal ligamentous ossification disease as the non-TOLF group. Additionally, we obtained 6 ligamentum flavum surgical specimens from each of the TOLF and non-TOLF groups. The specimens were collected under aseptic conditions and rinsed with phosphate buffer saline. Then, the ligamentum flavum tissues were isolated under a dissecting microscope. Freshly obtained ligamentum flavum tissues from TOLF patients were used for primary LFC isolation, and the rest were stored in liquid nitrogen for subsequent experiments. This study was approved by the Ethics Committee for Human Subjects of Peking University Third Hospital (PUTH-REC-SOP-06-3.0-A27, 2014003) and was conducted in a manner strictly in accordance with the Declaration of Helsinki.

LFC isolation and culture

LFCs were isolated from freshly obtained ligamentum flavum tissues from TOLF patients according to the previously reported method[20]. In brief, the obtained ligaments were cut into small pieces of approximately 0.5 mm^3 and washed twice with phosphate buffered saline. The clipped tissue pieces were then digested with 0.25% trypsin (Gibco, USA) followed by 250 U/mL type I collagenase (Solarbio, China). After centrifugation, the sediment was resuspended with Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) containing 10% fetal bovine serum (FBS; Gibco, USA) and 1% penicillin/streptomycin antibiotics (Gibco, USA), which was subsequently placed in 100mm culturing dishes in a humidified atmosphere with 5% CO_2 at 37°C. The second and third passage of LFCs were used for subsequent experiments. For osteogenic differentiation, LFCs were cultured in osteogenic medium consisting of DMEM supplemented with 50 μM ascorbic acid, 10 mM β-glycerophosphate and 100 nM dexamethasone (Sigma-Aldrich, USA).

Enzyme-linked immunosorbent assay (ELISA)

The levels of IL-17A in serum samples were measured by the ELISA kit (MEIMIAN, China). Briefly, the samples to be tested and the standards were added to the ELISA plate pre-coated with the specific

antibody, and then the manufacturer's instructions were followed. The absorbance of each well was detected at 450 nm using a microplate reader (Molecular Devices, USA). Ultimately, the IL-17A concentration for each sample can be calculated from the standard curve.

Cell proliferation assay

Cell counting kit-8 (CCK-8; Dojindo Co, Japan) assay was performed to detect the proliferation viability of LFCs. LFCs were seeded in 96-well plates (6000 cell/cm^2) and incubated at 37°C for 24 hours. Next, LFCs were treated with different concentrations of IL-17A (0, 6.25, 12.5, 25, 50 and 100 ng/mL) for 24 hours, followed by the addition of 10 μL of CCK-8 solution to each well. The plate was then incubated in the incubator for about 2 hours, and the absorbance per well was measured at 450 nm using a microplate reader (Molecular Devices, USA). Finally, the proliferation ability of LFCs under the influence of IL-17A was analyzed by absorbance. In addition, cell proliferation was assessed by using 5-Ethynyl-2'-deoxyuridine (EdU) detection kit (RiboBio, China) in accordance with the manufacturer's instructions. The EdU staining of LFCs was observed under a Nikon fluorescence microscope (Nikon, Japan). The proliferation of LFCs is reflected as the ratio of the number of EdU-positive cells to the number of Hoechst 33342-stained cells.

Western blot analysis

Total cellular proteins were extracted by lysing cells with RIPA and 1 mmol/L PMSF, and the nuclear proteins were obtained by using nuclear and cytoplasmic protein extraction kit (Beyotime, China). Next, the protein concentration was measured with a bicinchoninic acid (BCA) protein assay kit (Beyotime). After denaturation, the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, USA). After blocking with 5% skim milk for 2 hours, PVDF membranes were washed with TBST and incubated overnight at 4°C with the following primary antibodies: β-catenin (1:1000, 51067-2-AP, Proteintech), RUNX2 (1:1000, ab236639, Abcam), Osteopontin (1:1000, 22952-1-AP, Proteintech), Histone H3 (1:1000, 17168-1-AP, Proteintech), IL-17A (1:1000, 26163-1-AP, Proteintech), β-actin (1:1000, 66009-1-Ig, Proteintech). The membranes were then incubated with secondary antibodies for 2 hours. Finally, imaging was performed on an iBright CL1500 Imaging System Imaging System (Invitrogen, USA), and quantitative analysis was performed using iBright Analysis Software (Invitrogen).

Alkaline phosphatase (ALP) activity assay and Alizarin red staining

To assess the osteogenic differentiation potential of LFCs, ALP activity assay was used to assess early osteogenic activity, while Alizarin red staining was performed to detect matrix mineralization. LFCs were seeded in 6-well plates at a density of 1×10^5 per well and cultured in osteogenic medium under different conditions for 7, 14, 28 days. ALP activity was assessed with an Alkaline Phosphatase Assay Kit (Beyotime, China) and mineralization was assessed with an Alizarin Red S Staining Kit (Beyotime, China) according to the manufacturer's instructions.

small interfering RNA (siRNA) transfection

To inhibit the expression of β-catenin, LFCs were transfected with β-catenin siRNAs (si-β-catenin, RiboBio, China) or control siRNAs (si-Control, RiboBio) using riboFECT™ CP transfection reagent (RiboBio) according to the manufacturer's instructions. Then, western blotting was performed to evaluate the silencing effect of siRNA. The final target sequence of β-catenin used in this study is as follows: 5'-GCCACAAGATTACAAGAAA − 3'.

T cell culture and co-culture with LFCs

Naive $CD4^+$ T cells were isolated from human peripheral blood mononuclear cells using MojoSort™ Human CD4 Naïve T Cell Isolation Kit (BioLegend, USA). Then, obtained T cells were cultured with an equal number of magnetic beads for T cell expansion and activation using the Dynabeads Human T-Activator CD3/CD28 kit (Thermo Fisher Scientific, USA). For human T helper 17 (Th17) cell differentiation, T cells were cultured in the presence of the Th17-polarizing cytokines, IL-1β (20 ng/mL), IL-6 (30 ng/mL), IL-23 (30 ng/mL), and TGF-β1 (2.25 ng/mL) (BioLegend, USA), in addition to Anti-IFN-γ (1 μg/mL) and Anti-IL-4 (2.5 μg/mL) antibodies (BioLegend, USA). To investigate the effect of Th17 cells on LFCs, LFCs were co-cultured with naive $CD4^+$ T or Th17 cells in the 6-well Transwell plates (0.4 μm, Corning, USA). After co-culture, LFCs were harvested for proliferation and osteogenic potential assessment.

Statistical analysis

Data were described as the mean ± standard deviation (SD) and analyzed using GraphPad Prism 7.00 (GraphPad Software, USA). Student's *t* test (*t*-test) was used for comparison of two groups and the one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used for comparison of more than two groups. $P < 0.05$ was considered statistically significant.

Results

IL-17A expression is increased in the ossified ligamentum flavum

To investigate whether IL-17A is associated with TOLF, we first examined the expression of IL-17A in TOLF *versus* non-TOLF ligamentum flavum tissues by western blot assay. As shown in Fig. 1A-C, the expression of IL-17A was significantly increased in the ligamentum flavum tissue in the TOLF group compared with the non-TOLF group. In addition, we measured IL-17A levels in the plasma of individuals within the TOLF and non-TOLF groups by ELISA assay. Notably, the results showed that the IL-17A levels were comparable in the TOLF and non-TOLF groups (Fig. 1D). These results suggest that the elevated IL-17A in TOLF tissues may originate from the local microenvironment rather than the peripheral blood circulation.

IL-17A promotes the proliferation of LFCs

The proliferative activity of LFCs reflects, to some extent, the cellular viability and osteogenic potential of LFCs. To examine the effect of IL-17A on the proliferation of LFCs, we first detected the proliferative activity of LFCs under different concentrations of IL-17A treatment by CCK-8 assay. As shown in Fig. 2A, the cellular viability of LFCs increased with the increasing concentration of IL-17A. When IL-17A reached 50 ng/mL, the increase in cellular viability was significant compared to LFCs without IL-17A treatment. Therefore, a concentration of 50 ng/mL was used as the subsequent treatment concentration. Next, EdU staining assay was performed to further determine the proliferative activity of LFCs in response to IL-17A treatment. The results showed that the proportion of EdU-positive cells was significantly higher with IL-17A treatment compared to the control group (Fig. 2B, C). All these results indicate that IL-17A has the effect of promoting the proliferation of LFCs.

IL-17A promotes osteogenic differentiation of LFCs

To identify the effect of IL-17A on the osteogenic differentiation potential of LFCs, western blotting was performed to detect the expression of osteogenic-related markers (RUNX2, Osteopontin). The results showed that IL-17A significantly increased the expression of these markers in LFCs (Fig. 3A-C). Furthermore, as shown in Fig. 3D, LFCs treated with IL-17A exhibited higher ALP activity compared to the control group after 7 days of osteogenic induction. Similarly, the results of Alizarin red staining showed that IL-17A-treated LFCs had more pronounced calcium deposition than controls after 28 days of osteogenic induction (Fig. 3E, F). These results suggest that IL-17A facilitates osteogenic differentiation of LFCs.

IL-17A enhances the nuclear translocation of β-catenin in LFCs

It has been demonstrated that IL-17 can regulate osteogenic differentiation of mesenchymal stem cells by promoting the β-catenin signaling pathway[18]. To investigate whether IL-17A regulates the β-catenin signaling pathway in LFCs, we first examined the protein expression of total and nuclear β-catenin in LFCs under different concentrations of IL-17A treatment by western blot assay. The results showed that the total β-catenin protein expression levels of LFCs were not significantly different under different concentrations of IL-17A treatment (Fig. 4A, B). However, the protein expression of β-catenin in the nucleus was increased in an IL-17A concentration-dependent manner (Fig. 4C, D). These results imply that IL-17A may promote the nuclear translocation of β-catenin in LFCs.

IL-17A-induced nuclear translocation of β-catenin is involved in the facilitative effect of IL-17A on LFCs

Next, we further explored the role of the β-catenin signaling pathway in the effect of IL-17A on LFCs by downregulating β-catenin expression. As shown in Fig. 5A and B, the results of western blot showed a significant reduction of β-catenin expression after siRNA transfection, confirming the successful

knockdown of β-catenin in the nucleus of LFCs. The EdU staining assay showed that β-catenin knockdown reduced the proportion of EdU-positive cells under IL-17A treatment (Fig. 5C, D). Then, we examined the expression of osteogenic-related markers under the condition of β-catenin knockdown. The results of western blot assay showed that the expression of RUNX2 and Osteopontin was significantly decreased after β-catenin silencing (Fig. 5E-G). Moreover, both ALP activity (Fig. 5H) and calcium deposition (Fig. 5I, J) were significantly inhibited in IL-17A-induced LFCs after β-catenin silencing. The above results suggest that IL-17A-induced pro-proliferation effect and enhanced osteogenic differentiation potential in LFCs is mediated, at least partially, by the nuclear translocation of β-catenin.

Th17 cells may be the main source of IL-17A in TOLF

It is known that IL-17A is mainly synthesized and secreted by Th17 cells, but other types of cells of origin also exist, such as invariant natural killer T (iNKT) cells and myeloid cells[21]. To clarify the IL-17A-secreting cells in TOLF, we found by immunohistochemical staining that CCR6 (a specific marker of Th17 cells) positive cells were present in ossified ligamentum flavum tissues, while they were barely detected in normal ligamentous tissues (Fig. 6A). Subsequently, naive $CD4^+$ T cells were isolated from the peripheral blood of TOLF patients and induced to Th17 cells. The effect of Th17 cells on LFCs was revealed by co-culture system[22]. As shown in Fig. 6B and G, EdU staining showed that LFCs co-cultured with Th17 cells had a higher proportion of EdU-positive cells compared to LFCs co-cultured with naive $CD4^+$ T cells. Moreover, the expression levels of RUNX2, Osteopontin, and β-catenin in the nucleus were significantly increased in LFCs co-cultured with Th17 cells (Fig. 6C-F). As shown in Fig. 6H and I, the calcium deposition in LFCs was markedly increased after co-culture with Th17 cells.

To further determine whether co-culture effects were mediated by IL-17A secreted by Th17 cells, neutralizing antibodies to IL-17A were used to block the effects of IL-17A. As shown in Fig. 7A and B, the proportion of EdU-positive cells in LFCs co-cultured with Th17 cells was distinctly decreased after neutralization of IL-17A. The results of western blot assay showed that neutralizing IL-17A significantly inhibited the protein expression levels of RUNX2, Osteopontin and β-catenin in the nucleus of LFCs co-cultured with Th17 cells (Fig. 7C-F). Furthermore, Alizarin red staining showed that calcium deposition in LFCs co-cultured with Th17 cells was markedly decreased after IL-17A antibody treatment (Fig. 7G, H).

These results indicate that IL-17A in the local microenvironment of the ossified ligamentum flavum may be mainly derived from Th17 cells.

Discussion

TOLF is a type of heterotopic ossification that occurs in the spinal ligaments and has the typical characteristics of endochondral ossification[13]. Inflammation is a well-documented component of the osteogenic microenvironment during endochondral ossification[23–25]. A growing amount of evidence has demonstrated that inflammatory factors, such as TNF-α[26, 27] and IL-6[28], also play a significant role in the development of TOLF. In our study, we found that IL-17A levels were distinctly elevated in

ossified ligamentum flavum tissues, and it may be mainly derived from Th17 cells in the local microenvironment of the lesion. More importantly, IL-17A can promote the proliferation and osteogenic differentiation potential of TOLF patient-derived LFCs. This suggests that IL-17A-mediated inflammatory responses may play an essential role in the pathology of TOLF.

IL-17A is a cytokine with multifunctional properties. It mediates numerous pathophysiological processes such as inflammatory responses[29, 30], tumor immunity[31–33], and bone remodeling[34, 35] by acting on different types of downstream cells and prompting the synthesis and secretion of various pro-inflammatory mediators, chemokines, and proliferative cytokines. In the present study, we found for the first time that IL-17A has a role in enhancing osteogenic differentiation of LFCs. Numerous evidence has previously shown that IL-17A can promote bone formation[19, 36–38]. However, some other studies have concluded the opposite[39–41]. The contradictory findings resulting from IL-17A are attributed to the multi-targeting nature of IL-17A action and the complexity of its function.

On the one hand, IL-17A can act on many types of cells, such as mesenchymal stem cells and other cells with osteogenic potential. For example, it has been shown that IL-17A could promote osteogenic differentiation of human mesenchymal stem cells[42]. Moreover, Lavocat et al. reported that osteogenic differentiation of fibroblast-like synoviocytes was significantly enhanced by IL-17A stimulation[43]. However, IL-17A can also work on osteoclasts or immune cells[44, 45], which exhibit a very different effect, namely, promoting bone resorption. Interestingly, IL-17A functions in osteoblasts and regulates bone resorption by promoting the release of receptor activator of nuclear factor-kappaB ligand (RANKL) [46]. This is because the processes of bone formation and bone resorption are interactive.

On the other hand, IL-17A forms a complex regulatory network with its interacting factors. It has been reported that IL-17A could act synergistically with TNF-α to remarkably enhance matrix mineralization capacity of MSCs, whereas IL-17A stimulation alone could not effectively promote bone matrix formation in MSCs[47]. Moreover, Sritharan et al. found that treatment with the combination of IL-17A and IL-6 had a synergistic effect on promoting osteogenic differentiation in the murine osteoblast cell line (MC3T3-E1) [48]. In our study, we currently only detected the effect of IL-17A on LFCs, and whether it interacts with TNF-α or IL-6 in the pathogenesis of TOLF needs to be further investigated.

IL-17A is well-known for its key role in chronic inflammatory diseases such as Ankylosing spondylitis (AS) [49, 50]. Multiple lines of evidence[51, 52] suggest that IL-17A has a role in promoting new bone formation during the pathological process of AS, which usually involves multiple segments of the spine. Interestingly, more than half of the patients with TOLF also show multi-segmental involvement of the ligamentum flavum[53], which suggests to us that this part of TOLF is not only a localized lesion, but systemic factors may also be involved, similar to AS. This will change our current perception of TOLF. More importantly, it is expected to transform TOLF from traditional surgical treatment to targeted therapy. At present, it is poorly understood whether there is a link between TOLF and AS. However, it is certain that IL-17A plays an important role in the pathogenesis of both. Therefore, the potential relationship between

TOLF and AS and the possible similarities of their pathological mechanisms deserve further investigation.

However, some limitations remain in this study. First, the present study only confirms the effect of IL-17A on LFCs in *in vitro* experiments, which is not yet supported by *in vivo* experimental evidence. In addition, this study did not explore the effect of inflammatory factors such as TNF and IL-6 on LFCs when co-existing with IL-17A. In other words, it may not reflect the real complex pathological microenvironment *in vivo*. In view of the above limitations, the development of IL-17A as a therapeutic target for TOLF still needs further validation in animal experiments and clinical trials.

In conclusion, this is the first study to discover the involvement and possible mechanism of IL-17A in TOLF. We provided evidence to support that IL-17A derived from Th17 cells in the lesioned microenvironment involves in TOLF probably through regulation of nuclear translocation of β-catenin to promote proliferation and osteogenic differentiation of LFCs. Our study thus identifies that IL-17A may be a potential target for the prevention and treatment of TOLF.

Declarations

Author Contributions

Jialiang Lin: Conceptualization, Methodology, Data curation, Visualization, Writing – original draft; Shuai Jiang: Data curation, Resources, Formal Analysis; Qian Xiang: Software, Investigation, Validation; Yongzhao Zhao: Data curation, Software, Formal Analysis; Longjie Wang: Data curation, Resources; Dongwei Fan: Formal Analysis, Methodology; Woquan Zhong: Resources; Chuiguo Sun: Resources; Zhongqiang Chen: Resources; Weishi Li: Conceptualization, Funding acquisition, Supervision, Resources, Project administration, Writing – review & editing.

Declaration of competing interest

The authors declare no conflict of interest.

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Figures

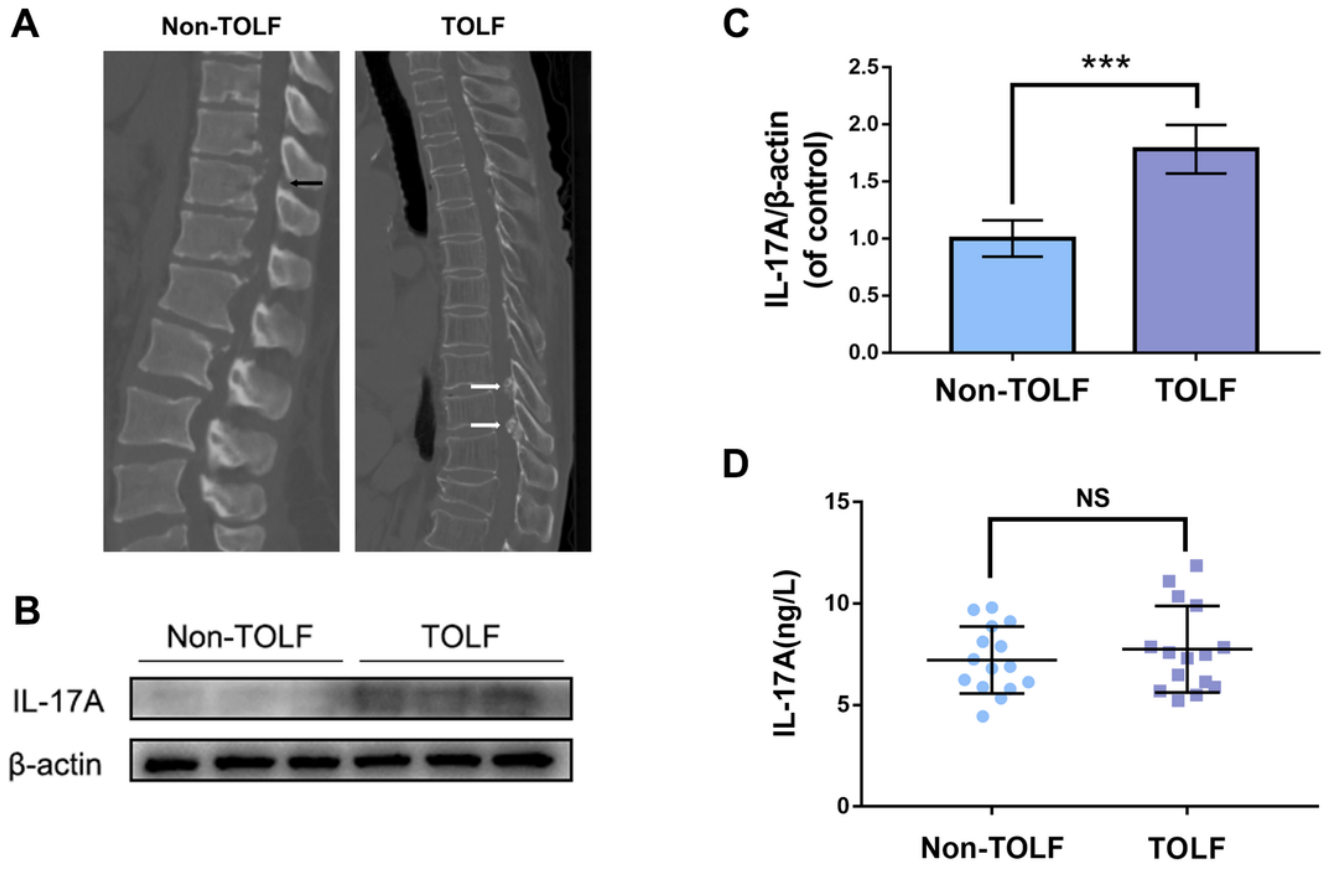

Figure 1

IL-17A is elevated in ossified ligamentum flavum tissue. **(A)** Representative sagittal CT images of patients with non-TOLF (Male, 20y, thoracic disc herniation; black arrow: normal ligamentum flavum, T10-11) and TOLF (Male, 65y, TOLF; white arrow: ossified ligamentum flavum, T9-11). **(B)** Representative western blot results of IL-17A expression in ligamentum flavum tissues of non-TOLF and TOLF patients. **(C)** Quantification of IL-17A immunoblots. **(D)** ELISA results of plasma IL-17A levels in non-TOLF and TOLF patients. Data are presented as the mean ± SD of three independent experiments. ***, *P* <0.001; NS, not statistically significant.

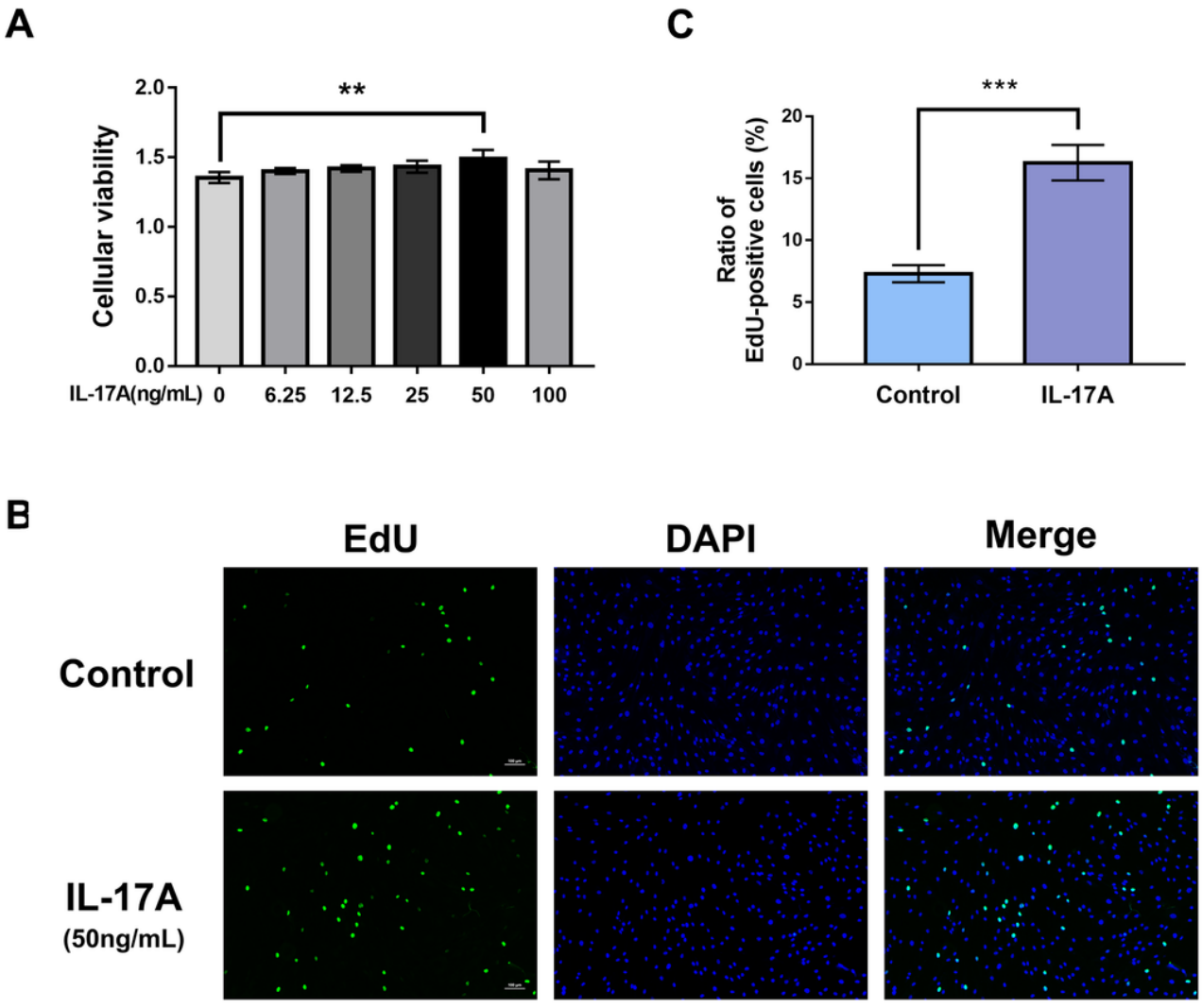

Figure 2

IL-17A promotes the proliferation of LFCs. **(A)** Detection of cellular viability of LFCs under different concentrations of IL-17A treatment by CCK-8 assay. **(B)** Cell proliferation analysis in normal and IL-17A-treated LFCs by EdU staining assay (scale bar: 100 μm). **(C)** Quantification of EdU-positive cells. Data are presented as the mean ± SD of three independent experiments. **, *P* <0.01; ***, *P* <0.001.

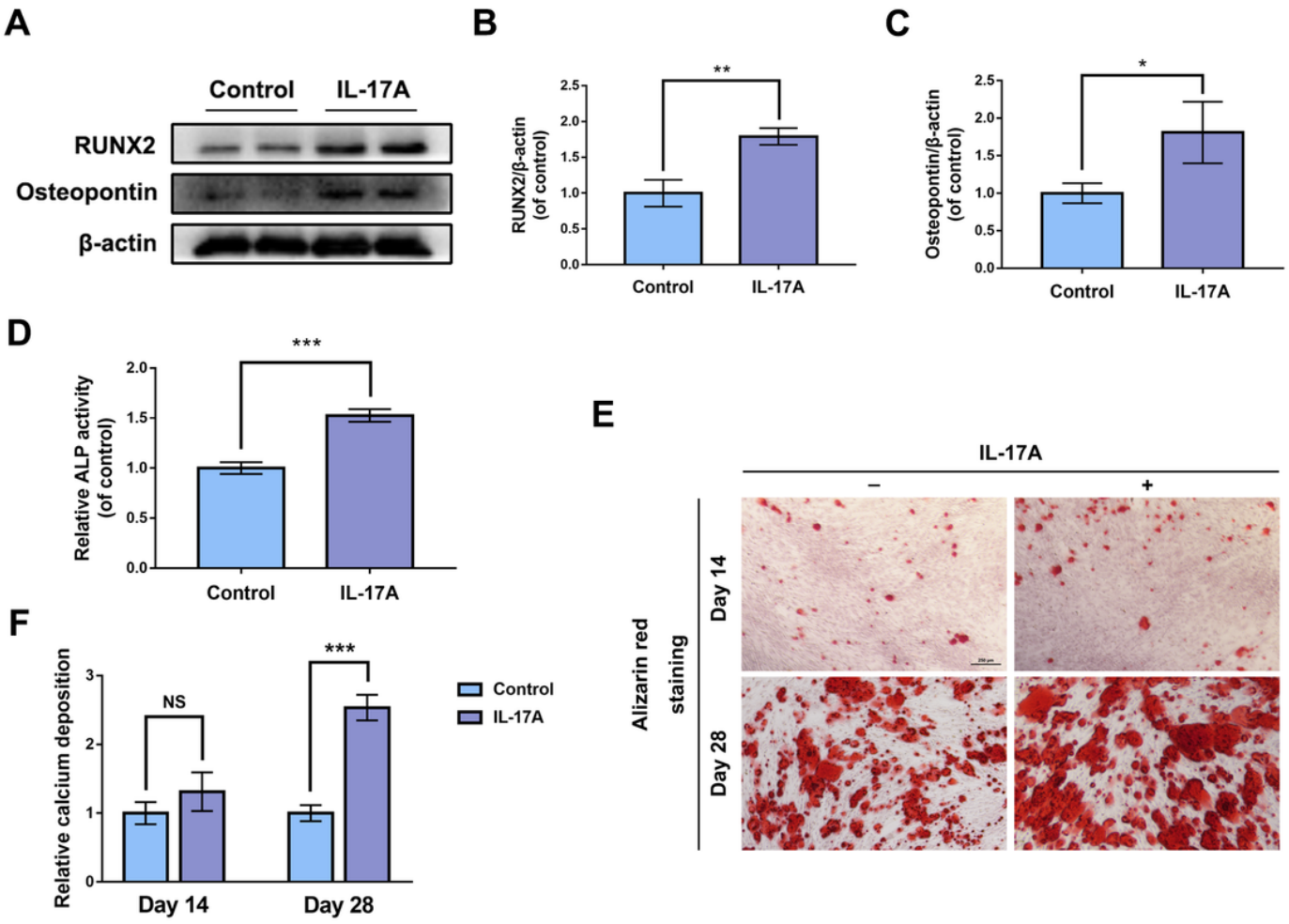

Figure 3

IL-17A promotes osteogenic differentiation of LFCs. **(A-C)** Protein level analysis of osteogenic indicators in normal and IL-17A-treated LFCs by western blot. **(D)** ALP activity analysis in normal and IL-17A-treated LFCs. **(E, F)** Mineralization analysis of normal and IL-17A-treated LFCs by Alizarin red staining after 14 and 28 days of osteogenic induction (scale bar: 250 μm). Data are presented as the mean ± SD of three independent experiments. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; NS, not statistically significant.

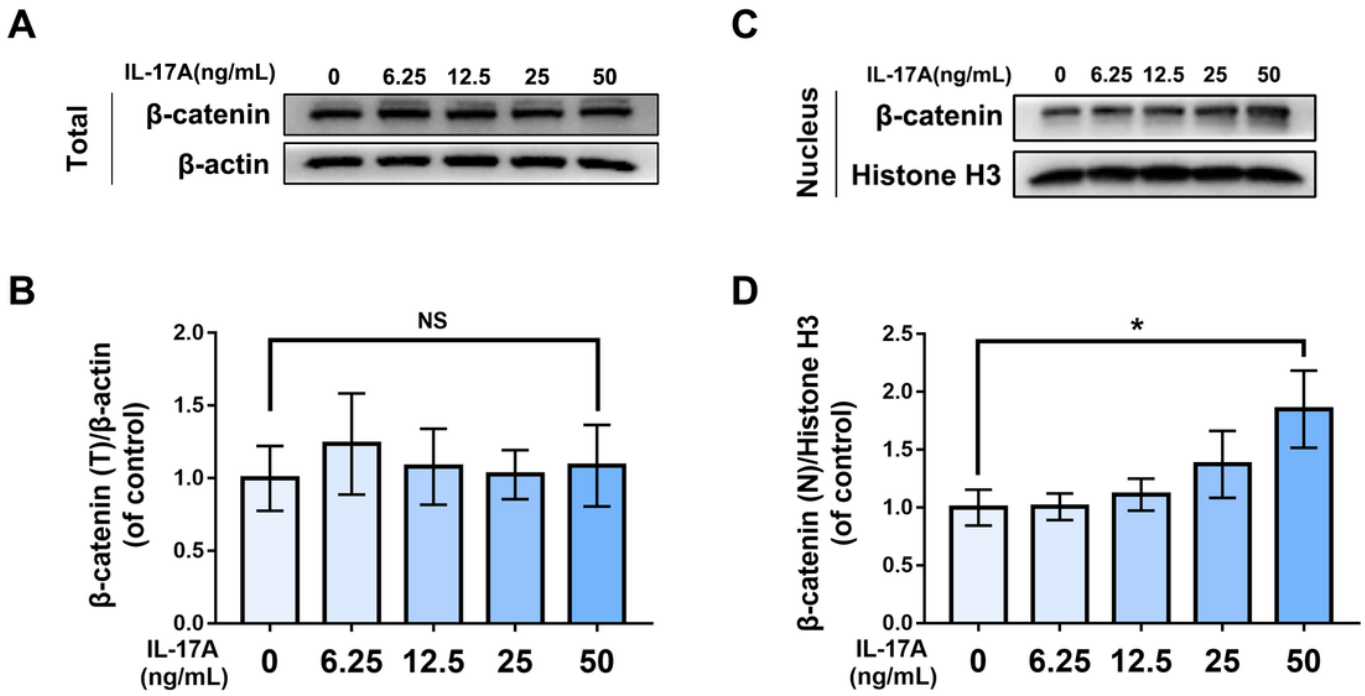

Figure 4

IL-17A contributes to the nuclear translocation of β-catenin in LFCs. **(A, B)** Protein level analysis of total β-catenin of LFCs under different concentrations of IL-17A treatment by western blot. **(C, D)** Protein level analysis of nuclear β-catenin of LFCs under different concentrations of IL-17A treatment by western blot. Data are presented as the mean ± SD of three independent experiments. β-catenin (T), total β-catenin; β-catenin (N), nuclear β-catenin; *, $P<0.05$; NS, not statistically significant.

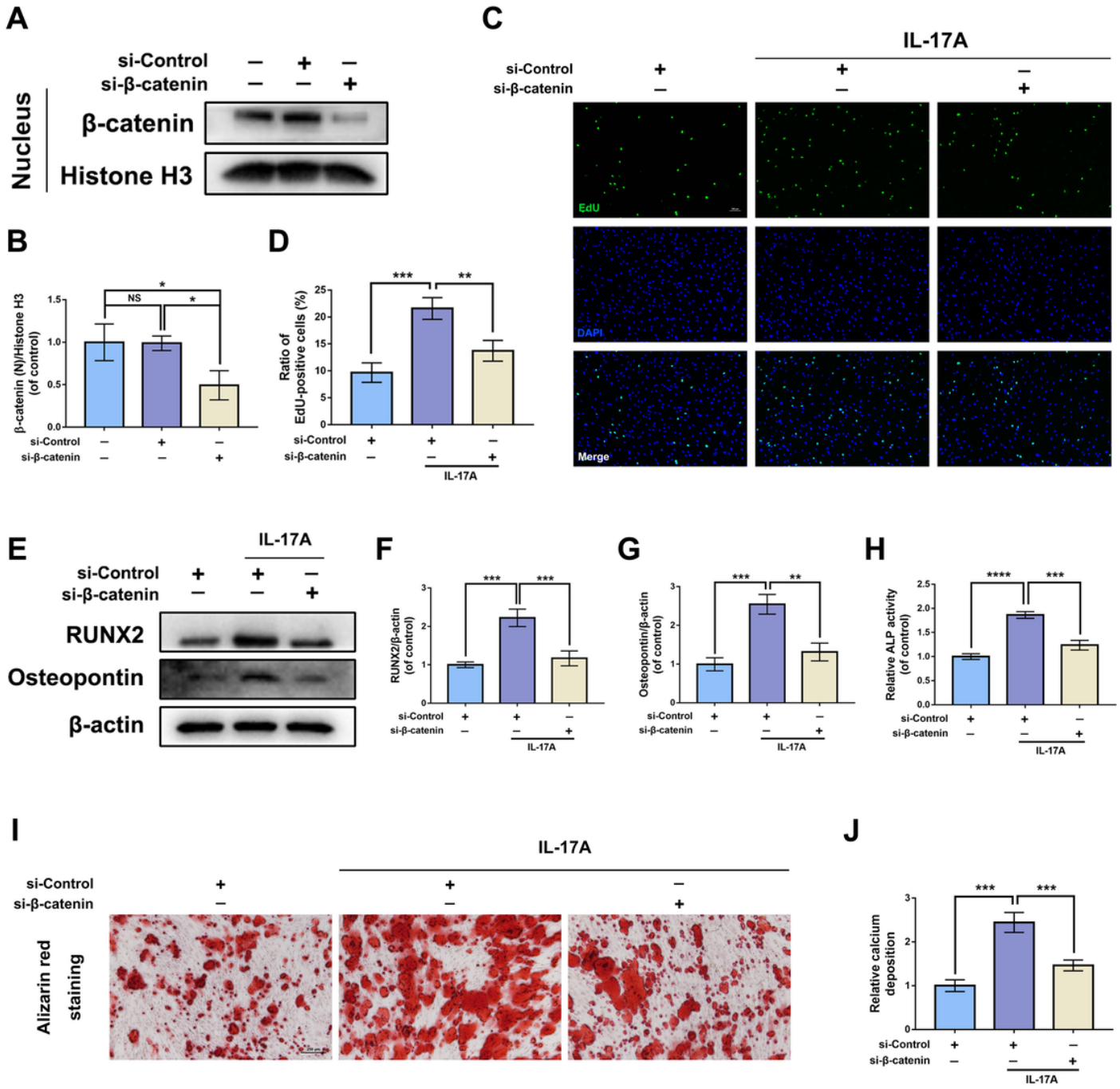

Figure 5

Nuclear translocation of β-catenin is involved in IL-17A-mediated proliferation and osteogenic differentiation of LFCs. **(A, B)** Analysis of β-catenin expression in the nucleus after siRNA transfection by western blot. **(C)** EdU staining of normal and IL-17A-treated LFCs with si-Control or si-β-catenin pretreatment (scale bar: 100 μm). **(D)** Quantification of EdU-positive cells. **(E-G)** Western blot analysis of osteogenic indicators in normal and IL-17A-treated LFCs with si-Control or si-β-catenin pretreatment. **(H-J)** ALP activity and mineralization analysis of normal and IL-17A-treated LFCs pretreated with si-Control or

si-β-catenin. (scale bar: 250 μm). Data are presented as the mean ± SD of three independent experiments. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$.

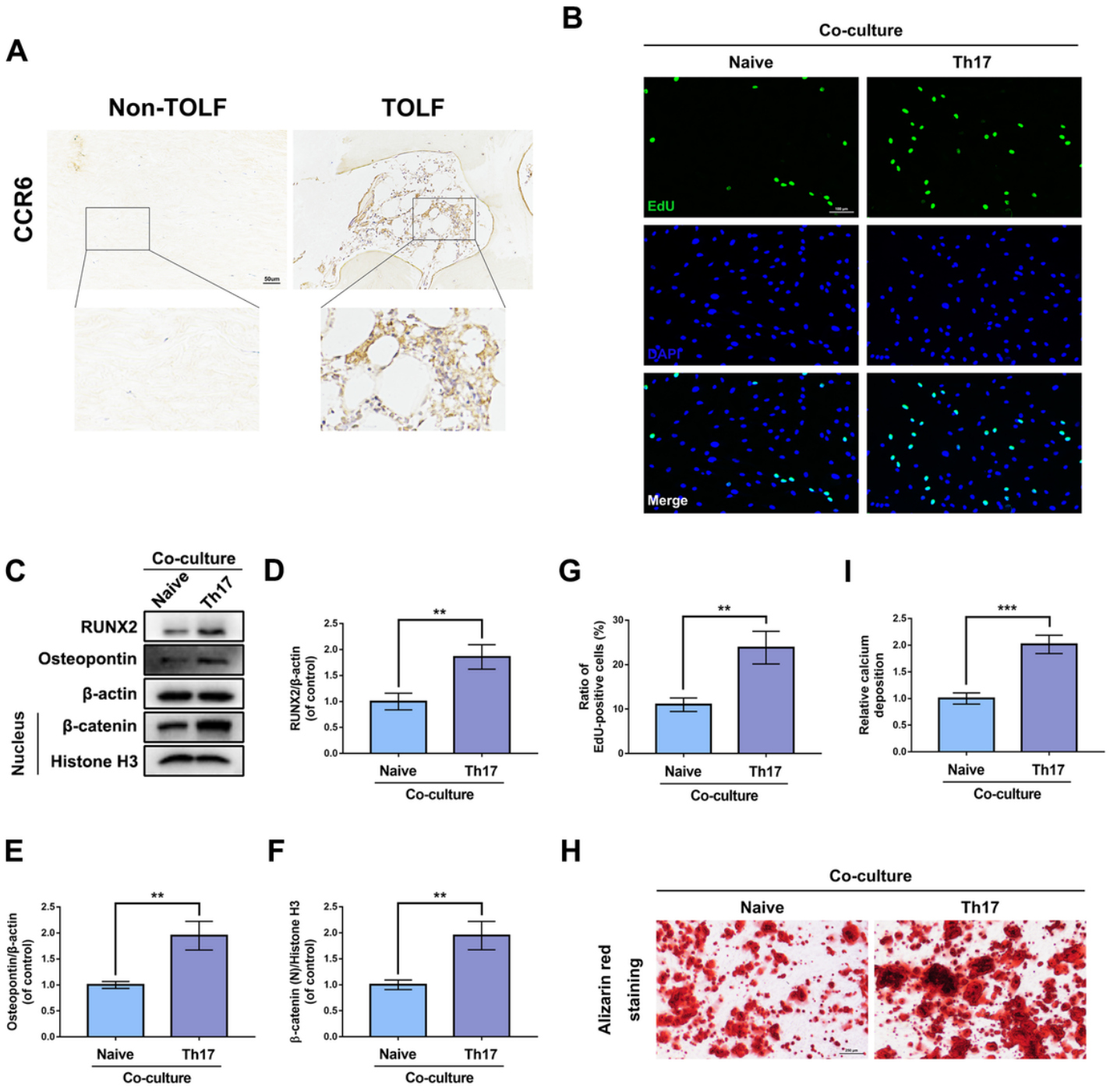

Figure 6

Co-culture with Th17 cells promotes the proliferation and osteogenic differentiation of LFCs. **(A)** Immunohistochemistry staining of CCR6 in normal and ossified ligamentum flavum tissues (scale bar: 50 μm). **(B, G)** EdU staining analysis of LFCs after co-culture with naive $CD4^+$ T cells or Th17 cells (scale bar: 100 μm). **(C-F)** Protein level analysis of osteogenic indicators and nuclear β-catenin of LFCs after co-

culture with naive CD4$^+$ T cells or Th17 cells. **(H, I)** Mineralization analysis of LFCs after co-culture with naive CD4$^+$ T or Th17 cells by Alizarin red staining (scale bar: 250 μm). Data are presented as the mean ± SD of three independent experiments. **, P<0.01; ***, P<0.001.

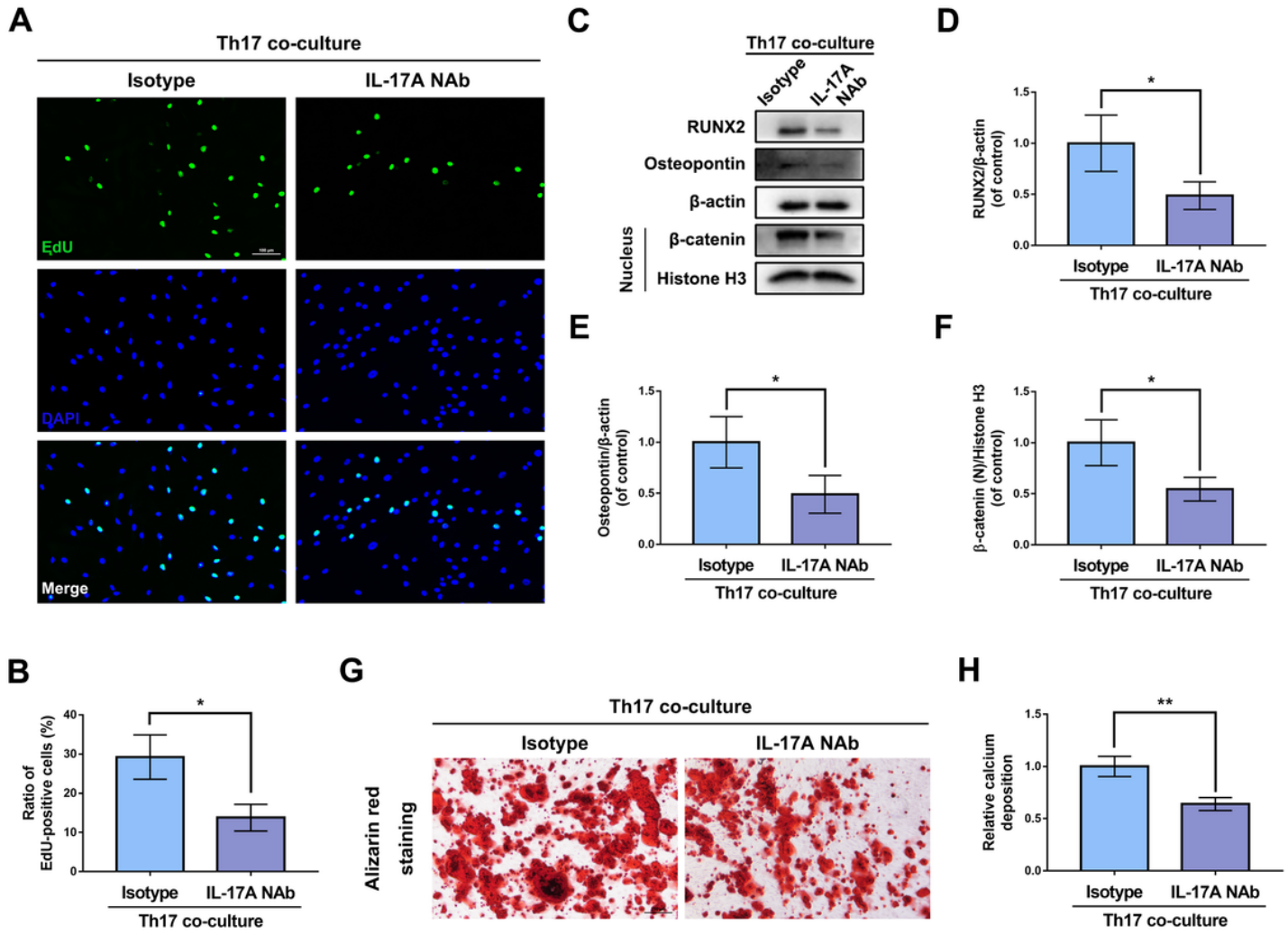

Figure 7

Neutralization of IL-17A attenuates the proliferation and osteogenic differentiation of LFCs induced by Th17 cells. **(A, B)** EdU staining analysis of co-cultured LFCs treated with the isotype antibody or IL-17A neutralizing antibody (scale bar: 100 μm). **(C-F)** Protein level analysis of osteogenic indicators and nuclear β-catenin of co-cultured LFCs treated with the isotype antibody or IL-17A neutralizing antibody. **(G, H)** Alizarin red staining and quantification of co-cultured LFCs treated with the isotype antibody or IL-17A neutralizing antibody (scale bar: 250 μm). Data are presented as the mean ± SD of three independent experiments. *, P<0.05; **, P<0.01.