



Vincristine Regulates C/EBP- β /TGF- β I to Promote A1 Astrocyte Polarization and Induce Neuropathic Pain

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Background: The neuropathic pain side induced by Vincristine severely limit its clinical application. However, the mechanism of neuropathic pain is not clear. This study aims to clarify the mechanism of C/EBP- β regulating TGF- β 1 mediated spinal astrocyte A1/A2 polarization in the neuropathic pain caused by vincristine.

Methods: Neuropathic pain model was established in rats by intraperitoneal injection of Vincristine (VCR). In vitro experiment, the astrocyte model was constructed by Vincristine, and si-C/EBP- β was regulated before VCR administration. Pain threshold of rats was measured by thermal withdrawal latency (TWL) and mechanical withdrawal threshold (MWT), Elisa was used to detect the expression level of inflammatory factors, qRT PCR and Western blotting were used to detect astrocyte polarization markers, C/EBP- β , TGF- β 1, p-smad2 and p-smad3.

Results: Following Vincristine administration, the TWL and MWT of rats exhibited a decrease. Additionally, there was an increase in A1 polarization of astrocytes, while A2 polarization remained relatively unchanged. Furthermore, the expression levels of pro-inflammatory factors were elevated, whereas no significant alterations were observed in anti-inflammatory factors. Notably, Vincristine promoted the expression of C/EBP- β and TGF- β 1. TGF- β 1 inhibitor alleviated VCR induced astrocyte A1 polarization and release of proinflammatory factors, ameliorated abnormal pain. Moreover, silencing C/EBP- β reversed the enhanced expression of TGF- β 1 induced by Vincristine, attenuated astrocyte A1 polarization and proinflammatory factor release.

Conclusion: Vincristine induced spinal cord inflammation by promoting A1 polarization of astrocytes via upregulating the C/EBP- β /TGF- β 1 signal pathway, thus leading to neuropathic pain. It was different from the traditional signal pathway, this study shown a new signal pathway for astrocyte A1 polarization, which may provide a possibility for clinical treatment of neuropathic pain.

Keywords: neuropathic pain, astrocytes, C/EBP- β , TGF- β 1, neuroinflammation

Introduction

Vincristine is a conventional chemotherapy drug that has good therapeutic effects on a variety of malignant tumours, especially in children with acute leukaemia.¹ However, its tendency to cause neuropathic pain as a side effect severely limits its clinical use. The International Association for the Study of Pain (IASP) defines neuropathic pain as pain caused by injury or disease of the somatosensory nervous system, which can lead to motor, sensory, and autonomic dysfunction, seriously affecting the quality of life of patients.² Spinal cord-related mechanisms play important roles in the development of neuropathic pain.³ Pathological changes in the spinal cord, such as inflammation, play prominent roles in neuropathic pain,⁴ but whether these changes are related to neuropathic pain caused by vincristine and the specific mechanism are not clear.

As a kind of glial cells, astrocytes play a key role in the structure and function of the spinal cord.⁵ Astrocytes can be activated in some pathological conditions, such as injury and disease. Like microglia, astrocytes can be polarized toward one of two phenotypes: the A1 proinflammatory phenotype and A2 anti-inflammatory phenotype.⁶ The A1/A2 polarization of astrocytes is closely related to the pathological process of neuroinflammation. In a variety of nervous system diseases, such as Alzheimer's disease, Parkinson's disease, and multiple sclerosis, astrocyte polarization and neuroinflammation can be observed,⁷ but whether A1 or A2 astrocyte polarization is related to neuroinflammation caused by vincristine is unclear.

Transforming growth factor beta 1 (TGF- β 1) is a multifunctional cytokine that can be synthesized in almost all cells. It regulates cell proliferation, growth, differentiation and movement and plays an important role in immune regulation and promoting fibrosis.⁸ Some studies have shown that TGF- β 1 can induce morphological changes in astrocytes and increase the expression of glial fibrillary acidic protein (GFAP) and other marker proteins.⁹ Other studies have shown that TGF- β 1 may interact with other signalling pathways, inhibit the A1 polarization of astrocytes or promote A2 astrocyte polarization.¹⁰ However, the effect of TGF- β 1 on the A1 and A2 polarization of astrocytes in the context of the neuropathic pain caused by vincristine needs to be further explored.

C/EBP- β is a transcription factor belonging to the CCAAT/enhancer binding protein (C/EBP) family, it is involved in regulating the expression of many genes and plays important roles in cell proliferation, differentiation, and apoptosis and metabolic regulation.¹¹ C/EBP- β can directly bind to the promoter region of the TGF- β 1 gene and affect the expression level of TGF- β 1 by regulating its transcriptional activity.¹² Thus, C/EBP- β plays an important role in the transcriptional regulation of TGF- β 1. Current studies on astrocytes and neuropathic pain usually focus on cytokines, chemokines and common pathways such as NF- κ B, Janus kinase (JAK), this study aimed to determine whether C/EBP- β regulates TGF- β 1-mediated A1/A2 astrocyte polarization in the context of vincristine-induced neuropathic pain, providing a novel signal pathway for astrocyte polarization and idea for the treatment of neuropathic pain.

Materials and Methods

Animals

SPF-grade Sprague–Dawley (SD) rats weighing 250–300 g were purchased from the Animal Experiment Center of Guizhou Medical University. The rats were provided free access to water and food and housed at an appropriate temperature on a 12-hour light–dark cycle. Adaptive feeding was performed for one week before the formal experiment. Our research protocol was approved by the Animal Care and Use Committee of the Affiliated Hospital of Guizhou Medical University. This study was carried out in strict accordance with the Care and Use of Laboratory Animals established by the US National Institutes of Health. The sample size for the animal experiments was calculated based on the Resource Equation Approach. Considering the reproducibility of the samples and the need for multiple biochemical assays, 16 rats were ultimately determined to be used in each group.¹³ The rats were randomly divided into three groups: the control group (Group sham), the vincristine group (Group VCR), and the TGF- β 1 inhibitor group (group P144). Six rats were used for behavioural test, five rats were used for WB, qRT-PCR and Elisa, and the remaining rats were used for HE staining and immunofluorescence. Each repeat was performed as a separate, independent experiment.

Neuropathic Pain Model

According to our previous research, 0.1 mg/kg vincristine (HY-N0488, Med Chem Express, Shanghai, China) was intraperitoneally injected once a day for the first five days and on days 8–12 to establish a neuropathic pain model. TGF- β 1 inhibitor Disitertide (product number HY-p0118, MCE) was administered at a dose of 6.5 mg/kg once a day for 14–20 days. All drugs were dissolved in a saline solution containing 1% DMSO and administered by intraperitoneal injection. Behavioural tests were carried out two hours after drugs administration on days 0, 3, 7, 10, 14 and 21 to observe whether neuropathic pain was successfully induced in the rats (Figure 1A). The rats were killed immediately after the behavioural test on the 21st day, and the spinal cord was collected for subsequent testing.

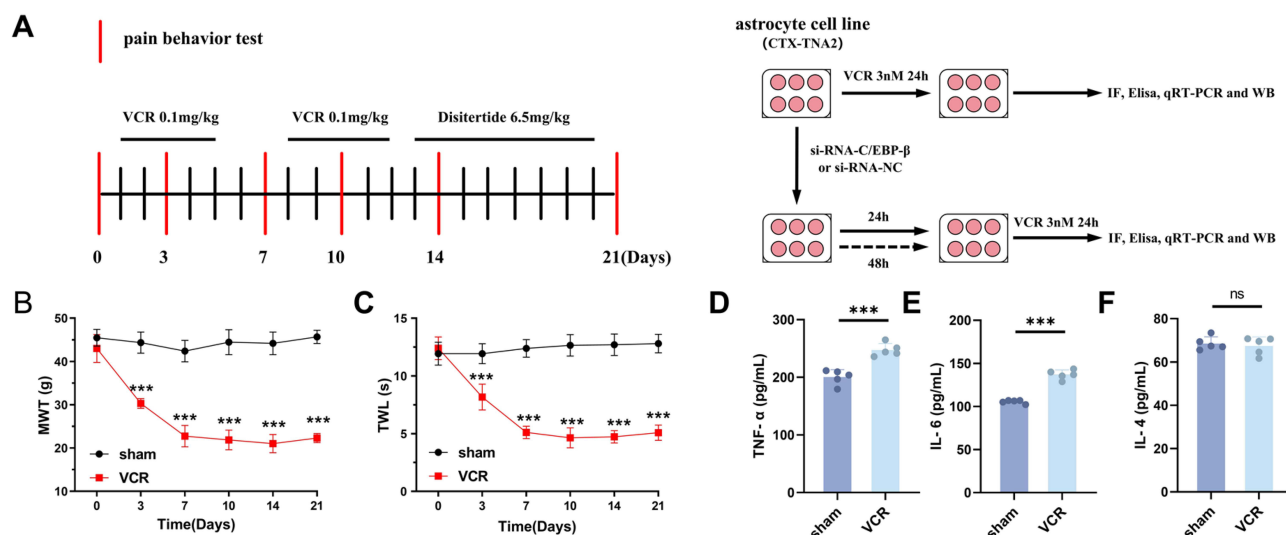


Figure 1 Vincristine induced neuropathic pain in rats and promoted inflammatory factors in the spinal cord. **(A)** The protocol of this experiment. **(B, C)** MWT and TWL of rats in each group. $n=6$ for each group. *** $p < 0.001$ compared with sham group. **(D–F)** Inflammatory factors of TNF- α , IL-6 and IL-4 in spinal cord of rats in each group. $n=5$ for each group. *** $p < 0.001$ compared with sham group. T -test was used in statistical tests.

Behavioural Test

The baseline thermal withdrawal latency (TWL) and mechanical withdrawal threshold (MWT) values of the rats were measured before vincristine was administered. The TWL and MWT were also evaluated on days 3, 7, 10, 14 and 21 after vincristine administration. For the Von Frey test, the rats were placed in a transparent plastic cage. The hind paws of the rats were stimulated perpendicular to the surface with a Von Frey electronic pain metre. The highest force required to cause retraction or licking of the paw was considered the pain threshold. Stimulation and measurement were performed every 30seconds for a total of 5 measurements, and the average value was taken as the MWT. For the thermal hyperalgesia test, a hot plate maintained at approximately $52 \pm 1^\circ\text{C}$ was used. Each rat was placed in an open, round, transparent plastic cylinder and allowed to adapt for 30 minutes, and the time from when the paw was placed on the hot plate to when the rate lifted or licked its hind paw, jumped, or tried to escape was recorded. To avoid tissue damage, the maximum time was set to 30s. If a rat did not respond within 30s, it was removed from the cylinder, and the TWL was recorded as 30s. Every 5 minutes 3 successive measurements were taken, and the average value was calculated.

Cell Culture

An astrocyte line (CTX-TNA2) (Subekang Cell Technology Co., Ltd., Shanghai) was used for the cell experiments.¹⁴ The cells were cultured with DMEM containing 10% FBS+1% penicillin/streptomycin. The cells were cultured in an incubator containing 5% CO_2 at 37°C . A CCK-8 kit (product number: HY-K0301, Med Chem Express, Shanghai, China) was used to measure cell activity. Suspended cells were inoculated in a 96-well plate (100 μL /well) according to the manufacturer's instructions. Vincristine solutions of different concentrations were added, and the cells were cultured for another 24 h. Ten microlitres of CCK-8 solution was added to each well, and the culture plate was placed in an incubator for 2 hours. The absorbance at 450 nm was measured with a microplate reader.

Cells were plated in six-well plates to establish a cell model. The astrocytes were cultured for 24 h in medium containing 3 nM vincristine, after which the cells were collected for Western blotting (WB), qRT-PCR and IF. The cells were transfected with C/EBP- β siRNA for 48 h (for WB and IF) or 72 h (for qRT-PCR) using GP transfer Mate (Shanghai Jima Pharmaceutical Technology Co., Ltd.) before the addition of vincristine. For the in vitro experiments, the cells were divided into four groups: the control group (group sham), vincristine group (group VCR), siRNA control+ vincristine group (Group NC-VCR) and si-C/EBP- β + vincristine group (Group si-VCR).

Immunofluorescence Staining

After behavioural testing, the rats were anaesthetized with 1% pentobarbital and perfused through the left ventricle with precooled 4% paraformaldehyde, and then spinal cord tissue was collected and fixed in paraformaldehyde. Then, paraffin sections (10 μ m) of the spinal cord tissue were prepared. The paraffin slices were placed in an electric thermostatic drying oven at 60°C for 1 h and then dewaxed in water. The slices were incubated in Tris EDTA antigen repair solution (product number C1038, Solarbio, Beijing, China) at 90°C for 10 minutes, and then 3% hydrogen peroxide was added for 10 minutes. The sections were fixed with 4% paraformaldehyde for 10 min. After they were incubated with 5% goat serum for 1 h, primary antibodies against C3 (1:200), C/EBP- β (1:200), TGF- β 1 (1:500) and GFAP (1:500) were dropped onto the slices, which were incubated overnight at 4°C. Then, a CoraLite Plus 488-conjugated secondary antibody (1:200, product number RGAR002, Proteintech, Wuhan, China) and a CoraLite Plus 594-conjugated secondary antibody (product number RGAM004, Proteintech, Wuhan, China) were added, and the slices were incubated for 1 h. DAPI solution was added, and the slices were incubated for 2 min. Finally, an anti-fluorescence quenching sealing agent was added, and a Zeiss microscope was used to take fluorescence images.

HE Staining

Fixed tissues were dehydrated with an automatic dehydrator, embedded and sectioned. The slices were then dewaxed in water. The sections were stained with haematoxylin for 10–20 min and then rinsed with tap water for 1–3 min. The samples were stained with eosin for 3–5 min and washed with water for 3–5 s. The sections were dehydrated in a graded alcohol series and sealed with neutral gum. A Panoramic 250 digital slice scanner produced by 3DHISTECH (Hungary) was used for image acquisition. Images (100X/400X) were collected of the area of interest to observe pathological changes.

Elisa

Tissues were mixed with normal saline at a ratio of 1:9 and then ground thoroughly. Cell culture medium and homogenized tissue were spun in a high-speed centrifuge at 10000 \times g for 10 min. The supernatant was collected for analysis. TNF- α , IL-6 and IL-4 levels were measured at 450 nm with the following kits according to the manufacturer's instructions: TNF- α (product number JM-01587R2), IL-6 (product number JM01597R2) and IL-4 (product number JM-01598R2) (JingMei Biotechnology, Jiangsu, China).

Qrt-Pcr

Spinal cord tissue and cultured astrocytes were collected, and appropriate amounts of TriQuick Reagent Total RNA Extraction Reagent (product number R1100, Solarbio, Beijing, China) were added according to the manufacturer's instructions. A reverse transcription kit was used to synthesize 1.0 μ g of RNA into single-stranded cDNA, and a real-time fluorescence quantitative PCR kit (product number: RR820A, Takara, Beijing, China) was used for PCR amplification. GAPDH was used as a housekeeping gene. The primers used in this study (Shanghai Sheng Gong Biological Co., Ltd., Shanghai, China) were as follows:

GAPDH:

Forward: CAAGTTCAACGGCACAGTCAAGG;

Reverse: ACATACTCAGCACCAGCATCACC;

C/EBP- β :

Forward: GCTGAGCGACGAGTACAAGATGC;

Reverse: CTTGTGCTGCGTCTCCAGGTTG;

TGF- β 1:

Forward: AGCAACAATTCCTGGCGTTACC;

Reverse: AGCCCTGTATTCCGTCTCCTTG;

C3:

Forward: CACCAGCAGACCGTAACCATCC;

Reverse: AGCAGCCTTGACTTCCACTTCC;

S100A10:

Forward: AAAGGACCCTCTGGCTGTGGAC;

Reverse: CAATGATGAGCCCCGCCACTAG.

Western Blotting

Astrocytes and rat spinal cord tissue were fully lysed with lysis buffer containing protease inhibitor, and then the protein concentration was quantified by the BCA method. A one-step PAGE gel rapid preparation kit (7.5%: product number PG211; 10%: product number PG212; EpiZyme, Shanghai, China) was used to prepare the gel. The proteins on the gel were transferred to a PVDF membrane, blocked for 15 min with protein-free fast sealing solution (product number PS108P, EpiZyme, Shanghai, China), and then incubated with primary antibodies against the following proteins at 4°C overnight: C3 (1:2000, product number 21337-1-AP, Proteintech, Wuhan, China), C/EBP- β (1:2000, product number 23431-1-AP, Proteintech, Wuhan, China), TGF- β 1 (1:1000, product number HA721143, HUABIO, Hangzhou, China), p-smad2 (1:2000, product number ET1612-32, HUABIO, Hangzhou, China), p-smad3 (1:2000, product number ET1609-41, HUABIO, Hangzhou, China), and GAPDH (1:10000, product number 60004-1-Ig, Proteintech, Wuhan, China). Then, the blots were incubated for 1 h with an HRP-labelled goat anti-rabbit secondary antibody (1:10000, product number RGAR001, Proteintech, Wuhan, China), developed with Fikett hypersensitive ECL solution (product number MA0186, Meilunbio, Dalian, China) and exposed in a darkroom.

Statistical Analysis

We used GraphPad Prism (version 8.0, GraphPad software) for statistical analysis of the data. A Q–Q chart was used to determine the distribution of the data. All the data were normally distributed, and the results are expressed as the means \pm standard deviations (SDs). The significance of differences between three or more groups was analysed by one-way analysis of variance (ANOVA) or Welch ANOVA. Fisher's least significant difference (LSD) test or Welch's variance analysis and the Game Howell test were used to compare multiple groups. Statistical significance was defined as $p < 0.05$.

Results

Vincristine Induces Spinal Cord Inflammation and Neuropathic Pain

We constructed a neuropathic pain model by intraperitoneally injecting vincristine into rats. The experimental procedure is shown in [Figure 1A](#). A Von Frey electronic pain metre and hot plate were used to determine the MWT and TWL to determine whether the rat neuropathic pain model was successfully constructed. Before vincristine administration, the baseline TWL and MWT values were measured for each group of rats on day 0, and there was no significant difference between the groups ($p > 0.05$). The TWL and MWT were significantly lower in group VCR than in group sham on days 3, 7, 10, 14, and 21 ($p < 0.001$) ([Figure 1B](#) and [C](#)). After behavioural testing on the 21st day, rat spinal cords were collected, and ELISA was used to measure the levels of inflammatory factors. The results revealed that the levels of the proinflammatory cytokines TNF- α and IL-6 in the spinal cord were significantly increased ($p < 0.001$), but there was no significant change in the level of the anti-inflammatory cytokine IL-4 ($p > 0.05$) ([Figure 1D–F](#)).

Vincristine Promotes the A1 Polarization of Spinal Cord Astrocytes but Has No Effect on A2 Astrocyte Polarization

Spinal cord astrocyte polarization plays an important role in the occurrence of neuropathic pain. Therefore, we investigated the polarization of spinal cord astrocytes in the vincristine-induced neuropathic pain model through immunofluorescence, qRT–PCR and WB. Immunofluorescence analysis ([Figure 2A](#) and [B](#)) revealed that, the expression of the A1 astrocyte marker C3 in astrocytes was significantly greater in group VCR than in group sham ($p < 0.001$). Compared with that in group sham, the expression of C3 in group VCR was significantly greater ($p < 0.01$), whereas the expression of the A2 astrocyte marker S100A10 was not significantly different ($p > 0.05$) ([Figure 2C, D](#) and [G](#)). These results suggest that in the model of neuropathic pain induced by vincristine, spinal cord astrocytes are polarized mainly toward the A1 phenotype, whereas A2 astrocyte polarization does not significantly change.

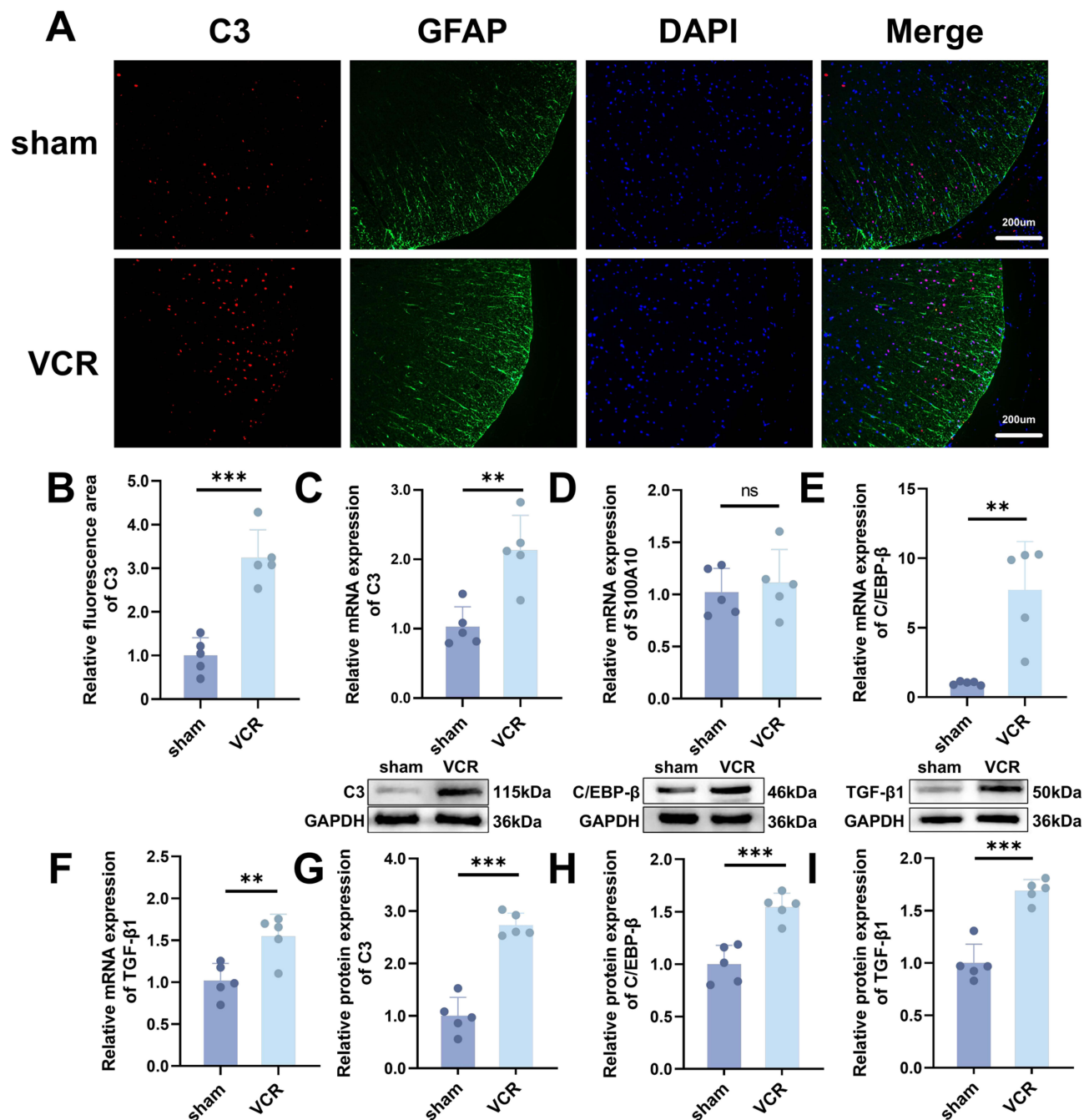


Figure 2 A1/A2 polarization status of spinal cord astrocytes in Vincristine-induced neuropathic pain in rats. **(A and B)** Representative immunofluorescence images of C3 and GFAP in spinal cord of rats in each group and the relative fluorescence area of C3. $n=5$ for each group. $***p < 0.001$ compared with sham group. **(C–F)** The relative mRNA expression of C3, S100A10, C/EBP-β and TGF-β1 in spinal cord of rats in each group. $n=5$ for each group. $**p < 0.01$ compared with sham group. **(G–I)** The relative protein expression of C3, C/EBP-β and TGF-β1 in spinal cord of rats in each group. $n=5$ for each group. $***p < 0.001$ compared with sham group. T -test was used in statistical tests.

Vincristine Increases the Expression of C/EBP-β and TGF-β1

To clarify the expression trends of C/EBP-β and TGF-β1 in the neuropathic pain model, qRT-PCR and WB were used. qRT-PCR showed that the mRNA expression of C/EBP-β and TGF-β1 in group VCR was significantly greater than that in group sham ($p < 0.01$) (Figure 2E and F). WB indicated that the protein expression levels of the C/EBP-β and TGF-β1 in group VCR were significantly greater than those in group sham ($p < 0.001$) (Figure 2H and I).

Inhibition of TGF- β 1 Alleviates Astrocyte A1 Polarization Caused by Vincristine

To clarify whether TGF- β 1 affects the polarization of A1 astrocytes, TGF- β 1 inhibitor was used to regulate the expression of TGF- β 1. The immunofluorescence results showed that the C3-positive area increased in group VCR compared with group sham. Compared with group VCR, group T, which received the TGF- β 1 inhibitor, presented a significant decrease in the C3-positive area ($p < 0.05$) (Figure 3A and B). qRT-PCR and WB suggested that the increase in the mRNA and protein expression of C3 induced by vincristine was inhibited to varying degrees after the TGF- β 1 was inhibited ($p < 0.05$) (Figure 3C and D). In addition, vincristine promoted the phosphorylation of smad2 and smad3 ($p < 0.05$) (Figure 3E and F). After the TGF- β 1 was inhibited, the phosphorylation levels of smad2 and smad3 decreased ($p < 0.05$). These findings suggest that TGF- β 1 is a key molecule in promoting the A1 polarization of astrocytes by vincristine.

Inhibition of TGF- β 1 Alleviates Spinal Cord Inflammation Caused by Vincristine and Alleviates Neuropathic Pain

To clarify that upregulating the expression of TGF- β 1 and promoting the A1 polarization of astrocytes are key for the induction of neuropathic pain by vincristine, we intraperitoneally injected vincristine as previously and then administered TGF- β 1 inhibitor to rats. The results of the Von Frey test and hot plate test revealed that the changes in the TWL and MWT induced by vincristine could be reversed by the TGF- β 1 inhibitor ($p < 0.01$) (Figure 4A and B). The HE staining results suggested that

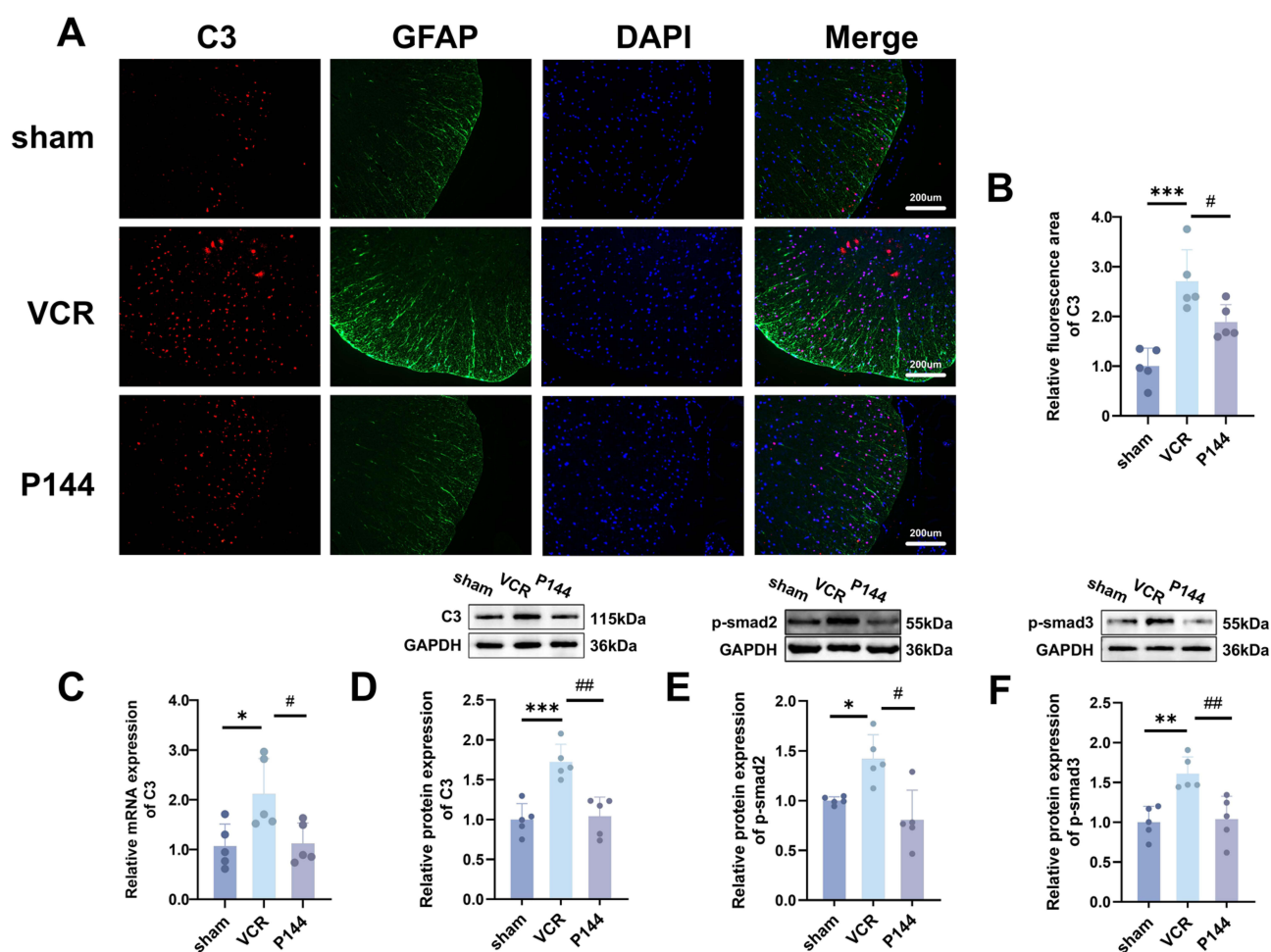


Figure 3 Effect of TGF- β 1 inhibitor on astrocytes A1 polarization of spinal cord in rats. (A and B) Representative immunofluorescence images of C3 and GFAP in spinal cord of rats in each group and the relative fluorescence area of C3. $n=5$ for each group. *** $p < 0.001$ compared with sham group. # $p < 0.05$ compared with VCR group. (C) The relative mRNA expression of C3 in spinal cord of rats in each group. $n=5$ for each group. * $p < 0.05$ compared with sham group. # $p < 0.05$ compared with VCR group. (D–F) The relative protein expression of C3, p-smad2 and p-smad3 in spinal cord of rats in each group. $n=5$ for each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with sham group. # $p < 0.05$, ## $p < 0.01$ compared with VCR group. One-way ANOVA was used in statistical tests.

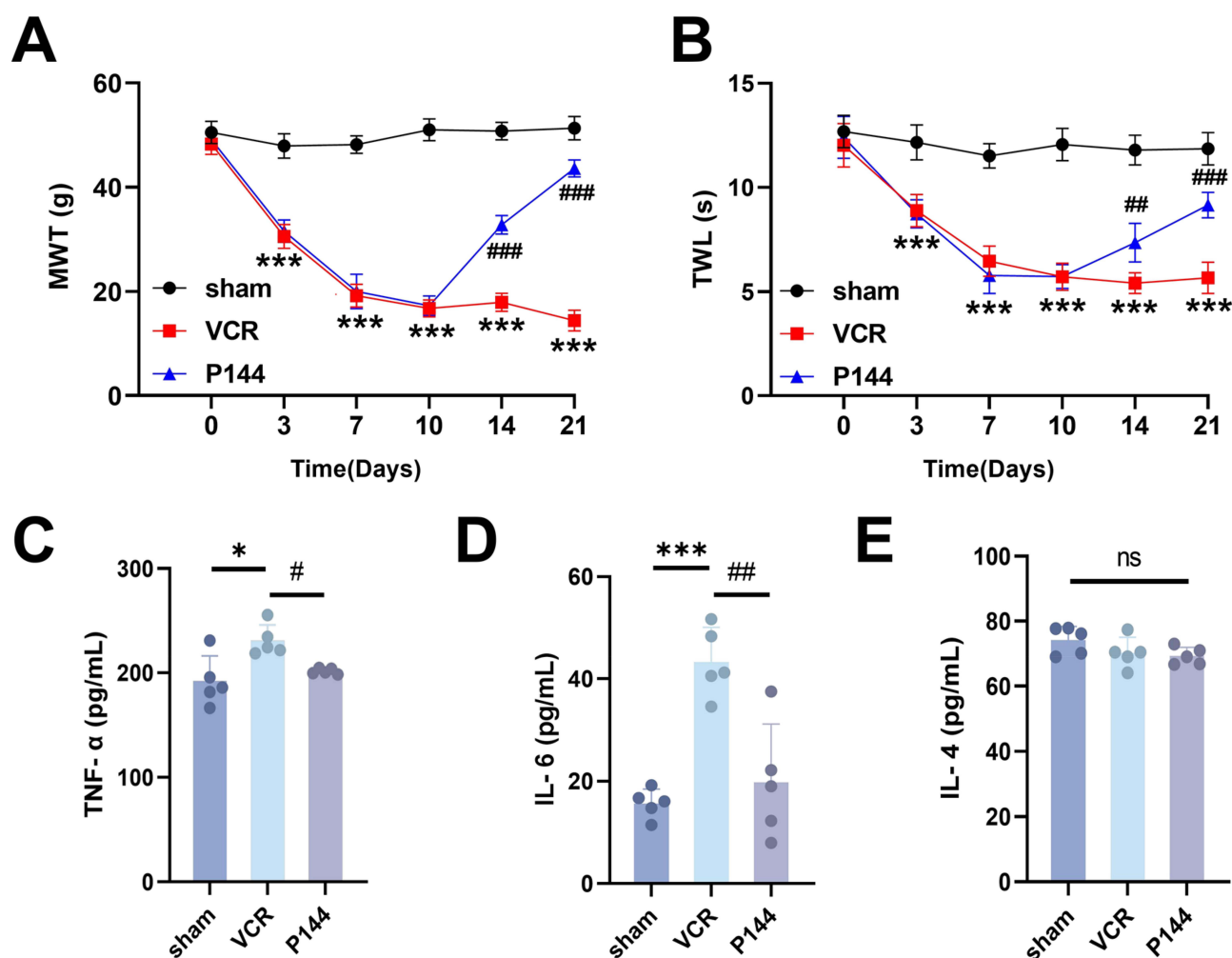


Figure 4 Effect of TGF- β 1 inhibitor on behavioural test and spinal cord injury in rats. (**A** and **B**) MWT and TWL of rats in each group. $n=6$ for each group. *** $p < 0.001$ compared with sham group. ### $p < 0.01$, #### $p < 0.001$ compared with VCR group. (**C**, **D** and **E**) Inflammatory factors of TNF- α , IL-6 and IL-4 in spinal cord of rats in each group. $n=5$ for each group. * $p < 0.05$, *** $p < 0.001$ compared with sham group. # $p < 0.05$, ### $p < 0.01$ compared with VCR group. One-way ANOVA was used in statistical tests.

vincristine significantly promoted the vacuolization of spinal cord neurons and impaired neuronal function, whereas the TGF- β 1 inhibitor significantly alleviated neuronal vacuolization and prevented spinal cord nerve injury ([Supplementary Material](#)).

The ELISA results suggested that the levels of the proinflammatory factors TNF- α and IL-6 in the spinal cord of neuropathic pain model rats were significantly increased, whereas the TGF- β 1 inhibitor significantly inhibited the expression of proinflammatory factors ($p < 0.05$) ([Figure 4C–E](#)). These results indicate that inhibiting TGF- β 1 can alleviate spinal cord neuroinflammation and nerve injury caused by vincristine and alleviate neuropathic pain.

Downregulating C/EBP- β Reverses Vincristine-Induced Overexpression of TGF- β 1

The promoter region of the TGF- β 1 gene has a binding site for C/EBP- β .¹² To confirm that C/EBP- β acts upstream of TGF- β 1 upregulation, we downregulated C/EBP- β before vincristine administration and then measured the expression of TGF- β 1 in astrocytes cultured with vincristine. The CCK8 assay was used to explore the optimal concentration of vincristine. At concentrations of 4, 6, 8, and 10 nM, vincristine had varying degrees of toxic effects on astrocytes ([Figure 5C](#)). We narrowed the drug concentration gradient and found that 3 nM was the optimal vincristine concentration. Thus, subsequent experiments were conducted using 3 nM vincristine ([Figure 5D](#)).

Immunofluorescence revealed that the average fluorescence intensity of TGF- β 1 and C/EBP- β increased significantly in cells cultured with vincristine. When siRNA was used to downregulate C/EBP- β and vincristine was administered to

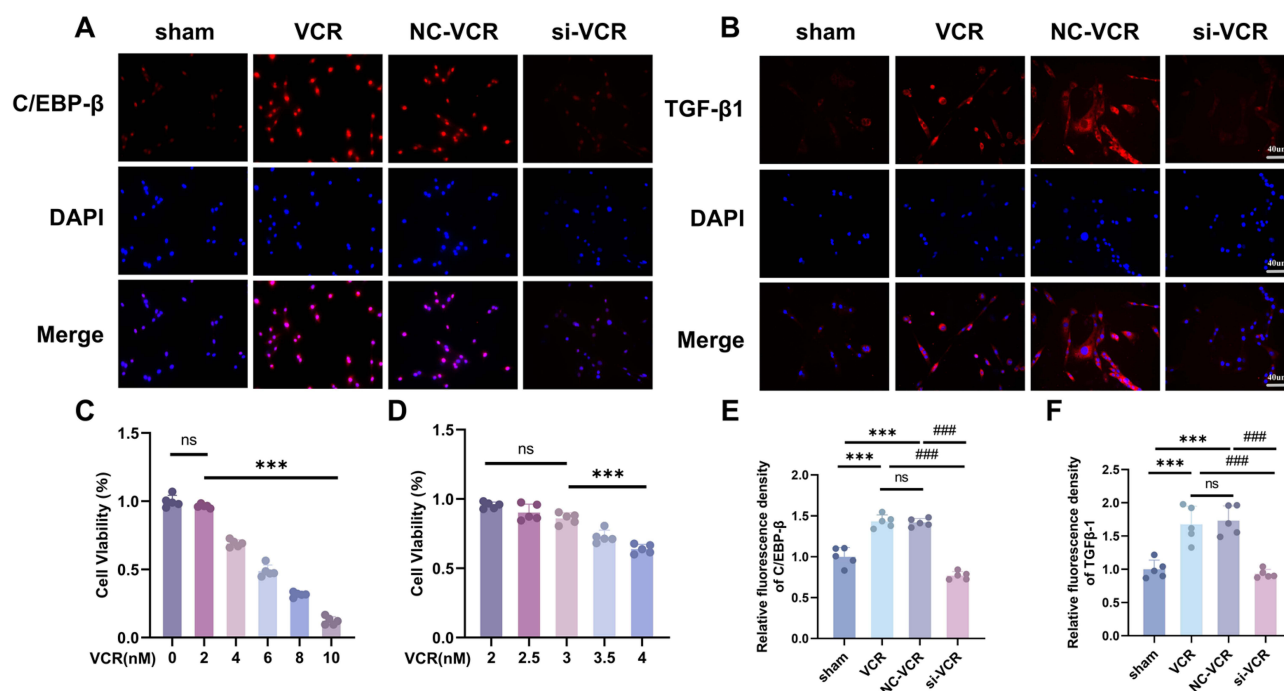


Figure 5 Effect of Interference with C/EBP- β on the mean fluorescence intensity of TGF- β 1. **(A and E)** Representative immunofluorescence images of C/EBP- β in astrocytes in each group and the mean fluorescence intensity of C/EBP- β . $n=5$ for each group. $***p < 0.001$ compared with sham group. $####p < 0.001$ compared with si-VCR group. **(B and F)** Representative immunofluorescence images of TGF- β 1 in astrocytes in each group and the mean fluorescence intensity of TGF- β 1. $n=5$ for each group. $***p < 0.001$ compared with sham group. $####p < 0.001$ compared with si-VCR group. **(C)** Astrocyte survival rate in 0nM to 10nM of Vincristine. $***p < 0.001$ compared with the group of 2nM. **(D)** Astrocyte survival rate in 2nM to 4nM of Vincristine. $***p < 0.001$ compared with the group of 3nM. One-way ANOVA was used in statistical tests.

cultured cells, the TGF- β 1 fluorescence intensity was significantly reduced (Figure 5A, B, E and F) ($p < 0.001$). qRT-PCR and WB also indicated that the mRNA and protein expression levels of TGF- β 1 were decreased after C/EBP- β was downregulated ($p < 0.01$) (Figure 6A–D). In addition, the TGF- β 1 downstream effectors p-smad2 and p-smad3 were significantly upregulated in vincristine-treated astrocytes (Figure 6E and F). However, p-smad2 and p-smad3 levels were significantly decreased after C/EBP- β was downregulated ($p < 0.01$). These results demonstrate that C/EBP- β can regulate the expression level of TGF- β 1 to some extent and affect the function of downstream effectors of TGF- β 1.

Downregulating C/EBP- β Inhibits Vincristine-Induced A1 Polarization of Astrocytes and Reduces the Expression of Proinflammatory Factors

Further experiments were conducted to determine whether downregulating C/EBP- β can inhibit the A1 polarization of astrocytes and reduce the release of proinflammatory factors by downregulating the expression of TGF- β 1. The immunofluorescence results revealed that the fluorescence intensity of C3, a marker of astrocyte A1 polarization, was significantly increased after vincristine administration, whereas C/EBP- β downregulating reversed the increase in the C3 fluorescence intensity induced by vincristine ($p < 0.001$) (Figure 7A and B). qRT-PCR and WB revealed that after C/EBP- β was downregulated, the mRNA and protein expression levels of C3, which was highly expressed in response to vincristine administration, were significantly reduced ($p < 0.05$) (Figure 7C and D). In addition, vincristine increased the expression of the proinflammatory cytokines TNF- α and IL-6 in astrocytes, whereas downregulating C/EBP- β significantly reduced the expression of these proinflammatory cytokines ($p < 0.05$) (Figure 7E and F). However, the expression level of the anti-inflammatory cytokine IL-4 was not affected by C/EBP- β downregulation ($p > 0.05$) (Figure 7G). These results indicate that vincristine causes neuropathic pain by increasing the expression levels of C/EBP- β /TGF- β 1 signalling pathway components, promoting the A1 polarization of astrocytes, and inducing spinal cord inflammation.

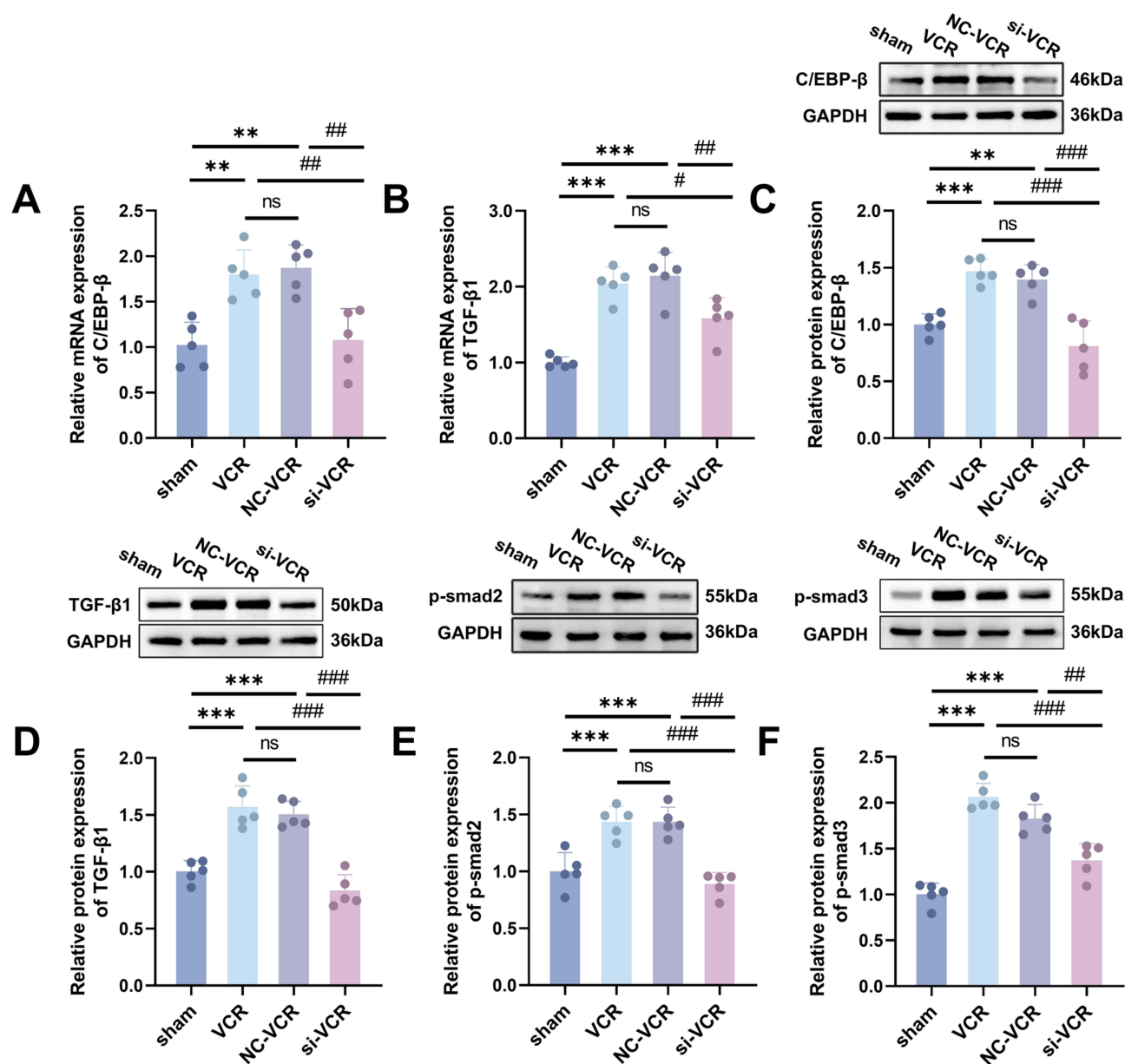


Figure 6 Effects of C/EBP- β interference on TGF- β 1 and downstream molecules. (A and B) The relative mRNA expression of C/EBP- β and TGF- β 1 in astrocytes in each group. $n=5$ for each group. $**p < 0.01$, $***p < 0.001$ compared with sham group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with si-VCR group. (C–F) The relative protein expression of C/EBP- β , TGF- β 1, p-smad2 and p-smad3 in astrocytes in each group. $n=5$ for each group. $**p < 0.01$, $***p < 0.001$ compared with sham group. $^{\#}p < 0.01$, $^{\#\#}p < 0.001$ compared with si-VCR group. One-way ANOVA was used in statistical tests.

Discussion

As a commonly used antitumour drug, vincristine may cause a series of side effects during the treatment process, of which neuropathic pain is the most significant.¹⁵ Neuropathic pain is a type of pain caused by nervous system injury or disease. Its characteristics include spontaneous pain, hyperalgesia and abnormal pain.¹⁶ Neuropathic pain is often difficult to control effectively with conventional analgesics and may seriously affect the quality of life of patients. The occurrence of neuropathic pain may be directly related to the neurotoxicity of drugs. Vincristine neurotoxicity may trigger a neuroinflammatory response, leading to the release of inflammatory mediators, such as cytokines and chemokines.¹⁷ These inflammatory mediators can further activate and recruit immune cells and aggravate nerve tissue damage and pain signal transmission¹⁸. In this study, the TWL and MWT decreased significantly, the expression of proinflammatory factors (TNF- α and IL-6) increased, and the expression of the anti-inflammatory factor IL-4 in the

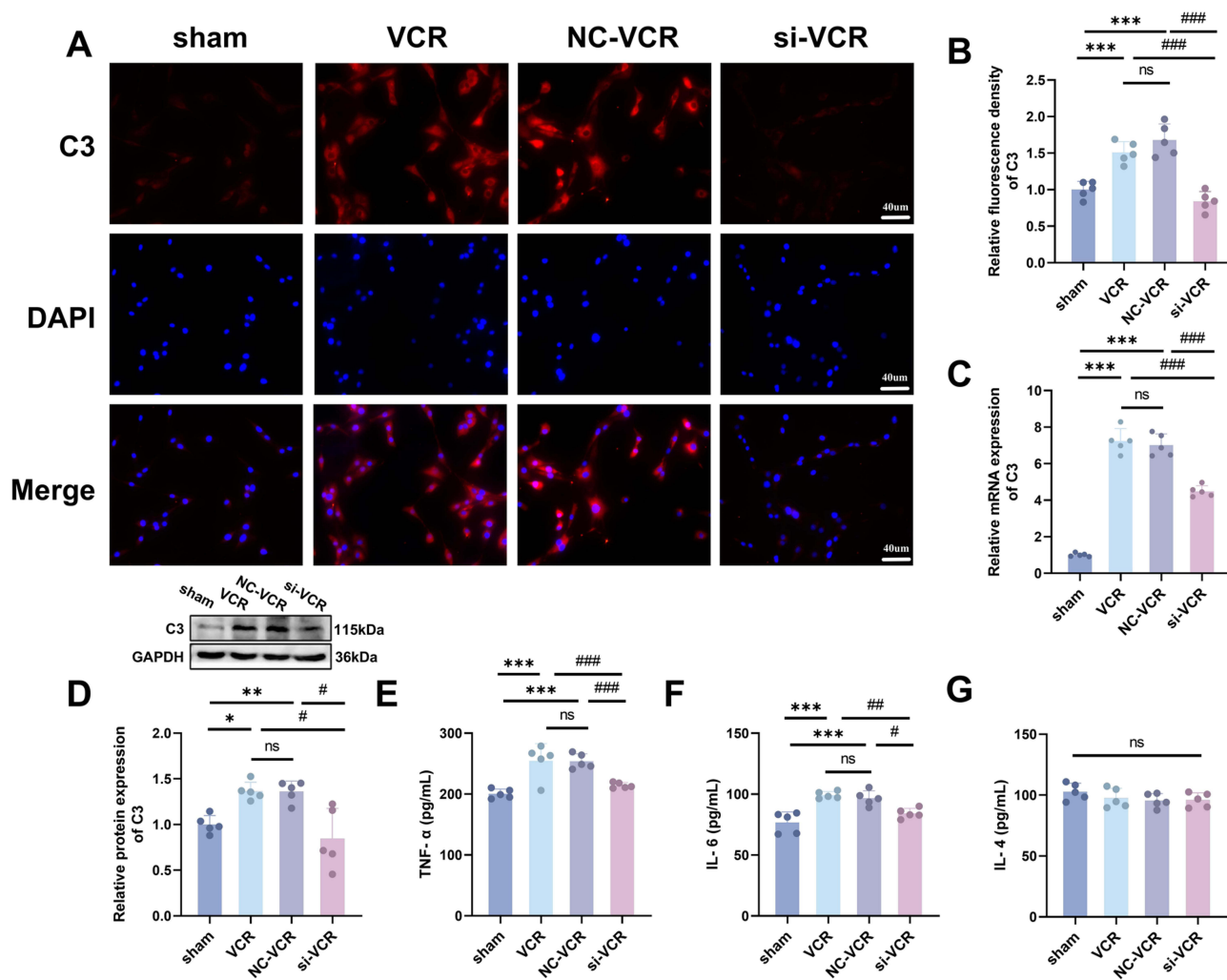


Figure 7 Effects of C/EBP- β interference on astrocytes A1 polarization and expression of inflammatory factors in each group. (A and B) Representative immunofluorescence images of C3 and the mean fluorescence intensity of C3 in astrocytes in each group. $n=5$ for each group. *** $p < 0.001$ compared with sham group. #### $p < 0.001$ compared with si-VCR group. (C and D) The mRNA and protein expression of C3 in astrocytes in each group. $n=5$ for each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with sham group. # $p < 0.05$, #### $p < 0.001$ compared with si-VCR group. (E–G) Inflammatory factors of TNF- α , IL-6 and IL-4 in astrocytes in each group. $n=5$ for each group. *** $p < 0.001$ compared with sham group. # $p < 0.05$, ## $p < 0.01$, #### $p < 0.001$ compared with si-VCR group. One-way ANOVA was used in statistical tests.

spinal cord decreased in rats in the vincristine group. Thus, the neuroinflammatory response plays an important role in the neuropathic pain caused by vincristine.

As the most abundant type of glial cell in the spinal cord, astrocytes play an indispensable role in supporting the structure and function of neurons, regulating neurotransmitter metabolism and promoting nerve development and regeneration.⁶ When the spinal cord is affected by injury or disease, astrocytes are activated and polarized. Activated astrocytes can be polarized toward one of two different phenotypes according to their functions: the neurotoxic (A1) phenotype and the neuroprotective (A2) phenotype.^{19–21} Inhibiting the A1 polarization of spinal cord astrocytes can reduce spinal cord injury and relieve neuropathic pain.²² Injecting A2 astrocytes into the spinal cord injury site in mice can significantly promote the recovery of motor function, protect the ascending pathway of the spinal cord and reduce neuroinflammation.²³ Our study revealed that vincristine promoted the A1 polarization of astrocytes and increased the expression of the proinflammatory factors TNF- α and IL-6. However, vincristine did not affect the A2 polarization of spinal astrocytes or the expression of the anti-inflammatory factor IL-4. Therefore, vincristine may cause neuroinflammation by promoting the A1 polarization of astrocytes.

TGF- β 1, a multifunctional growth factor, is involved in key events that regulate development, disease and tissue repair.²⁴ In the brain, TGF- β 1 is widely considered a cytokine related to injury, especially in relation to astrocyte scar formation after brain injury.²⁵ However, evidence in recent years has shown that, in addition to playing a role in brain injury, TGF- β 1 may be

a key regulator of cell survival and differentiation. TGF- β 1 activates TGF- β 1 receptor type II (T β RII) and TGF- β 1 receptor type I (T β RI). After ligands bind T β RII, T β RII can recruit T β RI and combine with it to form the T β RII/T β RI dimer and promote T β RI phosphorylation. Finally, p-T β RI can phosphorylate smad2 and smad3 TGF- β 1 to regulate astrocyte activation and polarization.²⁶ In this study, vincristine significantly increased the expression of TGF- β 1 in the spinal cord tissue of rats. Inhibiting TGF- β 1 can significantly reduce the phosphorylation of smad2 and smad3; inhibit the A1 polarization of astrocytes; reduce the release of proinflammatory factors, TNF- α and IL-6; increase the mechanical pain threshold and thermal pain threshold; and alleviate neuropathic pain. In addition to astrocytes, studies have shown that TGF- β 1 in microglia also plays an important role in neuropathic pain. Both TGF- β 1 and p-smad3 were upregulated simultaneously in the mouse model of chronic nerve injury, and the silence of TGF- β 1 could significantly relieve pain symptoms and produce anti-microglial effects. Therefore, regulation of TGF- β 1 may have inhibitory effects on microglia and astrocytes at the same time.²⁷

As a transcription factor, C/EBP- β can regulate the expression of many genes and participate in many biological processes, such as cell proliferation, differentiation, and metabolism, and the immune response.²⁸ For example, an increase in the nuclear C/EBP- β level promotes the interaction between C/EBP- β and the NF- κ B p65 subunit, promotes the activation of microglia and astrocytes, and reduces the transcription of the NF- κ B target genes TNF- α and IL-6.²⁹ Some studies have shown that C/EBP- β regulates the expression level of TGF- β 1, thereby affecting the downstream signalling pathway mediated by TGF- β 1.³⁰ However, few studies have reported whether the upregulation of TGF- β 1 caused by vincristine is related to C/EBP- β . In this study, we found that the expression of C/EBP- β in the spinal cord of rats treated with vincristine increased. After C/EBP- β was downregulated, the mRNA and protein expression of TGF- β 1 decreased significantly, the phosphorylation levels of smad2 and smad3 decreased, the A1 polarization of astrocytes was inhibited, and the release of the proinflammatory factors TNF- α and IL-6 was reduced. In addition, in the spinal cord, the demethylation of the Cxcr3 gene by C/EBP- α also promotes the binding of CXCR3 to CXCL10 to maintain neuropathic pain.³¹ Therefore, the CCAAT enhancer binding protein family has a potential relationship with neuropathic pain, but there are few studies in this area, which has great research potential.

There are some limitations of this study. First, we explored the role of spinal cord inflammation in neuropathic pain but did not explore other mechanisms of neuropathic pain. Second, we demonstrated that TGF- β 1 is an important cause of astrocyte polarization but failed to demonstrate the specific mechanism by which it activates astrocytes. Third, we measured the levels of some but not all markers of astrocyte polarization. Finally, we demonstrated the role of only C/EBP- β /TGF- β 1-mediated astrocyte A1 polarization in neuropathic pain induced by vincristine. Further research is needed to confirm whether other signalling pathways are also involved in this condition.

Conclusion

Clinical treatment of neuropathic pain usually uses sedative or analgesic drugs to relieve pain symptoms, but this long-term treatment will cause some side effects in patients. In our study, vincristine induced spinal cord inflammation by promoting A1 polarization of astrocytes via upregulating the C/EBP- β /TGF- β 1 signal pathway, thus leading to neuropathic pain. The discovery of this signal pathway may provide a new idea for the treatment of neuropathic pain by blocking the role of C/EBP- β in promoting transcription of TGF β -1.

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Disclosure

Jing Shi is the first correspondence author for this study. The authors report no conflicts of interest in this work.

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