RESEARCH ARTICLE

proBDNF/p75NTR promotes rheumatoid arthritis and inflammatory response by activating proinflammatory cytokines

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Abstract
P75 pan-neurotrophin receptor (p75NTR) is an important receptor for the role of neurotrophins in survival and death of neurons during development and after nerve injury. Our previous research found that the precursor of brain-derived neurotrophic factor (proBDNF) regulates pain as an inflammatory mediator. The current understanding of the role of proBDNF/p75NTR signaling pathway in inflammatory arthritis pain and rheumatoid arthritis (RA) is unclear. We recruited 20 RA patients, 20 healthy donors (HDs), and 10 osteoarthritis (OA) patients. Hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC) of proBDNF and proNGF were used to observe the expression of proBDNF/p75NTR in RA tissues.

Abbreviations: CFA, Complete Freund’s Adjuvant; CHO, Chinese hamster ovary; CIA, collagen-induced arthritis; CNS, central nervous system; ELISA, enzyme-linked immunosorbent assay; FMO, fluorescence minus one; GSMT, grip strength meter test; H&E, hematoxylin and eosin; HDs, health donors; I.P, intraperitoneally; ICFA, Incomplete Freund’s Adjuvant; IHC, immunohistochemistry; IL-10, interleukin-10; IL-1β, interleukin-1β; LPS, lipopolysaccharide; MFI, median fluorescence intensity; MTX, methotrexate; NGF, nerve growth factor; NT-3, neurotrophin-3; OA, osteoarthritis; p75NTR, P75 pan-neurotrophin receptor; PBMCs, peripheral blood mononuclear cells; proBDNF, precursor of brain-derived neurotrophic factor; proNGF, precursor of nerve growth factor; PWT, paw withdrawal threshold; RA, rheumatoid arthritis; RF, rheumatism factor; RIPA, radio immunoprecipitation assay; TNF-α, tumor necrosis factor α; Tregs, regulatory T cells; TrkA, tropomyosin receptor kinase A.

Chun-Rui Yang, Hong-Jun Ding, and Miao Yu contributed equally to this work.

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INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease which is characterized mainly by inflammation of synovial tissues, cartilage, and bone of the joints, but also potentially affects a variety of extra-articular organs. Subsequently, RA is associated with global progressive disability, potential systemic complications, high socioeconomic costs, and mortality. The pathogenesis of RA is complex and involves large numbers of different cell types and signaling pathways. The key factor in the occurrence and development of RA is driven by an inflammatory network, in which the imbalance of inflammatory responses, T and B cells, autoantibodies, cytokines, and other inflammatory mediators are crucial components. More recent studies have shown that brain-derived neurotrophic factor precursor (proBDNF) is an inflammatory mediator which plays a critical role in regulating pain. Injection of proBDNF into mice plantar induces pain behavior and synovitis. Another study suggests the role of proBDNF signaling pathway in the immune system in the pathogenesis of autoimmune-mediated inflammatory diseases. Currently, the relationship between proBDNF signaling pathway and RA remains elusive.

The discovery of proBDNF expression in the nervous system and the immune system was more than 30 years ago. Since then, proBDNF have been characterized in the peripheral nervous system, the central nervous system, and the peripheral tissues including skin, intestine, cardiovascular, macrophages, lymphocytes, fibroblasts etc. In the nervous system, proBDNF binds to its receptors, p75 pan-neurotrophin receptor (p75NTR) and sortilin, and exerts negative functions such as triggering neuronal apoptosis, axon pruning, neurite collapse, and synaptic plasticity. A number of studies have demonstrated that proBDNF/p75NTR signaling pathway not only plays a key role in pathogenesis of Alzheimer’s disease and depression, but also regulates various inflammatory diseases by activating the functions of monocytes, macrophages, or microglia. During peripheral inflammation or arthritis in rodents, proBDNF levels rise, and nociceptors consequently become hypersensitive. Neutralizing the upregulated endogenous proBDNF by anti-proBDNF antibody greatly attenuated inflammation, p75NTR, pain, proBDNF, rheumatoid arthritis
various types of pain behavior and inflammatory response. Therefore, peripheral proBDNF may be a potential pain mediator. Furthermore, Wang et al., found that lipopolysaccharide (LPS) injection induced the upregulation of proBDNF in the T cells of the mesenteric lymph node.14 Therefore, it is possible that proBDNF modulates functions of immune cells and plays a proinflammatory role in inflamed joints.

Defined as a member of the tumor necrosis factor (TNF)-receptor superfamily, p75NTR is also a nerve growth factor (NGF) receptor. Interestingly, while it binds to the mature NGF with low-affinity, p75NTR is the high-affinity receptor for the precursor of nerve growth factor (proNGF).15,16 Previous studies have demonstrated a role of proNGF–p75NTR axis promoted proinflammatory mechanisms contributing to chronic tissue inflammation.17 In patients with RA, the proNGF and p75NTR are highly expressed in fibroblast-like synoviocytes (FLS).

Since monocytes can express the receptor p75NTR4 and proBDNF binds to p75NTR which could activate NF-kB pathway and increase immune system functions in peripheral lymphocytes,18 it is possible that proBDNF/p75NTR signaling pathway perhaps modulate functions of immune cells and be involved in the pathogenesis of RA. Therefore, it is important to characterize the expression of proBDNF, p75NTR, and sortilin in peripheral blood mononuclear cells (PBMCs) of RA patients before examining whether inhibiting the proBDNF/p75NTR signaling pathway may provide therapeutic interventions for treating inflammatory arthritis pain or RA.

2 | MATERIALS AND METHODS

2.1 | Human subjects

Twenty patients, 40–60 years old, both male and female, were recruited from Tianjin Hospital in this study. The control population consisted of 20 healthy age- and gender-matched volunteers at the Second Hospital of Tianjin Medical University. The collection of human peripheral blood samples from the RA patients (n = 20) and healthy donors (HD, n = 20) (Table 1) was approved by the Ethics Committee of the Second Hospital of Tianjin Medical University and registered in Chinese Clinical trial (KY2020K003). The collection of human synovial membrane from the RA patients (n = 10) and osteoarthritis (OA) patients (n = 10) from a large (knee) joint was approved by the Ethics Committee (Table 2). We declared that our study was carried out in accordance with the latest version of the Declaration of Helsinki. Patients were enrolled to participate in the study after they had signed the informed consents for participation.

2.2 | Animals

A total of 70 male C57BL/6J mice (age 6–8 weeks, weight 23–27 g) were obtained from Tianjin Medical University Animal Service (Tianjin, China). In the experiments, all animals were housed in groups of three to four per standard cage with food and water in constant temperature (25°C) and humidity (50%) conditions at 12 h:12 h light/dark cycle. They were randomly divided into control group (n = 10) and model group (n = 60). Unfortunately, two model group mice died during collagen-induced arthritis (CIA) procedure. After 3 weeks, mice of the model group were randomly divided into CIA (n = 10), Vehicle (VEH, n = 9) controls, p75NTR selective inhibitor p75ECD-Fc 1 mg/kg (CIA+1 mg, n = 10), p75ECD-Fc 3 mg/kg (CIA+3 mg, n = 10), p75ECD-Fc 10 mg/kg (CIA+10 mg, n = 9), and methotrexate (CIA+MTX, n = 10). The experiments were approved by the Animal Research Ethics Committee of the Second Hospital of Tianjin Medical University and according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Number of Animal use permit: SYXK: 2019-0004).

| Variables                  | Healthy donors | RA group |
|---------------------------|----------------|----------|
| Number, n                 | 20             | 20       |
| Female sex, n (%)         | 14 (70)        | 15 (75)  |
| Male sex, n (%)           | 6 (30)         | 5 (25)   |
| Age, y, mean ± SD         | 60.1 ± 4.66    | 63.3 ± 7.22 |
| Disease duration, mean ± SD | N/A           | 6.68 ± 5.67 |
| RF positivity, mean ± SD IU/ml | 5.6 ± 5.3 | 394.27 ± 290.91 |
| Serum CRP, mean ± SD mg/L | 2.11 ± 1.95 | 96.26 ± 161.65 |
| ACR-EULAR score, mean ± SD | N/A           | 7.65 ± 1.46 |
| DAS28, mean ± SD          | N/A            | 4.91 ± 1.43 |

Abbreviations: CRP, C-reactive protein; RF, rheumatoid factor.

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Abbreviations: CRP, C-reactive protein; RF, rheumatoid factor.
2.3 | Reagents

Recombinant p75ECD-Fc was obtained and produced from Suzhou Auzone Biotech.

2.4 | Collagen-induced arthritis (CIA) model

The detailed CIA procedures were described in our previous paper. Bovine type II collagen (GeneRun) in acetic acid (1 mg/ml) was mixed with an equal amount of Complete Freund’s Adjuvant (CFA, Sigma) to prepare type II collagen and Complete Freund’s Adjuvant (II+CFA) or with an equal amount of Incomplete Freund’s Adjuvant (ICFA, Sigma) to make type II collagen and Incomplete Freund’s Adjuvant (II+ICFA). To induce RA, the back of mice was injected subcutaneously at 4 points with 0.025 ml (II+CFA) on 0 day and three intra-planter injections of 0.025 ml (II+CFA) on day 7. Generally, lesions occur after 2 weeks and additional II+ICFA injections were administered on day 14.

2.5 | Pharmacological interventions

CIA-induced RA models were dosed with the p75NTR selective inhibitor p75ECD-Fc (1, 3, and 10 mg/kg) intraperitoneally (I.P.), once a day until the end of the experiment (from 21 to 35 days). Vehicle (VEH) controls were treated with daily I.P. injections of sterile saline (0.01 ml/g) for 14 days, 21 days after CIA induction, while positive controls were treated with daily I.P. injections of methotrexate (MTX) (0.1 mg/kg).

2.6 | Arthritis score

Arthritis score was used to judge the degree of joint redness/swelling/deformation of mice with rheumatoid arthritis.19 The arthritis score was the average of the four individual joints scored per animal as follow: 0, normal; 1, ankle joints with erythema and slight swelling; 2, erythema and slight swelling from ankle joint to palm joint; 3, erythema and moderate swelling from ankle joint to palm joint; and 4, erythema and severe swelling from ankle joint to toe joint.

2.7 | Paw withdrawal threshold (PWT)

Mechanical allodynia was measured by examining the PWT by applying von Frey filaments (Bio EVF3, Bioseb company) with a up and down motion on the medial planar aspects of both the left and right hind paws of each mouse. Before the experiment, each animal was acclimatized in a metal cage for 15 min to limit its exploratory behavior. During each test, six trains of stimuli were applied randomly with von Frey filaments with different stimulus intensities ranging from 0.6, 1, 2, 4, 6 to 8 g equivalents of force. A positive reaction was recorded as “X” when obvious limb withdrawal, foot licking, and jumping behaviors were observed while a negative reaction was recorded as “O.” Each mouse was tested three times in 15-min intervals. Animals were excluded from the group, if they failed to respond to analgesia pressure >15 g.

2.8 | Grip strength meter test (GSMT)

As another pain behavioral assessment, the grip strength meter determines the maximum force displayed by an
animal in order to study neuromuscular functions. Before each test, the weight of each animal was determined. The grip strength was measured by grasping the distal end of the mouse’s tail, while its forelimb is grasping the metal grid plate of the grip strength meter (YLS-13A), and then stretching the animal out until a measurement (g) on the meter is display. Each mouse was tested five times in 1-min intervals. The mean value of grip strength of each animal was normalized to its body weight.

2.9 Immunohistochemistry and immunofluorescence

Fresh synovial tissue samples were fixed overnight in 10% formalin buffer at pH 7.0 and embedded in paraffin for histologic and immunohistochemical analyses. Serial histologic sections were stained with hematoxylin and eosin (H&E). The following parameters were evaluated: synovial hyperplasia, lymphoplasmacytic cell infiltrates, lymphoid follicle formation, fibrinoid necrosis, and vascular hyperplasia. Immunolabeling experiments were performed using a standard protocol, as previously described. Synovial tissue slices of 4 μm thickness were immunostained overnight with primary antibodies including: LCA (ZM-0183, ORIGENE), CD3 (ZM-0417, ORIGENE), CD4 (ZM-0418, ORIGENE), CD8 (ZM-0508, ORIGENE), CD20 (ZM-0039, ORIGENE), CD19 (ZM-0038, ORIGENE), proBDNF (from professor Xin-Fu Zhou’s laboratory), and p75NTR (NGFR/CD271, 14-9400-82, Invitrogen). Secondary antibodies used were goat anti-rabbit/mouse (PV-6000D, ORIGENE) or donkey anti-sheep IgG H&L (HRP, ab97125, abcam) secondary antibodies.

The other parts of fresh synovial tissue slices were used for immunofluorescence analysis. The antibodies used included anti-human CD45 antibody (Alexa Fluor 488, 368536, Biolegend), anti-human CD20 antibody (Alexa Fluor 594, 302354, Biolegend), anti-human CD14 antibody (Alexa Fluor 594, 325630, Biolegend), anti-human CD4 antibody (Alexa Fluor 488, 300519, Biolegend), and p75NTR antibody (NGFR/CD271, Alexa Fluor 700, 345118, Biolegend). The donkey anti-sheep IgG H+L secondary antibody (Alexa Fluor 488, A-11015, Biolegend) was used for proBDNF in this part for 1 h at 37°C. After thoroughly washed, they were cover-slipped with mounting buffer containing DAPI (P36935, Invitrogen) and visualized by fluorescence optical microscope.

Evaluation of the optical variables was performed using a semiquantitative score on a 0–3 scale, where 0 indicates the absence of feature and 3 represents them at highest level. A specific score was assigned for the extent of hyperplasia of the synovial lining layer: grade 0, staining of one to two cell layers; grade 1, staining of three to four cell layers; grade 2, staining of five to six cell layers; grade 3, staining of seven or more cell layers. Evaluation of the immunohistochemical variables was performed using a semiquantitative standard. Randomly select 10 high-power fields for each case, count the number of positive cells in the grid micrometer in each field, and the total number of positive cells in 10 fields/10 to get the average number of positive cells in each case.

2.10 Enzyme-linked immunosorbent assay (isolation of serum)

Human serum was assayed for p75NTR, sortilin, TNF-α, IL-1β, IL-6, and IL-10 in duplicate using ELISA assay kit (p75NTR, ab155436, abcam; sortilin, MM-50868H1, MEIMIAN; TNF-α, MM-0122H1, MEIMIAN; IL-1β, MM-0181H1, MEIMIAN; IL-6, MM-0049H1, MEIMIAN; and IL-10, MM-0066H1, MEIMIAN), according to the protocol provided by the manufacturer. CIA mice serum was assayed for TNF-α, IL-1β, and IL-10 in duplicate using ELISA assay kit (TNF-α, SEA133Mu, Cloud-Clone Corp.; IL-1β, SEAS63Mu, Cloud-Clone Corp.; and IL-10, SEA056Mu, Cloud-Clone Corp.). Briefly, ELISA plates were coated with protein G-purified mouse monoclonal antibody (2 μg/ml in coating buffer), blocked with PBS-BSA, loaded with serum at 1:5 dilution, detected with detection antibodies, and incubated with TMB, and OD values were obtained at 450 nm.

2.11 Quantitative real-time PCR

Total RNA was extracted from the spinal cord of rodents, and peripheral blood mononuclear cells (PBMCs) from participant donors, using TRIzol® reagent (Sigma), following the manufacturer’s recommendation. Briefly, animals were sacrificed by overdosing ether and rapid collected blood from heart. The whole spinal cord of each mouse was collected and rinsed in cold PBS. The RNA samples were dissolved in 30 μl of DEPC-H2O and subsequently stored at −80°C. The cDNA was obtained using the reverse transcription kit (Thermo- Fisher Scientific, CA, USA). Quantitative real-time PCR was performed with SYBR Green (Takara, Japan) on CFX96 TouchTM Deep Well Real-Time PCR Detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Gene expression level of p75NTR, sortilin, TNF-α, IL-1β, and IL-10 normalized to housekeeping gene β-actin/GAPDH was detected by quantitative real-time PCR. The reactions were performed in triplicates as follows: 95°C for 3 min, and 39 cycles of 95°C for 10 s and 60°C for 30 s. Relative RNA expression levels were determined using the 2-ΔΔCT method. The primer sequences used are shown in Tables 3 and 4.
2.12 | Western blotting analysis

Protein was extracted using radio immunoprecipitation assay (RIPA) lysis buffer supplemented with proteinase inhibitors from the synovial tissue of patients, PBMCs of patients, and the spinal cord of rodents. Protein concentrations were measured using the BCA Protein Assay Kit (Thermo Scientific, Waltham, MA). Equal amounts of protein were separated by 10% SDS/PAG, followed by immunoblotting with the primary antibodies (sheep anti-human proBDNF 1:200, from professor Xin-Fu Zhou’s laboratory; mouse CD271 antibody 1:1000, cat. #14-9400-82, Invitrogen, USA; mouse sortilin 1:1000, cat.#AF3154-SP, CoWin Biosciences, China; rabbit anti-GAPDH antibody, 1:5000, cat.#ab 9485, abcam, China). Membranes were then incubated with peroxidase-conjugated secondary antibody. Western blotting band were analyzed by the mean gray value with NIH Image J 7.0 and standardized to GAPDH control protein.

2.13 | Flow cytometry

To measure proBDNF and p75NTR expression in PBMCs from healthy volunteers and RA patients, human PBMCs were isolated from peripheral blood samples of healthy donors (HDS) and RA patients using lymphoprep separation liquid (Stemcell Technology, Norway). Freshly isolated PBMCs were then counted manually with a hemocytometer, and trypan blue exclusion was used to determine cell viability (>96%). PBMCs were stained with fluorescently conjugated monoclonal antibodies (mAbs) at 4°C for 30 min as follows, respectively: BV421 anti-human CD3 (cat.#ab562427, BD), ECD anti-human CD4 (cat.#ab6604727, BC), APC-CY7 anti-human CD8 (cat.#ab641400, BD), FITC anti-human CD20 (cat.#ab347673, BD), PC7 anti-human CD19 (cat.#abIM3628, BC), V500 anti-human CD45 (cat.#ab560777, BD), APC anti-human CD271 (p75NTR/NGFR, cat.#ab400119, Biolegend), and APC mouse IgG1 (cat.#ab400119, Biolegend). After the cells were rinsed, they were processed through the flow cytometer (Cystek, Fremont, CA, USA), and data were analyzed with FlowJo vX0.7 software. Single-stained cells or OneComp eBeads (eBioscience, San Diego, CA) were used for compensation calculations. Unstained cells, cells with 7-AAD, and fluorescence minus one (FMO) controls were used for cytometry and gating set up.

2.14 | Statistical analysis

Data were expressed as mean ± SD. Statistical Package for the Social Science v.13 and graphically presented
using Prism 8 softwares. Unpaired two-tailed Student's t test, one-way ANOVA followed by Dunnett's/Tukey's multiple comparison posttest, or two-way ANOVA with Bonferroni's multiple comparison post hoc test were used for mean comparisons, respectively. The Spearman's correlation test was used for analyzing the correlations of rheumatism factor (RF) with p75NTR, sortilin, TNF-α, IL-1β, or IL-10 levels in serum of RA patients. A value of \( p < 0.05 \) indicates statistical significance.

3 | RESULTS

3.1 | ProBDNF, p75NTR, and sortilin expression in patients with RA

Hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC) of LCA, CD3, CD4, CD8, CD20, CD19, proBDNF, and p75NTR (CD271) in synovial membrane were performed and evaluated. We found severe synovial inflammation of RA (\( n = 10 \)) synovial membrane compared to OA (\( n = 10 \)) controls in histologic sections, including synovial hyperplasia (\( t = 2.929, p = 0.009 \), See Figure S1A), lymphplasmacytic cell infiltrates (\( t = 9.798, p < 0.0001 \), See Figure S1A), vascular hyperplasia (\( t = 2.683, p = 0.0152 \), See Figure S1A), and fibrinoid necrosis (\( t = 1.524, p = 0.1449 \), See Figure S1A). Lymphocytes infiltration and exudation were the main pathological features, while T cells, B cells, plasma cells, and macrophages are the main inflammatory cells. In all cases of RA (\( n = 10, 100\% \)), lymphocytes focally aggregated, and the germinal center formed a lymphoid follicle-like structure. All the RA synovial membrane showed moderate or large amounts of lymphocytes which were marked by LCA (CD45a, See Figure S1B). Expression of LCA cells (CD45a+ cells) was higher in RA synovial membrane compared to OA controls (\( t = 16.56, p < 0.0001 \), See Figure S1B). CD3+T cells were highly expressed in RA synovial tissues more than CD20+B cells indicating that the infiltration and exudation of lymphocytes were dominated by T cells. While the expression of CD3+T cells (\( t = 17.02, p < 0.0001 \)), CD20+B cells (\( t = 4.532, p = 0.0003 \)), and CD19+B cells (\( t = 6.353, p < 0.0001 \), See Figure S1B) was significantly lower in OA synovial membrane than RA. Notably, CD4+T cells were mostly focally distributed in the infiltrating lymphocytes in RA, while CD8+T cells were few and scattered. We observed that CD4+T cells (\( t = 16.10, p < 0.0001 \)) and CD8+T cells (\( t = 11.72, p < 0.0001 \), See Figure S1B) were statistically different from those in the RA synovial tissues compared with OA. We next examined the expression of proBDNF/p75NTR in the synovial tissues.

The proBDNF/p75NTR immunoreactivity was distributed in the part of monocytes. As shown in Figure 1A,B, in contrast to the OA, the increased proBDNF (\( t = 13.94, p < 0.0001, \) Figure 1A) and p75NTR (\( t = 13.27, p < 0.0001, \) Figure 1B) could be detected in the part of inflammatory cells responding to peripheral inflammation. Interestingly, double labeling immunofluorescence showed that abundant proBDNF (green) positive staining was co-localized with p75NTR (red) in the RA synovial tissues (Figure 1C). There was significant difference between RA synovial membrane and OA synovial membrane. Furthermore, the increased proBDNF was also co-localized with CD14+B cells and part of CD20+B cells in the RA synovial tissues and upregulated p75NTR was also co-localized with part of CD4+T cells (Figure 1D). These findings suggested that inflammatory cells were likely to be the important source of the upregulated proBDNF and p75NTR.

In patients with RA (\( n = 20 \)), p75NTR (CD271) and sortilin were highly expressed in human peripheral blood mononuclear cells (PBMCs): the mean expression of p75NTR (\( t = 3.924, p = 0.0015, \) Figure 1E) and sortilin (\( t = 6.786, p = 0.0013, \) Figure 1F) mRNA were 11-fold and threefold higher than in HD (\( n = 20 \)) PBMCs. Consistently, western blot clearly indicated that PBMCs expressed higher proBDNF (\( t = 5.812, p = 0.002, \) Figure 1H), p75NTR (\( t = 4.168, p = 0.009, \) Figure 1I), and sortilin (\( t = 10.49, p = 0.01, \) Figure 1J) protein levels than HD PBMCs. These were in agreement with real-time PCR data. Furthermore, there was difference in representative western blot of proBDNF (\( t = 5.452, p < 0.0001, \) Figure 1L) and p75NTR (\( t = 5.487, p < 0.0001, \) Figure 1M) between RA (\( n = 10 \)) synovial membrane and OA (\( n = 10 \)) synovial membrane (Figure 1K–M). The results suggested that proBDNF/p75NTR could play important role in PBMCs and synovial membrane in patients with RA.

3.2 | Expression pattern of proBDNF/p75NTR in peripheral immune cells of RA patients

To explore the potential contribution of the immune cell imbalance in the internal environment and any subpopulation of lymphocytes involved, we examined the distribution of immune cells and the expression of the proBDNF/p75NTR on T/B cells from RA patients. Peripheral blood lymphocytes from 20 HD, 20 RA patients, and 20 treated patients were phenotypically analyzed by flow cytometry for the expression of CD45, CD19, CD20, CD3, CD4, and CD8 surface markers (Figure 2A). We next examined the median fluorescence intensity (MFI) of proBDNF/p75NTR(CD271) on T/B cells from RA patients (Figure 2).
We found that proBDNF/p75NTR (CD271) could be detected both on T and B lymphocytes. One-way ANOVA indicates that there is significant difference in cell subset in different groups, including CD45a\(^+\) cells \((F(2,37) = 15.27, p < .0001)\), CD3\(^+\)T cells \((F(2,37) = 5.866, p = .0066)\), CD4\(^+\)T cells \((F(2,37) = 16.85, p < .0001)\), and CD8\(^+\)T cells \((F(2,37) = 5.753, p = .0072)\). We investigated the MFI of p75NTR/CD271 in the CD45a\(^+\) cells \((p = .0084,\) Figure 2B), CD3\(^+\)T cells \((p = .0406,\) Figure 2C), CD4\(^+\) T cells \((p < .0001,\) Figure 2D), and CD8\(^+\) T cells \((p = .0055,\) Figure 2E).
Figure 2E) was significantly increased in RA group compared to HD. After treatment, the MFI of p75NTR/CD271 in the CD45a<sup>+</sup> cells (p < .0001, Figure 2B), CD3<sup>+</sup> T cells (p = .0085, Figure 2C), and CD4<sup>+</sup> T cells (p < .0001, Figure 2D) was downregulated compared to the untreated group. While the MFI of p75NTR/CD271 in the CD8<sup>+</sup> T cells (p = .2255, Figure 2E) was slightly decreased in the treated group. No difference was found
in the MFI of p75NTR/CD271 in the CD20+ B cells ($p = .0501$, Figure 2G) and CD19+ B cells ($p = .9697$, Figure 2F). Consistently, the MFI of proBDNF in the CD4+ T cells was significantly increased in RA group compared to HD and reached threefold ($p = .0024$, Figure 2H). After treatment, the MFI of proBDNF in the CD4+ T cells was significantly downregulated compared to the untreated group ($p = .004$, Figure 2H). The
FIGURE 2  Expression pattern of proBDNF/p75NTR in the peripheral blood during RA progression. (A) Representative images of flow cytometry for T and B lymphocytes in HD, RA patients, and therapy patients, gating of mononuclear cells isolated from the PBMCs. (B–G) PBMCs isolated from patients were stained with cell surface markers CD45, CD3, CD4, CD8, CD19, and CD20 to test the p75NTR expression by flow cytometry. P75NTR significantly increased in CD45⁺, CD3⁺, CD4⁺CD8⁻, and CD4⁻CD8⁺ cells in RA patients compared with the HD. These were increased in treated group compared with the untreated group. (H–K) Representative flow cytometry showed the proBDNF expression in CD4⁺CD8⁻, CD4⁺CD8⁺, CD19⁺, and CD20⁺. Note that proBDNF was highly expressed in CD4⁺CD8⁻ cells in RA groups, whereas proBDNF expression decreased from patients after treatment. *p < .05, **p < .01, ***p < .001; †p < .05, ‡p < .01, §§p < .001; all data are presented as mean ± SEM.
results suggested that proBDNF/p75NTR (CD271) were significantly upregulated in RA synovial and primarily produced by CD4\(^+\) T cells. It is suggested that the high expression of proBDNF/p75NTR (CD271) may promote the occurrence and development of RA, and play an important role in the pathogenesis of RA.

### 3.3 The serum levels of p75NTR and sortilin were positively correlated with DAS28

Clinical characteristics and clinical scores were documented on the day of sample collection. The correlation of DAS28 with ACR/EULA2009 is shown in Figure 3A \((r = 0.5685, p = .0089)\). There was no relationship between DAS28 and RF \((r = 0.2974, p = .2028, \text{ Figure } 3B)\). Next, we examined the expression pattern of p75NTR and sortilin with DAS28 or inflammatory cytokines in serum of RA patients. ELISA result showed that the p75NTR/sortilin level in the RA patients \((n = 20)\) was significantly increased in comparison with the HD \((n = 20)\) \((t = 3.142, p = .0032, \text{ Figure } 3C; t = 3.922, p = .0004, \text{ Figure } 3F, \text{ respectively})\). The analysis of correlation between the relative p75NTR/sortilin in the serum and DAS28/RF in patients are shown in Figure 3D,E,G,H. There was significant positive correlation between p75NTR/sortilin and DAS28/RF \((r = 0.5372, p = .0146, \text{ Figure } 3D; r = 0.4685, p = .0372, \text{ Figure } 3E; r = 0.4954, p = .0264, \text{ Figure } 3G; r = 0.5252, p = .0174, \text{ Figure } 3H, \text{ respectively})\). The results indicated that the trend of p75NTR/sortilin change in the PBMCs and serum was similar to that in RA synovial. The levels of p75NTR and sortilin were positively correlated with the RA severity. As shown, the expression of TNF-\(\alpha\), IL-1\(\beta\), and IL-6 gene was dramatically increased in the RA patients \((t = 5.421, p < .0001, \text{ Figure } 3I; t = 5.824, p < .0001, \text{ Figure } 3L; t = 3.391, p = .0016, \text{ Figure } 3O, \text{ respectively})\). Given the role of pro-inflammatory and suppress inflammatory cytokines, we then also analyzed IL-10 gene alteration in the RA patients. Interestingly, the gene expression of IL-10 was also upregulated in RA than in HD \((t = 3.923, p = .0004, \text{ Figure } 3R)\). The analysis of correlation between the inflammatory cytokines and DAS28/RF in patients is shown in Figure 3J,K,M,N,P,Q,S,T. They were slightly positive correlation but no significance. These findings suggested that upregulated p75NTR/sortilin and inflammatory cytokines participated in the occurrence and development of RA. Furthermore, the serum levels of p75NTR and sortilin positively correlated with DAS28 and RA severity.

### 3.4 The serum level of sortilin was positively correlated with the inflammatory cytokines

We analyzed the relationship between the serum levels of p75NTR/sortilin and the inflammatory cytokines. The data showed that the serum level of sortilin was positively correlated with TNF-\(\alpha\), IL-1\(\beta\), and IL-10 in RA patients \((r = 0.5674, p = .0091, \text{ Figure } 4E; r = 0.763, p < .0001, \text{ Figure } 4F; r = 0.689, p = .0008, \text{ Figure } 4G, \text{ respectively})\). The serum level of p75NTR was slightly positively correlated with TNF-\(\alpha\) \((r = 0.193, p = .4145, \text{ Figure } 4A)\) and IL-1\(\beta\) \((r = 0.2801, p = .2317, \text{ Figure } 4B)\) but no significance. The serum level of p75NTR was slight negatively correlated with IL-10 \((r = −0.02018, p = .9327, \text{ Figure } 4C)\) but no significance. However, the serum level of p75NTR was positively correlated with IL-6 \((r = 0.6397, p = .0024, \text{ Figure } 4D)\). These results suggested that the role of p75NTR/sortilin signaling pathway might enhance chronic inflammatory response in RA progression.

### 3.5 Effect of collagen-induced arthritis (CIA) model of joint inflammation and pain behavior

To research the effect of proBDNF/p75NTR in pathogenesis of RA, we established the CIA model in mice. The experimental schedules are depicted in Figure 5A. Injection of bovine type II collagen with Complete Freund's Adjuvant/with Incomplete Freund's Adjuvant into the back of mice on 0 day and intra-planter on 7/14 day induced joint swelling in mice knees. Immunization with collagen was associated with the expected increases in knee diameter (nearly twofold) than naïve animals \((p < .0001, \text{ Figure } 5B)\). CIA-induced arthritis induced pain behavior as measured by grip strength and paw withdrawal threshold. Grip strength to ankle compression occurred at lower strength in CIA group than control group \((p < .0001, \text{ Figure } 5C)\). Significant reduction in hind paw withdrawal thresholds was detected in CIA group compared with controls \((p < .0001, \text{ Figure } 5D)\). Immunization with collagen generated antibody in CIA-induced arthritis compared with controls \((p < .0001, \text{ Figure } 5E)\). Arthritic score was significantly increased in CIA group compared with controls \((p < .0001, \text{ Figure } 5F)\). The control animal displayed normal synovium, whereas CIA group displayed severe synovitis and moderate cartilage damage with mild pannus and bone resorption (Figure 5G).
FIGURE 3 The serum levels of p75NTR and sortilin positively correlated with DAS28 and RA severity. The p75NTR, sortilin, TNF-α, IL-1β, IL-6, and IL-10 levels were upregulated in serum of RA patients. (A–B) The correlation of DAS28 with ACR/EULA2009 or RF was shown. (C,F,I,L,O,R) The ELISA results of p75NTR, sortilin, TNF-α, IL-1β, IL-6, and IL-10 were significantly increased in RA patients compared with the HD. (D,G,J,M,P,S) The correlation among p75NTR, sortilin, TNF-α, IL-1β, IL-10, and DAS28. (E,H,K,M,Q,T) The correlation among p75NTR, sortilin, TNF-α, IL-1β, IL-10, and RF. ELISA, enzyme-linked immunosorbent assay; RF, rheumatoid factor; *p < .05, **p < .01, ***p < .001; ′p < .05, ′′p < .01, ′′′p < .001; all data are presented as mean ± SEM.
In order to investigate whether collagen-induced pain behavior and synovitis may be related to the proBDNF/p75NTR signal pathway. Following immunization with collagen injection, the p75NTR/sortilin and inflammatory cytokine mRNA expression were significantly upregulated in spinal cord compared to the controls (Figure 6A–E). Representative p75NTR and sortilin western blot and semi-quantitative analysis were consistent with mRNA expression (Figure 6F–I). Especially, p75NTR expression greatly increased in the spinal cord of CIA model. We further examined the inflammatory cytokines including TNF-α, IL-1β, and IL-10 expression in the serum of CIA model (Figure 6J–L). These findings strongly indicated that increased proBDNF/p75NTR exacerbated pain behavior may through activating the inflammatory reactions.

3.7 | P75ECD-Fc pretreatment attenuates inflammatory pain and decreases proBDNF/p75NTR in the spinal cord and serum of CIA mice

To establish the role of proBDNF/p75NTR signal pathway in the severe inflammatory pain in the CIA mice, we used p75ECD-Fc which greatly inhibited proBDNF/p75NTR signal pathway and attenuated the inflammatory...
pain. The recombinant p75ECD-Fc was from CHO cells and supplied by Suzhou Auzone Biotech. Its pharmacokinetics was characterized.\textsuperscript{21,22} Knee diameter was significantly reduced following treatment p75ECD-Fc (10 mg/kg) and MTX when respectively compared to the vehicle-treated CIA mice ($p < .0001$; $p = .0005$, Figure 5B). Treatment with p75ECD-Fc (1 mg/kg, 3 mg/kg) slightly reduced the increase in knee diameter, but no significance between treated groups and VEH groups ($p > .9999$; $p = .0883$, Figure 5B). Inhibited proBDNF/p75NTR signal pathway by i.p. injection of p75ECD-Fc (10 mg/kg) dramatically increased paw withdrawal...
threshold (PWT), supporting the assumption that p75ECD-Fc relieved pain hypersensitivity in CIA mice (Figure 5D). However, p75ECD-Fc and MTX pretreatment could not increase grip strength (Figure 5C). We examined the relative bovine type II collagen antibody expression level in different groups (Figure 5E). Significant reduction in the arthritic score was evident in mice treated with p75ECD-Fc (Figure 5F). Treatment with p75ECD-Fc (10 mg/kg) and MTX significantly inhibited all histological aspects of the disease (Figure 5G).

Next, we investigated whether treatment with p75ECD-Fc downregulated proBDNF/p75NTR and inflammatory cytokines in the spinal cord and the serum of CIA model. Treatment with different concentrated p75ECD-Fc (1, 3, 10 mg/kg) and MTX reduced the level of proBDNF/p75NTR mRNA expression and inflammatory cytokines including TNF-α and IL-1β mRNA expression in the spinal cord of CIA mice (Figure 6A–F). As shown in Figure 5E, there was no main effect of p75ECD-Fc (1, 3 mg/kg) on IL-10 mRNA expression. Similarly, the effect of p75ECD-Fc (10 mg/kg) and MTX injection downregulated proBDNF, p75NTR, and sortilin protein expression in CIA groups compared to VEH groups (Figure 6G–I). Furthermore, injection of p75ECD-Fc (1, 3, 10 mg/kg) decreased the p75NTR protein expression in the spinal cord. In the peripheral inflammation, the increased inflammatory cytokines (TNF-α and IL-1β) in the serum of CIA model were greatly reduced after treatment with p75ECD-Fc (10 mg/kg) and MTX injection (Figure 6J,K). However, elevated IL-10 expression was slightly decreased in the p75ECD-Fc (1, 10 mg/kg) treated groups (Figure 6L). Taken together, our results indicated that p75ECD-Fc could relieve inflammatory pain through biologically blocking all inflammatory responses.

4 | DISCUSSION

Although effector inflammatory cytokines involved in RA are known and could be effectively neutralized by current treatment strategies, cure is still rare in RA. Even drugs reduce disease activity, many patients suffer from serious pain. The present study demonstrated that the inflammatory cytokines or their signaling pathways are bound up with the underlying mechanisms in RA, then effective therapy by directly blocking therapeutic target could attenuate RA or improve the likelihood of curing RA. Several important findings emerged from our research. We first observed upregulation of proBDNF and its receptors in immune cells of both RA patients and CIA mice, including synovial membrane, blood, and spinal cords. Next, we discovered that active proBDNF/p75NTR signaling may contribute to sustain inflammatory responses and inflammatory pain in RA. Lastly, we confirmed a detrimental role of proBDNF/p75NTR signaling in the pathogenesis of RA and affirmed that proBDNF/p75NTR signaling might be a therapeutic target to relieve RA. ProBDNF/p75NTR signaling inhibition might therefore offer a novel therapeutic strategy for modulating immune system functions and improving pain in patients with RA.

4.1 | Upregulation of proBDNF/p75NTR in PBMCs and synovial tissue of RA patients with active disease and in spinal cords of CIA model

Current evidence showed that there was a close interaction between the immune and nervous system. Neurotrophins play multiple biologic roles including neuronal survival, shaping neurons, and synaptic plasticity. Five closely related factors have been identified: NGF, BDNF, neurotrophin-3 (NT-3), NT-4, and NT-5. Neurotrophins also are involved in the inflammatory responses of chronic arthritis, including RA. Nerve growth factor (NGF) expression was associated with inflammatory disease activity in RA and NGF/TrkA-p75NTR axis might contribute to inflammatory responses by increasing neuronal release of substance P and calcitonin gene-related peptide. A recent CIA rodent study displayed NGF receptor TrkA inhibition using AR786 could reduce pain behavior, joint damage, and synovial inflammation. A more recent paper showed that the serum level of BDNF and T-cell expression of its receptor NGFR were elevated in patients with RA. The knowledge of neurotrophins expression in the inflamed synovial compartment in arthritis patients might add the evaluation of the pathogenic mechanisms of RA and the development of novel therapeutic strategies. In this study, we investigated the modulation of the other signal pathway, proBDNF/p75NTR, during the inflammatory response in RA patients with active disease. Our study showed that patients with RA also had significantly elevated mRNA and protein level of proBDNF, p75NTR (NGFR) and sortilin in PBMCs compared with those of the HD. We found that the protein expression of proBDNF and p75NTR was significantly higher in the synovial tissue of RA patients compared with the OA patients. In our experimental mice models, the expression of proBDNF/p75NTR signaling was also upregulated in the spinal cords of CIA model. These results implicated that high expression of proBDNF/p75NTR signaling in the active immune system was associated with RA and may play an important role during RA progression.
FIGURE 6 Upregulation of proBDNF/p75NTR and effect of p75ECD-Fc injection on inflammatory reaction in mice. (A–E) Representative RT-PCR of p75NTR, sortilin, TNF-α, IL-1β, and IL-10 semiquantitative analysis of their expression in the spinal cord during the different groups. (F–I) Representative western blot images showing the expression of proBDNF, p75NTR, and sortilin in the spinal cord during the different group. (J–L) Representative ELISA of TNF-α, IL-1β, and IL-10 expression in the serum of the different groups. *p < .05, **p < .01, ***p < .001; #p < .05, ##p < .01, ###p < .001; all data are presented as mean ± SEM.
4.2 Expression pattern of proBDNF/p75NTR in the local tissue and immune system during RA progression

Although the immunopathogenesis of RA remains to be fully determined, the contribution of a variety of immune cells and cytokines to the progression of RA has been the subject of many studies, and the infiltration and exudation of lymphocytes are the key features in RA.\(^{29,30}\) Many kinds of mononuclear cells participate in the autoimmune inflammation, in which mainly including T, B lymphocytes, plasma cells, and macrophages in synovial tissue, and the T lymphocytes are dominated.\(^{31}\) CD4\(^+\) T cells, B cells, monocytes, and fibroblast-like synoviocytes have established relevance to RA pathogenesis.\(^{30,32,33}\) Therefore, defining key cellular subsets in the inflamed tissue and circulating lymphocytes are a critical step to clarify novel therapeutic targets for RA. We found that magnitude of inflammatory infiltrates and synovial papillary hyperplasia in RA synovitis. CD3\(^+\) T cells and CD4\(^+\) T cells were dominated in the synovial tissue of RA patients compared with the OA patients. The result was consistent with a previous report.\(^{34}\)

BDNF, a neurotrophic factor widely expressed in the CNS, is essentially synthesized as a precursor (proBDNF), which is then cleaved by proteases to mBDNF.\(^{35}\) BDNF and proBDNF can be released from B cells, T cells, and monocytes contributing to B-cell development and T-cell maturation.\(^{36–38}\) In contrast to BDNF, proBDNF can play an active role in cell apoptosis, cytokine processing, and inflammatory responses.\(^{3–5}\) Current evidence demonstrated that serum BDNF and proBDNF levels were significantly elevated in RA patients compared with the controls.\(^{28}\) High proBDNF expression was detected in the circulating lymphocytes and infiltrated inflammatory cells at the synovial tissue of RA patients. Our study showed that the protein and mRNA expression of proBDNF/p75NTR/sortilin were upregulated in PBMCs in RA patients compared with the HD. Furthermore, the protein expression of proBDNF/p75NTR was also upregulated in synovial tissue in RA patients compared with the OA patients. With respect to expression pattern of proBDNF/p75NTR to RA, we tested the proBDNF/p75NTR expression in the synovial tissue and circulating lymphocytes by representative immunofluorescence and flow cytometry. We found that the upregulated proBDNF and p75NTR coexisted in the synovial tissue and were also highly distributed in the inflammatory cells responding to peripheral inflammation. Our previous study has reported that proBDNF was highly expressed in the inflammatory cells.\(^{3}\) We further found that the expression of proBDNF MFI was significantly higher in CD4\(^+\) T cells in circulation from patients with RA compared with the HD. While the expression of p75NTR MFI was significantly higher not only in CD4\(^+\) T cells but also in CD8\(^+\) T cells in the circulation from patients with RA compared with the HD. When RA patients in remission, the upregulation of proBDNF/p75NTR signaling in the T lymphocytes was decreased. The recent studies also showed the upregulation of proBDNF/p75NTR signaling in the T lymphocytes and monocytes from the patients with sepsis and aorta dissection disease.\(^{13,39}\) These findings suggested that the active immune cells increased the expression of proBDNF/p75NTR in the local tissue and in the circulation, highlighting the role of proBDNF/p75NTR in immune cells during RA progression.

4.3 The relationship of p75NTR, sortilin, and inflammatory cytokines in RA patients and CIA model

RA is a well-established immune-mediated inflammatory disease characterized by upregulation of major pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, reflecting dysregulated innate and autoimmune pathophysiology.\(^{40}\) So, inflammation is an essential component of RA pain. It was well known that mBDNF in the spinal cord is involved in inflammatory and neuropathic pain, and neutralizing the increased spinal mBDNF could attenuate pain processing.\(^{41}\) ProBDNF mainly exerts its biological functions not only as an intermediate during the synthesis of mBDNF, but also binds to its receptors, p75NTR, and sortilin. When p75NTR forms complexes with sortilin, it has a high affinity for the proNGF and proBDNF and mediates apoptotic cell death.\(^{42}\) The increased p75NTR/sortilin was abundantly expressed in the serum of RA patients and positively correlated with DAS28/rheumatoid factor (Figure 3). The gene expression of inflammatory cytokines was also increased, including TNF-α, IL-1β, and IL-10 in the serum of RA patients and in the spinal cord of CIA rodents. Interestingly, the serum level of sortilin was positively correlated with the inflammatory cytokines. As our previous study showed that the increased proBDNF also co-localized with IL-1β and IL-6 positive staining.\(^{3}\) The upregulated p75NTR/sortilin after the active autoimmune system suggested that p75NTR/sortilin could enhance the expression of proinflammatory cytokines in stimulated PBMCs and activated spinal cord.

4.4 P75ECD-Fc pretreatment and MTX decrease the level of proBDNF/p75NTR

Notably, i.p. p75NTR antagonist administration, p75ECD-Fc, decreased the expression of proinflammatory cytokines in the spinal cord and serum. The low doses of p75ECD-Fc (1 mg/kg) administration would affect the proBDNF/p75NTR signaling and block all the reaction in this way,
resulting in slightly decreased proBDNF/p75NTR/sortilin and downregulated inflammatory cytokines. A high-dose p75ECD-Fc (10 mg/kg) dramatically reduced monocyte activation in the circulation, suggesting that p75NTR blockade could reduce inflammatory responses and pain. Moreover, the high-dose p75ECD acquired the same effect similar to MTX. MTX was a standard treatment in a number of autoimmune disorders such as RA, SLE, or psoriasis.41 These disorders are associated with impaired control of the immune response by circulating regulatory T cells (Tregs) and thus a proinflammatory balance in functional T-cell populations and in the pattern of cytokine secretion.44,45 There is evidence for the expression of p75NTR in leukocytes and mesenchymal stem cells, and in light of the evidence linking CNS and peripheral inflammation, it has been reported that the downstream effectors of p75NTR signaling include RhoA, JNK, and NF-κB pathways.8,18,48 This finding was consistent with previous studies, which demonstrated that blocking proBDNF or p75NTR could attenuate inflammatory responses.4,49 Thus, p75NTR may be a unique therapeutic target for immune modulation in RA.

4.5 Potential relationship between proBDNF/p75NTR and inflammatory pain

Neurotrophins in pain remain a promising area and have become potential therapeutic targets in the treatment of numerous pain states, including acute, chronic pain states, and persistent inflammatory pain.50,51 NGF binds to tropomyosin receptor kinase A (TrkA) and p75NTR which could induce the production of pain signal molecules, then produce pain and aggravation.27 Anti-NGF antibodies (such as tanezumab, a recombinant humanized monoclonal antibody) have been proven to be effective for OA pain and still actively undertaken the clinical trial.52 Numerous preclinical studies showed that BDNF has a pronociceptive effect in spinal dorsal horn by acting as a pain mediator at the synapse and thus inflammation-associated pain hypersensitivity could be alleviated by anti-BDNF treatments in rodents.53,54 More importantly, our previous study found that peripheral proBDNF was a potential pain mediator and anti-proBDNF pretreatment may alleviate the development of inflammatory pain. The involvement of p75NTR and sortilin processing in pain perception has been proposed.17,55 These findings highlighted the potential role of proBDNF/p75NTR/sortilin pathway for pain relief. In this study, we showed that proBDNF/p75NTR/sortilin interaction in monocytes contributed to inflammatory response and pain hypersensitivity in RA patients, and the interference of p75ECD-Fc with this interaction can reduce the inflammatory pain in CIA models.

Taken together, our study has reported a novel regulatory function of proBDNF/p75NTR/sortilin signal in immune cells mediating the RA pathogenesis. The pharmacological blockade of the signaling pathway by using p75ECD-Fc (p75NTR antagonist) may effectively attenuate disease progression not only at inflammatory response but also at inflammatory pain. Our finding prompts further functional as well as clinical studies on the role of proBDNF/p75NTR/sortilin signal and the potential target to treat RA.

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DISCLOSURES

The authors have declared that no competing interest exists.

AUTHOR CONTRIBUTIONS

Chun-Rui Yang and Xin-Fu Zhou were involved in conceptualization. Hong-Jun Ding and Miao Yu were involved in data curation. Chen-Yang Han was involved in formal analysis. Chun-Rui Yang and Rui Liang were involved in funding acquisition. De-Dong Li, Shuo Wang, and Wei-Zong Sun were involved in investigation. Xiao-Yang Zhang, Xiang-Lian Zhang, and Fan-Jie Meng were involved in methodology. Chun-Rui Yang, Hong-Jun Ding, and Miao Yu were involved in writing—original draft. Fiona-H Zhou, Bin Meng, and Xin-Fu Zhou were involved in supervision. Chun-Rui Yang was involved in writing—review and editing. All authors revising the manuscript critically for important intellectual content. All authors have read and agreed to the published version of the manuscript.

ETHICS APPROVAL

All studies involving human samples were performed in accordance with Ethics Committee of the Second Hospital of Tianjin Medical University and registered in Chinese Clinical trial.

DATA AVAILABILITY STATEMENT

The datasets generated during this study are available from the corresponding author upon reasonable request.

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**SUPPORTING INFORMATION**

Additional supporting information may be found in the online version of the article at the publisher’s website.

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