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Polarization of Helper T Lymphocytes Maybe Involved in the Pathogenesis of Lumbar Disc Herniation

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ABSTRACT

This study aimed to explore the expression of T helper type 1 (Th1)/T helper type 2 (Th2) in herniated nucleus pulposus (NP) and determine their association with sciatic pain.

NP was collected from 12 patients with lumbar disc herniation (LDH) (extrusion group) and 6 patients with a vertebral fracture (control group). The expression of Th1/Th2 and related cytokines in the NP was examined by flow cytometry, Western blot, and immunofluorescent staining. Subsequently, an LDH model was established in male Sprague–Dawley rats, and behavioral testings were carried out. The expression of Th1/Th2 and related cytokines in rat NP and the expression of macrophages in the dorsal root ganglia (DRG) were also examined.

The number of Th1 cells in rat NP dramatically increased on day 14 after the surgery, but significantly decreased on day 28. The number of Th2 cells increased on day 28. Chemokine ligand 3(CCL3) and CD86 proteins (M1-specific molecules) were expressed at a relatively low level in naive DRG, markedly increased on day 14 after the surgery, and decreased on day 28. Arg1 and CD206 protein (M2-specific molecules) were expressed at a relatively low level in naive DRG and markedly increased on day 28. The mechanical allodynia and heat hyperalgesia developed after NP application and finally partially alleviated.

The results suggested that the polarization of Th cells might be involved in the pathogenesis of LDH, and this might be achieved via the phenotypic shift of macrophages.

Keywords: Lumbar disc herniation; Macrophages; T helper type 1 (Th1); T helper type 2 (Th2)

INTRODUCTION

Lumbar disc herniation (LDH) is a common and costly disease in clinical practice. Its main symptom is sciatica. The mechanical compression theory could not explain all the clinical and pathological phenomena satisfactorily,[1] suggesting that other factors are also involved in the pathogenesis of sciatic pain. Macnab pointed out that inflammation was the main factor leading to pain in LDH.[2]

Currently, a lot of studies have proven that inflammation and autoimmunity play important roles in

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sciatica pain. Naylor et al proposed the theory of nucleus autoimmunity.[3] Kobayashi et al found macrophages in herniated nucleus pulposus (NP), which are considered to be important factors in the spontaneous regression of the herniated disc.[4] Cheng et al pointed out that increased levels of Th17 lymphocytes and IL-17 contributed to the pain associated with LDH.[5] Many cytokines, such as IL-1,[6] tumor necrosis factor (TNF)-α, IL-6,[7] IL-21,[8] phospholipase A2, and prostaglandin E,[9] have been found in painful intervertebral disc (IVDs) and proved to be associated with sciatica pain caused by LDH.

Helper T lymphocytes (Th cells) are a subpopulation of T lymphocytes that express the cluster of differentiation 4 (CD4) molecule; all Th cells arise from Th0 cells.[10] They can differentiate into Thl or Th2 cells depending on the type of antigen-presenting cells, nature of the antigen, route of antigen exposure, and stage of the immune response.[11-13] Th1 cells are characterized by the secretion of IL-2, IL-12, and IFN-γ, while Th2 cells are characterized by the secretion of IL-4, IL-5, and IL-10. IFN-γ is the signature cytokine of Th1 subset, playing a crucial role in maintaining the differentiation and proliferation of Th1. IL-4 is also responsible for Th2 cell clonal expansion.[14] In resting state, the Th1/Th2 cells are in a relatively balanced state. Once the balance between Th1/Th2 is disrupted, it may disturb the dynamic balance of cytokine network, thereby leading to the emergence and development of many diseases.[15] Allergic asthma is frequently characterized by an abnormally polarized Th2-type immune response against specific allergens, leading to inflammatory responses.[16] The elevated gene expression of Th1/Th2-associated transcription factors is correlated with disease activity in patients with systemic lupus erythematosus.[17] Insulin-dependent diabetes mellitus (IDDM)is a Th1- and Th2-mediated disease.[18]

However, knowledge about the relationship between the polarization of Th cells and LDH is scarce. The purpose of this study was to investigate the expression of Th1/Th2 in LDH and determine their association with sciatic pain.

MATERIALS AND METHODS

Patients

Twelve patients with LDH, who were diagnosed and treated in the Spine Centre of the Affiliated Hospital of Nantong University were chosen, including eight males and four females, with a mean age of 46 (29–68) years and an average duration of the disease of 8 months. These patients were diagnosed and proved to be prolapsed lumbar disc herniation (extrusion group) during the surgery (the NP broke through the posterior longitudinal ligament into the spinal canal).

Normal IVD tissue was obtained from six patients with acute burst fractures of the lumbar vertebra, including four males and two females, with an average age of 31 (28–35) years, but without a history of low back pain or signs of disc degeneration on magnetic resonance imaging. Written informed consent was obtained from all patients and controls. The study was approved by the ethics committee of the hospital affiliated to Nantong University (No. 2016-073).

During the surgery, the NP was removed and immediately placed in a cryopreservation tube under sterile conditions. The sample was preserved at -80°C.

Animals and Surgery

An LDH model was established in male Sprague–Dawley rats to investigate the association between the expression of Th1/Th2 cells and sciatic pain. A total of 130 male rats (Experimental Animal Center, Nantong University, China), weighing 200–250 g, were used in the study. The rats were kept in the animal housing facility with controlled room temperature (23±1°C) and unlimited access to food and water. All experimental procedures were approved by the Animal Care and Use Committee of Nantong University and performed in accordance with National Institutes of Health guidelines on animal care. Animal treatments were performed according to the Guidelines of the International Association for the Study of Pain.[19] Before the experiments, the animals were allowed to habituate to the housing facility for 2 days. For the LDH surgery, the rats were anesthetized and placed in a prone position. Laminectomy was performed to expose the left L5 spinal nerve and the associated dorsal root ganglia (DRG). The autologous NP was harvested using forceps from the C2-C3 and C3-C4 coccygeal vertebral disks and placed next to the left L5 nerve root just proximal to the DRG (NP group).[20] The rats in the sham group underwent the same surgical procedure except for implantation of the autologous NP.

The rats were sacrificed at designated time points by injecting pentobarbital (60 mg/kg) intraperitoneally. The NP was removed 0, 3, 5, 7, 10, 14, 21 and 28 days

after the surgery. The naïve DRG and the exposed DRG 14 and 28 days after the surgery were excised. The sample was placed in a cryopreservation tube and preserved at –80°C.

Behavioral Testing

The rats were habituated to the testing environment daily for at least 2 days before baseline testing. The room temperature and humidity remained stable for all experiments. Mechanical allodynia was determined by measuring the paw withdrawal threshold (PWT) in response to von Frey hair (Stoelting, IL, USA) stimulation. The withdrawal threshold was determined using the Dixon's up–down method.[21] Thermal hyperalgesia was determined by measuring the paw withdrawal latency (PWL) in response to the radiant heat stimulation using a plantar analgesia meter (IITC Life Science Inc., CA, USA). The baseline latencies were adjusted to 10–14 s with a maximum of 20 s as the cutoff to prevent potential injury. The latencies were averaged over three trials separated by 5-min intervals.

Cell Isolation

The NP samples were put into the homogenizer and homogenized after adding phosphate-buffered saline (PBS) and then digested with 0.25% collagenase II (Sigma, St Louis, USA) for 4 h at 37°C in a humidified incubator. The suspended cells were filtered through a 200-mesh filter, centrifuged for 5 min at 150 *g*, and washed twice with PBS. A total of 1×10^6 cells were acquired.

Western Blot

Tissue protein were immediately homogenized in a homogenization buffer containing 50 mM Tris-HCl (pH 7.5) (Sangon Biotech, Shanghai, China), 150 mM NaCl (Sangon Biotech, Shanghai, China), 0.1% NP-40 (Sangon Biotech, Shanghai, China), 5 mM EDTA (Sangon Biotech, Shanghai, China), 60 mM β-glycerophosphate (Sangon Biotech, Shanghai, China), 0.1 mM sodium orthovanadate (Sangon Biotech, Shanghai, China), 0.1 mM NaF (Sangon Biotech, Shanghai, China), and complete protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland), and then centrifuged at 12,000 *g* for 20 min to collect the supernatant. Protein concentrations were measured using a Bio-Rad protein assay (Bio-Rad, CA, USA). The supernatant was diluted in 5×sodium dodecyl sulfate (Sangon Biotech, Shanghai, China) loading buffer and boiled for 5 min. Then, 50 μg protein was added in each lane, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and transferred onto polyvinylidene difluoride membranes (Millipore, MA, USA). The membranes were blocked with 5% fat-free milk in Tris-buffered saline containing Tween 20 (TBST; 150 mM NaCl, 20 mM Tris, and 0.05% Tween 20) for 2 h at room temperature. Then, the membranes were washed with TBST three times and incubated with the primary antibodies overnight. The primary antibodies used in this study included mouse anti-IL-4 (1:500; Santa Cruz Biotechnology, TX, USA), rabbit anti-IFN-γ (1:500; Santa Cruz Biotechnology), rabbit anti-CD4 (1:500; Santa Cruz Biotechnology), mouse anti-glyceraldehyde 3-phosphate dehydrogenase (1:1000; Santa Cruz), mouse anti-β-actin (1:1000; Santa Cruz), mouse anti-CD206 (1:1000, Abcam, MA, USA), and rabbit anti-CD86 (1:1000, Abcam). Finally, horseradish peroxidase–conjugated anti-mouse or rabbit secondary antibodies (1:2000; Santa Cruz) were incubated for 2 h and then visualized using an enhanced chemiluminescence detection kit (Tiangen Biotech, Beijing, China). The intensity of the bands was analyzed using Quantity One V 4.62 Software (BIO-RAD, CA, USA). Values were calculated for at least three independent experiments.

Immunofluorescent Staining

The sections were blocked with blocking solution (PBS containing 10% normal goat serum, 0.1% Triton X-100, 0.02% sodium azide, and 0.05% Tween 20) at room temperature for 2 h. The slides were double labeled for IL-4 (1:100; Santa Cruz Biotechnology), IFN-γ (1:100; Santa Cruz Biotechnology), specific marker protein anti-CD4 (1:50; Sigma, Taufkirchen, Germany), anti-F4/80 (1:100 Cederlane, Burlington, Canada), IL-1β (1:100 Santa Cruz Biotechnology), and CD206 (1:100 Abcam) primary antibodies. In control sections, normal IgG substituted the primary antibody. On the following day, TRITC– and FITC– conjugated secondary antibodies (1:200, Jackson Immuno Research, PA, USA) were added and incubated for 2 h at 4°C in the dark. After placing coverslips, the sections were examined under the fluorescence microscope.

Flow Cytometry Assay

After filtration and centrifugation, a density of

3×10^5 cells/mL was seeded in the 6-cm dish and incubated with Roswell Park Memorial Institute 1640 medium for 4 h at 37.5°C, 5% CO2 in the presence of 25 ng/mL phorbol myristate acetate (PMA) (ENZO, BML-PE160-0001), 1 mg/mL ionomycin (ENZO, ALX-450-007-M001), and 1.7 mg/mL monensin (ENZO, 380- 026-M100). Irritants (PMA and ionomycin) and blocking agent (BFA) were gradually added to the samples, followed by 4 h of incubation. Next, the samples were loaded into the appropriate test tubes, centrifuged, and washed two times with PBS. Then, PerCP anti-human/rat CD4, PE anti-human/rat IL-4, and APC anti-human/rat IFN-γ (monoclonal antibodies, 1:1000, BD Bioscience Pharmingen, CA, USA) were added for intracellular cytokine staining for 30 min. The cells were washed again and suspended in PBS solution. The samples were measured, and data were recorded on FACScan cytometer equipped with CellQuest Pro software (BD Bioscience, CA, USA).

Statistical Analysis

Statistical analyses were performed using Sigma Stat (V3.5)(SYSTAT Software Inc., CA, USA). Data were expressed as mean ± standard error of mean (SEM). The expression levels from the western blot data were analyzed by one-way analysis of variance (ANOVA), followed by individual post hoc comparisons (Fisher's exact test) or pair-wise comparisons (*t* test). Behavior measures were normalized and analyzed by one- or two-way ANOVA with repeated measures on time factor followed by the Student–Newman–Keuls method for multiple comparisons. The criterion for statistical significance was set at 0.05 ($p<0.05$).

RESULTS

Balance between Th1/Th2 Was Disrupted in the Patients after LDH

Th1/Th2 cells and their related cytokines in the NP of normal controls and patients with LDH were analyzed by flow cytometry. The expression level of $CD4^+$ cells was significantly higher than that in the control group (Figures 1Aa and 1Ad). IL-4-producing cells (Th2 cells) significantly increased compared with those in the control group (Figures. 1Ab and 1Ae). IFN-γ-producing cells (Th1 cells) in each group were similar (Figures 1Ac and 1Af).

The protein levels of IL-4, IL-5, IL-12, and IFN-γ of the NP were measured by the western blot analysis. The expression of IL-4 and IL-5 in patients with LDH was higher than that in controls, while the levels of IFN-γ and IL-12 proteins were nearly the same as in the control group (Figure 1B).

The immunofluorescence assay revealed that the number of IFN-γ+ CD4 cells was less than that of IL-4+ CD4 cells (Figure 1C).

Evaluation of Nociceptive Tests after LDH

The LDH-induced neuropathic pain model was established in rats and used to explore the possible function of the balance between Th1/Th2 during neuropathic pain. The application of autologous NP to the left L5 nerve root induced rapid and persistent mechanical allodynia.[22] On the ipsilateral paw of the NP rats, the PWT dropped from 25.8±0.6 g before the surgery to 8.5±1.0 g 1 day after the surgery (Figure 2A). The PWT remained at the peak level for more than 14 days (5.4±0.6 g) and slightly recovered at 28 days (15.5±1.1 g). The sham group had no significant changes ($p>0.05$, vs baseline; Figure 2A).

Moreover, the application of autologous NP induced a delayed heat hyperalgesia. The paw withdrawal latency (PWL) decreased from 14.5±0.6 s to 5.6±1.4 s 3 days after the surgery was maintained at 21 days (9.1±1.2 s) and was recovered at 28 days (11.4±1.8 s) in the NP group. The PWL of sham group rats did not show significant changes ($p>0.05$, vs baseline; Figure 2B).

Balance between Th1/Th2 was Disrupted in Rats after LDH

The differentiation of T cells in the NP on days 14 and 28 after LDH was explored by flow cytometry. The number of Th1 cells dramatically increased on day 14 after LDH, but significantly decreased on day 28 after LDH. The number of Th2 cells increased on day 28 after LDH (Figure 3A).

Western blot analysis was performed to investigate the temporal pattern of IL-4, IL-5, IL-12, and IFN-γ protein expression. As shown in Figure 3B, the protein expression of IL-4 and IL-5 in the extrusive NP remarkably increased on day 10 and peaked at 4 weeks after LDH compared with the control group. The protein expression of IL-12 and IFN-γ in the extrusive

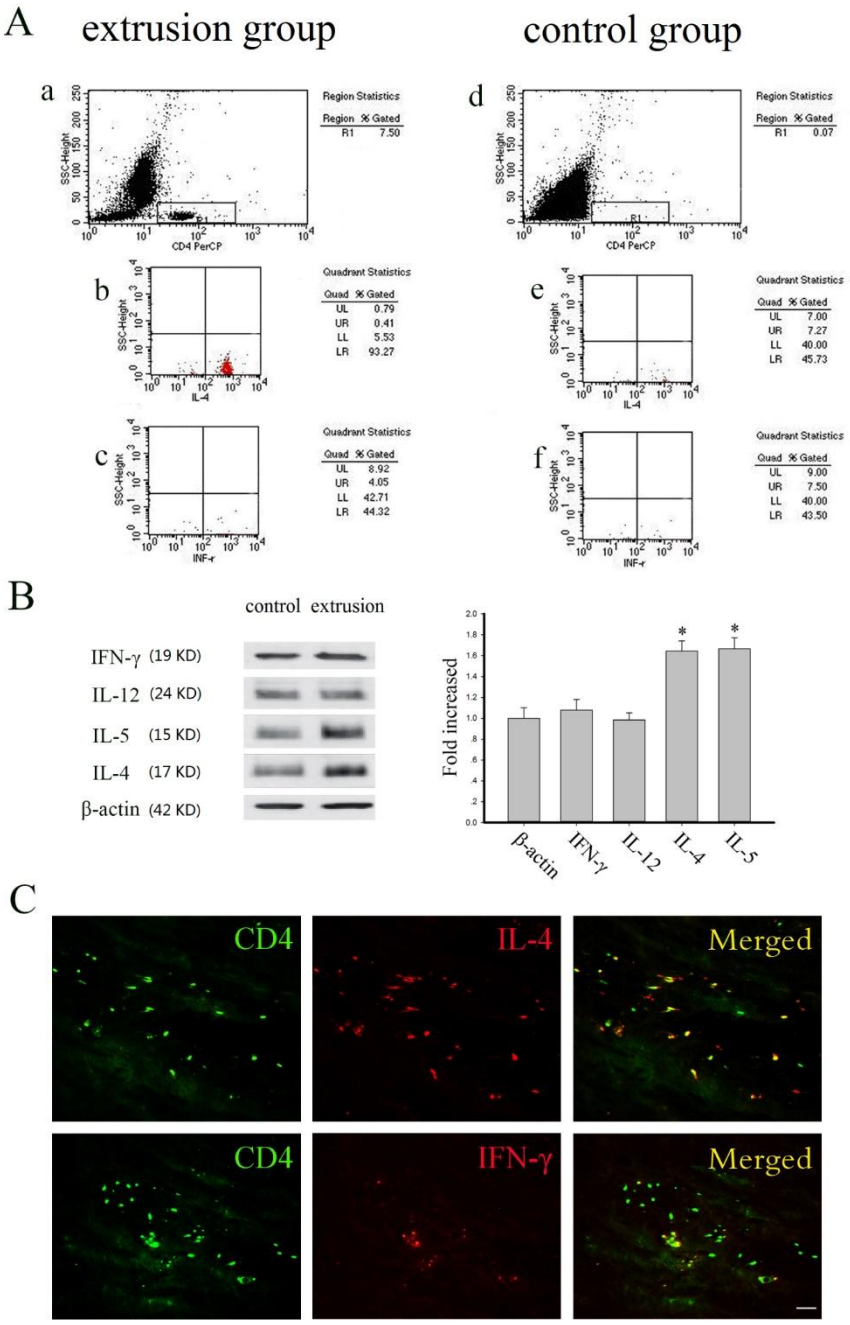

Figure 1. Th1 polarization in the patients' nucleus pulposus (NP) after lumbar disc herniation.
(A) Aa and Ad show the percentage of cluster of differentiation (CD) 4+cells in flow cytometry analysis in extrusion and control groups. Ac and Af show the percentage of Th cells that secreted IFN-γ among CD4+ Th cells in each group. Ab and Ae show the percentage of Th cells that secreted IL-4 among CD4+ Th cells in each group. The results shown are representative of six independent experiments with three NPs per group. (B) The protein expression of Th1-polarized specific molecules (IFN-γ and IL-12) and Th2-polarized specific molecules (IL-4 and IL-5) was evaluated by the western blot analysis. Each column shows the mean fold increase to baseline. Data are presented as the mean9±SEM of six experiments (*p<0.05). (C) The expression of IL-4 or IFN-γ protein on CD+ Th cells was visualized by the immunohistochemical analysis. The representative micrographs of IL-4, IFN-γ, CD4+, and merged images of NP are shown. Scale bars=30 µm.

NP remarkably increased on day 3, peaked at 2 weeks, and was recovered on day 28 after LDH.

Immunofluorescence showed the extensive co-localization of CD4 with INF-γ in the NP on day 14 after LDH. The extensive co-localization of CD4 with IL-4 was observed in the NP on day 28 after LDH (Figure 3C).

Phenotypic Shift of Macrophages from M1 to M2 in the DRG after LDH

Macrophages can be polarized into the classically activated (M1) macrophage phenotype or the alternatively activated (M2) macrophage phenotype as a response to the Th1 or Th2 cytokines.[23] The expression of the M1-specific molecules IL-1β, CC-chemokine ligand 3 (CCL3), CD86, M2-specific molecules arginase-1 (Arg1), IL-10, and CD206, was investigated to study the possible function of the polarization of Th cells in LDH.[28,32]

Western blot assay was employed to investigate the expression of CCL3, CD86, CD206, and Arg1. As shown in Figure 4A, CCL3 and CD86 proteins (M1-specific molecules) were expressed at a relatively low level in naive DRG; it markedly increased on day 14 after the surgery and decreased on day 28. Arg1 and CD206 proteins (M2-specific molecules) were expressed at a relatively low level in the naive spinal cord; it markedly increased on day 28 after LDH. The quantitative analysis is shown in Figure 4B.

Next cell types were explored by double-immunostaining. The immunohistochemical analysis showed that the population of IL-1β+, F4/80+ macrophages in LDH-operated DRG markedly increased on day 14 after the surgery and decreased on day 28. In contrast, the population of CD206+, F4/80+ macrophages in LDH-operated DRG markedly increased on day 28 after the surgery (Figure 4C).

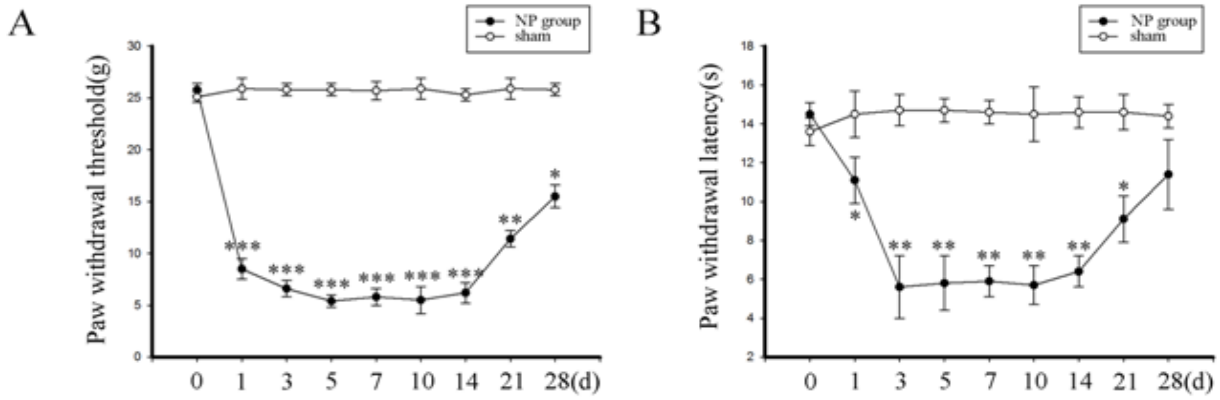

Figure 2. Time course of nucleus pulposus-induced mechanical allodynia and heat hyperalgesia in rats.
(A)The mechanical threshold of nucleus pulposus group significantly dropped after surgery, while the sham group had no significant changes.
(B) The paw withdrawal latency significantly decreased after surgery, while the sham group did not show significant changes. *p<0.05, **p<0.01, ***p<0.001 versus sham, Naive, baseline. Two-way ANOVA followed by the Bonferroni test (n=10 rats per group).

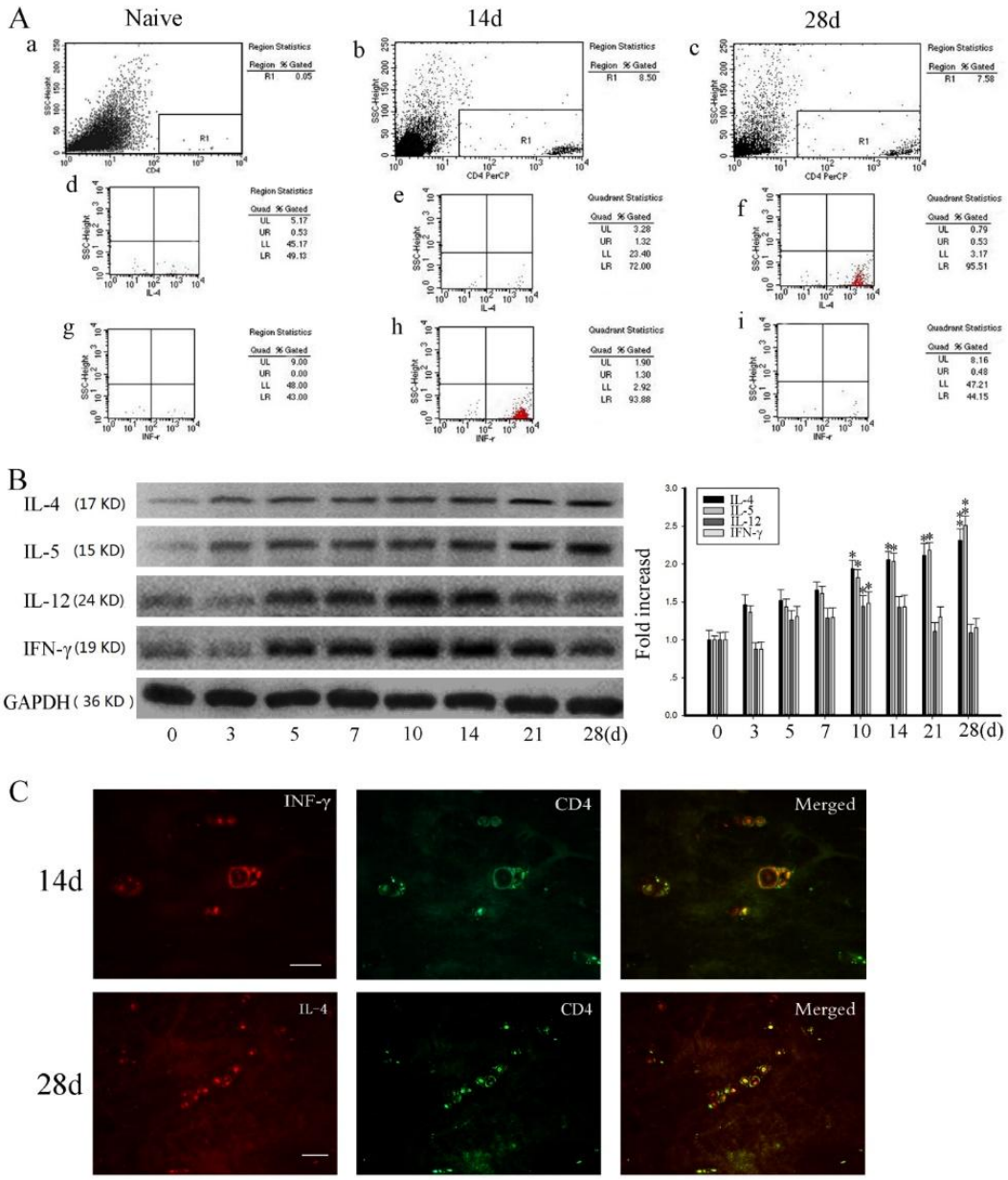

Figure 3. Th polarization in the rat nucleus pulposus (NP) after LDH.

(A)The NP was harvested on days 0, 14, and 28 from rats subjected to LDH. Aa-c show the percentage of CD4+ cells in flow cytometry analysis in each group. Ag– i show the percentage of Th cells secreting IFN-γ among CD4+ Th cells in each group, and Ad–f show the percentage of Th cells secreting IL-4 among CD4+ Th cells in each group. The results shown are representative of six independent experiments with three NPs per group.

(B)The protein expression of Th1-polarized specific molecules (IFN-γ and IL-12) and Th2-polarized specific molecules (IL-4 and IL-5) was evaluated by western blot. The NP was harvested on days 0, 3, 5, 7, 10, 14, 21, and 28 from rats subjected to LDH. Each column shows the mean fold increase to baseline. Data are presented as the mean ± SEM of six experiments (***p*< 0.01, ****p*< 0.05).**

(C)The expression of IL-4 or IFN-γ protein on CD+ Th cells was visualized by the immunohistochemical analysis.

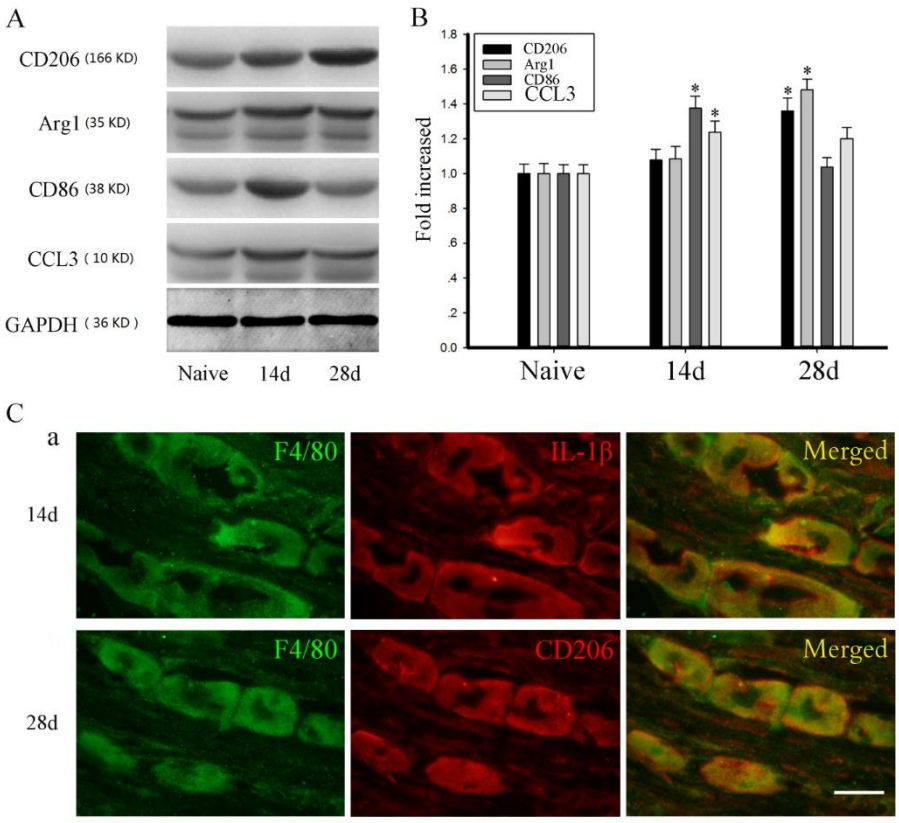

Figure 4. Phenotypic shift of macrophages from M1 to M2 after LDH.

(A) The DRG was harvested on days 14 and 28 from rats subjected to LDH. The protein expression of M1-polarized macrophage-specific molecules (CCL3 and CD86) and M2-polarized macrophage-specific molecules (Arg1 and CD206) was evaluated by the Western blot analysis.

(B) Each column shows the mean fold increase to baseline. Data are presented as the mean±SEM of five to six experiments (*p<0.05).

(C)The expression of IL-1β (Ca) or CD206 (Cb) protein on F4/80+ macrophages was visualized by the immunohistochemical analysis. The representative micrographs of IL-1β, CD206, and F4/80, and merged images of DRG are shown. Scale bars=20 µm.

DISCUSSION

The mechanism of sciatica caused by LDH is conventionally related to mechanical compression, inflammation, and autoimmunity. However, a previous study proved that the compression of nerves produced sensory and motor changes without pain, whereas pain was elicited with the manipulation of an inflamed nerve, in both animals and humans.[24] The inflammation and autoimmunity theory of LDH has been widely accepted recently by many scholars and also supported by many studies. Inflammatory cells (such as Th17, macrophages) and cytokines (such as IL-1, TNF-α, IL-6, IL-21, phospholipase A2, and prostaglandin E) have been proved to be associated with LDH-induced pain.[4-9]

Th cells are a subpopulation of T lymphocytes that express the cluster of differentiation 4 (CD4) molecule.[25] They can differentiate into Th1 or Th2 cells under various conditions. Th1 cells are characterized by the excretion of IL-2, IL-12, and IFN-γ, while Th2 cells are characterized by the excretion of IL-4, IL-5, IL-6, and IL-10. Th cells both in human and mice are similar.[11, 12] In the resting state (normal physiological state), the capacity of differentiation from Th0 cells to Th1 or Th2 cells is extremely weak, and the Th1/Th2 cells are in a relatively balanced state. Once the balance between Th1/Th2 is disrupted, it may damage the

dynamic balance of cytokine network, thereby leading to the emergence and development of many diseases.[15]

The NP is the body's largest avascular tissue in a closed structure.[26] It is surrounded by fibrosus annulus and cartilage end plate; therefore, losing the contact with the body's immune system and concealing the autoantigens. In patients with LDH, the NP is exposed to the body's immune system and may be able to induce local inflammation around the nerve root.

The present study demonstrated that Th2 cells and associated cytokines significantly increased in the NP of the extrusion group, whereas Th1 cells and associated cytokines in the extrusion and control groups were similar. The immunofluorescence assay indicated that the number of IFN-γ+ CD4 cells was less than that of IL-4+ CD4 cells (Figure 1). This implied that Th cells might differentiate into Th2 cells in the NP in the patients with LDH, and the Th1/Th2 balance shift might be involved in the pathogenesis of LDH-induced pain.

Next, an LDH model was established in male Sprague–Dawley rats, and behavioral tests were carried out. The expression of Th1/Th2 and related cytokines in the rat NP were shown in Figure 3. This further confirmed the hypothesis that the Th1/Th2 balance shift might be associated with the pathogenesis of sciatica. Moreover, as shown in Figure 2, the PWT dropped on day 1 after the surgery, remained at the peak for more than 14 days, and partially recovered on day 28; The PWL decreased on day 3 after the surgery, maintained for 21 days, and recovered on day 28. Together with Figure 3, these results showed some regularity: Thermal hyperalgesia and mechanical hypersensitivity developed with an increase in Th1 cells. However, thermal hyperalgesia and mechanical hypersensitivity were partially alleviated with an increase in Th2 cells.

Macrophages have been found in and around DRG and herniated NP [4,27] and considered to play an important role in herniated disk resorption.[4] Macrophages are classified into two distinct polarized phenotypes, but their phenotype can be altered.[28] The classical activation of M1-polarized macrophages is induced by bacterial lipopolysaccharide (LPS) and Th1 cytokines such as IFN-γ.[23] Activated M1 macrophages produce typical proinflammatory cytokines and chemokines, which initiate and drive inflammation.[23] In contrast, the activation of M2-polarized macrophages is promoted by Th2 cytokines such as IL-4.[23] M2 macrophages suppress and resolve inflammation.[29,30] In damaged nerves, macrophages and other leukocytes are recruited from blood vessels and produce a variety of inflammatory mediators associated with neuropathic pain.[31] The present study showed that the number of M1-polarized macrophages increased on day 14 and significantly reduced on day 28. The number of M2-polarized macrophages was still low on day 14, but increased on day 28. Combined with the characteristic of Th1/Th2 expression and behavioral testing, the results indicated some correlations between Th1/Th2 balance shift, phenotypic shift of macrophages, the results of behavioral testing, and that the polarization of Th cells might be involved in the pathogenesis of LDH through the phenotypic shift of macrophages.

The results of this study were encouraging. However, the study had several limitations. First, the number of subjects included was relatively small. Second, how Th1/Th2 and related cytokines influenced the phenotypic shift of macrophages and how macrophages influenced behavioral testing in the LDH model need further investigation. Third, the possible mechanism in patients with LDH needs to be confirmed.

In conclusion, the present study implied that the polarization of Th cells might be involved in the pathogenesis of LDH, and this might be achieved through the phenotypic shift of macrophages. Further studies on how to correct the imbalance between Th1 and Th2 cells may provide new insights into the therapeutic strategies of LDH.

ACKNOWLEDGEMENTS

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