Role of Link N in Modulating Inflammatory Conditions

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Aims: To elucidate the role of Link N in regulating inflammatory molecules from human mesenchymal stem cells (hMSCs) under interleukin (IL)-1β stimulation in vitro and under Complete Freund's Adjuvant (CFA)–induced arthritis of the temporomandibular joint (TMJ) in vivo. **Methods:** In vitro analysis of inflammatory cytokines and epithelial-mesenchymal transition (EMT) genes in hMSCs treated with Link N, IL-1β, and co-stimulation of IL-1β and Link N was undertaken using Luminex multiplex assays and real-time polymerase chain reaction, respectively. To determine the impact of Link N in ameliorating TMJ tissue homeostasis in arthritic conditions, histologic changes in CFA-induced arthritic TMJ tissues followed by application of Link N were examined. All data were analyzed using one-way analysis of variance with Bonferroni post hoc test. **Results:** Increased levels of IL-6; interferon gamma-inducible protein-10; and regulated upon activation, normal T cell expressed, and secreted (RANTES) were detected in response to IL-1β treatment, but these levels were significantly decreased in the co-stimulation group. In contrast, secreted IL-4, IL-10, and transforming growth factor β1–β3 proteins, as well as intracellular erb-b2 receptor tyrosine kinase 3 and Nodal homolog genes, were increased significantly in the co-stimulation group compared to the IL-1β group. Histologic analysis showed significant recovery for rat condyle thickness in the Link N–treated group when compared to the CFA-induced arthritis group. **Conclusion:** These findings indicate that Link N could modulate inflammation and EMT in vitro and repair arthritis-mediated TMJ disruption in vivo. Link N could be a potential therapeutic agent for TMJ disorder patients. *J Oral Facial Pain Headache 2019;33:114–122. doi: 10.11607/ofph.1952*

Keywords: *arthritis, Complete Freund's adjuvant, inflammation, Link N, temporomandbular joint*

Temporomandibular joint (TMJ) arthritis affects joint movement, leading to a malfunction of the masticatory system.[1] Under arthritic conditions, elevated production of proinflammatory cytokines could be detected and may contribute to TMJ pathology. Among all inflammatory cytokines, interleukin (IL)-1β, which can alter the production of extracellular matrix (ECM) compounds, plays a critical role in the pathogenesis of articular arthritis.[2,3] To replicate arthritis condition in vivo, administration of Complete Freund's adjuvant (CFA) has been used in various animal models to activate TMJ inflammation. Visible changes in the TMJ tissue and the release of proinflammatory cytokines (such as IL-1β and IL-6) have been reported.[4,5] The TMJ is a unique joint of the body. The fibrocartilage tissue is distributed in the TMJ disc and condylar cartilage.[6,7] With enriched composition of various ECM components, including collagen, hyaluronic acid, and aggrecan in the TMJ tissue, the TMJ possesses both tensile and compressive strength to assist joint movement and withstand compressive loads.[8] During TMJ inflammation, the structure of the fibrocartilage tissue is distorted, thereby affecting its function.

Link N is a small peptide consisting of 16 amino acids (DHLSDNYTLDHDRAIH). This peptide is released from the link protein, one of the components of ECM, and could act as a stabilizer to maintain the interaction between aggrecan and hyaluronic acid.[9,10] Link N has been used to repair intervertebral disc (IVD) degeneration

both in vivo and in vitro. After injection of Link N into a rabbit IVD, the disc height and its structures were restored. It was also found that Link N stimulated ECM protein synthesis and reduced proteinase messenger ribonucleic acid (mRNA) expression.[11] Similar Link N–mediated regenerative potential was also detected in cultured human IVD cells, showing that Link N could be an alternative agent for conservative treatment of IVD degeneration.[12] At the molecular level, Link N could bind to bone morphogenetic protein (BMP) type II receptors, thereby enhancing ECM protein expression through the Smad1/5 pathway.[13] As the IVD is also composed of fibrocartilage tissue,[7,14] Link N might be a good candidate peptide for treating TMJ arthritis.

Several studies recently attempted to use human mesenchymal stem cells (hMSCs) to more effectively treat arthritis-mediated TMJ damage.[15,16] As Link N can stimulate chondrogenic differentiation and upregulate ECM production but suppress osteogenetic differentiation in MSCs,[17] the application of Link N could be a good therapeutic scheme to restore TMJ fibrocartilage tissue. To date, the studies regarding the regulatory role of Link N for TMJ repair in inflammatory conditions are rarely emphasized, making it important to evaluate the effects of Link N administration in hMSCs under an inflammatory condition. This study therefore aimed to elucidate the role of Link N in regulating inflammatory molecules from hMSCs under IL-1β stimulation in vitro and under CFA-induced arthritis of the TMJ in vivo.

Materials and Methods

Cell Isolation and Culture

hMSCs were isolated from adipose tissue obtained from clinical surgery using protocols described elsewhere.[18] All procedures were approved by the Institutional Review Board of Tri-Service General Hospital, Taipei, Taiwan (TSGHIRB Number 2-105-05-212). In brief, the isolated tissues were transferred into Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 0.1% collagenase (Sigma). After 24-hour incubation, the tissue was transferred into DMEM containing 10% fetal bovine serum (FBS; Gibco) for 24 hours. After centrifugation at 500 g for 5 minutes, all cells were transferred to keratinocyte serum-free medium (K-SFM; Gibco) with 5% FBS, antioxidants N-acetylcysteine, and L-ascorbic acid 2-phosphate (Sigma) in a humidified atmosphere of 5% carbon dioxide at 37°C.

Peptide Synthesis

Link N was custom synthesized by Mission Biotech. The consensus sequence for the human link protein peptide consists of 16 amino acids (DHLSDNYTLDHDRAIH). The released peptides were purified up to > 95% purity using high-performance liquid chromatography (HPLC), characterized through electrospray ionization mass spectroscopy, and lyophilized until dry.

Cytokine Quantification

hMSCs (10th to 17th passage) were seeded into 25-cm^2 flasks and divided into four groups: *(1)* Link N (1 μg/mL) only; *(2)* IL-1β (1 ng/mL) only; *(3)* co-stimulation (Link N and IL-1β); and *(4)* untreated. hMSCs supernatants were collected for quantification of the levels of the following cytokines: IL-6; interferon gamma-inducible protein (IP)-10; regulated upon activation, normal T cell expressed, and presumably secreted (RANTES); matrix metalloproteinase (MMP)3; MMP13; IL-4; IL-13; and transforming growth factor (TGF)β1–β3. Quantification was carried out using a high-sensitivity human cytokine kit for simultaneous multi-analyte detection with Luminex technology (Milliplex, Millipore). The data were from three independent experiments (TGF: duplication; other markers: triplication).

PCR Array

Total RNA from each group was isolated using Quick-RNATM MiniPrep kits (Zymo Research). After RNA extraction and quality assessment, cDNA synthesis was performed using RT2 First Strand Kit (Qiagen Sciences) according to the manufacturer's instructions. The expression of epithelial-mesenchymal transition (EMT)–associated mRNAs was analyzed using the RT2 Profiler PCR Array (Qiagen Sciences). PCR arrays were run in a Roche LightCycler 480 System.

CFA-Induced TMJ Arthritis Model and Histopathologic Staining

All animal protocols were performed in accordance with those of the Institutional Animal Care and Use Committee of National Yang-Ming University. Eighteen Sprague-Dawley rats (8 weeks old, female) were used and divided into three groups of 6 rats each: *(1)* CFA-induced group; *(2)* CFA-induced + Link N group; and *(3)* untreated. In the CFA-induced group and the CFA-induced + Link N group, 50 μL of CFA (F5881; Sigma) was injected bilaterally into the superior space of the TMJ at days 0 and 7 to induce articular arthritis. In the CFA-induced + Link N group, 50 μL of Link N (0.01 mg/μL) was injected on day 14. All groups were sacrificed on day 35, and the TMJ tissue was collected to determine the morphologic changes using hematoxylin and eosin (H&E) staining. A total of 30 slides (thickness = 10 μm) from rat TMJs were collected, as each slide was 50 μm apart from

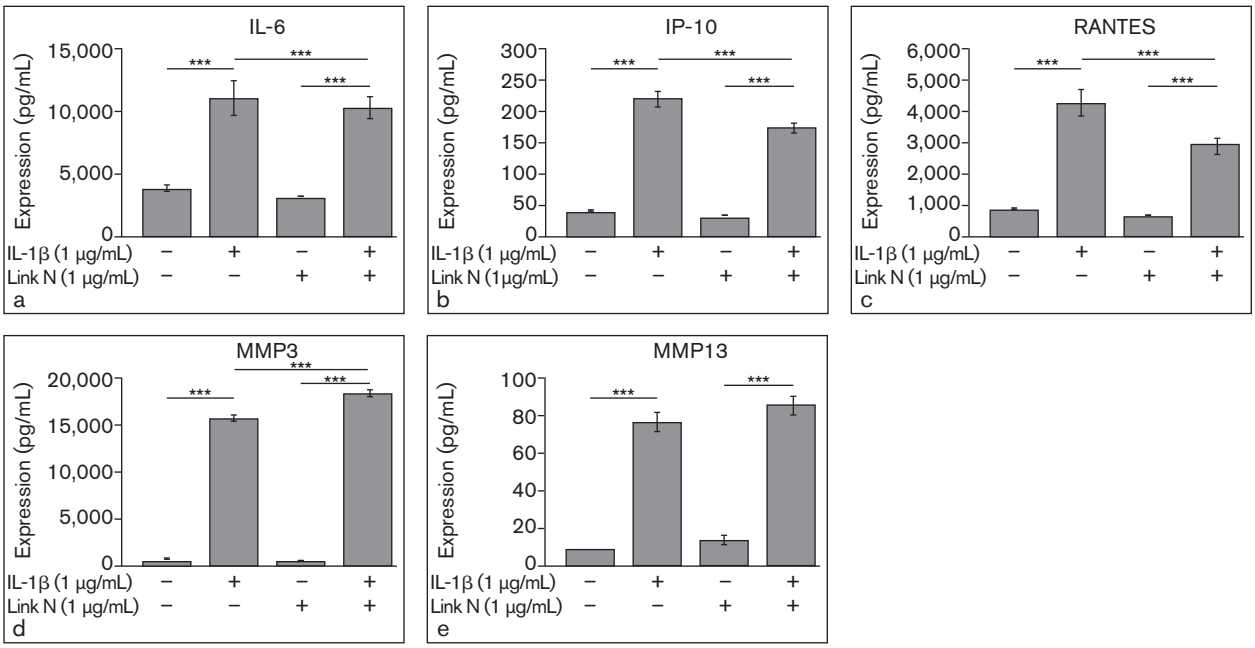

Fig 1 Expression of inflammatory cytokines 24 hours after IL-β stimulation. **(a)** IL-6, **(b)** IP-10, and **(c)** RANTES expressions were increased significantly in the IL-1β group compared to the untreated group. In the co-stimulation group, the IL-1β–mediated increase of IL-6 was significantly downregulated when compared to the IL-1β group. **(d)** MMP3 and **(e)** MMP13 expressions were increased significantly in both the IL-1β and co-stimulation groups compared to the untreated group. The Link N group did not differ from the untreated group in the expressions of any tested cytokines. Data are presented as mean ± standard deviation (SD) from three independent experiments with at least three replicates. ***$P < .001$.

each other. During tissue sectioning, slides were examined under a microscope in order to capture the section containing the most tissue component. The quantification analysis was performed using a spot software (SPOT Imaging, a division of Diagnostic Instruments).

Statistical Analyses

Statistical analyses were performed using SPSS version 18. The quantification of cytokines and condyle thickness are expressed as mean ± standard deviation (SD) for at least three replicates. All data were analyzed using one-way analysis of variance (ANOVA) with post hoc Bonferroni test, and *P* values less than .05 were considered statistically significant.

Results

To simulate inflammation, isolated hMSCs were initially exposed to IL-1β for 24 hours, and the detection of pro-inflammatory factors IL-6, IP-10, RANTES, MMP3, and MMP13 in culture medium using Luminex analysis was then carried out. The results showed a significant increase of the tested markers ($P < .001$). Link N treatment did not increase inflammatory cytokine secretion, providing evidence that Link N administration alone is safe enough to regulate inflammation (Fig 1). Interestingly, in the co-stimulation group, the expressions of IL-6 ($P < .001$), IP-10 ($P < .001$), and RANTES ($P < .001$) (Figs 1a through 1c), but not MMP3 or MMP13 (Figs 1d and 1e), were decreased significantly compared to the IL-1β group, indicating a potential role of Link N in modulating inflammation.

Next, the anti-inflammatory cytokine expression in IL-1β/Link N–treated hMSCs was evaluated. After 24 hours of treatment, levels of IL-4 ($P < .05$) and IL-10 ($P < .001$), but not IL-13, in the co-stimulation group had increased considerably compared to the Link N–alone group; while only IL-4 ($P < .01$) significantly increased in the co-stimulation group compared to the IL-1β group. After 96 hours of treatment, the IL-4 ($P < .05$) and IL-13 ($P < .05$) levels dropped in the IL-1β alone group compared to the untreated group. The expression of all three tested cytokines tended to upregulate, particularly for IL-10 ($P < .001$), in the co-stimulation group in comparison to the IL-1β–treated group (Fig 2).

To further assess chondrogenic activity in response to 96-hour Link N treatment under an IL-1β–mediated inflammatory condition, the expressions of TGFβ1–β3 were investigated. After individual stimulation with IL-1β, the expressions of TGF-β1 ($P < .001$) and TGF-β3 ($P < .05$) were significantly decreased compared to the untreated group, implying that IL-1β–mediated inflammation potentially down-

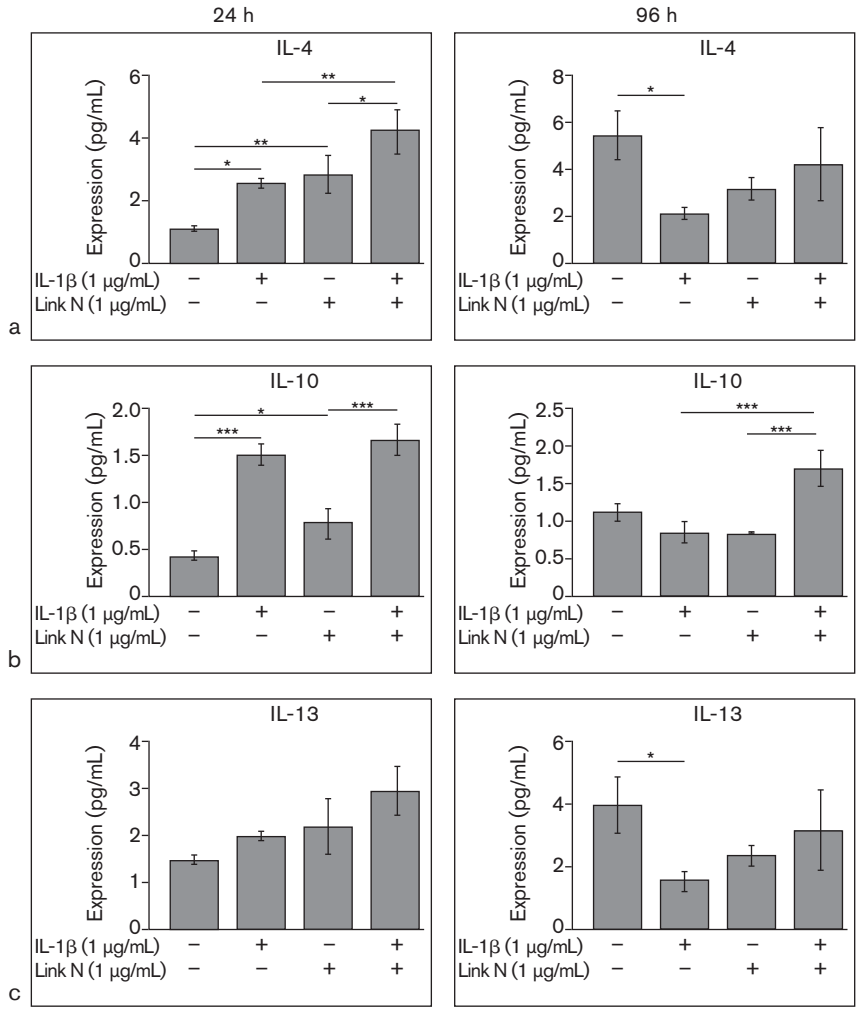

Fig 2 Expression of anti-inflammatory cytokines at 24 and 96 hours poststimulation. **(a)** IL-4 expression increased significantly in both the IL-1β and Link N stimulation groups compared to the untreated group at 24 hours after treatment. At both 24 and 96 hours after treatment, in the co-stimulation group, IL-4 further increased compared to the IL-1β group. **(b)** IL-10 expression increased significantly in both the IL-1β and Link N stimulation groups compared to the untreated group at 24 hours after treatment. After 24 and 96 hours, IL-10 was significantly upregulated in the co-stimulation group compared to the IL-1β group. **(c)** There was a moderate increase of IL-13 in the co-stimulation group compared to both the IL-1β and Link N groups at 24 and 96 hours after treatment. Data are presented as mean ± standard deviation (SD) from three independent experiments with at least three replicates. **P* <.05. ***P* < .01. ****P* < .001.

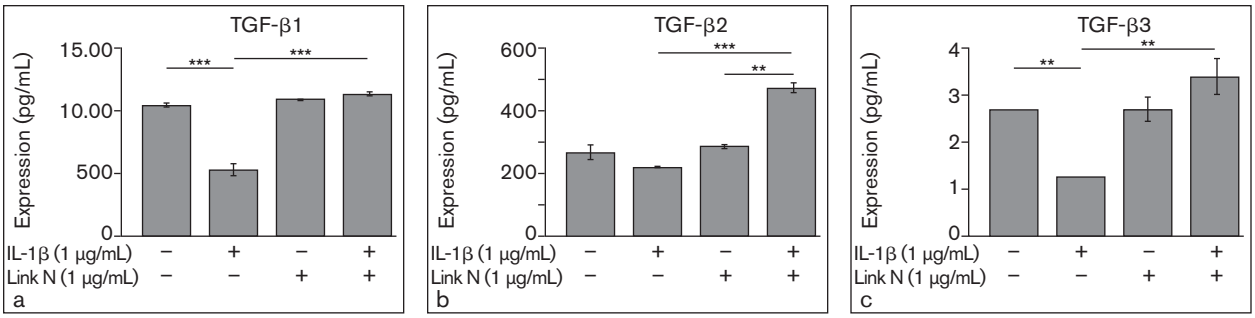

Fig 3 Expressions of chondrogensis regulators at 96 hours poststimulation. **(a)** TGF-β1, **(b)** TGF-β2, and **(c)** TGF-β3 expressions were downregulated in the IL-1β group compared to the untreated group, but were significantly increased in the co-stimulation group compared to both the IL-1β and Link N stimulation groups. Data are presented as mean ± standard deviation (SD) from three independent experiments with at least two replicates. **P* <.05. ***P* < .01. ****P* < .001.

regulated chondrogenic activity (Figs 3a through 3c). TGF-β1 (*P* < .001), TGF-β2 (*P* < .001), and TGF-β3 (*P* < .01) expressions were significantly greater in the co-stimulation group than in the IL-1β–alone group, demonstrating a beneficial impact of Link N in restoring chondrogenesis-associated molecules (Fig 3).

To screen IL-1β/Link N–related molecular cues, PCR array analysis for EMT-associated mRNA changes between different experimental groups was performed (Table 1). PCR array results showed that six genes, including MMP3, wnt family member 5A (WNT5A), transmembrane protein 132A

Table 1 Comparison of Gene Expression in Different Experimental Groups

	IL-1β	Co-stimulation (Link N + IL-1β)
Gene expression compared to untreated group		
Upregulated	MMP3, WNT5A, TMEM132A, BMP2, IL-1RN, KRT7	MMP3, WNT5A, TMEM132A, BMP2, IL-1RN, F11R, KRT7, ERBB3
Downregulated	JAG1, NODAL, KRT14	–
Gene expression compared to IL-1β group		
Upregulated	–	ERBB3, NODAL
Downregulated	–	–

IL-1β = interleukin-1β; MMP3 = matrix metalloprotein 3; WNT5A = wnt family member 5A; TMEM132A = transmembrane protein 132A; BMP2 = bone morphogenetic protein 2; IL-1RN = interleukin-1 receptor antagonist; KRT7 = keratin 7; JAG1 = Jagged 1; NODAL = Nodal homolog; KRT14 = keratin 14; F11R = F11 receptor; ERBB3 = erb-b2 receptor tyrosine kinase 3.

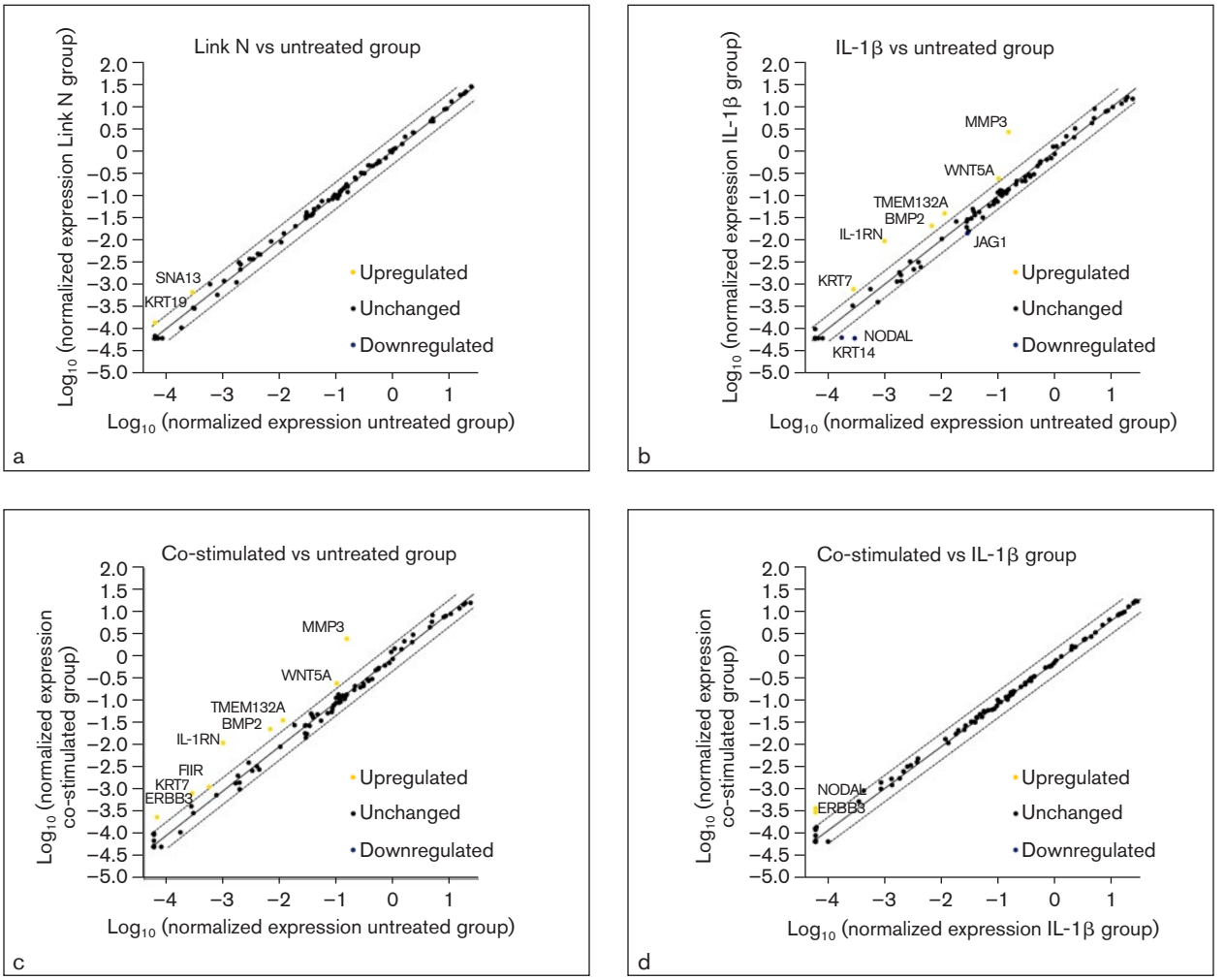

Fig 4 EMT gene expression at 24 hours poststimulation. **(a–c)** The mRNA expressions in the Link N, IL-1β, co-stimulation, and untreated groups were screened using a PCR array. SNAI3 and KRT19 were upregulated in the Link N group compared to the untreated group. Downregulated KRT14, NODAL, and JAG1 and increased MMP3, WNT5A, TMEM132A, BMP2, IL-1RN, and KRT7 were detected in the IL-1β group compared to the untreated group. In the comparison between the co-stimulation group and the untreated group, MMP3, WNT5A, TMEM132A, BMP2, IL-1RN, F11R, KRT7, and ERBB3 were upregulated. **(d)** The comparison of the co-stimulation group and the IL-1β group showed upregulated NODAL and ERBB3.

(TMEM132A), BMP2, interleukin-1 receptor antagonist (IL-1RN), and Keratin (KRT)7 were upregulated in the IL-1β group compared to the untreated group; in contrast, KRT14, Nodal homolog (NODAL), and Jagged (JAG)1 were decreased in response to IL-1β treatment (Fig 4b). It was noteworthy that a similar set of genes, as well as erb-b2 receptor tyrosine kinase 3 (ERBB3) and F11 receptor (F11R), were increased in the co-stimulation group compared to the untreated cells (Fig 4c), indicating that ERBB3 and F11R might

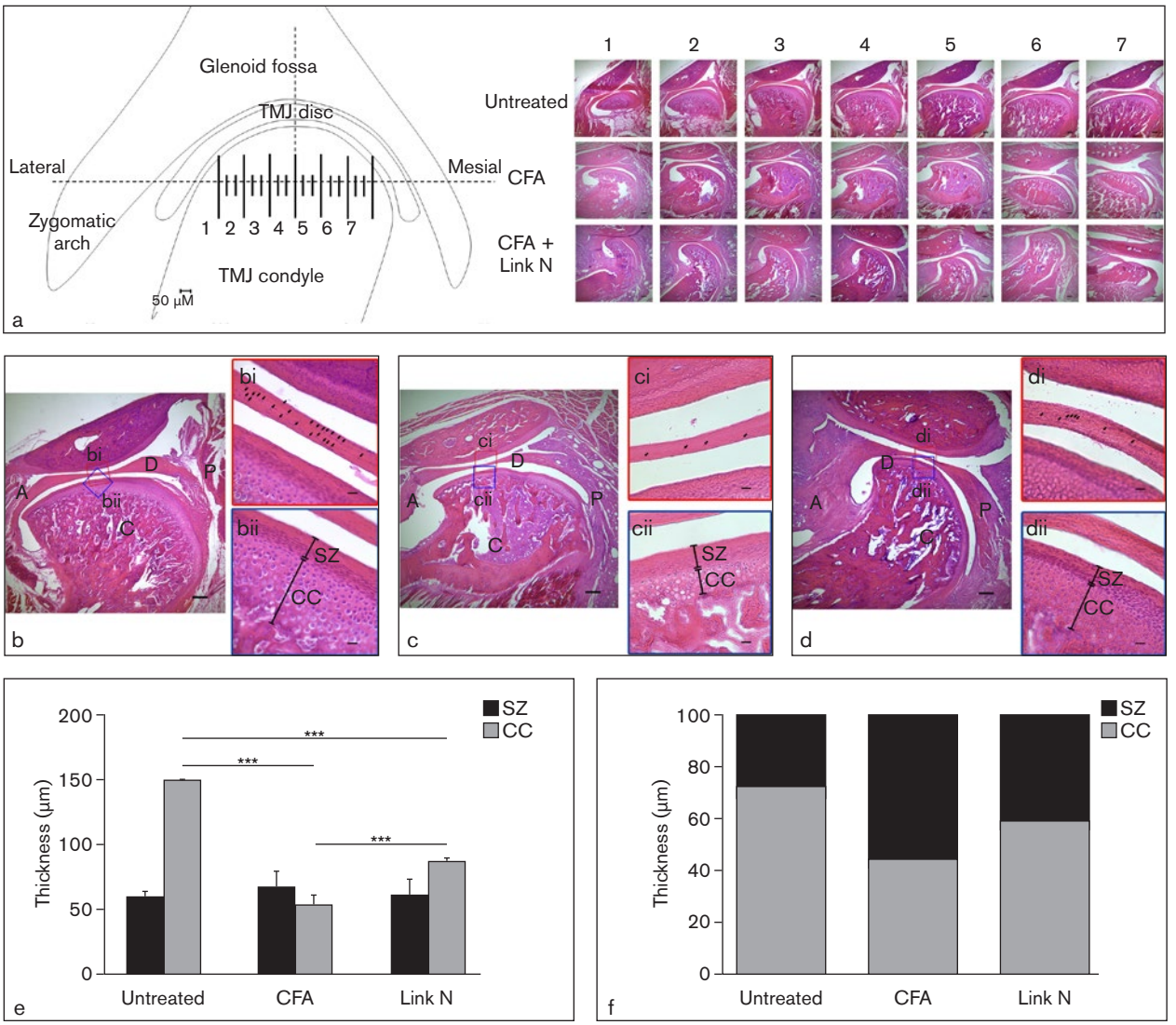

Fig 5 Histologic changes in TMJ tissues from CFA-induced arthritis and Link N–rescued rats. **(a)** The strategy of histologic slide collection of TMJ tissues was illustrated. The changes in tissue morphology from 1 to 7 in different groups were detected. Slide 4 represents the largest cross-sectional images. Representative sagittal view of **(b)** untreated (n = 6), **(c)** CFA-induced arthritic (n = 6), and **(d)** CFA + Link N (n = 6) groups. TMJ discs (red box) and TMJ condyles (blue box) are highlighted in bi and bii, ci and cii, and di and dii, respectively; superficial zone (SZ) and condylar cartilage (CC) are labelled. Arrowheads indicate thickness. **(ci, cii)** Injection of CFA into the TMJ led to a looser structure, as CC tissue was thinner than in the untreated group. **(di, dii)** Link N injection resulted in an increased cell intensity in SZ tissue and thicker CC. **(e)** Quantitative analysis for thickness of SZ and CC tissues showed that CC tissue in CFA-induced arthritis group decreased significantly compared to the untreated group. After Link N application, the thickness of CC tissue was restored significantly compared to the CFA-induced arthritis group. **(f)** The ratios of SZ to CC in condyles from different groups were examined. Scale bar: (a, b, c, d) = 250 μm; (bi, bii, ci, cii, di, dii) = 25 μm. D = disc; C = condyle; A = anterior; P = posterior. Arrowheads indicate cell position. Data are presented as mean ± standard deviation (SD).*P < .05. **P < .01. ***P < .001.

serve as Link N targets for rescuing IL-1β–stimulated inflammation. Nevertheless, a similar comparison between the IL-1β–treated and the co-stimulation groups surprisingly showed that only ERBB3, not F11R, was upregulated in the co-stimulation group (Table 1).

A well-established rat CFA-induced arthritis model[4,19,20] was used to further examine the role of Link N in restoring inflammation-mediated TMJ disruption in vivo. The CFA-induced arthritis model was validated via the detection of head swelling and histologic changes. In comparison with the untreated animal, rat CFA injection showed an enlarged head on day 1 (Appendix 1a and 1b) and elevated inflammatory cell infiltration in synovial tissue on day 35 (Appendix 1c and 1d; Appendix 1 available in the online version only, at www.quintpub.com). Sagittal views of the TMJ tissue in different conditions (untreated, CFA, and CFA + Link N) were examined. Slide 4 of Fig 5a represents the largest cross-sectional images. Normal TMJ tissues exhibited regular thickness and chondrocyte

cells in the disc (Fig 5bi), and the condyle subchondral bone was separated into two parts, superficial zone (SZ) tissue and condylar cartilage (CC) tissue (Fig 5bii).[7] After 35 days of CFA induction of an arthritis-like condition, the structure of the disc showed fewer cells (arrowheads) in comparison to the untreated group (Fig 5ci). The condyle layer did not change in the SZ tissue, but the CC tissue was thinner than in the untreated group (Fig 5cii). Link N application increased cell intensity in SZ tissue and thickness in CC tissue in the CFA-induced + Link N group compared to the CFA-alone group (Fig 5dii). Quantitative analysis of thickness of the CC tissue in different groups showed a significant decrease in the CFA-induced arthritis group compared to the untreated group, whereas Link N administration significantly restored CFA-induced CC reduction (Fig 5e). The analysis of the composition of the tissue layer in the condyle also confirmed the role of Link N in regulating tissue structural homeostasis in arthritic pathologic conditions (Fig 5f).

Discussion

In the present study, the regulatory role of Link N for inflammatory-associated TMJ tissue damage was investigated. The results indicate that Link N is safe enough to modulate IL-1β–stimulated inflammation. This effect is probably through the suppression of pro-inflammatory cytokines and enhancement of anti-inflammatory cytokines. Moreover, Link N application may also regulate EMT-related genes and cartilage differentiation markers, suggesting that the restoration of stem cell–mediated chondrogenesis could be a good target for repairing inflammation-associated TMJ disruption. In vivo analysis using a CFA-induced arthritis model further supported this statement, showing that Link N treatment could rescue TMJ tissue integrity under arthritic conditions. Taken together, Link N could indeed be a therapeutic agent for TMJ repair.

In addition to Link N, a previous study have shown that other factors, such as TGF-β, BMP-2, or insulin growth factor (IGF)-1, can enhance articular cartilage repair.[21] Furthermore, it was also found that when the hMSCs were cultured with Link N in chondrogenesis medium, the expression of ECM molecules was facilitated, as well as the transcription factors related to chondrogenesis. In contrast, no obvious chondrogenesis or proteoglycan production was detected in hMSCs cultured in serum-free medium containing Link N, implying that Link N is a chondrogenic enhancer but not an initiator.[17]

In agreement with previous reports,[11,12] the current study found that Link N treatment could downregulate the levels of pro-inflammatory cytokines IL-6, IP-10, and RANTES. IL-6 is a pleiotropic cytokine and plays an important role in inflammatory diseases.[22] IL-6 could be induced in IL-1β–stimulated arthritic joints through nuclear factor (NF)-κB and mitogen-activated protein kinase (MAPK) pathways.[23] IP-10 and RANTES are IL-1β–targeting chemoattractants, which are secreted by different cell types such as fibroblasts, neutrophils, and macrophages and act on T cells and monocytes.[24,25] However, MMP3 and MMP13 levels did not decrease in the co-stimulation group after 24 hours of treatment when compared to the IL-1β–treated group. This unexpected result may be attributed to differences in cell type or culture duration. Another explanation for this inconsistent observation could possibly be due to the requirements of MMP3 and MMP13 in regulating tissue regeneration.[26,27] The results may also suggest that inflammation is highly dynamic, and detection of multiple markers is required to better delineate inflammatory status. On the other hand, levels of anti-inflammatory cytokines, including IL-4, IL-10, and IL-13, increased in response to Link N treatment under the IL-1β condition to various degrees at 24 and 96 hours post-treatment, indicating that Link N application could potentially increase anti-inflammatory cytokines. IL-4, IL-10, and IL-13 have anti-inflammatory and chondroprotective potential in joint arthritis. These markers can stimulate proteoglycan production and proliferation in cells and inhibit cell apoptosis.[23,28] The changing expression of anti-inflammatory cytokines at different time points after treatment of Link N could reflect short- and long-term roles of Link N in differentially regulating IL-1β–stimulated inflammation.

Another interesting finding in the present study is the potential involvement of the TGF family Link N–mediated effect. During chondrogenic differentiation, TGFβ proteins stimulate ECM protein synthesis, serving as major regulators in chondrogenesis and osteogenesis.[29–31] Moreover, TGFβ proteins are also capable of ameliorating proteoglycan production in IL-1β–induced ECM disruption.[32] Previous studies have also shown that the TGF-β signaling pathway promotes expression of fibrochondrocytic genes, including sex-determining region Y (SRY)-box 9 (Sox9) gene, aggrecan (ACAN) gene, and collagen type II (Col2) gene.[33,34] In addition, as a previous study detected inflammation at 96 hours after treatment of inflammation inducers,[35,36] the Link N–mediated response for chondrogenesis at 96 hours was examined in the current study.

EMT and the reciprocal mesenchymal to epithelial transition (MET) play important roles in normal development and stem cell behavior.[37,38] These processes are a series of cell conversions between epithelial and mesenchymal phenotypes, but are not limited to epithelial/mesenchymal cells,[39,40] as they have also been detected in stem cell, chondrocyte, and TMJ

fibroblasts.[15] It was recently shown that EMT could be induced by inflammation: Inflammatory conditions elevated, indicating that EMT is responsible for triggering the release of proinflammatory factors and eliciting immunosuppressive mechanisms.[41] The molecular cues to regulate Link N–mediated rescue for inflammation were revealed using EMT-associated PCR array analysis. The results showed that KRT19 or snail homolog (SNAI)3 were greatly expressed in the Link N–treated group. It was previously shown that KRT19 and SNAI3 were both involved in mesodermal development, which has the potential to develop into chondrocytes.[42–44] On the other hand, KRT14, NODAL, and JAG1 molecules contribute to cell proliferation[45–47] and are all downregulated upon IL-1β treatment, suggesting that inflammation modulates stem cell propagation. With the addition of Link N into inflammatory conditions, ERBB3 and NODAL were upregulated in the Link N–treated group under inflammatory conditions in the present study. Previous studies have shown that the ERBB protein family is involved in chondrocyte development and expressed in differentiated TMJ chondrocytes in mice.[48,49] On the other hand, NODAL belongs to the TGFβ family and can induce chondrogenic differentiation of adipose-derived hMSCs.[50] While TGFβ is one of the cytokines inducing EMT,[51] current results suggest that Link N modulates the TGFβ-EMT molecular axis to modulate an inflammation-like condition by activation of anti-inflammatory factors in company with suppression of pro-inflammatory cytokines. Interestingly, Link N treatment alone does not induce significant inflammation, suggesting it is safe for clinical use, although more studies are required to examine its immunogenic response in humans.

Link N has been postulated to be a growth factor that could induce a disc- or cartilage-like ECM formation.[17] In vitro data of the present study demonstrated that Link N regulates inflammation and enhances chondrogenic differentiation, which is necessary for TMJ repair. Histologic analysis showed Link N injection restored rat TMJ tissue in a CFA-induced arthritis model, in accordance with former reports in a rabbit IVD degeneration model.[11] In another study, fibrocartilage stem cells (FCSCs) residing within the SZ tissue in the TMJ were thought to be essential for tissue remodeling.[7] To the best of the authors' knowledge, this work is the first study using Link N in TMJ tissue. Future work needs to be performed to elucidate the role of Link N in stimulating FCSCs for TMJ tissue regeneration.

Conclusions

Link N may modulate inflammatory responses and have an additional effect on chondrogenic differentiation. The most significant finding of the present study was that Link N administration could restore TMJ histology in a rat arthritis model, thus making Link N a great candidate factor to assist in TMJ fibrocartilage repair.

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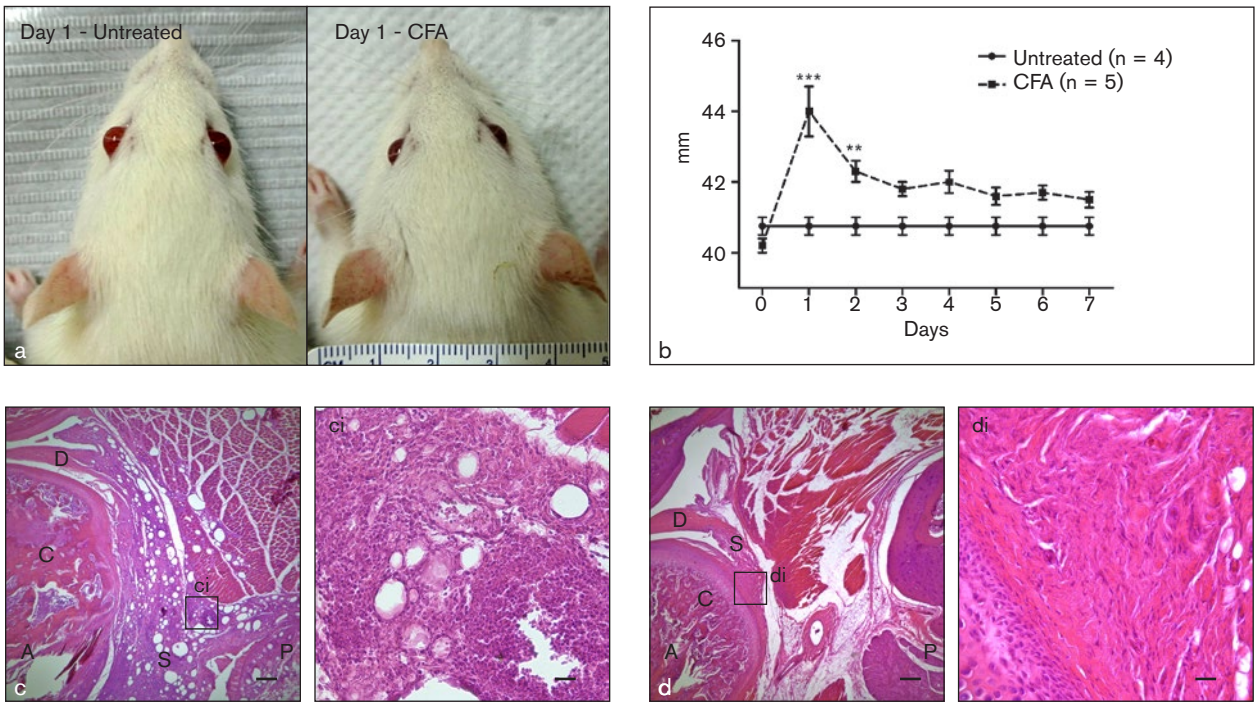
Appendix



Appendix 1 Signs of arthritis in CFA-induced arthritis model. **(a, b)** Severe head swelling was observed in the TMJ region of the CFA-induced arthritis group compared to the untreated group on day 1, and head width was recorded for 7 days in both the CFA-injected and untreated rats. Inflammatory cell infiltrate was observed in synovial tissue (blue box) of **(c)** the CFA-induced arthritis group, but not in **(d)** the untreated group. Scale bar: (a) = 1 cm; (c, d) = 250 μm; (ci, di) = 25 μm. D = disc; C = condyle; A = anterior; P = posterior; S = synovial tissue. Data are presented as mean ± standard deviation (SD). $^{**}P < .01$. $^{***}P < .001$.