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LETTER

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Revealing the presence of lymphatic vessels within intervertebral discs: Novel insights into disc degeneration

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Dear Editor,

The intervertebral disc (IVD) is a crucial fibrocartilaginous structure that connects adjacent vertebrae. It plays a fundamental role in maintaining spinal stability and absorbing mechanical stress. Each disc is composed of three parts: the inner nucleus pulposus (NP), the outer annulus fibrosus (AF), and the superior and inferior cartilage endplates (CEPs). Degeneration of IVDs is a primary contributor to various spinal disorders, such as cervical spondylosis, low back pain, and lumbar disc herniation, all of which lead to a significant reduction in the quality of life of patients and impose a substantial economic burden. In China, IVD degeneration (IVDD) is the third most common chronic disease following hypertension and diabetes, highlighting the widespread impact of IVDD on public health.[1]

Non-degenerated adult IVDs are devoid of a vascular system within their internal structure; that is, they lack both blood and lymphatic vessels.[2] Nutrient supply to these discs primarily relies on diffusion from the capillaries into the CEPs and the outer layers of the AF. This limited nutrient exchange significantly restricts the self-repair and self-regenerative abilities of IVDs, making them more vulnerable to degeneration. The absence of vascular and lymphatic systems in IVDs is considered a major factor contributing to the pathogenesis of IVDD.[3]

A hallmark of IVDD is the formation of microvessels within the degenerated disc tissue, particularly in the AF.[4] Although the ingrowth of microvessels may temporarily provide a compensatory nutrient supply to the disc, it may exacerbate inflammatory cell infiltration, intensifying the inflammatory response and accelerating disc degeneration.[5] The reason for the failure of clearance of these cells remains unknown in the context of IVDD.

Although the presence of blood vessels in degenerated discs has been extensively reported, studies investigating the presence and role of lymphatic vessels in IVDs are relatively limited. As integral components of the vascular system, lymphatic vessels are essential for maintaining tissue fluid homeostasis, removing metabolic waste, and regulating immune responses. In other tissues, lymphangiogenesis has been shown to support tissue repair by facilitating the clearance of excess interstitial fluid and immune cells. Therefore, lymphatic vessels may play a similar role in maintaining disc health and mitigating degeneration.[6]

In this study, we demonstrated the presence of lymphatic vessels in the AF of healthy discs, contradicting the conventional belief that non-degenerated adult IVDs are devoid of a vascular system. We used spatial transcriptomic analysis and immunohistochemistry (IHC) and immunofluorescence staining to provide evidence of lymphatic vessels within the AF. Furthermore, we found that an increase in the number of blood vessels in degenerated discs significantly decreased the number of lymphatic vessels. These findings suggest that lymphatic vessels play a crucial role in regulating the nutritional metabolism and immune microenvironment of IVDs, offering novel insights into the mechanisms underlying IVDD progression. Altogether, the results of this study suggest that regulating the lymphatic system is a promising therapeutic strategy for IVDD.

CONSTRUCTION OF A SPATIAL TRANSCRIPTOMIC MAP OF HUMAN IVD

Magnetic resonance imaging (MRI) revealed a high signal intensity on T2-weighted images of the cervical disc in patients with Hirayama disease, indicating no disc degeneration (Figure 1A). Subsequently, the spatial transcriptomic profiles of AF and the expression of vascular genes within the AF were analyzed using the advanced 10× Genomics Visium platform. For this analysis, intraoperative AF specimens were collected from patients with Hirayama disease.[7] Hematoxylin and eosin (H&E) staining of AF specimens from a 19-year-old patient with Hirayama disease (Figure 1B) and spatial feature mapping (Figure 1C) showed that the AF region comprised a total of 4,991 spots with a mean depth of 481,583 reads, corresponding to a median of 6 genes per spot. Furthermore, t-distributed stochastic neighbor embedding (tSNE) analysis showed that cells in the AF were categorized into 12 subgroups (Figure 1D). Consistent with the findings of previous studies,[8] we found high expression levels of the AF markers Col1a1 (Figure 1E), Comp (Figure 1F), and Sparc (Figure 1G) in AF specimens.

PRESENCE OF LYMPHATIC VESSELS IN THE IVD

IVD tissues collected from patients with Hirayama disease were included in the control group, whereas those collected from patients with cervical spondylosis were included in the IVDD group. The lymphatic marker genes prospero homeobox protein 1 (Prox1) (Figure 1H), desmoplakin (DSP) (Figure 1I), and junction plakoglobin (JUP) (Figure 1J) were detected in healthy AF specimens (control group).[9] H&E staining showed that the IVDs in the control group had a compact structure (Figure 1K), whereas those in the IVDD group had a loose structure and tended to disperse easily when sectioned (Figure 1L). Furthermore, we detected the expression of lymphatic vessel-related proteins using IHC staining. The results confirmed the expression of lymphatic vessel endothelial hyaluronan receptor 1 (LYVE1) and Prox1 in AF tissues (Figure 1M). Prox1 is a specific transcription factor involved in the development of lymphatic endothelial cells (LECs). It induces the transformation of blood endothelial cells into a lymphatic phenotype, initiating the early development of LECs. IHC staining of degenerated IVD tissues (IVDD group) showed significantly reduced expression of Prox1 and LYVE1 (Figure 1N). IHC staining of caudal IVDs of young (2 months old) and aged (24 months old) rats showed consistent results (Figures 1O and 1P). In particular, the expression levels of Prox1 and DSP were higher in young rats than in aged rats.

PRESENCE OF BLOOD VESSELS IN THE IVD

The vascular system communicates with the lymphatic system, forming an intricate network structure. Therefore, we analyzed the expression of vascular genes in AF specimens in the control and IVDD groups. The results showed that C-X-C motif chemokine ligand 1 (CXCL1) (Figure 1Q), CXC chemokine receptor 4 (CXCR4) (Figure 1R), and vascular endothelial growth factor A (VEGFA) (Figure 1S) were highly expressed in the AF. IHC staining showed that the vascular markers CD31 and endomucin (EMCN) were expressed in the AF (Figure 1T). As shown in Figure 1U, the expression levels of CD31 and EMCN were significantly increased in degenerated IVDs. IHC staining of caudal IVDs of young (Figure 1V) and aged (Figure 1W) rats showed consistent results. In particular, the expression levels of CXCL1 and VEGFA were higher in aged rats than in young rats.

DECREASED NUMBER OF LYMPHATIC VESSELS IN DEGENERATED HUMAN IVDs

Immunofluorescence staining was used to examine the structure of lymphatic vessels in IVD tissues in the control and IVDD groups. As shown in Figure 1X, lymphatic vessel markers were fluorescently labeled in the IVD tissues of the

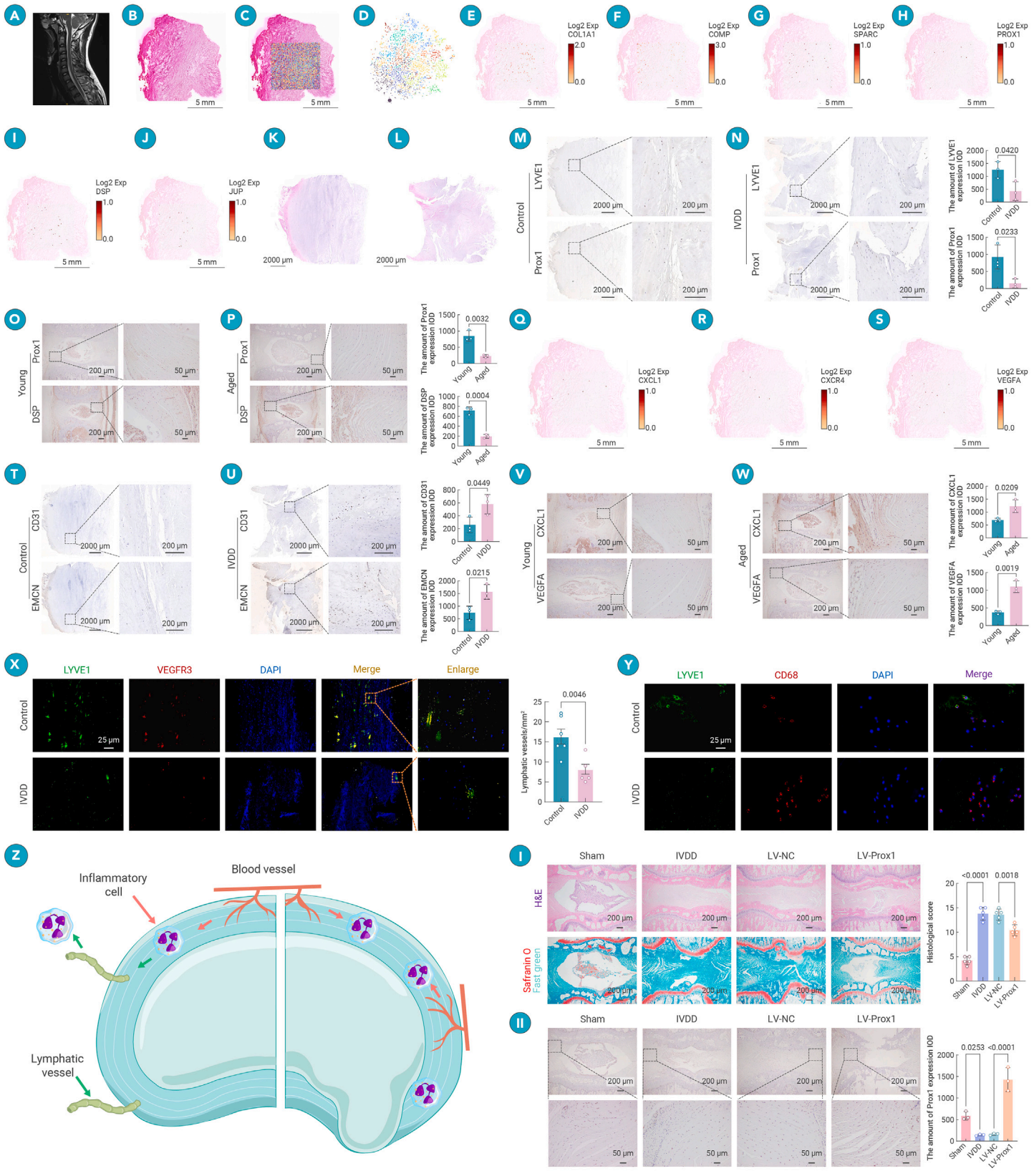

Figure 1. Lymphatic vessels alleviate the inflammation of intervertebral discs (A) Cervical MRI of patient with Hirayama disease. (B) The H&E staining image of the AF. (C) A spatial feature plot of AF. (D) The tSNE plots of all cells from spatial transcriptomics of AF. (E–G) The spatial feature plots of Col1a1 (E), Comp (F), and Sparc (G) genes. (H–J) The spatial feature plots of Prox1 (H), DSP (I), and JUP (J) genes. (K) H&E staining of IVD in patients with Hirayama disease. (L) H&E staining of IVD in patients with cervical spondylosis. (M) Representative IHC images of LYVE1 and Prox1 in human healthy AF section. (N) Representative IHC images of LYVE1 and Prox1 in human degenerated AF section (left). Quantitative analysis of LYVE1 and Prox1 IHC results for the two groups of human IVDs (right). (O) Representative IHC images of Prox1 and DSP in the AF of 2-month-old rats (left). Quantitative analysis of Prox1 and DSP IHC results for the two groups of rat IVDs. (Q–S) The spatial feature plots of CXCL1 (Q), CXCR4 (R), and VEGFA (S) genes. (T) Representative IHC images of CD31 and EMCN in human healthy AF section. (U) Representative IHC images of CD31 and EMCN in human degenerated AF section (left). Quantitative analysis of CD31 and EMCN IHC results for the two groups of IVDs (right). (V) Representative IHC images of CXCL1 and VEGFA in

(legend continued on next page)

two groups, presenting tubular structures in the tissues. However, in the IVDD group, the expression levels of Lyve1 and VEGFR3 were decreased, suggesting a reduction in the number of lymphatic vessels. The results of immunofluorescence analysis validated the presence of lymphatic vessels in both healthy and degenerated IVDs.

LYMPHATIC VESSELS RECYCLED INFLAMMATORY CELLS IN THE IVD

IVDD is characterized by the ingrowth of blood vessels in the IVD. Inflammatory cells can migrate from blood vessels to the IVD, causing an inflammatory response.[10] We speculate that one of the functions of lymphatic vessels in the IVD is to recycle inflammatory cells and attenuate the inflammatory response of the IVD. We used green fluorescence to label the lymphatic vessel marker LYVE1 and red fluorescence to label the macrophage marker CD68 to assess the distribution of inflammatory cells and lymphatic vessels in the IVD. Healthy IVD tissues had several lymphatic vessels and fewer inflammatory cells, which were distributed near the lymphatic vessels (Figure 1Y), whereas degenerated IVD tissues had fewer lymphatic vessels and a higher number of inflammatory cells. These cells might not have been effectively recycled, leading to inflammation in the IVD tissues.

The abovementioned results suggest that healthy IVDs have more lymphatic vessels and fewer blood vessels. Inflammatory cells in these IVDs are effectively recycled; therefore, the level of inflammation is low. However, degenerated IVDs have more blood vessels, fewer lymphatic vessels, and extensive inflammatory cell infiltration, which leads to increased inflammation (Figure 1Z).

OVEREXPRESSION OF PROX1 DELAYED THE PROGRESSION OF IVDD *IN VIVO*

The abovementioned results indicate that lymphatic vessels play an active role in the prevention and treatment of IVDD. To validate the function of lymphatic vessels *in vivo*, we established a rat model of caudal IVDD through needle puncture[3] and subsequently administered these rats with lentivirus (LV)-Prox1 with a microsyringe to overexpress the Prox1 gene, which is abundant in healthy IVDs. H&E and safranin O/fast green staining showed that the arrangement of the AF was disordered, and the area of the NP decreased or even vanished in the degenerated IVDs (Figure 1I). Treatment with LV-Prox1 preserved part of the NP and restored the arrangement of the AF to some extent, decreasing the histological score of the IVD. These results suggest that promoting lymphangiogenesis in the IVD may delay the progression of IVDD. IHC staining showed that Prox1 expression decreased in the degenerated AF in rats with caudal IVDD. However, treatment with LV-Prox1 reversed this decrease (Figure 1II).

This study has some limitations that should be acknowledged. First, we performed spatial transcriptomic sequencing only on healthy human IVDs; however, the spatial transcriptomic profiles of degenerated IVDs remain to be investigated. Second, we only preliminarily discussed that lymphatic vessels in IVDs can attenuate inflammatory responses. Future studies should focus on other functions of lymphatic vessels, such as nutrient supply. Third and last, *in vivo* validation in this study remains limited, and the role of lymphatic vessels *in vivo* warrants further investigation.

In conclusion, the spatial transcriptomic profiles of human IVDs analyzed in this study provide a foundational reference for investigating the vascular and lymphatic systems within the human IVD. This study presents novel evidence of the presence of lymphatic vessels within the AF and highlights their potential role in regulating disc health and degeneration. The findings of this study indicate that the vascular system plays a crucial role in IVDD, suggesting that both blood and lymphatic vessels influence disc health and are promising therapeutic targets for IVDD.

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DECLARATION OF INTERESTS

The authors declare no conflicts of interest.

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the AF of 2-month-old rats. (W) Representative IHC images of CXCL1 and VEGFA in the AF of 24-month-old rats (left). Quantitative analysis of CXCL1 and VEGFA IHC results for the two groups of rat IVDs (right). (X) The lymphatic vessels in the IVDs were detected by immunofluorescence. LYVE1 was labeled with green fluorescence, VEGFR3 was labeled with red fluorescence, and the nuclei were stained blue (left). The number of lymphatic vessels in normal and degenerative IVDs was statistically analyzed (right). (Y) Detect the distribution of lymphatic vessels and macrophages in the IVDs using immunofluorescence. LYVE1 was labeled with green fluorescence, CD68 was labeled with red fluorescence, and the nuclei were stained blue. (Z) In normal IVDs, lymphatic vessels recycle inflammatory cells and alleviate the inflammatory response of the IVDs. In degenerative IVDs, there is an increase in the number of blood vessels and inflammatory cells infiltrating. (I) H&E staining of the rat caudal IVDs (top). Safranin O/fast green staining of the rat caudal IVDs (bottom). Histological scoring of the rat caudal IVDs (right). (II) Representative IHC images of Prox1 in the AF of rats (left). Quantitative analysis of Prox1 IHC results (right). (I and II) Sham group: IVD with no treatment. IVDD group: IVD was punctured with a needle. Lentivirus-NC group: acupuncture+5 μL LV-NC. Lentivirus-Prox1 group: acupuncture+5 μL LV-Prox1.