The Synovial Lymphatic System Plays a Critical Role in the Pathogenesis of Osteoarthritis

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Introduction: Osteoarthritis (OA) is characterized by articular cartilage loss, synovial inflammation and subchondral bone remodeling, associated with accumulation of numerous catabolic mediators in the synovial space that can break down cartilage components. How these components are cleared and if the “clearance” process contributes to OA pathogenesis is not known. We hypothesized that the synovial lymphatics remove (drain) catabolic mediators, limiting OA progression. Using immunohistochemical staining (IHC), whole slide digital microscopy (WSD), and near infrared indocyanine green (NIR-ICG) lymphatic imaging, we recently reported alteration in synovial lymphatic vessels and decreased lymph drainage in mouse OA knees and in specimens from OA patients. However, the cause-effect relationship between lymphatics and OA and the mechanisms responsible for impaired lymph drainage in OA joints have not been determined. Here we used a mouse model of OA and lymphatic endothelial cells (LECs) to investigate: 1) the effects of blocking lymphangiogenesis on OA progression and joint lymph drainage; 2) changes of signaling pathways in LECs from OA joints; 3) effects of pro-inflammatory cytokines on LEC function; and 4) involvement of the negative regulator of inflammation, ITCH, on lymph drainage in OA joints and LEC function.

Methods: WT mice received meniscal-ligamentous (MLI) surgery to induce knee OA, or sham surgery as control. Sham mice were treated with anti-VEGFR3 neutralizing antibody (anti-R3), a blocker of lymphangiogenesis, or the isotype IgG (N=8/group) and lymph drainage and lymphatic pumping pressure were assessed using NIR-ICG and a lymphatic pressure-measuring system, both of which we developed recently. OA mice received anti-R3 or IgG (N=10/group). Tissue damage, accumulation of the aggrecan degradation product, NITEGE, and lymph drainage of knee joints were examined by histology, IHC/WSD and NIR-ICG. Primary LECs from OA and sham joints were isolated from synovium using magnetic beads conjugated with anti-podoplanin antibody for RNAseq-pathway analysis. The expression of catabolic mediators, including cytokines, chemokines, MMPs and iNOS, in OA LECs was examined by qPCR. The effect of IL-1 and TNF on LEC function was determined by assessing the levels of catabolic mediators by qPCR. MLI surgery was performed on ITCH−/− mice and WT littermates. Lymph drainage, tissue damage and LEC function of knee joints were examined.

Results: NIR-ICG revealed that anti-R3 significantly decreased lymph drainage in OA knee joints, which was associated with the accumulation of the aggrecan degradation product, NITEGE in synovium by IHC/WSD and more severe tissue damage by histology. Goodness of fit indicated a strong correlation among lymph drainage (% ICG clearance), NITEGE expression (positively stained area), and tissue damage (OARSI score) in OA joints (Fig.1). Anti-R3 also reduced lymphatic pumping pressure (data not shown). About 1000 genes were differentially expressed in OA LECs vs. sham LECs, resulting in 12 dysregulated pathways, 9 of which involve elevated NF-kB signaling. Increased expression of catabolic
mediators in OA LECs (Fig. 2A) was confirmed in IL-1 or TNF-treated murine LECs (Fig. 2B). IL-1-treated ITCH-/LECs expressed elevated catabolic mediators (Fig. 3A). ITCH-/ OA mice had less lymph drainage than WT mice (Fig. 3B).

**Discussion:** We used a MLI-induced mouse model of OA and identified a novel role of synovial lymphatic vasculature in the pathogenesis of OA. First, blockage of lymphangiogenesis with a neutralizing VEGFR3 antibody significantly increased OARSI scores and NITEGE expression, and simultaneously decreased lymph drainage. Thus, normal lymphatic function is required for limiting the progression of OA disease.

Second, LECs isolated from OA joints expressed high levels of catabolic factors, including pro-inflammatory cytokines, chemokines, MMPs and iNOS, suggesting that LECs have an inflammatory phenotype. Last, depletion of the negative regulator of inflammation, ITCH, potentiated IL-1-mediated LEC inflammation and accelerated OA-associated lymphatic dysfunction, further supporting a critical role of inflammatory signaling in the pathogenesis of OA. Based on these findings, we propose a new model for the role of synovial lymphatic vasculature in OA pathogenesis; in the early stages of OA, tissue damage causes lymphangiogenesis and LEC inflammation, during which LECs: 1) release cytokines/chemokines/MMPs to the surrounding soft tissues and target synovial cells, and 2) produce large amounts of NO via iNOS, resulting in reduced lymphatic transport through inhibition of adjacent lymphatic smooth muscle cells.

**Significance:** The synovial lymphatic system plays a critical role in the pathogenesis of OA. Agents that can increase production of anti-inflammatory molecules and thus improve lymphatic function could become new therapies for OA.
Fig. 1. Blockage of lymphangiogenesis increases the severity of tissue damage in OA joints. (A, D) NIR-ICG to assess lymph drainage as ICG clearance. (B, E) Hematoxylin/Orange G staining and tissue injury assessed by OARSI score. (C, F) IHC of NITEGE expression by NITEGE+ stained area. Values are mean ± SD of 10 joints. Significance was determined by ANOVA followed by Bonferroni's test (*p<0.05). (G) Correlation among ICG clearance, OARSI score and NITEGE+ staining area (N=25); p<0.001.
Fig. 2. *Expression of catabolic factors id elevated in LECs from OA joints.* Synovial LECs were isolated by beads conjugated with anti-FcγR chain Ab from mice at 12-wks post MLI- or sham surgery (A). A murine LEC line was treated with different doses of IL-1 or TNF for 24 hrs (B). The expression levels of catabolic factors were examined by qPCR. Values are mean±SD of 3 joints (A) or triplicates (B).

Fig. 3. *ITCH limits expression of IL-1-stimulated catabolic factors in LECs and impaired lymph drainage in OA joints.* (A) LECs isolated from WT and ITCH-/- mice were treated with IL-1 for 24 hrs and the expression levels of catabolic factors were determined by qPCR. Values are mean±SD of triplicates. Fold changes were calculated using WT Ctrl as 1. (B) NIR-ICG images were analyzed in WT and ITCH-/- mice 6-wks post-MLI surgery and ICG clearance was measured. N=3 joints. *p<0.05 vs. WT mice.