Elevated ILC3s-related Inflammatory Factors May Promote Tendinopathy

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Research

Keywords: Tendinopathy, ILC3s, IL-17A, IL-22

DOI: https://doi.org/10.21203/rs.3.rs-95724/v1

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Abstract

**Background:** The prevalence of tendinopathy has risen dramatically over the last few decades and has become a common and serious orthopedic problem in sports injury and elderly populations. Immune cells have been shown to be associated with tendinopathy, with the percentage of Group 3 Innate Lymphoid Cells (ILC3s) having been shown to be upregulated in tendon tissue compared with peripheral blood.

**Materials and Methods:** We used flow cytometry to investigate the percentage of circulating ILC3s in patients with tendinopathy and controls patients; Realtime-PCR was performed to detect the mRNA levels of ILC3s surface markers, CD45, IL-23R, ICOS and ILC3s-related inflammatory factors and transcription factors IL-17A, IL-22 and RORC in tendon samples.

**Results:** Our results showed that the proportion of ILC3s in the peripheral blood circulation of patients with tendinopathy has no significant difference from that of controls by flow cytometry for Lin⁻IL-2R⁺IL-23R⁺ cells; however, the surface marker of ILC3s was upregulated in tendon tissue. In addition, IL-23, a characteristic activating factor of ILC3s, was also upregulated. Relative mRNA expression levels of IL-17A and IL-22 were also upregulated in tendinopathy tendon tissue compared to control patients.

**Conclusions:** These results suggest that ILC3s may be a potential immune factor for tendinopathy.

**Introduction**

Shoulder pain is a common and serious orthopedic problem in sports injuries and the elderly population and is caused by overuse of tendon injuries, i.e., tendinopathy[1]. Although it has been shown that proinflammatory mediators, such as immune cells[2], play an important role in tendon diseases, their intrinsic pathogenesis remains unclear. In particular, the interaction between immune cells and the initiation and development of tendon injury has not yet been fully elucidated.

The development and progression of tendon sheath lesions is associated with an imbalance of inflammatory factors, immune cells, and chemical mediators. Previous studies have shown that vigorous immune cells infiltrate the site of tendon injury, and these immune cells subsequently release cytokines, such as IL-1β, IL-6 and TNF-α, which are involved in the processes of tendon injury and repair[3,4]. IL-17A, a member of the IL-17 cytokine family, is a proinflammatory mediator involved in the development of a variety of immune-related diseases[5]. It has been reported that IL-17A expression is increased in "early tendinopathy" compared to control samples. Additionally, after IL-17A treatment, tenocytes secreted more proinflammatory cytokines and produced more type III collagen[6].

The previous concept suggested that IL-17A is mainly secreted by Th17 cells; however, newly discovered Group 3 Innate Lymphoid Cells (ILC3s), which are a type of non-B, non-T innate lymphocytes, can also secrete large amounts of IL-17A, and their immune response process is earlier than the adaptive immune response[7]. Innate lymphocytes play important roles in tissue homeostasis, repair and remodeling[8]. Unlike adaptive immune cells, ILCs are nonspecific antigens, lack recombinant antigen-specific receptors,
and lack dendritic cell phenotypic markers and myeloid cell markers[8]. ILCs can be divided into three groups and are based on the designations of helper T cells: (1) Group 1 ILCs (ILC1s) that produce IFN-γ predominately[9]; (2) Group 2 ILCs (ILC2s) that produce type 2 cytokines, especially IL-5 and IL-13 predominately[10]; (3) Group 3 ILCs (ILC3s) that produce IL-17 and/or IL-22 predominately[11]; and regulatory ILCs (ILCreg) that produce IL-10 and TGF-β[12]. As a counterpart of Th17 cells, ILC3s have also been confirmed to be involved in the development and progression of various diseases, such as atherosclerosis and inflammatory bowel disease [13,14]. In addition, there has been evidence that ILC3s are closely associated with fibrosis[15]. Interestingly, in donors without systemic inflammatory diseases, the proportion of ILC3s in tendon soft tissue was higher than its proportion in peripheral blood, and this portion of ILC3s secreted large amounts of IL-17 and IL-22 under the stimulation of IL-23[11], creating conditions for the involvement of ILC3s in the process of tendinopathy. However, no article has reported that the process of tendinopathy is associated with ILC3s. In this study, we aimed to test our hypothesis that ILC3s in peripheral blood and related factors of ILC3s in tendon tissue are associated with tendinopathy.

**Materials And Methods**

**Patients**

This study was conducted at the Third Affiliated Hospital of Soochow University (Changzhou, China). It was reviewed and approved by the Ethics Committee of the Third Affiliated Hospital of Soochow University (Changzhou, China) and was conducted according to standard surgical procedures with informed consent. Fifteen tendon samples were collected from patients with shoulder cuff tears undergoing shoulder surgery. The mean age was 52 years (range, 35-67). Only patients with no clinical evidence of subscapularis tendinopathy on preoperative MRI scans or macroscopic subscapularis tendon injury on arthroscopy were included — they represent a true preclinical cohort according to these criteria. All patients in this cohort met the following criteria: 1) a history of shoulder pain and dysfunction, 2) no history of surgery in the affected shoulder; 3) no imaging signs of fracture in the shoulder, and 4) no history of rheumatoid or osteoarthritis. Another independent control package consisted of 10 subscapularis tendon samples from patients undergoing shoulder arthroscopy for stabilization of the shoulder, with arthroscopic confirmation of no tendon tear, no history of shoulder surgery, no previous history of rheumatoid or osteoarthritis, no radiographic evidence of shoulder fracture, and a control mean age of 32 years (25-42).

**Cell preparation and flow cytometry quantification**

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient centrifugation (GE Healthcare, Tokyo, Japan) and immediately quantified by flow cytometry, and for flow cytometry quantification, the cells were stained with fluorescein-conjugated monoclonal antibodies against the following in strict accordance with the instructions. The following antibodies were used in this study: Anti-Lineage-FITC CD2, CD3, CD14, CD16, CD19, CD56, and CD235a (eBioscience (San Diego,
CA, USA), Anti-IL-23R-PE (BioLegend), and Anti-IL-2R-APC (BD Biosciences). After incubation, the samples were washed twice with phosphate-buffered saline (PBS) and resuspended with 300 microliters of PBS. For the control group, the corresponding isotype-matched antibody was used for each staining. Labeled cells were quantified by flow cytometry (BD Biosciences). Flow cytometry data were analyzed with Flowjo10 software.

**Tissue collection and preparation**

Arthroscopic tendon repair was performed using a standard three-door technique as previously described. The subscapularis tendon was collected arthroscopically from the superior border of the tendon 1 cm lateral to the glenoid cavity, and the suprascapular tendon was removed 1.5 cm from the tear edge before surgical repair. The tissues were divided into two aliquots, and one of the tissue specimens was placed in 4% formalin and fixed for 4 to 6 hours, followed by paraffin embedding for Hematoxylin & Eosin (H&E) staining. One milliliter of TRIzol (Invitrogen, USA) was then added to another specimen, which was cryopreserved at −80 °C for extraction of total RNA.

**Histological examination**

The tendons were fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. The tissue was cut into 4-μm-thick sections and stained with hematoxylin and eosin (H&E) for evaluation of inflammation. Inflammation in H&E-stained tendon sections was evaluated by five independent, blinded readers according to a semiquantitative scoring system. The results were calculated as the percentage of positive cells in each group. Data are presented as medians (range). All experiments were performed three times.

**RNA extraction and fluorescence quantitative PCR**

Total RNA from tendon tissue was extracted with TRIzol in strict accordance with the instructions, and the concentration and purity of RNA were measured by a NanoDrop 2000c and diluted to 500 ng/microliter per specimen with RNase-free water. Subsequently, equal amounts of RNA were analyzed by quantitative fluorescence PCR using the SYBR Green Premix EX Taq kit. Each RNA sample was tested for relative expression of CD45, RORC, IL-23, IL-22, and IL-17A mRNA and normalized to β-actin as a housekeeping gene, with comparative threshold cycles calculated with the Ct value method. All sequences of primers are shown in Table 1. Each sample was analyzed in duplicate with the CFXA96 Cycler (Thermal).

**Statistical analysis**

All data were statistically analyzed using Prism 5 (Graph Pad Software, La Jolla, CA, USA), and the data are expressed as the mean ± standard deviation. Student's unpaired t-test was used for difference analysis, Spearman's analysis was used for correlation analysis between two variables, and the difference was statistically significant when the p value was less than 0.05.
Results

Lesion tendons show infiltration of inflammatory cells compared to control tendons

To verify the infiltration of inflammatory cells in the diseased tendons, H&E staining was performed on the tendons of healthy controls and patients with tendinopathy in this study, and the results are shown in Fig. 1. The tendons of patients with tendinopathy showed more significant infiltration of inflammatory cells.

There was no significant difference in the proportion of ILC3s in the peripheral blood circulation of patients with tendinopathy compared with controls

To verify whether ILC3s differ in the peripheral blood of patients with tendinopathy, we examined the relative and absolute numbers of ILC3s in the periphery circulation of 15 patients with tendinopathy and 10 healthy controls. The gating strategy of ILC3s in PBMCs is shown in Fig. 2A. In this study, the proportion of ILC3s was expressed by the percentage of Lin-IL2R+IL-23R+. Our findings show that ILC3s are associated with Lin- (B) relative to healthy controls, there were no significant differences in the proportions of lymphocytes (C) and total PBMCs (D).

Expression levels of ILC3s-Associated surface Markers in tendon samples

We then examined the expression of CD45 antigen on all leukocytes, and the results are shown in Fig. 3A, where the mRNA expression level of CD45 was upregulated in the diseased tendons relative to healthy controls. Additionally, the expression of IL-23R, the surface marker receptor of ILC3s, was also consistent with CD45, showing a trend of upregulation (Fig. 3B). We also examined the expression of ICOS, a marker associated with lymphocyte activation, and showed that the mRNA expression level of ICOS in diseased tendons was also upregulated relative to healthy controls. Since IL-23 is considered to be an activator of ILC3s, we also examined the expression levels of IL-23, consistent with the expression of its receptor IL-23R, and showed that the mRNA expression levels of IL-23 were upregulated in diseased tendons.

Expression levels of ILC3s-related inflammatory factors and transcription factors in tendon samples

Since IL-17A and IL-22 are characteristic inflammatory factors secreted by ILC3s, RORC is a characteristic transcription factor of ILC3s. We then examined the mRNA expression levels of IL-17A, IL-22, and RORC (Fig. 4), and the results showed that the expression levels of IL-17A (A), IL-22 (B), and RORC (C) in the diseased tendons showed a tendency to be upregulated compared with healthy controls.

Discussion

In this study, we observed that although the proportion of ILC3s in the peripheral blood circulation of tendinopathy patients was not significantly different from that of healthy controls, the expression levels of surface molecules characteristic of ILC3s (CD45, ICOS, IL-23R), related inflammatory factors (IL-17A
and IL-22), and their characteristic transcription factors (RORC) tended to be upregulated in diseased tendon tissues.

Soft tissue lesions of the shoulder such as tendinopathy cause pain, loss of function, joint failure, and the development of secondary osteoarthritis, resulting in a huge social and economic burden[16]. Identification of key immune cell populations that act as master regulators in this inflammatory process will advance our understanding of its pathogenic mechanisms. Accumulating evidence supports the contribution of inflammation in the development of tendinopathy[17]. Recent studies have highlighted the importance of the innate immune response during the persistence of inflammation[18]. As a newly discovered class of innate immune cells, the role of ILC3s in the development of tendinopathy remains to be examined.

Researchers have found that ILC3s are involved in promoting the development of rheumatoid arthritis[19]. IL-17A secreted by Th17 cells is also a characteristic cytokine of ILC3s, and when IL-17A stimulates tenocytes, tenocytes produce large amounts of TNF-α, MIP-1α, IL-6, IL-8 and MCP-1, and tenocyte apoptosis is increased[6]. Meanwhile, ILC3s have been found to be present in tendon soft tissues.

We collected peripheral blood and tendon tissues from patients who met the diagnostic criteria for tendinopathy and control patients and then examined the infiltration of inflammatory cells in tendon tissues from these tendinopathy patients and control patients (Fig. 1) and the proportion of ILC3s in the peripheral blood circulation (Fig. 2A). Interestingly, although the proportion of ILC3s in the peripheral blood circulation of tendinopathy patients was not significantly different from that of control patients (Fig. 2B-D), HampE sections showed that more inflammatory cells were infiltrated in the tendon tissue of tendinopathy patients (Fig. 1). Considering that tendinopathy is a local inflammatory response, and previous inflammation suggested that the proportion of ILC3s in tendon soft tissue was higher than its proportion in the peripheral blood, we then examined the relevant surface markers of ILC3s in tendon tissue. ILC is defined by characteristic antigens expressed on its surface, characteristic cytokines secreted by it, and specific transcription factors. For example, human ILC1s express CD56 to secrete IFN-γ, and their transcription factor is T-beta [20]; ILC2s express CRTH2 to secrete IL-13, and their transcription factor is RORA [21]; and ILC3s express IL-23R, secrete IL-17A, and their transcription factor is RORC [22]. As shown in Figure 3A and B, the expression of CD45, a common leukocyte differentiation marker, was upregulated; additionally, the expression of IL-23R, a surface marker of ILC3s, was also upregulated relative to tendon tissues from control patients. Furthermore, as an activation marker of leukocytes, the expression of ICOS in tendon tissues from tendinopathy patients also tended to be consistent with that of IL-23R (Fig. 3C). The expression of IL-23, a cytokine that activates ILC3s, was also upregulated in tendon tissues from patients with tendinopathy (Fig. 3D).

The flow staining protocol for ILC3s consists of a variety of protocols. In this study, our staining protocol defines ILC3s as Lin⁻IL-2R⁺IL-23R⁺, which is commonly used in the flow cytometry detection of ILC3s[22]. These Lin⁻IL-2R⁺IL-23R⁺ cells may include two populations of cells, namely, NKp44⁺ ILC3s and NKp44⁻ ILC3s; however, according to previous studies, NKp44⁺ mainly secretes IL-17A, and NKp44⁻ ILC3s mainly
secretes IL-22. Therefore, we then examined the expression of these two cytokines. As shown in Fig. 4 A and B, the expression levels of IL-17A and IL-22 were upregulated in the tendon tissue of patients with tendinopathy, suggesting that both NKp44$^+$ ILC3s and NKp44$^-$ ILC3s were upregulated in the tendon tissue of patients with tendinopathy. As a characteristic transcription factor of ILC3s, RORC also maintained a consistent trend of upregulation with IL-17A and IL-22 (Fig. 4C).

**Conclusion**

Based on these results, we propose that ILC3s are potential immune factors for tendon sheath lesions. A better insight into the mechanisms by which ILC3s are involved in lesion development may advance the development of cell-targeted therapeutic modalities for early tendon disorders in humans.

**Abbreviations**

ILC3s: Group 3 Innate Lymphoid Cells; IL-23: Interleukin-23; IL-17A: Interleukin-17A; IL-22: Interleukin-22; RORC: RAR Related Orphan Receptor C; ICOS: Inducible T Cell Costimulator.

**Declarations**

- **Acknowledgements** Not applicable
- **Funding** This work was supported by grants from the National Natural Science Foundation of China (grant 81801568), the National Science Foundation for Post-doctoral Scientists of China (grant 2018M642314), the Postdoctoral Science Foundation of Jiangsu Province (grant 2018K235C), the Changzhou Science and Technology Project (Applied Based Research, No. CJ20180035), and the Young Talent Development Plan of Changzhou Health Commission (CZQM2020009).
- **Availability of data and materials** The datasets generated/analyzed during the current study are available
- **Authors’ contributions** Peng Xu performed all the experiments and prepared the initial draft of the manuscript. Yumin Wu performed the data analysis and supervised all the studies. All authors reviewed, edited, and approved the final content of the manuscript.
- **Competing interests** The authors declare that they have no competing interests.
- **Consent for publication** All patients consent to publish patient-identifiable information and the obtained data.
- **Ethics approval and consent participate** This study was conducted in accordance with the Declaration of Helsinki. This study was conducted with approval from the Ethics Committee of the Third Affiliated Hospital of Soochow University. Written informed consent to participate was obtained from all the participants.
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Tables

Table 1. The primer sequences for RT-PCR
| Gene  | Sequence (5’-3’) | Accession |
|-------|-----------------|-----------|
| D45   | Fwd: GTGAGGCGTCTGTACTGATG | NM_002838 |
|       | Rev: ACGGCTGACTTCCAGATATG |           |
| ICOS  | Fwd: GGCATGAGAATGGTCCAAGT | NM_012092 |
|       | Rev: CATGAAGTCAGGCTCTGTT |           |
| IL-23R| Fwd: TGATGGATGCTAFGAGTATT | AF461422.1|
|       | Rev: -AGTCT TCTGGGTGGCA GTGAT |           |
| IL-23 | Fwd: ACAGAGAGAATCAGGCTCA | NM_016584.2|
|       | Rev: GGTACACAGGGGTGATCA |           |
| IL-22 | Fwd: CAGGCTCAGCAACAGGCTAA | NM_020525.4|
|       | Rev: TGATCTCTCCACTCTCTCAAGC |           |
| IL-17A| Fwd: CCTGGAGGCCATAGTGAAAGG | NM_002190.2|
|       | Rev: TTCCGGTTATGGATGTTCAGG |           |
| RORC  | Fwd: CCGAGATGCTGTCAAGTTCG | NM_001001523.1|
|       | Rev: GTTCCTGTTGCTGTTCGG |           |
| β-Actin| Fwd: TGGCACCCAGCACAATGAA | XM_005249820.1|
|       | Rev: TAAGTCATAGTCGGCTAGAAGC |           |