The Role of the Immune System in Tendon Healing: A Systematic Review

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Introduction

Tendons are highly specialized structures composed mainly of specialized fibroblasts surrounded by an abundant extracellular matrix (ECM) [1,2]. The specialised fibroblasts include tenoblasts and tenocytes, and these account for 90-95% of the cellular elements in tendons [3]. The ECM is a complex collagen based structure based on proteoglycans including glycosaminoglycans, and several other small molecules. [1,2] The normal mechanical and structural features of tendons depend on a complex and dynamic remodelling process [1,4]. The dysregulation of these features results in tendon inflammation, injury or tendinopathy [5–7], resulting in considerable pain which negatively impacts the life of the patients restricting pain free activities. [5–7].

Healing from acute tendon injury occurs in three progressive partially overlapping phases: an acute inflammatory phase, a proliferative phase and a remodelling phase. While the role of inflammation is still being studied [4,8], emerging evidence supports a major role of the immune system, both in the etiopathogenesis and treatment of the tendinopathy [9,10]. The first inflammatory phase lasts three to seven days from the injury, and is characterised by the presence of monocytes and macrophages at the site of injury [4,8]. Elastic deformation and mechanical stimuli are an integral part of this process, and type III collagen is increasingly produced within the tendon and its extracellular matrix [11]. This is followed by the proliferation phase with the release of vascular endothelial growth factor to allow neovascularization and stimulate the formation of granulation tissue [3,4]. In the final remodelling phase, the tissue proceeds to reorganize its structure quantitatively and qualitatively [3]. This process can take up to two years to complete healing.

The role of the immune system in the dysregulation of healing probably results from a chronic low grade inflammation [4] related to polymorphonucleocyte, mast cells, macrophages and lymphocytes; the presence of these 'immune cells' has recently been highlighted in tendons[12–14]. Controversially, it is increasingly clear that, even when absent or poorly present, this does not equate to the absence of these immune cells' action on inflammation [9]. In addition, tendon injuries are accompanied and preceded by the secretion and action of several chemical mediators of inflammation by tenocytes including pro-inflammatory and anti-inflammatory cytokines, and several growth factors such as TNF-a, IL-1b, IL-6, IL-10, VEGF, TGF-b, 10,23 and 25 COX-2, and PGE2. [11,15,16] Although the inflammation driven by the cytokines might have a role in the healing

process, its role in the development, healing and resolution of tendinopathy, tendon rupture and other inflammatory processes remains controversial [17,18].

This systematic review reports the most up-to-date evidence on the role of immune cells on tendon healing with a focus on its clinical relevance.

2. Methods

2.1 Literature search Strategy

This systematic review was conducted according to the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) [19] and MOOSE guidelines [20]. A comprehensive search was performed on three medical electronic databases (PubMed, Embase and Cochrane Library) by two independent authors (E.C. and W.S.K.) from their inception to 10th June 2019. Our main aims were to: (1) understand the role of inflammation and immune response in tendon healing, (2) identify factors associated with anti-inflammatory intervention, (3) evaluate their effects through the review of animal and *in vitro* studies, and (4) critically summarize the evidence available. To achieve the maximum sensitivity of the search strategy, we combined the terms: ‘‘tendon”, as well some common terms of tendon conditions such as “tendon injury OR (tendon damage) OR tendonitis OR tendinopathy OR (chronic tendonitis) OR tendinosis OR (chronic tendinopathy) OR enthesitis)” AND “healing” AND “(immune response) OR (macrophages) OR (immune cells) OR (monocytes) OR (lymphocytes) OR (immunology) ” as either key words or MeSH terms. The reference lists of all included articles, previous literature reviews on the topic and top hits from Google Scholar were reviewed for further identification of potentially relevant studies. To avoid overlapping with other ongoing reviews, we first searched PROSPERO site for any similar review, and then prospectively registered our study

2.2 Selection Criteria

Eligible studies included those investigating inflammation and immune response in tendon healing. Primary screening of the titles and abstracts was performed by including studies of any level of evidence published in peer-reviewed journals reporting clinical or preclinical results in English. Also, Italian, French, Spanish, Portuguese articles were included since the senior author was able to evaluate them (N.M.). Moreover, articles discussing the effect of several cytokines and immune response actors, both pathologically and physiologically were reviewed. Exclusion criteria included studies investigating the treatment response of tendon to regenerative treatments including platelet rich plasma (PRP), mesenchymal stem cells (MSCs) etc, or new drugs related to healing of the tissue. Additionally, we excluded studies in which data were not accessible, missing, without an available full text, or not well reported. We also excluded duplicates, and the

studies with poor scientific methodology assessed as described below. Abstracts, case reports, conference presentations, reviews, editorials and expert opinions were excluded. Two authors (E.C. and W.S.K.) performed the search and evaluated the articles independently. An experienced researcher in systematic reviews (N.M.) solved cases of doubt. At the beginning of the procedure, each investigator read the abstracts of all the articles, selected the relevant ones according to both inclusion and exclusion criteria, and then compared the results with the other investigators. After four weeks, the same studies were read again to establish the agreement of the investigators about articles' selection. No disagreement was observed among the investigators. One investigator extracted the data from the full text articles to Excel spreadsheet structured tables to analyze each study in a descriptive fashion. Another investigator independently double checked the extraction of primary data from all the articles. Doubts and inconsistencies solved by discussion.

2.3 Data Extraction and Criteria Appraisal

All data were extracted from article text, tables and figures. Data were extracted using the Population, Intervention, Comparison, Outcome (PICO) framework and included title, year of publication, study design, sample size, study population, patient characteristics, intervention and comparator (where applicable), outcomes, funding and conclusions. Two investigators independently reviewed each article (E.C. and L.R.). Discrepancies between the two reviewers were resolved by discussion and consensus. The final results were reviewed by another experienced investigator (N.M.).

2.4 Risk of Bias Assessment

The assessment of the risk of bias of all *in vivo* selected full-text articles was performed according to the SYRCLE's risk of bias tool [21] for preclinical studies and the Cochrane Collaboration's risk of bias tool [22] for clinical studies (Supplementary material Tables 1a-1b). This assessment used "Low," "Moderate" and "High" as judgement keys: "Low" indicated a low risk of bias, "Moderate" indicated that the risk of bias was moderate, and "High" indicated a high risk of bias. The assessment was performed by two authors (E.C. and L.R.) independently. Inter-rater agreement was 92%. Any discrepancy was discussed with the senior investigator (N.M.) for the final decision.

2.5 Study Quality Assessment

The quality of evidence was assessed according to Collaborative Approach to Meta-Analysis and Review of Animal Data from Experimental Studies (CAMARADES) checklist with supporting guidance from the CAMARADES website [23], giving one point for each of (1) publication in a peer-reviewed journal; (2) statement of temperature control; (3) random allocation to groups; (4) allocation concealment; (5) blinded assessment of outcome; (6) use of anaesthetic without significant internal protection of blood vessel; (7) appropriate animal model (aged, healthy, diabetic, or hypertensive); (8) sample size calculation; (9) compliance with animal welfare regulations; (10) statement of potential conflict of interests. Each study was assessed and scored on a scale from 0 (lowest) to 10 (highest) points. The assessment was performed by two authors (E.C. and L.R.) independently. Inter-rater agreement was 94%. Any discrepancy was discussed with the senior investigator (N.M.) for the final decision.

3. Results

A total of 225 studies were identified from the databases according to the aforementioned inclusion and exclusion criteria. Overall, 112 articles were screened through abstract and title reading after removal of duplicates. Eventually, after full text reading and reference list check, we selected 68 articles to include in the present manuscript**.** A PRISMA [19] flow chart of the selection process and screening is provided (Figure A)

Figure A.

We ultimately included 53 articles [12,24,33–42,25,43–52,26,53–62,27,63–72,28,73–82,29,83,84,30–32] after applying our search strategy, inclusion and exclusion criteria. The articles included investigate the role of immune cells, the pathway triggered by their action and other immune mediators involved in the healing response of tendons after an injury.

The onset and progression of tendinopathy is related to an imbalance of inflammatory factors, immune system cells and chemical mediators, hormones, mechanical stimuli and other yet unknown agents. Morita et al[85] described over 20 cytokynes as actors of the immune and inflammatory process involved in tendon healing. While emerging evidence supports their role in every physiological phase of healing, their imbalance can ultimately lead to a failed healing response.[43] Chemokines such as CCL5, CCL2, CCL3, CXCL10 are involved in the pathogenesis of tendinopathy inducing inflammation [44], even after mechanotrasduction[8,45]. The most investigated proinflammatory cytokines including IL-1β, IL-6 and TNF-α, are also able to elicit the immune response. [85] The immune cells are reported to be main actor of all the aforementioned processes both producing mediating factors and acting through cell mediated processes.

Mast cells

Mast cells exert were reported as inducer of the proinflammatory response on human tendon-derived cells *in vitro*.[48]

Macrophages

Macrophages are immune cells involved in both inflammatory and repair processes [46,47]. They are crucial for healing, and initially secrete pro-inflammatory agents in response to tissue damage including IL-

1β, TNF-α bioactive prostaglandins, reactive oxygen intermediates and many proteases [49–51]. These factors act as important initiators of the tendinopathic cascade [52,53], which may drive matrix metalloproteinase (MMP) mediated catabolism of tendon extracellular matrix.[53,54] They can be categorized into two broad subsets including the M1 (classically activated) and the M2 (alternatively activated) macrophages [47,55]. Although the M1 and M2 (and its subset such as M2a, M2b, M2c, and M2d) dichotomy is insufficient to describe their diverse phenotypes and functions [47,55], M1 polarised macrophages appear to show a pro-inflammatory response pattern, while M2 macrophages regulate inflammatory responses by producing immunosuppressive cytokines such as IL-1 receptor antagonist (IL-1Ra), IL-10, IL-4 and IL-13. [47,56]

The literature suggests that tenocytes influence the phenotype macrophages are directed towards following their initial activation during inflammation. The macrophages polarization might be controlled through soluble factors [28,84]. Changes in macrophage phenotype and epithelial-to-mesenchymal transition genes have been noted following Achilles tenotomy and during repair [33]. In an equine tendon repair model, a phenotype switch towards M2-type macrophage polarization along with reduced expression for the Lipoxin A4 receptor was seenin chronic injury suggesting incomplete inflammation resolution[28]. Emerging evidence supports the role of macrophages as key players in tendon homeostasis and in tendon repair [28,32,46,55]. In particular, the anti-inflammatory effect of the M2 subset on classically activated M1 macrophages limit their action, promoting tissue repair. [47,55]

In animal models, rodents with surgically induced tendon injury have been used to evaluate the presence of inflammatory cells by immunohistochemistry [46,57]. In a rat Achilles tendon injury model, , a sequential pattern of inflammatory cell infiltration with a rapid and transient accumulation of neutrophils followed by an increase in MQ infiltration 1–28 days post-injury was observed[46]. Similarly, Wong et al (2009) documented temporal changes in inflammatory cell subsets in a murine immobilised surgical adhesion model of injury, reporting peak neutrophil and macrophages accumulation 1–5 days and 21 days post-surgery respectively [57]. The requirement of macrophages for adult tissue repair is supported by wound healing studies in murine macrophages-knockout models, with impaired healing responses observed in macrophages deplete wounds [49–51].

The complex network of factors influencing macrophage polarization, both *in vitro* and *in vivo*, can be affected by MSCs, raising the possibility of a regenerative medicine solution for tendon healing. [41,58–66] In animal models of tendon injury, MSC treatments increased the presence of M2 macrophages and their associated anti-inflammatory factors, which subsequently resulted in improved healing. [34,67,68] MSC-stimulated macrophages seem to have marked anti-inflammatory properties compared with wild type control macrophages, with a higher levels of IL-10 and IL-6 and lower level of IL-12 and TNF-α expression [41,69]. The M2-like stimulated macrophages in particular can modulate an improved and faster tendon healing with better mechanical and histological feature. [41]

MSCs facilitate monocyte to macrophage transition, skew naive macrophages to an M1 state, and attenuate already activated M1 macrophages while enhancing M2 activation [70]. Although the exact mechanisms behind MSCs and macrophages interaction across different activation stages are not fully understood, Németh et al (2009) suggested a role by inflammation signalling factors such as PGE2 and its receptors EP2 and EP4 [65]. Other studies have noted metabolic changes in the expression of IDO1, SIRUTIN1, AMPK and GLUT1. [70] Macrophages are essential for the orchestration and promotion of satisfactory wound healing as well as the resolution of inflammation in response to pathogenic challenge or tissue damage. Additional studies are required to further elucidate the complexities of MSC modulated macrophage polarization.

There is no clear picture of the influence of macrophages on tendon healing. Some studies report that macrophage depletion or deficiencies are associated with improved quality of the healing tissue [24,32], sometimes together with a decreased mass of tissue [32]. Although these studies looked at the effect of absence of macrophages during the entire healing process, studies where macrophages were specifically inhibited during early inflammation [82,83] e.g. with NSAIDs [81] demonstrated a positive effect.

Lymphocytes

The possible role of lymphocytes in tendon healing and tendinopathy is still not understood. Although their presence in healthy and tendinopathic tendons has been reported [12,71], further studies are needed to validate the function of lymphocytes in tendinopathy.

Mechanical load and immune cells

Mechanical load appears to influence the metabolism and healing of tendons [26,32,35,72–79]. It upregulate both anabolic and catabolic pathways through regulation of inflammation and immune reaction. In animal studies, loading prolonged the early inflammatory response and increased the cross-sectional area in tendons [35,72,80]. Other effects included macrophage polarization (M1>M2) with a delayed regeneration phase type of inflammation with more M2 macrophages and Treg cells [35]. Studies on macrophages polarization reported that the mechanical stress also influenced the immune cell differentiation and action. [26,35,78,79] Andersson et al (2012) loaded by unrestricted cage activity and demonstrated an increased strength of the healing tendon [72], but this increase was due to a greater mass of the healing tissue measured by increased cross-sectional area, without any significant improvement in mechanical quality.

Discussion

The literature contains conflicting data on the presence and role of immune cells in tendon healing inflammation. This review systematically analysed the current evidence on the presence and possible role of both proinflammatory and anti-inflammatory cytokines in tendon healing.

Macrophages and other immune cells are also derived from adipose tissue, and an intricate relationship exists between them and the adipocyte-derived proinflammatory cytokines [15,86]. In particular, MCP-1 induces macrophage infiltration of adipose tissue. In turn, activated macrophages release additional proinflammatory cytokines, notably TNF-a expression is significantly increased [15,86]. This reduces the expression of adiponectin, an adipocyte derived anti-inflammatory hormone. This altered balance between chemotactic mediators and macrophages results in a state of persistent local inflammation within the adipose tissue [15]. Moreover, increased adipose mass alters the relationship between leptin and suppressor T cells. Leptin, an adipocyte-derived

hormone responsible for the central control of energy balance, also seems to inhibit the proliferative capacity of suppressor T cells [86].

Macrophages polarization and action seem to be influenced by mechanical loading [26,35,78,79,87]. In particular, emerging evidence supports their role to be dualistic setting the basis for a U curve interpretation of its role, where overloading the tendon will result in a failed healing and reinjury and underloading in a less effective healing.

The development of a better understanding of the role of specific cell subpopulations in the pathogenesis of tendinopathy and during tendon healing is vital to identify potential therapeutic targets and develop more effective future treatments for patients. Studies of equine tendinopathy suggest that chronic inflammation may develop from inadequate resolution of inflammation. [28,88]

Exercise still represent one of the best ways to positively influence tendon healing by negatively affecting the inflammatory environment, as reported in several preclinical studies focusing on the role of early mobilization of injured tendons [73–77]. The mechanism of cytokine expression is still not fully understood but seems to rely on the stimulatory effect exerted by trauma leading to microdamage and vessel leakage [73,74]. While rat models exposed to loading by unrestricted cage activity showed an increased strength of the healing tendon [72], this increase was due to an increased mass of the healing tissue without a significant improvement in mechanical quality. Even though there is no clear consensus on how much load will be appropriate for tendon healing, early and progressive physical therapy after tendon injury, tendon surgery, and in tendinopathy should be advised.

5. Limitations

The main limitation of this systematic review is the heterogeneity and quality of the included studies. Most of the studies were preclinical studies, with no clinical randomized controlled trials. Despite applying strict methodological evaluation through quality and risk of bias tools, treatment variables including dose, drug delivery and population used differed across the included studies. The findings of our review will however hopefully help direct future investigations.

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