INNATE AND ADAPTIVE IMMUNE SYSTEM CELLS IMPLICATED IN TENDON HEALING AND DISEASE

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Abstract

Tendons perform a critical function in the musculoskeletal system by integrating muscle with skeleton and enabling force transmission. Damage or degeneration of these tissues lead to impaired structure and function, which often persist despite surgical intervention. While the immune response and inflammation are important drivers of both tendon healing and disease progression, there have been relatively few studies of the diverse immune cell types that may regulate these processes in these tissues. To date, most of the studies have focused on macrophages, but emerging research indicate that other immune cell types may also play a role in tendon healing, either by regulating the immune environment or through direct interactions with resident tenocytes. The present review synthesises the literature on innate and adaptive immune system cells that have been implicated in tendon healing or disease, in the context of animal injury models, human clinical samples or *in vitro* experiments.

Keywords: Immune, tendon, innate immune system cells, adaptive immune system cells, tendinopathy, inflammation.

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List of Abbreviations

ALOX15	arachidonate 15-lipoxygenase
aSMA	alpha smooth muscle actin
CD	cluster of differentiation
COX-2	cyclooxygenase-2
CX3CL1	C-X3-C motif chemokine ligand 1
CX3CR1	C-X3-C motif chemokine receptor 1
DAMP	damage-associated molecular pattern
ECM	extracellular matrix
Egr	early growth response
Ig	immunoglobulin
IL	interleukin
INF-γ	interferon gamma
MHC	major histocompatibility complex
Mkx	Mohawk homeobox
MRI	magnetic resonance imaging
NK	natural killer
NMDA	N-methyl-D-aspartate
PAMP	pathogen-associated molecular patterns
PDGFRα	platelet-derived growth factor receptor A
PEG2	prostaglandin E2
PRP	platelet-rich plasma
PRR	pattern recognition receptors
Scx	scleraxis BHLH transcription factor
Rag2	recombination activating gene 2
TGFβ	transforming growth factor beta
Tppp3	tubulin-polymerisation-promoting protein family member 3
Tregs	regulatory T cells

Introduction

Tendons are essential anatomical structures that transmit muscle forces to bones to enable movement and sustain mechanical loads (Franchi *et al.*, 2007). Due to their poor intrinsic regenerative capacity, injury frequently results in permanent scar formation and loss of function. Tendon rupture can arise from trauma; however, a rupture is more frequently preceded by degeneration, which may be initiated by repetitive over-use, causing micro-damage to the tendon structure (Andarawis-Puri *et al.*, 2012a;

Andarawis-Puri *et al.*, 2012b). Tendon dysfunction is broadly categorised under the term tendinopathy. In general, diseased tendons are distinguished by increased cellularity, altered cell phenotype and morphology, disrupted collagenous ECM, increased vasculature, increased water content, increased glycosaminoglycans and neurovascular infiltration (Fenwick *et al.*, 2002; Lozano *et al.*, 2019). Interestingly, tendons that appear pathological by MRI or ultrasound are not always painful (Farnqvist *et al.*, 2020; Rio *et al.*, 2014). Tendinopathies are also not equally distributed among all tendons: tendons of the upper and lower limbs (such as the rotator cuff, patellar and Achilles tendons) are among the most commonly affected (Figueroa *et al.*, 2016; Longo *et al.*, 2009). For more insight on the aetiology, pathophysiology, diagnosis and management of tendinopathy, please see the excellent and comprehensive review by Millar *et al.* (2017).

While there are many risk factors that can influence the progression from preclinical tendinopathy to chronic tendinopathy, including environmental factors and genetic predisposition, one critical factor that has been relatively underappreciated is immune cell dysfunction (Millar *et al.*, 2021). Historically, tendinopathy was sub-divided clinically as inflammatory (termed tendinitis) or non-inflammatory (termed tendinosis). The contribution of inflammation to tendon degeneration was largely ignored prior to 2012, which may be due to an overly narrow definition of inflammation (Mosca *et al.*, 2018). In fact, accumulating evidence now suggest that tendon degeneration and fibrotic tendon healing could be a consequence of failed immune polarisation, resulting in prolonged or chronic type I inflammation. For tissue regeneration, a finely tuned balance between inflammation and its resolution is crucial (D'Addona *et al.*, 2017). Indeed, while chronic inflammation is harmful for proper tendon healing, this pro-inflammatory phase is still an essential component of the immune response and early suppression of the acute response also impairs functional tendon healing (Blomgran *et al.*, 2017; D'Addona *et al.*, 2017).

Despite the importance of the immune environment in tendinopathy and poor tendon healing, there is still very little known about the cells that orchestrate the immune response in the context of tendons. Therefore, the purpose of the present review was to provide updated information regarding the immune system cells involved in tendon inflammation and healing. For an in-depth review of selected type I and type II immune cytokines in tendinopathy and wound healing and advances in immunomodulatory drugs, see the review on the topic by Arvind and Huang (2021). The present review will briefly describe tendon structure, function and resident cell types (tenocytes, epitenon/endotenon cells, progenitor cells, immune system cells) followed by an overview of the known innate and adaptive immune system cells that have been implicated in tendon injury and repair.

Tendon structure and function

Healthy tendons are composed of a dense ECM that is primarily comprised of highly organised, cross-linked type I collagen fibres (~ 70 % by dry weight) (Kastelic *et al.*, 1978). The main tendon structure is divided into fascicles, which contain collagen fibre bundles. In turn, collagen fibres are composed of fibrils, which are further subdivided into microfibrils. These collagenous components are largely arranged in parallel to the long axis of the tendon, which contributes to the tendon's mechanical properties (Franchi *et al.*, 2007). Although collagen fibrils are homogeneous at birth,with a small diameter, fibril diameters increase rapidly during postnatal tendon maturation, resulting in a heterogeneous field of small and large collagen fibrils at the end of growth. While collagen fibrils directly contribute to tendon tensile load bearing, loads are also transferred across discontinuous fibrils through interfibrillar shear and sliding (Szczesny and Elliott, 2014; Szczesny *et al.*, 2017). In addition to type I collagen, tendon ECM also contains other components, including the small leucine-rich proteoglycans (for example decorin and biglycan) and minor collagens (for example collagen type II, V and XII) (Buckley *et al.*, 2013). In general, the arrangement of the different collagen types directly contributes to tendon function by providing resistance, flexibility and elasticity while transmitting forces, dissipating energy and preventing mechanical failure (Franchi *et al.*, 2007). Recent evidence also suggest tendon extrafibrillar components may not directly contribute to tensile properties after tendon maturation and growth (Szczesny *et al.*, 2017).

Although tendon fascicles form the bulk of the tendon structure, individual fascicles are surrounded by a specialised tissue called the endotenon, while the entire tendon structure is enclosed by a similar tissue called the epitenon. While relatively little is known about endotenon and epitenon tissues, they are thought to play an important role in establishing the organisation of the ECM in the developing tendon *via* cadherin-mediated cell-cell junctions (Richardson *et al.*, 2007). Moreover, the epitenon and endotenon largely contain most of the blood vessels that supply the tendon (Edwards, 1946). However, tendon vascularity can also vary greatly across subjects and tendon types (Cook *et al.*, 2005) (Fig. 1).

Resident tendon cell types

The resident cell type within the tendon fascicle is the specialised fibroblastic cell called tenocyte that synthesise and maintain tendon ECM. In the mature tendon, tenocytes are longitudinally oriented and

reside in rows. Tenocytes communicate through long protrusions connected by gap junctions composed of proteins such as connexins 32 and 43. To date, only four transcription factors have been identified for tenocytes, including *Scx*, *Mkx*, *Egr1* and *Egr2*. While *Scx* is strongly expressed during embryonic and early postnatal stages, expression levels decrease with tendon maturation and heterogeneity in *Scx* expression emerges (Best *et al.*, 2021; De Micheli *et al.*, 2020; Howell *et al.*, 2017; Nichols *et al.*, 2019). Although tenocytes were originally considered to be a relatively homogeneous population, there is growing appreciation that sub-populations of tenocytes express distinctive markers and may have specialised functions (De Micheli *et al.*, 2020; Kendal *et al.*, 2020). While tenocytes are proliferative in the first 1-2 weeks after birth, mitotic capacity is lost with maturation, in parallel with dramatic increases in matrix deposition and mechanical properties (Ansorge *et al.*, 2011; Grinstein *et al.*, 2019). While there is some activation of *Scx*-expressing tenocytes after injury, the proliferative capacity of adult tenocytes is relatively limited, especially compared to neonatal tenocytes (Best and Loiselle, 2019; Gumucio *et al.*, 2020; Howell *et al.*, 2017).

The cells that reside within the endotenon/epitenon are different from tenocytes in terms of characteristic marker expressions. During development, epitenon cells appear after the induction of tenocyte progenitors and express the marker Tppp3 (Staverosky *et al.*, 2009). While this marker is lost in later stages of embryonic development, it re-emerges in mature tendons and identifies a sub-population of epitenon cells with stem and regenerative potential (termed tendon stem and progenitor cells) (Harvey *et al.*, 2019). In general, epitenon cells express laminin, aSMA and PDGFRα. These cells are highly activated after tendon injury, proliferate and contribute to both scar formation and new Scx$^+$ tenocytes production (Best *et al.*, 2021; Dyment *et al.*, 2014; Gumucio *et al.*, 2014; Gumucio *et al.*, 2020; Harvey *et al.*, 2019; Taylor *et al.*, 2011).

Resident immune system cells

Although the tendon was previously thought to be devoid of immune system cells, several studies have reported the presence of both innate (*e.g.* macrophages) and adaptive (*e.g.* T cells) immune cell types within normal tendons (Garcia-Melchor *et al.*, 2021; Howell *et al.*, 2021; Kendal *et al.*, 2020). While the function of these immune system cells in the context of healthy tendon development and homeostasis has not been explored in detail, tendon mechanical properties are unchanged in Rag2$^{-/-}$ mice devoid of T and B cells (data not published), indicating a minimal role for these cells in tendon development. The presence of immune system cells in tendons suggests that these cells may function as first responders in case of damage. It is also possible that dysregulated resident immune system cells may induce degenerative changes independent of overt mechanical damage. Since immune system cells have been shown to be mechanoresponsive (Göhring *et al.*, 2021; Jin *et al.*, 2019; McWhorter *et al.*, 2015), it is intriguing whether resident immune system cells may regulate the local immune environment in response to tendon loading.

Although resident immune system cells are localised in close proximity to resident tenocytes/epitenon cells, direct interactions between cell types are only beginning to be elucidated. Co-culture and mixed culture experiments suggest there are likely reciprocal interactions between tenocytes and immune system cells (Garcia-Melchor *et al.*,

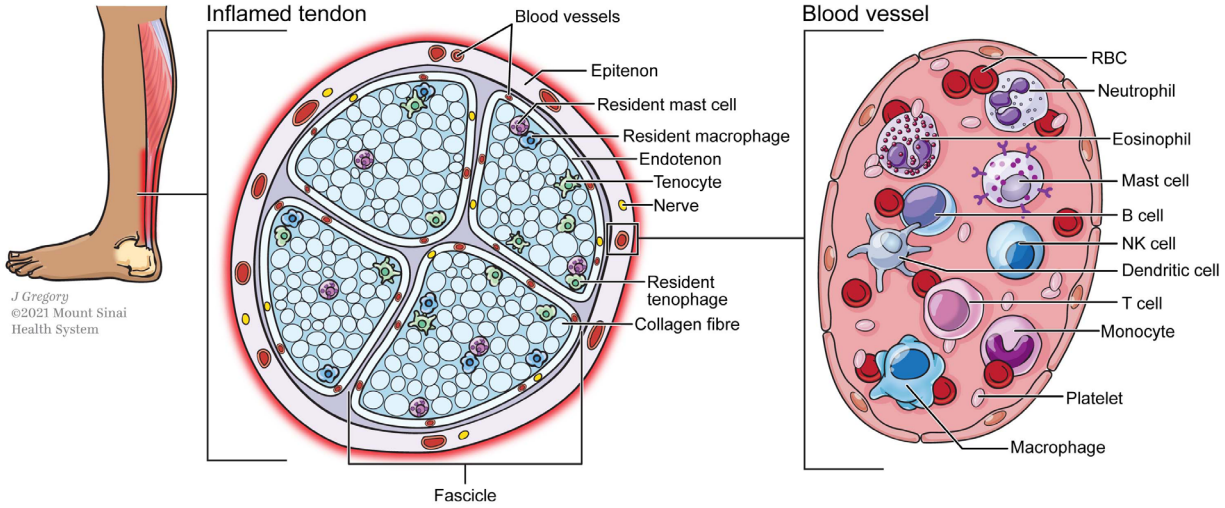

Fig. 1. Overview of innate and adaptive immune system cells involved in tendon inflammation. A small population of resident immune system cells such as mast cells and macrophages can be found in normal tendons. Those cells expand during injury or disease. Other immune system cells that contribute to tendon inflammation, disease progression or healing infiltrate the tendon from peripheral sources and interact with resident tendon and epitenon cells. The temporal dynamics of recruited immune system cells may vary according to injury model.

2021; Stolk *et al.*, 2017); however, further studies are required to determine the extent of these interactions under healthy and diseased conditions.

Innate immune system cells in tendinopathy

Typically, the first cells that trigger the acute inflammation in a wounded and/or infected tissue are innate immune system cells (Fig. 2). These are cells that either circulate in the blood or are resident in tissues. Innate immune system cells have a rapid, non-specific response to microbes and injured cells. In fact, most innate immune system cells have PRR, which bind and recognise both PAMP present on microbes as well as DAMP (Kumar *et al.*, 2011; Marshall *et al.*, 2018). DAMPs, also known as alarmins, are nuclear, mitochondrial or cytosolic proteins released by cells upon infection, necrosis or injury (Roh and Sohn, 2018). Once innate immune system cells bind proteins that trigger an immune response, they release a large number of cytokines, which in turn stimulate the blood flow to recruit more immune system cells to the site, thereby increasing inflammation (D'Addona *et al.*, 2017; Marshall *et al.*, 2018). Interestingly, tenocytes release pro-inflammatory cytokines upon injury, contributing to oedema and hyperaemia (D'Addona *et al.*, 2017; Millar *et al.*, 2017). This section summarise the known research on innate immune cell populations that have been implicated in some way in tendon healing. Unsurprisingly, the vast majority of the research has centred on macrophages, with relatively limited information on other innate immune system cells such as neutrophils, mast cells, *etc.*. Characteristic markers that have been used to identify these innate immune system cells by flow cytometry are indicated in Table 1.

Macrophages

Macrophages are granulocytic phagocytic innate immune system cells whose main function is to engulf pathogens, cell debris and apoptotic bodies (Marshall *et al.*, 2018). Macrophages either circulate in the bloodstream seeking inflamed areas to penetrate through trans-endothelial migration or permanently reside in specific tissues (Weber, 2008). Macrophages, differently from neutrophils (another important phagocytic population), are long-lived cells. For this reason, they play a more prominent role in adaptive immunity as crucial antigen-presenting cells (cells that process and present antigens on their surface to activate B and T lymphocytes) (Marshall *et al.*, 2018).

Historically, activated macrophages were thought to exist in two forms, either as M1 or M2 macrophages. M1 macrophages were considered the "typical" pro-inflammatory macrophages that clear pathogens, debris and apoptotic bodies, while releasing cytokines to increase the inflammation (Mantovani *et al.*, 2002; Millar *et al.*, 2017; Sunwoo *et al.*, 2020). On the other hand, M2 macrophages inhibit the inflammatory response, which promotes angiogenesis, tissue remodelling, fibrosis and healing (D'Addona *et al.*, 2017; Del Buono *et al.*, 2011; Mantovani *et al.*, 2002; Sica and Mantovani, 2012; Zhang *et al.*, 2008). Consensus among immunologists during the past few years has established that activated macrophages exist as a continuum, from M1- to M2-like macrophages (Murray *et al.*, 2014). Macrophage polarisation can be induced in the presence of specific signals, such as IL-10, IL-4, INF-γ, IL-13, glucocorticoid hormones and vitamin D (Mantovani *et al.*, 2002). Interestingly, M1- and M2-like macrophages not only differ in their effector function but also in receptor expression as well as cytokine and chemokine production (Mantovani *et al.*, 2002; Murray *et al.*, 2014; Sica and Mantovani, 2012). In the context of tendon healing, prolonged activity of M1-like macrophages is thought to be detrimental to healing while M2-like macrophages are generally pro-regenerative. This is supported by recent studies showing that the immune-modulating activities of mesenchymal stem cells in tendon repair are due

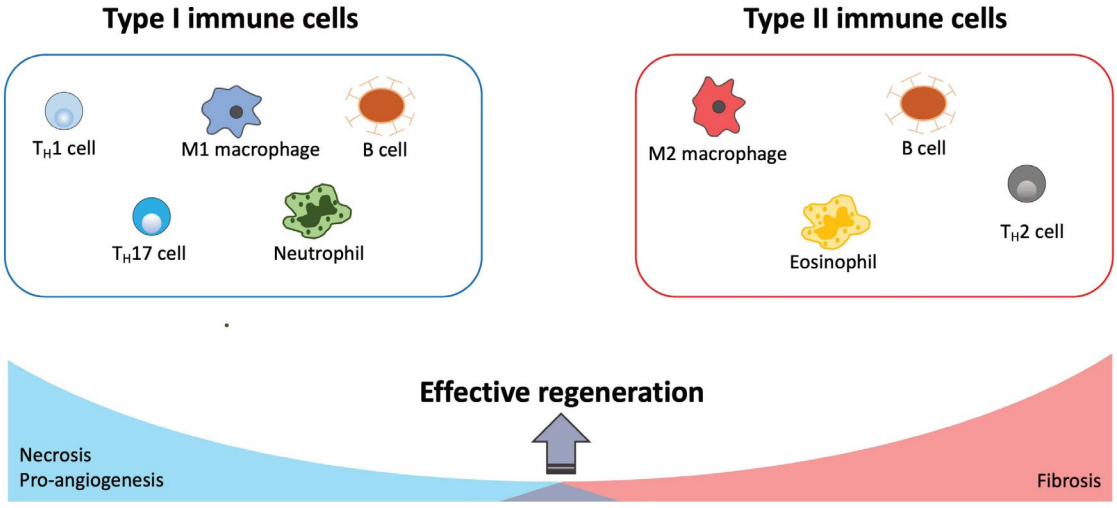

Fig. 2. Representative immune system cells that regulate type I and type II immune responses. Effective tissue regeneration requires a proper balance between type I and type II immune responses.

Table 1. Commonly used cell markers to identify murine immune system cells. Markers listed are not necessarily unique or exhaustive. Optimal marker choice depends on the cell subtype, the tissue that it is found in and its experimental application.

Cell type	Common cell surface markers in mice
Innate immune system cells	
Macrophage	CD11b, F4/80, CD68
Monocyte	CCR, CX3CR1, LY6C
Tenophages	CX3CR1, CX3CL1
Neutrophils	CD11b, GR1, LY6G
Mast cells	CD117/C-Kit, IL-3 Rα/CD123 and Fc epsilon RI
Eosinophils	Cd11b, Singlec-F
Platelets	CD41, CD62p
Dendritic cells	CD11c, MHC-II
Adaptive immune system cells	
T cells	CD3
Cytotoxic	CD3, CD8
Helper	CD3, CD4
B cells	CD19, CD80, CD73, PD-L2/CD273
NK cells	NK1.1/NKp46, NKG2D

to their effects on M2-like macrophage polarisation (Chamberlain *et al.*, 2019). Mechanistically, this interaction appears to be driven by extracellular vesicles secreted by mesenchymal stem cells that induce macrophage polarisation (Chamberlain *et al.*, 2019). Thus, injection of vesicle-educated macrophages promotes improved functional healing after mouse Achilles tendon rupture (Chamberlain *et al.*, 2019).

While the dynamics of M1- to M2-like macrophage polarisation is likely critical to tendon healing, most tendon studies generally focus on total macrophage populations. While macrophages are normally scarce in healthy tendons (Best *et al.*, 2019; Howell *et al.*, 2021), their numbers increase during disease. In human supraspinatus tendons, the number of $CD68^+$ tissue-resident macrophages increases in early and intermediate-advanced stages of tendinopathy compared to healthy tendons (Dakin *et al.*, 2015; Del Buono *et al.*, 2011). The presence of $CD206^+$ macrophages and the activation of ALOX15 and CD206 pathways is also associated with resolution of tendon pain following treatment (Dakin *et al.*, 2015). However, temporal regulation of macrophage accumulation is variable and depends on the injury model, the subtype of macrophage analysed (and markers used) and the anatomical tendon analysed. For example, in tendon grafts for reconstruction of rat anterior cruciate ligaments, recruited macrophages have been identified in the tendon 4 d after surgical reconstruction, while resident macrophages accumulated 11 d after surgery (Kawamura *et al.*, 2005). In contrast, collagenase-induced Achilles tendon injury in mouse showed an increase in recruited macrophages at 1 d post-injury while resident macrophages increased at 28 d (Marsolais *et al.*, 2001). In general, injury models show consistent upregulation of macrophage numbers during disease or injury (Noah *et al.*, 2020; Wojciak and Crossan, 1993). Regardless of the temporal dynamics post-injury, there is consensus that these cells play an important role in both acute and chronic tendon inflammation (Jomaa *et al.*, 2020). In addition, macrophages have also been shown to directly stimulate tenocyte proliferation and promote ECM deposition (de la Durantaye *et al.*, 2014; Sunwoo *et al.*, 2020).

Several studies ablating macrophages (either by genetic targeting or clodronate delivery) have confirmed the important function of macrophages in tendon healing. In adult tendon, depletion of macrophages reduces cell proliferation (de la Durantaye *et al.*, 2014; Godbout *et al.*, 2010) and matrix accumulation after injury (de la Durantaye *et al.*, 2014). However, functional outcomes are mixed, with some studies showing improvement or no change in mechanical properties. Using genetic ablation of macrophages, Howell *et al.* (2021) have recently shown that macrophage ablation in neonatal mice results in failed regeneration, indicated by impaired function, reduced cell proliferation and reduced neo-tendon formation. However, one limitation of all these ablation studies is the inability to precisely target M1- or M2-like populations, which have distinct functional activities in the healing cascade.

Monocytes

Monocytes are a type of myeloid agranular white blood cell that can differentiate into either macrophages or dendritic cells (Marshall *et al.*, 2018). Generally, monocytes infiltrate an inflamed area within 24 h of acute inflammation, together with macrophages and neutrophils (D'Addona *et al.*, 2017). One of the main function of monocytes is to renew tissue-resident macrophages and transport antigens to secondary lymphoid tissues, without differentiating into macrophages (Jakubzick *et al.*, 2017; Kapellos *et al.*, 2019). Inflammatory monocytes typically give rise

to M1-like macrophages while anti-inflammatory monocytes give rise to M2-like macrophages (Auffray *et al.*, 2007). Notably, macrophages can also arise from cells other than monocytes, such as embryonic progenitors (Stremmel *et al.*, 2018).

While the role of monocytes in tendon healing has been analysed to a lesser extent compared to macrophages, resident monocytes are present in healthy human tendons and accumulate in case of an injury (Kendal *et al.*, 2020). During the early and intermediate stages of tendinopathy, monocytes are elevated in tendons and contribute to increased macrophage levels (Crowe *et al.*, 2019; Dakin *et al.*, 2015). In general, monocyte accumulation patterns follow macrophage patterns after injury, with increased levels observed 3-7 d post-injury depending on injury model (Markworth *et al.*, 2021; Noah *et al.*, 2020).

Chemotactic monocyte factors have been implicated in both pro-inflammatory and anti-inflammatory events in tendons (Crowe *et al.*, 2019). For example, lipoxins produced by both macrophages and monocytes are essential to dampen the inflammatory response and promote tendon healing (Millar *et al.*, 2017). However, monocytes can release alarmins such as S100A8 and S100A9, which participate in a positive feedback mechanism that enhance leukocyte recruitment and release of more pro-inflammatory cytokines (Crowe *et al.*, 2019).

Tenophages

Recently, the presence of macrophage-like tenocytes in healthy tendons, named tenophages, has been proposed (Lehner *et al.*, 2019). These tendon-resident cells express the fractalkine receptor CX3CR1, together with its ligand CX3CL1. Moreover, *in vitro* stimulation of these tenophages induces the production of various pro-inflammatory molecules that are involved in tissue healing and repair (Lehner *et al.*, 2019). Due to limited evidence, the identity of these cells remains an open question; however, the concept of a specialised sub-population of tenocytes is consistent with the growing consensus that tenocytes are heterogeneous and harbour distinctive functions.

Neutrophils

Neutrophils are among the first granulocytic innate immune system cells that respond to macrophage activation (Jomaa *et al.*, 2020). Similar to other white blood cells, neutrophils migrate from the bloodstream to damaged and/or infected tissues through the leukocyte adhesion cascade, following a gradient of chemoattractants (Rosales, 2020). Neutrophils have a variety of anti-microbial functions, which include phagocytosis of invading microorganisms and other mechanisms promoting pathogen death. Toward this end, neutrophils can release cell granule microbicidal contents (termed degranulation), produce reactive oxygen species and form neutrophil extracellular traps (Chaplin, 2010; Rosales, 2020). In the last decade, additional neutrophil functions have been elucidated. Indeed, these phagocytic cells are important mediators in the immune cell response, as they produce cytokines and chemokines that regulate both the innate and adaptive immune system (Chaplin, 2010; Rosales, 2020). For example, neutrophils can recruit and activate T cells at inflamed sites (Rosales, 2020). Neutrophils can also migrate into secondary lymphoid organs and act as antigen-presenting cells to directly activate lymphocytes (Rosales, 2020).

In the context of tendon injury, neutrophils have been detected in various tendinopathy models, although peak neutrophil accumulation varies depending on the model. In an ovine model of superficial digital flexor tendon injury, it was found that neutrophils are highly activated as late as 5 months post-injury in adults, compared to regenerative foetal counterparts (Ribitsch *et al.*, 2021). With collagenase-induced Achilles tendon injury, the neutrophil population in the tendon peaks 1 d post-injury before gradually returning to baseline by 7 d (Marsolais *et al.*, 2001). Other models such as tenotomy show extended temporal dynamics, with persistence of neutrophils from 7-28 d post-injury (Crowe *et al.*, 2019; Millar *et al.*, 2017; Noah *et al.*, 2020). These differences in neutrophil dynamics may be due to differences in injury severity between model systems. Detection of neutrophils at relatively late stages of healing may also suggest their contribution toward chronic inflammation or dysregulated immune cell function. Limited research on neutrophil serine proteases (such as elastase and cathepsin G) found that neutrophil elastase is capable of solubilising tendon collagen type I, while cathepsin G has little effect (Starkey *et al.*, 1977). Therefore, direct secretion of proteases that disrupt the tendon ECM may be another mechanism by which neutrophils can promote the tendon degeneration cascade once induced.

Mast cells

Mast cells are granulocytic phagocytic innate immune system cells that reside in most connective tissues and all vascularised areas (Krystel-Whittemore *et al.*, 2016). In innate immunity, they have important anti-viral, anti-parasitic and bacterial responses through degranulation and release of pro-inflammatory cytokines (Krystel-Whittemore *et al.*, 2016). In healing tendons, an elevated mast cell concentration has been observed in a variety of contexts, including overused rat calcaneal tendons (Pingel *et al.*, 2013), tendinopathic human patellar tendon biopsies (Behzad *et al.*, 2013; Scott *et al.*, 2008), injured rabbit flexor tendons (Berglund *et al.*, 2010) and other human tendons (Del Buono *et al.*, 2011; Jomaa *et al.*, 2020).

The role of mast cells in tendon healing has not been fully elucidated. While mast cells can stimulate fibroblast proliferation and collagen deposition as well as mediate wound healing (Garbuzenko *et al.*, 2002), other studies using conditioned media have

suggested that mast cells may stimulate the release of excessive pro-inflammatory proteins (COX-2, PEG2) resulting in reduced type I procollagen production by tenocytes (Behzad *et al.*, 2013). Despite these data, it is generally accepted that mast cells do play a role in collagen turnover; however, this has not yet been shown specifically in the context of tendon inflammation and healing (Alim *et al.*, 2020). While the role of mast cells in tendon collagen deposition remains unclear, treatment of injured mouse patellar tendons with sodium cromolyn (a mast cell inhibitor) improves tendon collagen organisation and reduces hypercellularity during healing *in vivo* (Sharma *et al.*, 2011). Furthermore, mast cells are also implicated in neurogenic inflammation and pain associated with tendinopathy, since mast cells produce glutamate receptors and can, thus, communicate with the peripheral nervous system (Alim *et al.*, 2017; 2020). Indeed, larger numbers of degranulating mast cells and mast cells expressing the glutamate receptor NMDA-1 have been reported in rat tendon healing (Alim *et al.*, 2017).

Eosinophils

Eosinophils are granulocytic innate immune system cells that can be found both circulating in the blood and resident in the lamina propria of the gastrointestinal tract (Rosenberg *et al.*, 2013). Eosinophils have a known role in fighting parasitic, bacterial and viral infections. They are also involved in thrombosis, plaque formation, inflammatory bowel diseases and gastrointestinal diseases (Rosenberg *et al.*, 2013).

To date, there is very limited evidence for eosinophils activity in the context of tendon healing and disease as these cells are rarely present in chronically inflamed tendons (Jomaa *et al.*, 2020). However, high levels of eosinophils in the blood are associated with eosinophilic fasciitis, which is a connective tissue disorder that is characterised by tendon retraction, subdermal sclerosis and joint contraction (Das *et al.*, 2017). Also, eosinophils can stimulate ECM contraction and may interact with mesenchymal cells to promote ECM remodelling (Zagai *et al.*, 2004). Therefore, data suggests that eosinophils might play a role in tendinopathies that is worth further investigation.

Platelets

Platelets are anuclear, discoidal cells that are derived from megakaryocytes (Thon and Italiano, 2012). These cells function in haemostasis, host defence, tissue repair and resolution of inflammation (van der Meijden and Heemskerk, 2019). In general, most of the research on platelets for tendon healing focused on the therapeutic potential of PRP delivery, rather than studies of native platelet function in tendon healing. The beneficial activity of PRP is thought to derive from the high concentration and enrichment of platelets, which harbour growth factors and cytokines that promote regenerative healing responses. PRP delivery was shown to ameliorate tendon inflammation and promote regenerative tendon healing (Andia *et al.*, 2018; Chen *et al.*, 2012; de Almeida *et al.*, 2012; de Vos *et al.*, 2010; Nishio *et al.*, 2020; Solchaga *et al.*, 2014; Virchenko and Aspenberg, 2006). These results have been observed in both Achilles and patellar tendon injuries in mice, rats and humans (de Almeida *et al.*, 2012).

The specific mechanism of action of PRP in the context of tendon remodelling is still being investigated. So far, it has been shown that cell morphology, cellularity, vascularity and collagen arrangement are improved in injured patellar tendons compared to controls with PRP administration (Nishio *et al.*, 2020). Moreover, PRP increases macrophage infiltration in injured patellar tendons, although different PRPs appear to recruit different subtypes of macrophages (Nishio *et al.*, 2020). Notably, PRP effects on tendon healing may depend in part on mechanical loading, since tendon unloading by botulinum toxin-induced paralysis leads to decreased transverse area and reduced mechanical properties (Virchenko and Aspenberg, 2006). However, independent of loading, tendon stem cells and platelets from PRP treatments appear to work synergistically to promote tendon healing (Chen *et al.*, 2012). One limitation to PRP treatment is the variability in PRP formulations and the undefined nature of PRP itself. Therefore, it is not surprising that clinical outcomes have been mixed (Bianco *et al.*, 2019; Halpern *et al.*, 2012).

Dendritic cells

Dendritic cells are crucial immune system cells that have important functions in both the innate and adaptive immune response. Dendritic cells act as phagocytic innate cells; however, as they mature, they acquire antigen-presenting abilities and link the innate immune system to the adaptive immune system by activating T cells (Mellman and Steinman, 2001).

Despite their importance in innate and adaptive immunity, dendritic cells are seldom studied in tendon healing. In Achilles tendons and their associated popliteal lymph nodes, dendritic cells accumulate 1 week post injury, peaking at 2 weeks post injury (Noah *et al.*, 2020). Dendritic cells were also found in chronically tendinopathic human samples (Kendal *et al.*, 2020). The functional requirement for dendritic cells in tendon healing and whether dendritic cells promote or resolve inflammation after tendon injury remain open questions.

Adaptive immune system cells in tendinopathy

In contrast to the innate immune response, which broadly targets pathogens, the adaptive immune response targets specific antigens (Chaplin, 2010). Adaptive immunity toward unique external molecules

depends on the interaction between the antigen and receptors on T and B lymphocytes, which form through somatic gene rearrangement (Chaplin, 2010). Therefore, a vast repertoire of T and B cell receptors can be produced that are highly specific for unique antigens and create immunological memory after exposure to a particular pathogen (Chaplin, 2010).

Although historically less studied in the context of wound healing, there is growing appreciation for the role of adaptive immune system cells in regulating inflammation immune system cells and in directly activating resident cells after injury. Studies in muscle, for example, have revealed a requirement for Tregs in muscle regeneration through stimulation of resident satellite cells (Burzyn *et al.*, 2013; Cho *et al.*, 2019). Other T cell subpopulations (such as Th1 and Th2 helper T cells) have also been implicated in poor or regenerative healing across various musculoskeletal tissues (Bozec *et al.*, 2014; Burzyn *et al.*, 2013; Gyarmati *et al.*, 1983; Horowitz *et al.*, 1984; Li *et al.*, 2007). While this is still an emerging area in tendon research, the potential roles of adaptive immune system cells (T cells, B cells and NK cells) in tendon disease and healing are highlighted in Fig. 2. Characteristic markers identifying T and B cells are indicated in Table 1.

T cells

$CD3^+$ T cells are lymphoid cells with distinctive subtypes, including $CD8^+$ cytotoxic T cells and $CD4^+$ T cells. Cytotoxic T cells act primarily to kill cells infected by intracellular microbes (Chaplin, 2010). Notably, tendon healing has been previously shown to be unaffected by $CD8^+$ cell depletion in rats, although these cells appear to be important for cancellous bone healing (Bernhardsson *et al.*, 2019). $CD4^+$ T cells include several helper T cells (such as Th1, Th2, Th17 and others) as well as Tregs. Unlike macrophages, which are defined based on cell surface markers, helper T cell subpopulations are defined by well-established transcription factors (Table 1). Similar to macrophages, T cell subpopulations can also be classified as pro- or anti-inflammatory. In general, Th1 and Th17 cells are associated with inflammation while Th2 and Treg cells resolve or suppress inflammation (Biton *et al.*, 2016; Rankin *et al.*, 2010).

Most studies in tendon research are descriptive characterisations of T cells, their sub-types and temporal dynamics. After tendon injury, T cell recruitment has been observed as early as 3-7 d (Noah *et al.*, 2020; Wojciak and Crossan, 1993). Analysis of $CD4^+$ T cells shows peak presence in mouse Achilles tendons 2 weeks after injury and repair, while $CD8^+$ T cells continue to accumulate at 4 weeks (Noah *et al.*, 2020). In rats, $CD4^+$ T cells are elevated in the flexor tendon synovial sheath and epitenon 3 d post crush injury (Wojciak and Crossan, 1993). The mechanical loading environment may also be a regulator of T cell recruitment as Botox-induced paralysis after tendon transection results in the absence of Tregs by 10 d post-injury compared to loaded samples (Blomgran *et al.*, 2016). Similar to animal injury models, T cells are also elevated in human tendinopathic tissues, suggesting a role in disease progression (Kragsnaes *et al.*, 2014; Millar *et al.*, 2010; Schubert *et al.*, 2005). Intriguingly, recent studies using *in vitro* co-culture systems have revealed a positive inflammatory feedback loop between tenocytes and T cells, although the T cell subtype was not determined (Garcia-Melchor *et al.*, 2021). On the other hand, other studies have surprisingly concluded that T cell numbers are insignificant in injured and control human tendons (Gotoh *et al.*, 1997; Scott *et al.*, 2008).

The accumulation of T cells during tendon injury and disease suggests a role in healing but there are few mechanistic studies that directly test the requirement for T cells or T cell subpopulations. Jomaa *et al.* (2020) suggested that excessive recruitment of T cells to injured tendons might lead to ECM damage, which occurs in autoimmune disorders. In contrast, cell culture studies showed that $CD4^+$ T cells and T cell-derived cytokines such as IL2, TGFβ and IL1 regulate epitenon cell proliferation, adhesion and ECM production (Wòjciak and Crossan, 1994). Since $CD4^+$ T cells were not characterised in this study and potentially comprise both pro-inflammatory and anti-inflammatory subpopulations, it is not clear which T cells are driving these responses. While these studies suggested a pathological role for T cells in tendon healing, Tregs are required for tendon regeneration in neonatal mice, as depletion of Tregs results in poor structural and functional healing. In contrast to adult Tregs, neonatal Tregs facilitate regeneration, in part by polarising macrophages from a pro- to anti-inflammatory profile (data not published). Adoptive transfer of neonatal Tregs into adult hosts results in improved adult macrophage polarisation leading to functional recovery. Indeed, different injury models have also shown that IL33, which promotes Treg expansion, has a protective effect in a variety of tissues, although it can be pathological as well (Li *et al.*, 2019; Liew *et al.*, 2016).

B cells

B cells are characterised by the production of Ig either in a transmembrane form (B cell receptors) or secreted form (antibodies), following activation with either a T-cell-dependent or independent mechanism. Mature B cells can exist in the form of plasma cells or memory cells. Plasma cells actively produce antibodies when they encounter an antigen, while memory cells are stored for future antigen encounters. When this occurs, they convert to plasma cells and quickly start producing antibodies against the foreign molecule.

To date, there are almost no studies on B cells in tendon research. Despite a handful of studies showing B cell accumulation in some animal models of tendon injury and human tendon disease tissues, their function in healing remains completely

unknown (Noah *et al.*, 2020; Schubert *et al.*, 2005). In other tissues such as skin, B cell subsets can drive or suppress inflammation and interact with T cells, while application of mature B cells enhances regenerative healing (Debes and McGettigan, 2019). Additional studies will be required to determine temporal dynamics of B cells in tendon healing, as well as mechanistic function (if any).

NK cells

Although NK cells are part of the lymphoid lineage, they do not have antigen-specific receptors. Rather, NK cells have inhibitory receptors whose main function is to mediate killing of cells that have downregulated MHC-I proteins on their surface. This is evolutionarily advantageous since viruses often reduce the production of MHC-I in infected cells (Chaplin, 2010). Although NK cells have been found in chronically inflamed human Achilles tendons, their role in inflammation and tendon healing has not been determined (Kragsnaes *et al.*, 2014).

Discussion

The immune response is a critical driver of tendon healing and pathology; however, the immune system cells that promote and modulate inflammation in these contexts are poorly characterised. While much of the existing research focused on macrophages, given their importance in inflammation, there is a vast array of other immune cell types that likely also play important and distinctive roles. One challenge in reconciling different studies is the variability in animal injury models (in terms of injury severity, anatomical tendon targeted and species) as well as immune cell markers and methodology used (flow cytometry compared to immunohistochemistry for example), which may result in different temporal dynamics reported or conflicting interpretations. In terms of clinical samples, there are additional confounding factors such as painful symptoms that may not be necessarily correlated with structural hallmarks of degeneration. The studies by Dakin *et al.* (2015) clearly show that distinctive immune system cells and activated immune pathways can distinguish patients experiencing pain.

In addition to immune modulation, specific immune system cells may also directly interact with resident tendon cell types such as tenocytes, epitenon cells or resident stem/progenitor cells. In other tissues, such as muscle, regeneration depends in part on factors secreted from T cells that directly activate muscle satellite cells (Kuswanto *et al.*, 2016). The interactions between immune system cells and resident cells have largely focused on immune regulation [such as the pro-inflammatory feedback loop between the cells that may drive a degenerative cascade, as reported by Garcia-Melchor *et al.* (2021)] but immune system cells may also be the source of tenogenic growth factors such as TGFβ ligands, which have been implicated in both fibrotic and regenerative tendon healing (Kaji *et al.*, 2020; Katzel *et al.*, 2011). Resident cell types may also respond to the same immune signal in different ways. Inflammation, for example, may induce proliferation of scar-forming cells while inhibiting resident stem cells or inducing aberrant differentiation. Finally, while the present review focused on individual immune cell types and known findings for each cell type in tendon healing, the immune landscape is likely driven by complex interactions between multiple immune cell populations that change across time. The growing use of sophisticated technologies such as single-cell RNA sequencing will allow interrogation of multiple immune cell populations at once.

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Discussion with Reviewer

Reviewer: The review includes data from both tendinopathy and tendon injury/tenotomy models. Do you think that the processes in a freshly ruptured tendon are similar to those in a tendon affected by a tendinopathy in terms of immune cell involvement and temporal dynamics?
Authors: Acute rupture of healthy tendon by laceration injury is likely distinctive from the progressive degeneration that characterises a tendinopathy. The immune response to a laceration injury is likely more intense in early stages but perhaps regulation of immune system cells and the interactions between immune system cells and resident tendon cells will follow similar mechanisms. It may be that the immune landscape between acute rupture and tendinopathy will be more similar once the tendon has healed but maintains some level of chronic inflammation, which was found in an adult Achilles tendon transection model (unpublished data from our the authors' laboratory). Since there is no perfect animal model for human tendinopathy, it is a very challenging (but important) question to tackle.

Reviewer: Given the authors now have an excellent overview of the current literature on immune system cells in healthy and diseased tendons, which cell type do they consider the most promising target for new therapies to treat diseased tendons and why?
Authors: In researching for the present review, what was most striking was how little we understand the role of specific immune system cells in driving tendinopathy and tendon healing. While macrophages are an appealing target given their role in both inflammation and its resolution, emerging research suggests important interactions between T cell populations and macrophages. More basic science studies are needed in this area before we can focus on therapeutic targets since there will likely be unforeseen effects on different immune system cells.

Reviewer: Can the authors comment on the ongoing debate of tendinopathy being an inflammatory disease *versus* it being a mechanically degenerative one with inflammation being a secondary event?
Authors: We think these two factors are likely very challenging to uncouple. Certainly, mechanical overuse could induce degenerative cascades but inflammation could also be the first degenerative event that precedes any obvious damage to the ECM. Given that there are resident immune system cells and there are studies that show immune system cells can also be mechanoresponsive, it is entirely possible these cells can respond to loading. There is also evidence that systemic inflammation occurs with ageing, and this could be yet another immune factor that induces degeneration-absent overuse. Once initiated, mechanical and immune factors likely create a degenerative feedback loop that exacerbates the condition. Have immune system cells been traditionally overlooked because they represent such a small proportion of cells even in acute injury models? However, from the depletion studies we have carried out (macrophage and Treg depletion), it is clear they are functionally important in the healing process and exert an outsized role relative to their numbers in the injury environment.

Reviewer: Can the authors comment on the most suitable animal model to mimic a tendinopathy?
Authors: Honestly, we are not sure. Our lab relies on acute tendon injury models, which are likely the least representative of tendinopathy, a much more gradual process often associated with age. Other existing models induce tendinopathy through mechanical overloading [Dr Sowslowsky's downward treadmill running induced rotator cuff model, Dr Flatow and Dr Andarawis-Puri's patellar tendon fatigue loading model or other models (collagenase or TGFβ injections)]. There is value in all these models and by combining knowledge from various models we can arrive at some consensus.

Reviewer: The authors note that one challenge in comparing different studies is the variability in animal injury models as well as immune cell markers and analytic methods used. What approach would the authors suggest being preferably used to obtain data that can be more easily compared between different research groups?
Authors: Immunostaining for specific immune system cells has been quite challenging (except for F4/80 staining for macrophages). Additional challenges are the rarity of certain immune cell populations and their temporal response. Flow cytometry and single-cell RNA sequencing are likely the gold standards in terms of immune cell phenotyping, despite not providing spatial information. It certainly would be very helpful to have consensus in the field in terms of timepoints and markers.

Editor's note: The Scientific Editor responsible for this paper was Juerg Gasser.