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Tenascin-C in fibrosis in multiple organs: translational implications

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ABSTRACT

Systemic sclerosis (SSc, scleroderma) is a complex disease with a pathogenic triad of autoimmunity, vasculopathy, and fibrosis involving the skin and multiple internal organs (*1*).Because fibrosis accounts for as much as 45% of all deaths worldwide and appears to be increasing in prevalence (*2*), understanding its pathogenesis and progression is an urgent scientific challenge. Fibroblasts and myofibroblasts are the key effector cells executing physiologic tissue repair on one hand, and pathological fibrogenesis leading to chronic fibrosing conditions on the other. Recent studies identify innate immune signaling via toll-like receptors (TLRs) as a key driver of persistent fibrotic response in SSc. Repeated injury triggers the *in-situ* generation of "damage-associated molecular patterns" (DAMPs) or danger signals. Sensing of these danger signals by TLR4 on resident cells elicits potent stimulatory effects on fibrotic gene expression and myofibroblast differentiation triggering the self-limited tissue repair response to self-sustained pathological fibrosis characteristic of SSc. Our unbiased survey for DAMPs associated with SSc identified extracellular matrix glycoprotein tenascin-C as one of the most highly up-regulated ECM proteins in SSc skin and lung biopsies (*3*, *4*). Furthermore, tenascin C is responsible for driving sustained fibroblasts activation, thereby progression of fibrosis (*3*). This review summarizes recent studies examining the regulation and complex functional role of tenascin C, presenting tenascin-TLR4 axis in pathological fibrosis, and novel anti-fibrotic approaches targeting their signaling.

INTRODUCTION

Fibrosis commonly results from a wound healing response to repeated injury or tissue damage, irrespective of the underlying etiology (*5*). Excessive accumulation of collagen-rich extracellular matrix (ECM) in fibrosis eventually results in disrupted tissue architecture and frequently causes significant organ dysfunction. The fibrotic process in SSc is easily recognized in the skin, but fibrosis affecting the internal organs contributes to high mortality (*1*). Importantly, as fibrosis progresses, it leads to further tissue damage and inflammation, generating endogenous damage-associated molecular patterns (DAMPs) resulting in a positive feedback loop (*4, 6*).

Our laboratory is interested in dissecting the cellular and molecular networks underlying self-sustaining fibroblast activity in SSc and the impact of innate immune signaling in initiating and maintaining this process. We demonstrated that tissue damage that occurs from chronic injury causes local generation and accumulation of endogenous DAMPs which in turn activate innate immune signaling in resident fibroblasts through TLR4 (*3, 4, 6-8*). This results in enhanced matrix production and TGF-β secretion, establishing a self-amplifying vicious cycle of fibrosis (*4, 9*). Our unbiased immunofluorescence analysis for DAMPs associated with SSc identified tenascin-C as one of the prominent ECM proteins in SSc skin and lung biopsies as well as in circulation (*3, 9*). Several earlier reviews have summarized the structure, regulation, splice variants, expression patterns, and functions of tenascin-C (*10*). This review aims to highlight the current understanding of the functional role of tenascin C in fibrosis, present recent views of the tenascin-C-TLR4 axis in fibrosis, and discuss the potential therapeutic strategies targeting tenascin-C.

TENASCIN-C STRUCTURE, FUNCTION, AND REGULATION

The human tenascin-C is a multifunctional hexameric extracellular matrix (ECM) glycoprotein comprises four domains: a tenascin assembly (TA) domain, epidermal growth factor-like (EGF-L) repeats, up to 17 FNIII-like repeats, and a fibrinogen-like globe (FBG) domain (*11*). Each of these domains can interact with a different subset of binding partners, including cell surface receptors and other extracellular components. Tenascin-C gene is on chromosome 9q33 and contains 29 exons of which 9 (each coding for a fibronectin type III domain) can be alternatively spliced. Alternative splicing results in several different forms of tenascin-C, containing variable numbers of FN III repeats. Eight of the FNIII repeats are constitutively expressed (FNIII 1–8), and 9 can be alternatively spliced (FNIIIA1-D) (*10*). Tenascin-C FN III domain has a high affinity towards many growth factors including vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), TGF-β, and insulin-like growth factor binding proteins (IGFBPs) (*12*). Tenascin-C FBG binds to integrins and activates TLR4 (*13*, *14*). This enables tenascin-C to involve in a wide range of functions including cell migration, cell attachment, cell spreading, focal adhesion, cell survival, matrix assembly, and proinflammatory cytokine synthesis. An overview of factors regulating tenascin-C expression is presented in Giblin et al (*11*).

Normally tenascin-C is under tight spatial and temporal regulation, with prominent expression during embryogenesis but restricted in most healthy adult tissues, and transient re-expression during wound healing and dynamic tissue remodeling. In contrast, persistent tenascin-C

accumulation occurs in response to a chemical or mechanical injury in a variety of chronic pathological conditions such as cancer, rheumatoid arthritis (RA), and fibrosis (*10*).

Profibrotic signals induce tenascin C

Tenascin-C expression is induced by a variety of inflammatory cytokines, growth factors including TGF-β and PDGF, oxidative stress, as well as integrins and mechanical stress (*15*). Signaling pathways that lead to activation of various transcription factors, including TCF/LEF, NfκB and c-Jun, Ets and Sp1, and Prx-1, have been shown to induce tenascin-C transcription (*15*). In human dermal fibroblasts, TGF-β induced tenascin-C transcription via Smad3/4, Sp1, Ets1, and CBP/p300 (*16, 17*). On the other hand, platelet-derived growth factor (PDGF) regulates tenascin-C gene expression via PI3K/AKT signaling (*15, 18, 19*). In murine cardiac fibroblasts, tenascin-C expression was induced by angiotensin II, TGFβ1, and PDGF (*20*). In chicken embryonic fibroblasts, PDGF and TGF-β act in an additive manner with tensile strain to promote tenascin-C transcription and thus an increase in these factors might indirectly stimulate tenascin-C production in response to mechanical load (*20*). In contrast, anti-inflammatory steroid hormones glucocorticoids potently down-regulates tenascin-C expression in bone marrow stromal cells (*15*). A zinc finger transcription factor GATA6 is another repressor of human tenascin-C transcription (*21*). Exogenous expression of GATA6 in dermal fibroblasts negatively modulated tenascin-C expression by binding to GATA site within the tenascin-C promoter and inhibited its induction by IL-4 and TGF-β (*21*). Paired-related homeobox 1 (Prx1) and Prx2 transcription factors induce tenascin-C gene transcription by interacting with a homeodomain binding site (HBS) of tenascin-C promoter and focal adhesion kinase (FAK) (*22-25*).

TENASCIN-C IN FIBROSIS REGULATION: INSIGHTS FROM ANIMAL MODEL

Various studies highlighted the deleterious role of tenascin-C in promoting tissue fibrosis in animal models of disease. For instance, the contribution of tenascin C to liver fibrosis was demonstrated by El-Karef and colleagues in immune-mediated hepatitis model, induced by intravenous injections of concanavalin A where tenascin C KO mice showed less fibrotic and inflammatory responses compared to wild type (WT) littermates (*26*). Moreover, recombinant tenascin-C treatment enhanced hepatic stellate cells (HSC) migration and increased type I collagen production via TGF-β1 and α9β1 integrin signaling (*27*). Therefore, tenascin-C can promote liver fibrosis by augmenting the inflammatory and fibrotic responses by upregulating cytokines, hepatic stellate cells recruitment, and TGF-β expression during the progression of hepatic fibrosis (*26*). Moreover, tenascin-C deficiency is accompanied by improved liver regeneration and reduction in liver damage after hepatic ischemia/reperfusion injury (IRI) probably as a result of impaired leukocyte recruitment and decreased expressions of interleukin IL-1β, IL-6, and CXCL2 and matrix metalloproteinase-9 (MMP-9) (*28*).

In acute lung injury (ALI) model induced by intratracheal bleomycin instillation, tenascin C was significantly induced in alveolar septal walls of damaged tissues of WT mice triggering fibrosis. In contrast, tenascin-C KO mice are protected from interstitial fibrosis with reduced lung collagen and myofibroblasts differentiation and reduced intranuclear Smad2/3 suggesting impaired TGF-β activation in tenascin-KO mice (*29, 30*).

Tenascin-C is upregulated in kidney injury/renal fibrosis model induced by unilateral ureteral obstruction (UUO) and ischemia/reperfusion injury (IRI) (*31*). Interestingly, tenascin-C was predominantly localized in the renal interstitium and is enriched at the focal sites of kidney fibrosis, creating a unique microenvironment in the fibrotic kidney. Furthermore, tenascin-C-enriched ECM scaffold acts as a fibrogenic niche that favors fibroblast expansion, and depletion of tenascin-C attenuates fibroblast expansion *ex vivo* and reduces kidney fibrosis *in vivo (29)*. Altogether, these studies establish a major role of tenascin-C in organizing fibrogenic niche to provide a favorable microenvironment for fibroblast activation and proliferation in kidney fibrosis. In contrast, in snake venom-induced model of proliferative glomerulonephritis, tenascin-C KO mice showed impaired renal regeneration, with increased proteinuria and scar tissue formation, decreased mesangial cells proliferation, and increased fibrotic gene expression suggesting a contradictory role of tenascin-C in this context (*32*).

During myocardial tissue repair, interstitial fibroblasts in the vicinity of the injured cardiomyocytes are the major source of tenascin-C (*33*). Tenascin-C synthesized by interstitial cells at an early phase induces differentiation to myofibroblasts and promotes migration into damaged areas in an autocrine and paracrine fashion. In rat myocardial infarction model, tenascin-C is expressed in interstitial fibroblasts within 24 hours of coronary ligation and disappears by day 14 (*34*). During the healing process, α-smooth muscle actin (α-SMA)-positive myofibroblasts appeared to colocalize with tenascin C in the damaged myocardium. Electrical injury to the myocardium demonstrated normal myocardial repair in tenascin-C KO mice with delayed appearance of myofibroblasts in KO (*35*). In angiotensin II-induced cardiac fibrosis model, tenascin-C deficiency blunted the development of fibrosis (*36*). The authors demonstrated enhanced inflammatory and fibrotic responses by tenascin-C along with accelerated macrophage

migration and increased production of proinflammatory/profibrotic cytokines and integrin αVβ3/FAK-Src/NF-κB. On the other hand, tenascin-C deficiency exacerbated fibrosis, rather than attenuated, in angiotensin-induced model of cardiac hypertrophy by promoting rapid accumulation of CCR2þ/Ly6Chimonocyte/macrophage subset into the myocardium (*37*). These contradictory findings may be due to the difference in genetic background of the mouse strains or the type of injury. In a chronic pressure overload-induced left ventricular fibrosis model in mice, lack of tenascin-C is coupled with a marked reduction in fibrosis and cardiac hypertrophy (*38*). Tenascin-C has context-dependent diverse functions and, thus, may exert both harmful and beneficial effects in damaged hearts. However, tenascin-C appears to deteriorate adverse ventricular remodeling by proinflammatory and profibrotic effects in most cases. Recently Saori Yonebayashi et al generated a transgenic mouse overexpressing tenascin-C in the heart in embryos and adults which did not show any distinct histological or functional abnormalities (*39*). However, the expression of proinflammatory cytokines/chemokines and the mortality rates were significantly up regulated during the acute stage after myocardial infarction (*39*). Thus tenascin- C overexpressing Tg mice may be applied to investigate the effect of tenascin-C overexpression in several tissue/organ pathologies.

Tenascin-C regulation in skin aging

Although tenascin-C is implicated in collagen synthesis and TGF-β signaling in fibroblasts, the role of tenascin-C during aging has not been studied until recently. In this study the authors showed that the tenascin-C levels were significantly reduced in skin tissues from aged mouse (24 months old) compared with young mice (3 months old) (*40*). Consistently, tenascin-C expressions were reduced in sun-protected elderly females (buttock skin from aged adult >72

years old) compared to young individuals (aged 30-35 years old). Moreover, the expression of procollagen I levels in sun-protected skin tissues gradually decreased during the intrinsic aging process. Based on these findings, the authors concluded that tenascin-C contributes to maintain ECM integrity to prevent skin aging (*40*). As this study is based on the results from only three elderly females, further research on this matter is crucial to conclude on this important issue.

DOES ABERRANT TENASCIN-C EXPRESSION OR FUNCTION HAVE AN IMPORTANT ROLE IN FIBROSING DISEASES?

To date, the evidence linking tenascin-C expression and human fibrotic disorders are largely correlative. A meta-analysis of three distinct transcriptome datasets comprising skin biopsies from a total of 80 SSc patients and 26 healthy controls showed significantly elevated tenascin-C mRNA in SSc skin biopsies mapping to the previously-defined inflammatory intrinsic gene expression subsets compared to healthy controls (*3, 41*). Notably, among skin biopsies classified as inflammatory (78% of the total in this group), tenascin-C levels showed a strong correlation with the skin score (r=0.73, p=0.03, Spearman's rank correlation).

Tenascin-C protein levels are elevated in SSc skin, explanted skin fibroblasts from SSc, and in circulation (Figure 1) (*3*). The antibodies used in these studies specifically detected the FNIII-B and FNIII-C epitopes of the large tenascin-C isoforms. This finding was consistent with another observation where serum from both limited and diffuse cutaneous SSc (lcSSc and dcSSc) patients showed significantly higher tenascin-C FNIII-C isoform (*42*). Importantly, pulmonary fibrosis showed higher prevalence in patients with elevated serum levels, and in patients with

pulmonary hypertension and heart failure (*42, 43*). In SSc lung biopsies, Tenascin-C showed increased expression around the distal airways of SSc lung biopsies with a subepithelial distribution compared to normal lung (*43*). Elevated levels of tenascin-C have been observed in the sera of patients with cryptogenic pneumonia as well as in bronchoalveolar lavage fluids from patients with usual interstitial pneumonia, sarcoidosis, and other fibrotic lung diseases (*43-46*). These studies highlighted above implicate tenascin-C as a possible biomarker of skin and lung fibrosis in SSc and related diseases.

Increasing numbers of studies have examined the clinical utility of elevated serum tenascin-C levels in patients with atrial fibrillation, dilated cardiomyopathy, diabetes mellitus, rheumatic heart disease, and congenital heart disease, and various cardiomyopathies (*47-55*). Further study highlights the impact of tenascin-C B variants as biomarker reflecting the extent of cardiac remodeling in heart failure patients (*56, 57*). Most of the cardiac studies associate high serum tenascin-C levels with poor prognosis or increased rates of adverse cardiovascular events and mortality (*58, 59*). In a multivariate analysis, tenascin-C splice variant B variant, but not C, was found to be an independent predictor of pulmonary hypertension (*60*).

Elevated tenascin-C is seen in the kidney biopsies, serum, and urine levels in patients with chronic kidney disease (CKD) (*55, 61, 62*). Moreover, urinary tenascin-C levels were correlated with the severity of kidney dysfunction and fibrosis. In diseased kidney tissues, the tenascin-C protein was primarily localized in the tubulointerstitium with sparse staining in the glomeruli. A mechanistic study further demonstrated the involvement of αvβ6 integrin and activated FAK in renal tubular cells (*61*). This study underscores tenascin-C as a pathogenic mediator impairing tubular integrity and a noninvasive biomarker of kidney fibrosis.

TENASCIN-C-TLR4 -DEPENDENT RESPONSES IN INFLAMMATORY AND FIBROTIC DISEASES

Danger signals that are generated in response to chronic tissue injury contribute to the progression of many autoimmune diseases via TLR4 activation. This notion is consistent with previous studies showing that tenascin-C mediates persistence of synovial inflammation and tissue destruction in arthritis (*63*). Midwood et al showed that tenascin-C induced cytokine synthesis in synovial fibroblasts and macrophages via activation of TLR4 by the C-terminal FBG domain (*63, 64*). Intra-articular injection of the FBG domain in WT mice triggers synovial inflammation, along with cartilage and bone destruction, but not in TLR4 null mice (*63*).

In addition, tenascin-C KO mice showed rapid resolutionof acute joint inflammation and are protected from erosive arthritis (*63*).

To explore the mechanism of activation of TLR4 by this endogenous ligand, the authors used peptide mapping and systematic mutagenesis of the FBG domain to identify 3 distinct sites within this part of the tenascin-C molecule that contributed to TLR4 activation, including a cationic ridge on the surface of the domain which was essential for FBG-TLR4 interaction in a solid phase binding assay, induction of cytokine synthesis in primary human macrophages and macrophage cell lines in vitro, and for the induction of synovial inflammation in vivo, following intra articular injection into mice (*64*).

Early hints suggested that FBG mediated activation of TLR4 occurs via a different mode of action than activation of this receptor by the pathogenic ligand LPS. For example, FBG-induced cytokine synthesis occurs independently of the canonical co-receptors CD14 and MD2, that are necessary for LPS-TLR4 binding (*63*). Further examination of the TLR4-dependent signaling

pathways and biological readouts elicited in macrophages by the tenascin-C fibrinogen-like globe (FBG) domain revealed commonalities between those elicited by lipopolysaccharides (LPS) but also some intriguing differences (*13*), that may be explained by the different co-receptor requirements for each ligand. Interestingly, TLR4 activation elicited by LPS and FBG generated two distinct macrophage phenotypes. FBG promoted a profibrotic macrophage phenotype, whereas LPS promoted a matrix-degrading phenotype. For example, whilst both ligands induced the expression of a wide range of collagen genes, each ligand drives a different subset of gene expression, with FBG inducing COL2A1, COL8A1, COL23A1 and COL24A1 respectively. Moreover, only in macrophages activated with FBG was COL1, COL2 and COL3 phosphorylated, a post-translational modification that may stabilise de novo matrix. Finally, LPS activated macrophages were efficient at collagen degradation, mediated by specific up-regulation of collagen degrading proteases including MMP1 and MMP13, whilst FBG activated macrophages could not degrade collagen, but instead were more efficient at processing gelatin, consistent with higher levels of the gelatinase MMP14 (*13*). These findings provide evidence that different microenvironmental cues can elicit distinct biological responses via the same receptor. Whether TLR4 activation elicited by LPS versus DAMPs will generate similarly divergent responses in fibroblasts, remains an important unanswered question with relevance to fibrosis.

Both TLR4 and tenascin-C have been implicated in fibrosis across a number of disease states and tissues. For example, in maladaptive cardiac tissue remodeling such as MI, myocarditis, and dilated cardiomyopathy (*34, 53, 65-67*), tenascin-C exacerbates autoimmune myocarditis and inflammation and TLR4 deficiency terminated the effect (*68*). In human cardiac myofibroblasts, tenascin-C up-regulates IL-6 expression via interaction between FBG domain andTLR4 (*69*).In patients with diabetic kidney disease (DKD), TLR4 and tenascin-C levels are increased in renal tissues and tenascin-C levels are negatively correlated with the glomerular filtration rate (*62*). In high glucose-treated rat mesangial cells, tenascin-C, TLR4, and p-NF-κB p65 and miR-155-5p were increased, while tenascin-C knockdown reduced the levels, suggesting tenascin-C upregulated miR-155-5p expression through the TLR4/NF-κB p65 pathway to promote inflammation and fibrosis in DKD (*70-72*).

TENASCIN-C-TLR4-MEDIATED PERSISTENT ORGAN FIBROSIS IN SSC

As discussed in the earlier section that tenascin-C expressions are significantly elevated in both the affected tissue and circulation in SSc patients (*3, 42, 43*). We showed that tenascin-C eliciteda broad spectrum of profibrotic responses in normal fibroblasts (Figure 2) and in TLR4-deficient skin fibroblasts or in presence of TLR4 inhibitor, tenascin-C failed to elicit stimulation of fibrotic responses. These combined pharmacological and genetic approaches together establish asufficient and necessary role for tenascin-C-TLR4 in profibrotic cellular responses (*3*). Skin fibroblasts treated with TGF-ß and PDGF preferentially synthesize tenascin-C variant detected using an antibody against FN III-B domain, while SSc fibroblasts constitutively produce the same tenascin-C isoform (*3*). Additional experiments demonstrated the involvement of Smad2/3and PI3K-dependent intracellular signaling pathways underlying tenascin-C stimulation induced

by TGF-ß and PDGF respectively (*6, 15*). In this regard this is worth mentioning a recent publication where the authors revealed that tenascin family members (comprised of tenascin-C, tenascin-R and tenascin-W) share their ability to regulate latent TGF-β activation through their highly conserved C-terminal FBG domain (*73*). Herein, the authors demonstrated that latent TGF-β activation is a common feature of the tenascin family FBG-like domains that may regulate tissue homeostasis under healthy and pathological conditions. Although in our publicly available transcriptome datasets comprising skin biopsies from a total of 80 SSc patients and 26 healthy controls showed either undetected or no change in expressions of other tenascin family members except tenascin-C mRNA which showed significant elevated expressions in SSc skin biopsies (*3*).

We further explored the potential pathogenic role for tenascin-C in fibrosis *in vivo* using tenascin-C KO mice. Bleomycin treatment in WT mice and tenascin-C KO mice showed a progressive increase in both skin and lung fibrosis at day 15. However, tenascin-C KO mice showed attenuated fibrosis and enhanced fibrosis resolution in response to bleomycin from day 15 to day 24 (*3*). Based on our observations, we proposed that reduced TLR4 signaling in mice lacking tenascin-C accounts for accelerated resolution of fibrosis. Thus, tenascin C generated in injured microenvironments induces TLR4-mediated innate immune signaling might drive the persistent activation of fibrotic cells in tissues and underlies the switch from a self-limited repairresponse to non-resolving pathological fibrosis characteristic of SSc. Interestingly, fibroblast-specific ablation of TLR4 was found to protect mice from development and persistence of cutaneous and pulmonary fibrosis. While these findings reveal important disease-relevant differences in how fibroblasts vs. monocytes respond to TLR4 stimulation, and support a previously unrecognized inflammation-independent pathogenic

role of fibroblast TLR4 signaling, the role of TLR4 in fibrosis is cell type, organ, and injury specific (*8*).

Earlier studies demonstrated that full-length EGF-like repeats (EGFL) of tenascin-C induced prolonged vasoconstriction and EGFR inhibitor attenuated the effect (*74*). Importantly, EGFR

kinase inhibitor erlotinib protects mice from LPS-induced septic shock and death by selectively blocking TLR4 signaling implying that EGFR kinase activity is required for TLR4 signaling (*75-78*). These observations involving TLR4 and EGFR gain significance given clinical observations linking EGFR signaling to fibrosis and SSc. A multicohort analysis of SSc skin transcriptomes identified a 415-gene SSc signature with transcriptional profiles of 314 ligand stimulations across different cell lines showed a positive correlation with multiple EGFR ligands (*79*). These results together indicate a novel pathogenic role for EGFR signaling in SSc, possibly mediated via the EGFL domain of tenascin-C binding to EGFR and crosstalk with TLR4.

TENASCIN-C ALTERNATIVE SPLICING IN TISSUE REMODELING

A recent report provides insight into the disease-specific roles of tenascin-C alternative splicing (*80*). Tenascin-C small isoforms (containing only FnIII 1–8) are restricted to areas of cell differentiation during organogenesis, while large variants (containing one or more alternatively spliced FnIII repeat) are associated with regions of active tissue remodeling and cell migration. This group systematically examined the production of tenascin-C in immune and stromal cells and demonstrated how disease-associated tenascin-C isoforms selectively drive pro-inflammatory cell behavior, via removal of FnIII AD1 and AD2 domains with immuno-regulation (*25*). Interestingly, the proto-oncogene serine/arginine-rich splicing factor 6 (SRSF6) was shown to be an essential regulator of tenascin-C alternative splicing. Transgenic mice overexpressing SRSF6 in collagen-producing cells spontaneously develop SSc-like skin hyperplasia (*81*). This was accompanied by accumulation of the ‘‘large’’ tenascin-C isoform in the skin. Consistently, we observed that the expression of SRSF6 was highly elevated in SSc skin biopsies and levels correlated with tenascin-C accumulation (Bhattacharyya S and Varga J;

unpublished). Moreover, RNA sequencing indicated an increased abundance of alternatively spliced tenascin-C mRNA isoforms in SSc skin biopsies. While these intriguing observations implicate SRSF6 and alternative splicing of tenascin-C in the pathogenesis of skin fibrosis, further investigation of differential tenascin-C isoform expression and regulation in SSc and their role in pathogenesis are warranted. Moreover, the potential impact of tenascin-C spliced variants for risk stratification in patients' needs further investigation.

THERAPEUTIC STRATEGIES TARGETING TENASCIN-C

Several novel approaches for selectively targeting tenascin-C have been tried in cancer patients. The alternatively spliced domains of tenascin-C could be targeted using specific antibodies. For example, the F16 antibody targets the A1 domain of tenascin-C whereas the 81C6 antibody recognizes domain D (*82, 83*). Another D domain-specific novel human antibody R6N induced cancer regression in tumor-bearing mice, without showing signs of toxicity (*84*). A recent approach established human tenascin-C specific nanobodies that recognize an epitope in the constitutive FNIII domains thereby potentially detecting more tenascin-C isoforms for delivery of therapeutic compounds in the tissues with high tenascin-C content (*85*). All these studies provide a rationale for future clinical evaluation of tenascin-C-specific targeting. Another approach that generated dsRNA ATN-RNA against tenascin-C by using sequence homology, which in brain tumors and breast cancer cell lines significantly diminished the cell proliferation, migration, and reverses mesenchymal cells to epithelial cell phenotype (*86*). Accordingly, tenascin-C could be considered as the universal target, where its overexpression is usually associated with poor prognosis. Alternately, as TLR4 activation involves the terminal FBG

domain of tenascin-C, selectively targeting the FBG domain using specific monoclonal antibodies represents another promising antifibrotic therapy (*64*). Administration of anti-FBG monoclonal antibodies that neutralized FBG activation of TLR4, without impacting pathogenic TLR4 activation, to rats in collagen-induced arthritis inhibited disease progression and prevented joint damage (*87*). Therefore, the identification and targeting of the biological processes and effector molecules that are modulated by tenascin-C FBG domain may reveal new therapeutic opportunities for suppressing inflammation and fibrosis. Further studies on preclinical and clinical data underlying the therapeutic potential of targeting TLR4 DAMPs are warranted.

SUMMARY AND FUTURE PERSPECTIVE

Accumulating evidence supports the paradigm that activation of TLR4 in resident nonimmune cells within injured tissue microenvironments generated DAMPs, such as tenascin-C that are recognized as danger signals in SSc. Persistence of this DAMP-TLR4 signaling promotes progression of fibrosis (Figure 3). Although the precise contribution of tenascin-C driving sustained fibrosis in SSc remains unclear, results from both preclinical and clinical studies establish our premise that pharmacological targeting of tenascin-C-TLR4 pathway might have therapeutic potential to prevent the self-amplifying cycle of fibrosis. There is potential concern that disrupting TLR4 signaling might impair innate immune defenses against pathogens triggered via PAMP activation. However, since the tenascin C-mediated TLR4 activity is mostly pathogenic, and unlikely to be involved in anti-microbial host defenses, we anticipate that immunocompromise is unlikely to complicate such therapy for long-term. It will be crucial to elucidate if targeting selective TLR4 activation by tenascin-C is effective in SSc, or if it is more appropriate to target shared downstream signaling pathways common to many DAMPs. Several monoclonal antibodies against tenascin-C alternatively spliced domains, dsRNA, and nanobodies

have been attempted for therapeutic intervention. Alternately, targeting TLR4 interacting tenascin-C FBG domain-specific monoclonal antibodies represents another promising antifibrotic therapy. In the same way, targeting tenascin-C EGFL-EGFR interaction with TLR4

might also have therapeutic potential for SSc. Additional studies on differential tenascin-C isoform expression and their role in disease pathogenesis, and the potential impact of different tenascin-C isoforms for risk stratification in patients also need thorough investigation. In summary, this review implicated tenascin-C as TLR4 DAMP and its role in persistent tissue fibrosis in SSc and other forms of pathological inflammatory and fibrotic diseases and novel approaches for therapeutic intervention.

REFERENCES

1. Y. Allanore, O. Distler, Systemic sclerosis in 2014: Advances in cohort enrichment shape future of trial design. *Nat Rev Rheumatol* **11**, 72-74 (2015).
2. V. J. Thannickal, Y. Zhou, A. Gaggar, S. R. Duncan, Fibrosis: ultimate and proximate causes. *J Clin Invest* **124**, 4673-4677 (2014).
3. S. Bhattacharyya *et al.*, Tenascin-C drives persistence of organ fibrosis. *Nat Commun* **7**, 11703 (2016).
4. S. Bhattacharyya, J. Varga, Endogenous ligands of TLR4 promote unresolving tissue fibrosis: Implications for systemic sclerosis and its targeted therapy. *Immunol Lett* **195**, 9-17 (2018).
5. J. H. W. Distler *et al.*, Shared and distinct mechanisms of fibrosis. *Nat Rev Rheumatol* **15**, 705-730 (2019).
6. S. Bhattacharyya, K. S. Midwood, H. Yin, J. Varga, Toll-Like Receptor-4 Signaling Drives Persistent Fibroblast Activation and Prevents Fibrosis Resolution in Scleroderma. *Adv Wound Care (New Rochelle)* **6**, 356-369 (2017).
7. S. Bhattacharyya *et al.*, FibronectinEDA promotes chronic cutaneous fibrosis through Toll-like receptor signaling. *Sci Transl Med* **6**, 232ra250 (2014).
8. S. Bhattacharyya *et al.*, TLR4-dependent fibroblast activation drives persistent organ fibrosis in skin and lung. *JCI Insight* **3**, (2018).
9. S. Bhattacharyya, J. Varga, Emerging roles of innate immune signaling and toll-like receptors in fibrosis and systemic sclerosis. *Curr Rheumatol Rep* **17**, 474 (2015).
10. K. S. Midwood, M. Chiquet, R. P. Tucker, G. Orend, Tenascin-C at a glance. *J Cell Sci* **129**, 4321-4327 (2016).
11. S. P. Giblin, K. S. Midwood, Tenascin-C: Form versus function. *Cell Adh Migr* **9**, 48-82 (2015).
12. L. De Laporte, J. J. Rice, F. Tortelli, J. A. Hubbell, Tenascin C promiscuously binds growth factors via its fifth fibronectin type III-like domain. *PLoS One* **8**, e62076 (2013).
13. A. M. Piccinini, L. Zuliani-Alvarez, J. M. Lim, K. S. Midwood, Distinct microenvironmental cues stimulate divergent TLR4-mediated signaling pathways in macrophages. *Sci Signal* **9**, ra86 (2016).
14. L. Zuliani-Alvarez, K. S. Midwood, Fibrinogen-Related Proteins in Tissue Repair: How a Unique Domain with a Common Structure Controls Diverse Aspects of Wound Healing. *Adv Wound Care (New Rochelle)* **4**, 273-285 (2015).
15. F. Chiovaro, R. Chiquet-Ehrismann, M. Chiquet, Transcriptional regulation of tenascin genes. *Cell Adh Migr* **9**, 34-47 (2015).
16. M. Jinnin *et al.*, Tenascin-C upregulation by transforming growth factor-beta in human dermal fibroblasts involves Smad3, Sp1, and Ets1. *Oncogene* **23**, 1656-1667 (2004).
17. A. Mettouchi *et al.*, The c-Jun-induced transformation process involves complex regulation of tenascin-C expression. *Mol Cell Biol* **17**, 3202-3209 (1997).
18. M. Jinnin *et al.*, Upregulation of tenascin-C expression by IL-13 in human dermal fibroblasts via the phosphoinositide 3-kinase/Akt and the protein kinase C signaling pathways. *J Invest Dermatol* **126**, 551-560 (2006).
19. M. Jinnin *et al.*, Platelet derived growth factor induced tenascin-C transcription is phosphoinositide 3-kinase/Akt-dependent and mediated by Ets family transcription factors. *J Cell Physiol* **206**, 718-727 (2006).
20. K. S. Midwood, T. Hussenet, B. Langlois, G. Orend, Advances in tenascin-C biology. *Cell Mol Life Sci* **68**, 3175-3199 (2011).

21. A. Ghatnekar, M. Trojanowska, GATA-6 is a novel transcriptional repressor of the human Tenascin-C gene expression in fibroblasts. *Biochim Biophys Acta* **1779**, 145-151 (2008).
22. F. S. Jones, R. Meech, D. B. Edelman, R. J. Oakey, P. L. Jones, Prx1 controls vascular smooth muscle cell proliferation and tenascin-C expression and is upregulated with Prx2 in pulmonary vascular disease. *Circ Res* **89**, 131-138 (2001).
23. R. A. Norris, M. J. Kern, The identification of Prx1 transcription regulatory domains provides a mechanism for unequal compensation by the Prx1 and Prx2 loci. *J Biol Chem* **276**, 26829-26837 (2001).
24. R. A. Norris, M. J. Kern, Identification of domains mediating transcription activation, repression, and inhibition in the paired-related homeobox protein, Prx2 (S8). *DNA Cell Biol* **20**, 89-99 (2001).
25. D. M. McKean *et al.*, FAK induces expression of Prx1 to promote tenascin-C-dependent fibroblast migration. *J Cell Biol* **161**, 393-402 (2003).
26. A. El-Karef *et al.*, Deficiency of tenascin-C attenuates liver fibrosis in immune-mediated chronic hepatitis in mice. *J Pathol* **211**, 86-94 (2007).
27. J. C. Ma *et al.*, Tenascin-C promotes migration of hepatic stellate cells and production of type I collagen. *Biosci Biotechnol Biochem* **80**, 1470-1477 (2016).
28. N. Kuriyama, S. Duarte, T. Hamada, R. W. Busuttil, A. J. Coito, Tenascin-C: a novel mediator of hepatic ischemia and reperfusion injury. *Hepatology* **54**, 2125-2136 (2011).
29. W. A. Carey, G. D. Taylor, W. B. Dean, J. D. Bristow, Tenascin-C deficiency attenuates TGF-ss-mediated fibrosis following murine lung injury. *Am J Physiol Lung Cell Mol Physiol* **299**, L785-793 (2010).
30. S. Estany *et al.*, Lung fibrotic tenascin-C upregulation is associated with other extracellular matrix proteins and induced by TGFbeta1. *BMC Pulm Med* **14**, 120 (2014).
31. H. Fu *et al.*, Tenascin-C Is a Major Component of the Fibrogenic Niche in Kidney Fibrosis. *J Am Soc Nephrol* **28**, 785-801 (2017).
32. N. Nakao, N. Hiraiwa, A. Yoshiki, F. Ike, M. Kusakabe, Tenascin-C promotes healing of Habu-snake venom-induced glomerulonephritis: studies in knockout congenic mice and in culture. *Am J Pathol* **152**, 1237-1245 (1998).
33. K. Imanaka-Yoshida, Tenascin-C in cardiovascular tissue remodeling: from development to inflammation and repair. *Circ J* **76**, 2513-2520 (2012).
34. K. Imanaka-Yoshida *et al.*, Tenascin-C modulates adhesion of cardiomyocytes to extracellular matrix during tissue remodeling after myocardial infarction. *Lab Invest* **81**, 1015-1024 (2001).
35. M. Tamaoki *et al.*, Tenascin-C regulates recruitment of myofibroblasts during tissue repair after myocardial injury. *Am J Pathol* **167**, 71-80 (2005).
36. N. Shimojo *et al.*, Tenascin-C may accelerate cardiac fibrosis by activating macrophages via the integrin alphaVbeta3/nuclear factor-kappaB/interleukin-6 axis. *Hypertension* **66**, 757-766 (2015).
37. L. Song *et al.*, Bone Marrow-Derived Tenascin-C Attenuates Cardiac Hypertrophy by Controlling Inflammation. *J Am Coll Cardiol* **70**, 1601-1615 (2017).
38. B. K. Podesser *et al.*, Tenascin-C promotes chronic pressure overload-induced cardiac dysfunction, hypertrophy and myocardial fibrosis. *J Hypertens* **36**, 847-856 (2018).
39. S. Yonebayashi *et al.*, Generation of Transgenic Mice that Conditionally Overexpress Tenascin-C. *Front Immunol* **12**, 620541 (2021).
40. Y. E. Choi *et al.*, Effects of Tenascin C on the Integrity of Extracellular Matrix and Skin Aging. *Int J Mol Sci* **21**, (2020).
41. M. E. Johnson *et al.*, Experimentally-derived fibroblast gene signatures identify molecular

pathways associated with distinct subsets of systemic sclerosis patients in three independent cohorts. *PLoS One* **10**, e0114017 (2015).
42. K. Inoue *et al.*, Serum levels of tenascin-C in collagen diseases. *J Dermatol* **40**, 715-719 (2013).
43. M. Brissett, K. L. Veraldi, J. M. Pilewski, T. A. Medsger, Jr., C. A. Feghali-Bostwick, Localized expression of tenascin in systemic sclerosis-associated pulmonary fibrosis and its regulation by insulin-like growth factor binding protein 3. *Arthritis Rheum* **64**, 272-280 (2012).
44. K. Hisatomi *et al.*, Elevated levels of tenascin-C in patients with cryptogenic organizing pneumonia. *Intern Med* **48**, 1501-1507 (2009).
45. R. Kaarteenaho-Wiik *et al.*, Tenascin immunoreactivity as a prognostic marker in usual interstitial pneumonia. *Am J Respir Crit Care Med* **154**, 511-518 (1996).
46. R. Kaarteenaho-Wiik, P. Mertaniemi, E. Sajanti, Y. Soini, P. Paakko, Tenascin is increased in epithelial lining fluid in fibrotic lung disorders. *Lung* **176**, 371-380 (1998).
47. Y. Shiomi *et al.*, Serum tenascin-C levels in atrium predict atrial structural remodeling processes in patients with atrial fibrillation. *J Interv Card Electrophysiol* **59**, 401-406 (2020).
48. T. Yokokawa *et al.*, Significance of myocardial tenascin-C expression in left ventricular remodelling and long-term outcome in patients with dilated cardiomyopathy. *Eur J Heart Fail* **18**, 375-385 (2016).
49. B. Gellen *et al.*, Serum tenascin-C is independently associated with increased major adverse cardiovascular events and death in individuals with type 2 diabetes: a French prospective cohort. *Diabetologia* **63**, 915-923 (2020).
50. S. Yonebayashi, K. Tajiri, S. Li, A. Sato, Tenascin-C: an emerging prognostic biomarker in diabetes. *Ann Transl Med* **8**, 1699 (2020).
51. Z. Karatas, T. Baysal, F. Sap, H. Altin, H. Cicekler, The role of tenascin-C and oxidative stress in rheumatic and congenital heart valve diseases: an observational study. *Anadolu Kardiyol Derg* **13**, 350-356 (2013).
52. H. Kitaoka *et al.*, Serum tenascin-C levels as a prognostic biomarker of heart failure events in patients with hypertrophic cardiomyopathy. *J Cardiol* **59**, 209-214 (2012).
53. K. Imanaka-Yoshida, I. Tawara, T. Yoshida, Tenascin-C in cardiac disease: a sophisticated controller of inflammation, repair, and fibrosis. *Am J Physiol Cell Physiol* **319**, C781-C796 (2020).
54. M. Franz *et al.*, Serum levels of tenascin-C variants in congestive heart failure patients: comparative analysis of ischemic, dilated, and hypertensive cardiomyopathy. *Clin Lab* **60**, 1007-1013 (2014).
55. M. Kasprzycka, C. Hammarstrom, G. Haraldsen, Tenascins in fibrotic disorders-from bench to bedside. *Cell Adh Migr* **9**, 83-89 (2015).
56. M. Franz *et al.*, De novo expression of fetal ED-A(+) fibronectin and B (+) tenascin-C splicing variants in human cardiac allografts: potential impact for targeted therapy of rejection. *J Mol Histol* **45**, 519-532 (2014).
57. F. Terasaki *et al.*, Higher serum tenascin-C levels reflect the severity of heart failure, left ventricular dysfunction and remodeling in patients with dilated cardiomyopathy. *Circ J* **71**, 327-330 (2007).
58. Y. Yoshikane *et al.*, Serum tenascin-C predicts resistance to steroid combination therapy in high-risk Kawasaki disease: a multicenter prospective cohort study. *Pediatr Rheumatol Online J* **19**, 82 (2021).
59. K. Imanaka-Yoshida, Tenascin-C in Heart Diseases-The Role of Inflammation. *Int J Mol Sci*

22, (2021).
60. I. Rohm *et al.*, Increased Serum Levels of Fetal Tenascin-C Variants in Patients with Pulmonary Hypertension: Novel Biomarkers Reflecting Vascular Remodeling and Right Ventricular Dysfunction? *Int J Mol Sci* **18**, (2017).
61. H. Zhu *et al.*, Tenascin-C promotes acute kidney injury to chronic kidney disease progression by impairing tubular integrity via alphavbeta6 integrin signaling. *Kidney Int* **97**, 1017-1031 (2020).
62. J. H. Horstrup *et al.*, Elevation of serum and urine levels of TIMP-1 and tenascin in patients with renal disease. *Nephrol Dial Transplant* **17**, 1005-1013 (2002).
63. K. Midwood *et al.*, Tenascin-C is an endogenous activator of Toll-like receptor 4 that is essential for maintaining inflammation in arthritic joint disease. *Nat Med* **15**, 774-780 (2009).
64. L. Zuliani-Alvarez *et al.*, Mapping tenascin-C interaction with toll-like receptor 4 reveals a new subset of endogenous inflammatory triggers. *Nat Commun* **8**, 1595 (2017).
65. L. Timmers *et al.*, Toll-like receptor 4 mediates maladaptive left ventricular remodeling and impairs cardiac function after myocardial infarction. *Circ Res* **102**, 257-264 (2008).
66. S. Morimoto *et al.*, Diagnostic utility of tenascin-C for evaluation of the activity of human acute myocarditis. *J Pathol* **205**, 460-467 (2005).
67. A. Tamura *et al.*, Tenascin expression in endomyocardial biopsy specimens in patients with dilated cardiomyopathy: distribution along margin of fibrotic lesions. *Heart* **75**, 291-294 (1996).
68. T. Machino-Ohtsuka *et al.*, Tenascin-C aggravates autoimmune myocarditis via dendritic cell activation and Th17 cell differentiation. *J Am Heart Assoc* **3**, e001052 (2014).
69. A. Maqbool *et al.*, Tenascin C upregulates interleukin-6 expression in human cardiac myofibroblasts via toll-like receptor 4. *World J Cardiol* **8**, 340-350 (2016).
70. Y. Zhou *et al.*, Metformin regulates inflammation and fibrosis in diabetic kidney disease through TNC/TLR4/NF-kappaB/miR-155-5p inflammatory loop. *World J Diabetes* **12**, 19-46 (2021).
71. C. Wu *et al.*, The function of miR-199a-5p/Klotho regulating TLR4/NF-kappaB p65/NGAL pathways in rat mesangial cells cultured with high glucose and the mechanism. *Mol Cell Endocrinol* **417**, 84-93 (2015).
72. S. C. W. Tang, W. H. Yiu, Innate immunity in diabetic kidney disease. *Nat Rev Nephrol* **16**, 206-222 (2020).
73. A. Aubert *et al.*, Latent TGF-beta Activation Is a Hallmark of the Tenascin Family. *Front Immunol* **12**, 613438 (2021).
74. M. Fujimoto *et al.*, Epidermal growth factor-like repeats of tenascin-C-induced constriction of cerebral arteries via activation of epidermal growth factor receptors in rats. *Brain Res* **1642**, 436-444 (2016).
75. S. Chattopadhyay *et al.*, EGFR kinase activity is required for TLR4 signaling and the septic shock response. *EMBO Rep* **16**, 1535-1547 (2015).
76. D. Hsu *et al.*, Toll-like receptor 4 differentially regulates epidermal growth factor-related growth factors in response to intestinal mucosal injury. *Lab Invest* **90**, 1295-1305 (2010).
77. C. E. Trussoni, J. H. Tabibian, P. L. Splinter, S. P. O'Hara, Lipopolysaccharide (LPS)-Induced Biliary Epithelial Cell NRas Activation Requires Epidermal Growth Factor Receptor (EGFR). *PLoS One* **10**, e0125793 (2015).
78. S. De *et al.*, Erlotinib protects against LPS-induced endotoxicity because TLR4 needs EGFR to signal. *Proc Natl Acad Sci U S A* **112**, 9680-9685 (2015).
79. S. Lofgren *et al.*, Integrated, multicohort analysis of systemic sclerosis identifies robust

transcriptional signature of disease severity. *JCI Insight* **1**, e89073 (2016).
80. S. P. Giblin, A. Schwenzer, K. S. Midwood, Alternative splicing controls cell lineage-specific responses to endogenous innate immune triggers within the extracellular matrix. *Matrix Biol* **93**, 95-114 (2020).
81. M. A. Jensen, J. E. Wilkinson, A. R. Krainer, Splicing factor SRSF6 promotes hyperplasia of sensitized skin. *Nat Struct Mol Biol* **21**, 189-197 (2014).
82. S. S. Brack, M. Silacci, M. Birchler, D. Neri, Tumor-targeting properties of novel antibodies specific to the large isoform of tenascin-C. *Clin Cancer Res* **12**, 3200-3208 (2006).
83. D. A. Reardon *et al.*, A pilot study: 131I-antitenascin monoclonal antibody 81c6 to deliver a 44-Gy resection cavity boost. *Neuro Oncol* **10**, 182-189 (2008).
84. L. Nadal *et al.*, Novel human monoclonal antibodies specific to the alternatively spliced domain D of Tenascin C efficiently target tumors in vivo. *MAbs* **12**, 1836713 (2020).
85. S. Dhaouadi *et al.*, Novel Human Tenascin-C Function-Blocking Camel Single Domain Nanobodies. *Front Immunol* **12**, 635166 (2021).
86. D. Wawrzyniak *et al.*, Down-regulation of tenascin-C inhibits breast cancer cells development by cell growth, migration, and adhesion impairment. *PLoS One* **15**, e0237889 (2020).
87. S. R. Aungier *et al.*, Targeting early changes in the synovial microenvironment: a new class of immunomodulatory therapy? *Ann Rheum Dis* **78**, 186-191 (2019).

Figure 1

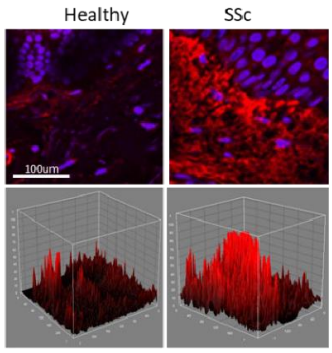

Figure 1: Elevated tenascin-C expression in SSc biopsies. Upper panel, immunofluorescence of SSc and healthy control skin biopsies using antibodies to tenascin-C. Scale bars: 100 µm. **Lowel panel,** 3D surface area plots

Figure 2

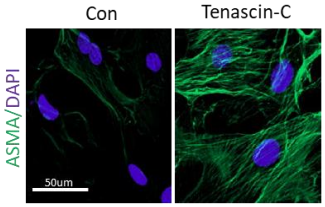

Figure 3: Tenascin-C promotes myofibroblasts differentiation in normal fibroblasts. Human skin fibroblasts were incubated in medium with tenascin-C for 72 hours. Immunofluorescence showed marked increase in myofibroblasts differentiation compared to untreated control. Scale bar, 50 µm.

Figure 3

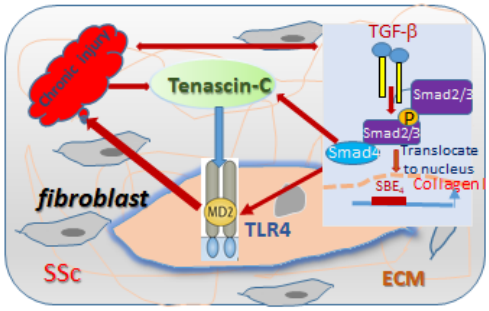

Figure 3: Tenascin-C-TLR4-TGF-β-driven vicious cycle of fibrosis in SSc. Tissue damage occurs from chronic injury causing local generation and accumulation of endogenous danger signal tenascin-C which in turn activate innate immune signaling in resident fibroblasts via TLR4. Chronic injury and activated TLR4 result in enhanced extracellular matrix (ECM) production and TGF-β secretion, TGF-β induces the phosphorylation of Smad2/3 proteins to form the R-Smad/Smad4 complex, which upon entering the nucleus binds to Smad-binding element (SBE_4) and other transcription factors and cofactors to upregulate the expression of both collagen I and tenascin-C, establishing a self-amplifying vicious cycle of fibrosis (adapted from review 6)