

# **Endogenous toll-like receptor signaling drives persistent fibroblast activation and prevents fibrosis resolution**

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## **ABSTRACT**

Sterile inflammation, fibroblast activation and persistent accumulation of activated myofibroblasts are hallmarks of organ fibrosis, including systemic sclerosis (SSc). The cues that elicit, and maintain, fibroblast activation in these conditions are not well understood, hindering the development of effective anti-fibrotic therapies. Recent studies reveal that aberrant activation of toll-like receptor (TLR) in resident tissue fibroblasts and myofibroblasts is a key contributor to progressive fibrosis in SSc. New data further highlight that endogenous TLR stimuli generated by tissue damage within SSc lesions are predominantly responsible for this aberrant TLR activity, creating stimulatory feedback loops that underlie the persistence of fibrosis and failure of its resolution. This review describes emerging mechanisms by which TLR4 stimulation mediates pro-fibrotic signalling, enabling direct, cell-autonomous, and self-sustaining fibroblast activation. We discuss how amelioration of fibrosis by targeting this TLR4 loop may be translated into innovative therapeutic approaches for treating fibrotic disease, and highlight how predictive biomarkers of on-going TLR4 signalling can enable a precision medicine approach to treating SSc, a disease that exhibits marked clinical and molecular heterogeneity.

## Introduction

### **Organ fibrosis is the hallmark of SSc**

Systemic sclerosis (SSc) is a chronic autoimmune disease characterized by interstitial and perivascular fibrosis. In contrast to more common organ-based fibrotic diseases such as liver cirrhosis, fibrosis in SSc synchronously affects the skin (scleroderma) and multiple internal organs. The disease occurs more commonly in women, has a worldwide distribution, and shows a complex multigenic inheritance pattern <sup>1</sup>. There are no therapies effective in halting or resolving fibrosis. Current immunomodulatory treatments that are highly effective in rheumatoid arthritis and other inflammatory diseases generally show only modest and variable efficacy in SSc, and at best slow disease progression. Fibrosis, defined as aberrant accumulation of extracellular matrix disrupting tissue structure and function, is the distinguishing hallmark of SSc. The fibrotic process in SSc is easily recognized in the skin, but it is fibrosis affecting the internal organs, most commonly the lungs, that contributes to the high mortality of SSc <sup>2</sup>. Additionally, fibrosis in the myocardium, gastrointestinal tract, tendons and muscles, and renal interstitium can also be frequent and prominent, and contributes to disease burden and mortality <sup>3</sup>.

Vascular injury and evidence of cellular and humoral autoimmunity, including activated T and B cell, dendritic cells and macrophages, are prominent in SSc, particularly in patients with early-stage disease. However, later stages of SSc are dominated by tissue fibrosis, and inflammatory cell infiltration in target organs is sparse. Understanding how the autoimmune/inflammatory, vascular, and fibrotic processes network in SSc to dictate disease evolution remains a major challenge in the field <sup>4</sup>. Current thinking suggests a model for pathogenesis whereby exposure of

genetically susceptible individuals to certain forms of environmental, microbial or toxic injury elicits early (sterile) inflammation, that together with vascular injury, trigger a cascade leading to fibroblast activation and non-resolving fibrosis (Fig. 1).

### **Inflammatory signaling is tightly linked to fibrogenesis in SSc**

Activation of tissue-resident quiescent fibroblasts and their *in situ* phenocconversion into biosynthetic and apoptosis-resistant myofibroblasts, as well as transition of various mesenchymal progenitor cell types (endothelial cells, pericytes, adipose-derived progenitors) into myofibroblasts accounts for the persistence of fibrosis, even in the absence of inflammation. The origins of fibrotic myofibroblasts, and the cues and mechanisms involved in their transdifferentiation and persistence, have been the subject of controversy, with data deriving largely from various animal models of organ fibrosis, and differing among distinct types of organ fibrosis <sup>5</sup>; nevertheless, the fundamental pathogenic roles of transforming growth factor- $\beta$  (TGF- $\beta$ ), Wnt/ $\beta$ -catenin, cytokines such as IL-13, oxidative stress and biomechanical signals, across multiple types of organ fibrosis are supported by substantial experimental evidence <sup>6</sup>.

Genetic studies during the past decade have contributed to a better understanding of the immunity-fibrosis link in SSc pathogenesis. These studies have identified a substantial number of polymorphic loci associated with the risk of SSc <sup>7</sup>. The most prominent of these genetic associations are within the major histocompatibility complex (MHC). The great majority of the non-MHC variants associated with SSc are related to immune cell activation and function, including T and B cell signaling, along with innate immunity, type I interferon and toll like receptor (TLR) signaling. It is remarkable that a majority of SSc-associated genetic loci are

shared in common with other autoimmune diseases, particularly systemic lupus erythematosus <sup>8</sup>. An especially intriguing genetic finding is the association of SSc with both synonymous and non-coding variants of TNFAIP3, which encodes the deubiquitinase enzyme A20, a prototypic intracellular inhibitor of TLR signaling <sup>8-10</sup>. At the genomic level, pioneering transcriptome microarray studies carried out by the Whitfield lab demonstrated that ~50% of skin biopsies from patients with diffuse cutaneous SSc (dcSSc) show a very robust inflammatory gene signature that appears to be independent of disease duration <sup>11</sup>. Many of the genes up-regulated in these skin biopsies are related to the “interferon signature” and/or are involved in innate immunity and TLR signaling. It is noteworthy that inflammatory gene signatures are equally prominent in SSc skin biopsies from patients with both early and late-stage disease, indicating that evidence of enhanced inflammatory gene expression in lesional tissue is not simply a function of disease stage <sup>11</sup>. Finally, network analysis integrating SSc-associated genetic risk loci and differentially regulated genes in the skin underlines the intimate relationship between innate immunity, fibrotic responses elicited by TGF- $\beta$  and Wnt/ $\beta$ -catenin, and extracellular matrix gene expression <sup>12</sup>.

Notwithstanding compelling genetic and genomic evidence pointing to the fundamental pathogenic role for inflammatory/autoimmune processes in SSc, and the sometime dramatic beneficial clinical response seen following myeloablative therapy of SSc <sup>13</sup>, the contribution of bone marrow-derived inflammatory cells to SSc fibrosis continue to be enigmatic and controversial, and biopsies from SSc patients with established disease are largely pauci-immune. Our laboratory has been interested in dissecting the cellular and molecular networks underlying self-sustaining fibroblast activity in SSc, and the impact of inflammation in triggering and maintaining this process. Our recent studies have provided novel insights into fibrosis, and

revealed therapeutic targets for preventing fibrosis progression and promoting resolution. In particular, the results point to an unexpected function for innate immune signaling in fibroblasts triggered by endogenous damage-associated molecular patterns (DAMPs) and mediated by TLRs. Despite substantial recent progress, however, the pathogenic roles, mechanisms and regulation of DAMP-TLR signaling in the context of fibrosis and SSc remain inadequately defined. In this review, we present current understanding of the DAMP-TLR4 axis in fibroblasts, and its pathogenic roles in unresolving fibrosis in SSc. We also discuss the application of these insights to the development of therapeutic and precision medicine approaches for targeting TLRs in fibrosis.

## **A brief overview of toll-like receptor signaling**

The first line of host defense is the innate immune system comprising a diverse set of pattern recognition receptors (PRRs). These receptors recognize both extrinsic pathogen-derived molecule patterns (PAMPs) from gram negative bacteria (lipopolysaccharide, LPS) or viral nucleic acids, as well as damage-associated endogenous molecules (DAMPs) such as the extracellular matrix (ECM) proteins tenascin-C and fibronectin-EDA that are generated within injured microenvironments<sup>14</sup>. The best-characterized PRRs in addition to TLRs include nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) and the cGAS-STING pathway. Upon PAMP or DAMP recognition, PRRs stereotypically induce activation of NF- $\kappa$ B and interferon response factors (IRFs), resulting in the secretion of interferons and proinflammatory cytokines<sup>15</sup>.

The mammalian TLR family consists of 12 members, with each possessing a leucine-rich repeat (LRR) ligand-binding domain and a signaling Toll/Interleukin-1 (IL-1) receptor homology (TIR) domain. Some TLRs (TLR1, 2, 4, 5, 6 and 10) localize to the cell surface, while TLR3, 7, 8, 9, 11 and 13 are located within intracellular compartments <sup>16</sup>. Binding of TLR ligand to the extracellular leucine-rich repeat region causes TLR dimerization and conformational changes of the intracellular TIR domains, and recruitment and activation of adaptor molecules MyD88, and/or TRIF, which then transduce signals. The MyD88-dependent signaling pathway recruits IRAKs and activates TRAF6 and the I $\kappa$ B kinase (IKK), leading to NF- $\kappa$ B activation. This pathway is negatively controlled by the deubiquitinase A20. The MyD88-independent TRIF signaling pathway culminates in the activation of both IRF3 and NF- $\kappa$ B. Toll-like receptor 4 is the only TLR that engages both MyD88 and TRIF for intracellular signaling <sup>17</sup>.

### **Endogenous TLR ligands**

In response to LPS, the hallmark PAMP, TLR4 forms a complex with its co-receptor MD2. Structural studies show that five of the six LPS lipid chains bind to the hydrophobic pocket of MD2, while the remaining lipid chain associates directly with TLR4 <sup>18</sup>. There is an extensive, and growing, list of DAMPs that have been shown to serve as endogenous TLR4 ligands <sup>19</sup>. These DAMPs, representing a diverse group of molecular responses to tissue damage, include single or double-stranded self-nucleic acids, fatty acids, serum amyloid A (SAA), ECM fragments, and alternatively-spliced “oncofetal” variants of normal extracellular matrix components. In contrast to LPS, DAMPs may not require the same co-receptors and accessory molecules to achieve signaling-competent TLR4 conformation. It is worth noting that many of the DAMPs were implicated as putative endogenous TLR4 ligands based on co-

immunoprecipitation or functional cell-based assays *in vitro*, or using mutant mice deficient in TLRs or their adaptor proteins *in vivo*. No crystal structures of DAMP-TLR4 complex have been reported so far to confirm direct DAMP-TLR4 interactions, or the requirements of specific co-receptors for the formation of active TLR signaling complexes.

One of the best studied endogenous TLR4 ligands linked to SSc fibrosis is the large modular glycoprotein tenascin-C<sup>20,21</sup>. An intriguing recent study asked whether the TLR4-dependent signaling pathways and biological readouts elicited in macrophages by the tenascin-C fibrinogen-like globe (FBG) domain (Fig. 3) were similar to those elicited by LPS<sup>22</sup>. Pursuing a comparative analysis of the signaling pathways and biological outcomes, the authors found that TLR4 activation elicited by LPS and FBG generated two distinct macrophage phenotypes, with only partially-overlapping sets of activation markers, secreted effector molecules, and phosphoproteomic profiles<sup>22</sup>. FBG promoted a TLR4-dependent “profibrotic” macrophage phenotype, whereas LPS promoted a phenotype with enhanced matrix-degrading capacity. These observations 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.

## **Altered TLR4 signaling in SSc fibrosis**



In light of the genetic and genomic evidence suggesting a pathogenic role for dysregulated innate immunity and TLR activity in SSc, we undertook a series of studies focusing on the TLR ligand-receptor axis in patients with SSc, and in animal models of disease. These studies demonstrated that levels of both TLR4, as well as certain endogenous TLR ligands, are elevated in skin and lung tissues from patients with SSc, and correlate with clinical disease parameters<sup>23 24</sup>(Fig. 2). For instance, tissue levels of TLR4 and its co-receptors MD2 and CD14 are elevated in SSc, and levels predict severity and/or progression of skin disease<sup>23,24</sup>. Prominent TLR4 immunostaining was seen in skin and lung biopsies from patients with SSc, and TLR4 largely co-localized with myofibroblasts, as well as infiltrating macrophages and vascular cells within lesional tissue (Fig. 2A)<sup>23</sup>. Moreover, treatment of fibroblasts by LPS or by endogenous TLR4 ligands elicit potent fibrosis-related gene expression and phenoconversion into alpha smooth muscle-actin-positive myofibroblasts. Conversely, genetic targeting of TLR4 or its endogenous “damage-associated” ligands, or pharmacological disruption of signaling with drugs that block TLR4 or its co-receptor MD2, ameliorates progressive tissue fibrosis in various disease models.

Transcriptome analysis of SSc skin biopsies revealed substantial molecular heterogeneity among different patients<sup>25,26</sup>. Cluster analysis reveals distinct subsets that are termed fibroproliferative, inflammatory, limited, and normal-like. The inflammatory intrinsic subset, which account for a substantial portion of skin biopsies from patients with diffuse cutaneous SSc and was independent of disease duration, demonstrated up-regulation of many genes involved in innate immunity. In contrast, the fibroproliferative, limited and normal-like subsets did not show such associations<sup>11</sup>. Relative paucity of immune cells within biopsies from patients with established SSc suggests that the inflammatory gene signatures might originate primarily from resident

stromal cells. To explore this hypothesis, we generated an experimentally-derived “fibroblast TLR4 (LPS)-regulated gene signature” using normal skin fibroblasts transfected with TLR4<sup>23, 27</sup>. Comparison of this fibroblast TLR4 gene signature with those generated from human monocytes indicated only a partial overlap of differentially-expressed genes in the two cell types (hypergeometric test;  $p=0.02$ ) (Table 1). The fibroblast TLR4 gene signature showed significant enrichment with pathways related to wound healing, matrix remodeling and TGF- $\beta$  signaling, revealing important cell type-specific differences between TLR responses in bone marrow-derived inflammatory versus stromal cells (Table 1). Most interestingly, we found that SSc skin biopsies displaying a strong fibroblast TLR4 gene signature mapped to the inflammatory intrinsic gene subset (Bhattacharyya S. and Varga J., unpublished). These findings suggest that elevated inflammatory gene expression in SSc skin biopsies, particularly in patients with late-stage disease, might originate from activated fibroblasts. From a translational perspective, a strong fibroblast TLR4 signature in skin biopsies might represent a predictive biomarker that identifies SSc patients with on-going fibroblast activity; such patients might be potentially more responsive to therapies that block TLR signaling (see below). A similar predictive strategy might also be relevant for SSc-associated interstitial lung disease (ILD). The leading cause of death in patients with SSc, ILD is a frequent complication that can show sustained stability or rapid progression<sup>1</sup>. Lung biopsies from patients with SSc-ILD show up-regulation of genes related to TLR and TGF- $\beta$  signaling, similar to lesional skin<sup>27</sup>.

We carried out a comparative analysis of SSc skin and lung transcriptomes to identify pathways shared between these two target organs. Pathways involved in positive and negative regulation of TLR signaling were most highly shared (Bhattacharyya S and Varga J; unpublished). The role of

TLR4 in lung fibrosis appears to be complex and injury- and cell type-dependent, and the available evidence is contradictory. For instance, while bleomycin-induced lung fibrosis was ameliorated in TLR4-null and IRF5-null mice <sup>28,29</sup>, a recent study demonstrated worse lung fibrosis and higher mortality in the absence of TLR4, attributed to failure of alveolar epithelial cell regeneration in the absence of TLR4 <sup>30,31</sup>. The findings suggest that epithelial cell TLR4 signaling might have a protective role during injury, and its absence results in worse fibrosis in organs such as the lung where post-injury regeneration is critically dependent of epithelial stem cell renewal. However, the conflicting data from these publications remain difficult to reconcile at the moment.

## **Mechanisms for the profibrotic responses elicited by TLR4**

In skin fibroblasts, TLR4 activation by LPS elicited global changes in gene expression <sup>23</sup>. The response was dominated by genes involved in ECM remodeling, tissue repair, and wound healing, while changes in inflammatory genes were relatively modest. These results suggest distinct roles for TLR4 in inflammatory cells, where TLR4 serves to elicit a powerful inflammatory response designed to deal with invading microbial pathogens, versus tissue-resident stromal/parenchymal cells, where TLR4 might have evolved primarily to promote robust repair of injured tissue <sup>16,23</sup>. We found that TLR4 activation in explanted fibroblasts led to enhanced collagen synthesis and increased expression of genes involved in tissue remodeling and extracellular matrix homeostasis. Moreover, TLR4 dramatically enhanced the sensitivity of fibroblasts to the stimulatory effect of TGF- $\beta$ . These profibrotic responses were abrogated by both genetic and pharmacological disruption of TLR4 signaling *in vitro*, and bleomycin-induced skin fibrosis was attenuated in mice harboring a mutated TLR4. Mechanistically, TLR4-

dependent profibrotic fibroblast responses involved suppression of the endogenous TGF- $\beta$  antagonist BAMBI (bone morphogenetic protein and activin membrane-bound inhibitor), resulting in unopposed TGF- $\beta$ /Smad signaling<sup>23,32</sup>. Moreover, levels of miR-29, a microRNA known to function as a negative regulator of fibrosis, were down-regulated by both TLR4 activation and by TGF- $\beta$ <sup>23,33</sup>. Additional transcriptional mechanisms and epigenetic reprogramming underlying the persistent profibrotic TLR4 responses remain to be characterized.

## **Damage-associated molecular patterns acting as endogenous TLR4 ligands are implicated in SSc fibrosis**

Sterile tissue injury results in the generation of DAMPs that enable cells to recognize and respond to danger<sup>14</sup>. Persistent DAMP exposure however contributes to the pathogenesis of many inflammatory and autoimmune diseases. DAMPs encompass an extensive repertoire of molecules released by dying or damaged cells such as high mobility box 1 (HMGB1) and heat shock proteins (HSPs), self-nucleic acids including single and double-stranded DNA, RNA, mitochondrial DNA and nucleic acid-antibody complexes; SAA, fragments of ECM macromolecules, and “oncofetal” ECM macromolecule isoforms generated through alternative splicing. We performed an unbiased survey of putative endogenous TLR4 ligands detected in SSc skin biopsies<sup>16</sup>. Using immunohistochemistry, we identified low molecular weight hyaluronic acid, alternately-spliced fibronectin (Fn<sup>EDA</sup>) isoform and tenascin-C as most highly up-regulated in the fibrotic skin<sup>16,20,23,33</sup>. Normally, alternately spliced Fn<sup>EDA</sup> and tenascin-C isoforms are detected in developing tissues during embryogenesis, and subsequently decline. However, the ‘embryonic’ splicing pattern of these molecules is re-established transiently during

tissue repair and angiogenesis; in contrast, persistent re-expression of these isoforms is a hallmark of cancer and fibrosis<sup>34,35</sup>.

## **The alternately-spliced EDA fibronectin isoform acts as an endogenous TLR4 ligand with potent profibrotic activity**

Fibronectin is generated by alternate splicing from a 47-exon gene<sup>34</sup>. Isoforms that include or exclude the EDA and EDB exons arise due to alternative splicing from a single fibronectin pre-mRNA. In adults, the protein occurs in two major forms: dimeric soluble plasma fibronectin (pFN) which lacks the EDA and EDB domains; and multimeric cellular fibronectin (cFN) that includes the EDA or EDB domains and is deposited within the ECM (**Fig. 3**). During wound healing, the EDA and EDB isoforms accumulate at the wound base<sup>36</sup>. The inclusion or exclusion of EDA domain defines the ability of fibronectin to activate TLR4; recombinant EDA but not EDB can induce TLR4-dependent NF- $\kappa$ B signaling and cytokine synthesis<sup>37-39</sup>.

Since the EDA isoform of fibronectin had been previously shown to be required for the myofibroblast phenoconversion of normal fibroblasts<sup>40</sup>, we sought to investigate its expression and regulation in SSc, and its possible mechanisms of action in fibrosis. A series of studies revealed that Fn<sup>EDA</sup> was selectively elevated in SSc skin biopsies, as well as in lesional tissues from mice with experimentally-induced cutaneous fibrosis, and explanted SSc fibroblasts constitutively produced increased Fn<sup>EDA</sup> *ex vivo* (Fig. 2B)<sup>33</sup>. Moreover, circulating Fn<sup>EDA</sup> levels were elevated in patients with SSc. In subsequent studies we examined how Fn<sup>EDA</sup> was regulated, and how it affected normal fibroblasts. In normal fibroblasts, TGF- $\beta$  induced an isoform-specific preferential up-regulation of Fn<sup>EDA</sup><sup>33</sup>. Serving as a *bona fide* endogenous TLR4

ligand, Fn<sup>EDA</sup> elicited potent profibrotic responses, with enhanced synthesis of collagens and expression of the myofibroblast marker alpha smooth muscle actin. Moreover, similar profibrotic effects were elicited in fibroblasts cultured within a 3D human skin equivalent <sup>33</sup>. Mice with genetic deletion of Fn<sup>EDA</sup> showed no spontaneous phenotype, but were largely resistant to the induction of skin fibrosis <sup>33</sup>. Other studies had shown that mice carrying a functionally hypomorphic TLR4 mutant <sup>23</sup>, or lacking TLR4 <sup>28</sup> were similarly resistant to fibrosis. On the basis of these observations, we propose a model for persistent cutaneous fibrosis, where resident fibroblasts are chronically exposed to Fn<sup>EDA</sup> acting as an endogenous TLR4 stimulus within the fibrotic microenvironment. In response, fibroblasts undergo TLR4-mediated activation and reprogramming, resulting in unopposed TGF- $\beta$  signaling, enhanced profibrotic responses, and myofibroblast phenoconversion. Moreover, TLR4-dependent profibrotic responses in these cells include preferential production of the EDA isoform of fibronectin, along with other endogenous TLR4 ligands, which in turn drive further TLR4 activation. This potentially generates a cell-autonomous and inflammation-independent fibrosis amplification loop underlying persistent tissue fibrosis.

In addition to its alternate splicing, another mechanism for fibronectin to become a signaling-competent DAMP has been reported. It was shown that exposure of fibroblasts to FNIII1c, a stable unfolded intermediate of FNIII1, can elicits TLR4-dependent inflammatory signaling and induce cytokine synthesis <sup>41,42</sup>. This region of the fibronectin molecule is exposed via tensional forces within the rigid fibrotic microenvironment. These observations have clear implications for fibrosis, since fibrotic tissue is characterized by increased stiffness and mechanical forces <sup>43</sup>. We speculate that in SSc, the stiff matrix of fibrotic skin and lungs might drive exposure of the EDA domain of fibronectin, which, combined with increased generation of the EDA isoform via

alternate splicing, results in augmented Fn<sup>EDA</sup> bioavailability and profibrotic activity as an endogenous TLR4 ligands.

### **Tenascin-C accumulates within fibrotic lesions and drives TLR4-dependent profibrotic responses**

Tenascin-C is a multifunctional hexameric ECM glycoprotein that undergoes extensive alternate splicing to generate multiple isoforms<sup>35</sup>. Normally tenascin-C is under tight spatial and temporal regulation. Tenascin-C is prominent in tissues during embryogenesis but undetectable in most healthy adult tissues, and only transiently re-expressed during wound healing and dynamic tissue remodeling<sup>35</sup>. By contrast, persistent expression of the large isoform of tenascin-C is seen in non-healing wounds, autoimmune diseases and cancer<sup>35,44</sup>. The first tenascin-C exon sequence isolated from human glioblastoma identified a clone with 8 consecutive FNIII like repeats between FNIII 5 and 6, providing genetic evidence of alternative splicing (Fig. 3)<sup>35</sup>.

Our unbiased survey identified tenascin-C as one of the most highly up-regulated ECM proteins in SSc skin biopsies<sup>16</sup>. Further studies revealed elevated tissue levels of tenascin-C in SSc skin and lung biopsies, as well as in circulation; moreover, serum levels showed positive correlation with the Modified Rodnan skin score, a measure of skin fibrosis<sup>20</sup>. The antibodies used in these studies detect the FNIII-B and FNIII-C epitopes of the large tenascin-C isoforms (Fig. 2A).

Normal fibroblasts treated with TGF- $\beta$  and PDGF preferentially synthesize the large (~250 kDa) tenascin-C variant detected with an antibody against FN III-B domain, while SSc fibroblasts constitutively produce the large tenascin-C isoform (Fig. 2B)<sup>20</sup>.

Recent studies provide insight into the physiological and pathological regulation of tenascin-C alternate splicing. The proto-oncogene serine/arginine-rich splicing factor 6 (SRSF6) is an essential regulator of tenascin-C alternate splicing<sup>45</sup>. Transgenic mice overexpressing SRSF6 in collagen-producing cells spontaneously develop marked scleroderma-like skin hyperplasia<sup>45</sup>. This was accompanied by aberrant alternative tenascin-C splicing, and accumulation of the ‘large’ tenascin-C isoform in the skin. By regulating alternative splicing of tenascin-C, SRSF6 thus has a powerful effect on skin homeostasis in mouse models. Remarkably, we recently observed that the expression of SRSF6 was highly elevated in SSc skin biopsies, and levels correlated with tenascin-C accumulation (Bhattacharyya S, Roberson E, Varga J; unpublished). Moreover, RNA sequencing indicated increased abundance of alternatively-spliced tenascin-C mRNA isoforms in SSc skin biopsies. While these intriguing observations implicate SRSF6 and alternate 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.

Explanted skin fibroblasts isolated from SSc biopsies show constitutive tenascin-C production, indicating that tenascin-C accumulation in SSc might at least in part result from its cell-autonomous overproduction<sup>20</sup>. In SSc patients, circulating and tissue levels of tenascin-C were up-regulated in patients with both early- and late-stage disease, suggesting that persistent up-regulation of tenascin-C might play a role in maintaining tissue fibrosis. It is noteworthy that tenascin-C levels showed a positive correlation with TLR4, as well as with IL-6, a read-out for TLR4 signaling, in individual SSc skin biopsies<sup>20</sup>. Treatment of skin fibroblasts with tenascin-C elicited TLR4-dependent profibrotic responses, including up-regulation of TLR4 itself. Mice



lacking tenascin-C were viable and fertile, but showed reduced skin and lung fibrosis in response to bleomycin. Moreover, lack of tenascin-C was associated with reduced hypodermal fibrosis in the *Tsk/+* mouse. Moreover, bleomycin-induced skin fibrosis showed accelerated resolution in mice lacking tenascin-C. While the EDA isoform of fibronectin is a potent TLR4 agonist, it might be insufficient to compensate for the loss of tenascin-C in tenascin-C null mice, since its expression is reduced in lesional skin. We propose that reduced cutaneous TLR4 signaling in mice lacking tenascin-C accounts for attenuated fibrosis, and more important, accelerated resolution. Pathological tissue fibrosis in SSc might be similarly perpetuated via a TLR4-dependent fibrosis amplification loop driven by endogenous DAMPs that accumulate and persist within injured microenvironments. Since little is currently known about tenascin-C splicing in inflammation and tissue remodeling, it will be of great interest to determine if alternatively spliced tenascin-C isoforms, or the FBG domain of tenascin-C, are necessary and sufficient to activate fibroblast TLR4 signaling, and whether they can be targeted for anti-fibrotic therapy (see below).

## **Multiple mechanisms to prevent aberrant TLR4 activation**

To forestall injury resulting from aberrant innate immune activation, a number of cellular mechanisms that inhibit TLR signaling have evolved <sup>46</sup>. These negative regulatory mechanisms include suppression of TLR4 expression; alternate splicing of TLR adaptors; the inhibitory surface molecule radioprotective 105 (RP105); ubiquitin ligase and deubiquitinase enzymes such as A20 that modulate the activity of key TLR signaling intermediates; transcriptional regulators; and microRNAs <sup>46</sup>. Impaired negative regulation of TLR signaling results in unchecked TLR activation that might contribute to chronic inflammatory and fibrotic diseases.

In many cell types, RP105 forms a complex with the TLR co-receptor MD1, which then interacts directly with TLR4-MD2 and inhibits downstream TLR4 signaling. Mice lacking RP105 show enhanced sensitivity to arthritis<sup>47</sup>. Additionally, RP105-null mice develop exaggerated cardiac dysfunction, neointima formation upon injury, and aggravates vein graft disease while atherosclerosis is attenuated<sup>48-51</sup>. Treatment of skin and lung fibroblasts with recombinant RP105 abrogated TLR4-dependent inflammatory as well as profibrotic responses (Bhattacharyya S, Varga J, unpublished data). Furthermore, bleomycin treatment of RP105-deficient mice resulted in accelerated skin and lung fibrosis (Bhattacharyya S, Varga J; unpublished), indicating that RP105 suppresses fibrotic signaling via attenuated TLR4-dependent fibroblast activation. The expression and function of RP105 in SSc remains to be investigated.

The level of cellular TLR4 expression regulates the sensitivity to TLR4 ligands. Not surprisingly, LPS is a potent stimulus for enhanced TLR4 expression, whereas proresolving mediators such as RvD2 suppress TLR4 expression, preventing sustained inflammatory responses<sup>52</sup>. Both up-and down-regulation of TLR4 involve microRNAs, including inhibitory miR146a<sup>53</sup>. Whether miRNA-mediated modulation of the expression of TLR4, along with MD2, is involved in fibrosis and SSc, have not been explored.

Cell-autonomous regulation of inflammatory signaling involves the cytosolic ubiquitin-editing enzyme A20<sup>54</sup>. In a variety of cells A20 controls both TLR-dependent and TLR-independent inflammatory responses. Genetic studies have uncovered consistent associations of A20 locus variants with a range of autoimmune and inflammatory diseases. Rare loss-of-function mutations in *TNFAIP3* leading to A20 haploinsufficiency cause an early-onset autoinflammatory syndrome<sup>55</sup>. Altered A20 expression or function causes persistent inflammation and other pathologies, and

functional A20 variants show robust association with disease susceptibility and severity in SSc. Consensus clustering of SSc-associated genetic variants with differentially expressed genes shows that sub-networks enriched for genes related to TLR pathways are connected to clusters enriched for genes associated with TGF- $\beta$  and ECM remodeling, with A20 as a central hub within the network <sup>56</sup>. We recently showed that in fibroblasts, A20 plays a fundamental role in limiting TGF- $\beta$  activity and preventing excessive collagen production <sup>57</sup>.

Impaired A20 expression, regulation or function due to genetic variants, epigenetic modulation and/or environmental influences might contribute directly to the development or progression of fibrosis in SSc, and represents a promising target for therapy. Treatment of fibroblasts with adiponectin, an adipocyte-derived peptide that is reduced in patients with SSc, blocked TLR-dependent fibrotic responses <sup>58,59</sup>. Remarkably, adiponectin induces sustained up-regulation of A20 in fibroblasts. Pharmacologic agents that induce A20 expression, such as vitamin E ( $\gamma$ -tocotrienol), adiponectin, hold promise as anti-fibrotic therapies via restoring endogenous A20 expression, particularly when targeted in a tissue-restricted manner, such as by topical application <sup>57,60,61</sup>. Connectivity mapping identified ikarugamycin and quercetin as A20-inducing agents with potential as anti-inflammatory drugs. In preclinical studies these compounds were shown to attenuate inflammatory responses by inducing A20 in cystic fibrosis airway epithelial cells <sup>62</sup>.

In the absence of A20, mice spontaneously develop sclerodermatous skin changes including thickened dermis and loss of dermal white adipose tissue, and show premature lethality due to widespread inflammation <sup>63</sup>. Deficiency of A20 in intestinal epithelial cells is associated with

IBD, in myeloid cells causes joint inflammation, in dendritic cells causes colitis, in mast cells exacerbate inflammation<sup>64,65</sup>, in B cells autoimmunity but only in old mice, and in vascular allografts aggravates severity of transplant arteriosclerosis<sup>66-69</sup>. These observations highlight the diverse and cell type-, age- and disease-specific functions of A20. The effect of fibroblast-specific A20 loss in fibrosis in vivo remains to be investigated.

## **TLR4 signaling represents a potential therapeutic target in SSc**

Currently, there are limited therapeutic options for patients with SSc. A major hurdle in the introduction of more effective treatments arises from disease heterogeneity--either in the form of differing disease subtypes or different rates of fibrosis progression between individual patients and even within the same patient between different organs (such as skin and lung). Application of a targeted precision medicine approach is therefore highly appropriate for SSc. For instance, we found that our experimentally-derived “fibroblast TLR4 gene signature” was highly expressed in one patient subset only, mapping to the inflammatory intrinsic subset of SSc biopsies (Bhattacharyya S. and Varga J., unpublished data); this signature therefore might serve as a predictive biomarker identifying a subset of SSc patients who might be responders to anti-TLR4 therapy in future clinical trials.

As dysregulated TLR4 signaling underlies the pathology of diverse conditions, substantial effort has been committed to the design and development of selective inhibitors<sup>70-73</sup>. A prime disease indication pursued for TLR4 inhibitors is sepsis. Eritoran (Eisai Co., Ltd., Japan), a structural inhibitor of the lipid A portion of LPS, has been shown to inhibit TLR4 signaling in vitro and in vivo. TAK242 (*Takeda Pharmaceutical Company Limited*, Japan), covalently binds to Cys747 of the TIR domain of TLR4, rendering TLR4 completely inactive. Both of these TLR4 inhibitors

reached Phase III clinical trials for the treatment of septic shock, but unfortunately failed to demonstrate acceptable efficacy and safety<sup>74-76</sup>. Notably, TAK242 showed potent anti-fibrotic effects, preventing bleomycin-induced organ fibrosis (Bhattacharyya and Varga, unpublished observation) (Fig. 5).

Recent studies implicating TLR4 and its co-receptor MD2 in SSc have focused attention of TLR4/MD2 as a novel therapeutic target<sup>16</sup>. Since endogenous TLR4 ligand require MD2 as a co-receptor for signaling and initiation of fibrotic responses<sup>14,37</sup>, disrupting ligand-MD2-TLR4 complex formation represents a logical anti-fibrotic strategy. Based on this hypothesis, Yin and colleagues undertook extensive structure-activity-relationship studies to identify potent and selective drug candidates that disrupt TLR4-MD2 interactions. The most efficient compound in this series, T5342126, competes with MD2 for binding to TLR4, and disrupts LPS/TLR4-stimulated NF- $\kappa$ B-activity *in vitro*. Importantly, T5342126 showed potent anti-fibrotic effects, preventing stimulation of Type I collagen synthesis and myofibroblast differentiation induced in normal fibroblasts by microbial LPS or by EDA fibronectin or tenascin-C (Bhattacharyya S and Varga J; unpublished) Furthermore, the TLR4 inhibitor attenuated the constitutively activated phenotype of SSc fibroblasts. Treatment of mice with T5342126 not only prevented, but also reversed, organ fibrosis in multiple disease models (Bhattacharyya S, Yin H and Varga J unpublished). Additional agents targeting MD2 include caffeic acid phenethyl ester (CAPE) and rifampin, which prevent LPS-induced proinflammatory mediators, although their effects on fibrosis have not been examined<sup>77,78</sup>. A viable therapeutic strategy for disrupting MD2-TLR4 complex formation with antibodies directed against TLR4 or the TLR4-MD-2 complex showed promising results in pre-clinical studies<sup>71</sup>.

Selectively preventing TLR4 activation by disease-associated DAMPs represents a highly appealing innovative approach to TLR4 inhibition. By carefully choosing a target unique to the response to tissue damage, and not to pathogen-mediated activation of the immune response may have the advantage of preserving an intact host response to infection. Several novel approaches for selectively targeting tenascin-C have been tried in cancer patients <sup>79</sup>. The alternatively-spliced domains of fibronectin and 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; the EDA domain of fibronectin is the target of F8 antibody <sup>80</sup>. Alternately, as TLR4 activation involves the terminal FBG domain of tenascin-C (Fig. 3) <sup>21,81</sup>, selectively targeting the FBG domain using antibody represents another promising anti-fibrotic therapy. Further studies on preclinical and clinical data underlying therapeutic potential of targeting TLR4 DAMPs are warranted.

The precise mode of TLR4 interaction with DAMPs leading to active signaling remains to be elucidated. Although the fibronectin EDA domain has been shown to bind to cell surface TLR4 <sup>37</sup>, there are no data indicating whether tenascin-C binds directly to TLR4. Furthermore, the TLR4-dependent tenascin-C response in macrophages is not inhibited by MD2 blockade <sup>21</sup>. In contrast, we observed that the MD2-TLR4 inhibitor T5342126 abrogated tenascin-C-induced profibrotic responses in fibroblasts, suggesting potential cell specificity. The identification of TLR4 co-receptors and their precise roles in DAMP-TLR4 signaling might help delineating the molecular events leading to ligand/ cell-type specific pathological responses to DAMPs. A comparative analysis of co-receptors, kinases, and transcription factors involved in DAMP signaling versus PAMPs may highlight key differences that could hold promise to novel

therapeutic approaches by selectively silencing DAMP-TLR4 signaling without compromising the host immune defense.

## Summary and future perspective

Emerging insights from *in vitro* experiments, experimental disease models using transgenic mice, and clinical information highlight a previously unsuspected pathogenic role of DAMP-TLR4 signaling in fibrosis. Activation of fibroblast TLR4 by disease-associated DAMPs appears to convert a controlled and self-limited tissue repair process into unresolving fibrosis.

Transcriptome analyses highlight the association of aberrant TLR4 signaling with skin fibrosis in the ‘inflammatory’ intrinsic gene subset of SSc, suggesting that patients mapping to this subset might be the optimal responders to therapy targeting the TLR4 axis. It is noteworthy that current drugs targeting TLR4, such as eritoran and TAK-242, failed to achieve their primary endpoint in septic shock clinical trials, highlighting an urgent need for new treatment strategies. Anti-TLR4 agents might also be considered as anti-fibrotic therapies in a drug “repurposing” strategy. We have highlighted promising approaches of targeting TLR4 signaling with small molecule TLR4 inhibitors. Alternatively, ablating the expression or function of individual DAMPs or blocking their recognition by TLR4, or restoring endogenous TLR inhibitors such as A20 or RP105 by pharmacologic agents might hold promise for anti-fibrotic therapies. Much remains to be learned regarding the unique and redundant functions of various TLR4 DAMPs, their specific co-receptors, negative regulators and repertoire of molecular pathways. Moreover, it will be of great interest to investigate the genetic associations between A20 and specific SSc endophenotypes, as well as disease outcomes and response to therapy. In summary, this review highlighted an emerging novel paradigm implicating TLR4-DAMP signaling in persistent fibroblast activation

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underlying non-resolving fibrosis in SSc and possibly other forms of pathological fibrosis, appealing opportunities for therapy and novel approaches for identifying a homogeneous population of patients most likely to benefit from these therapies (Fig. 6).

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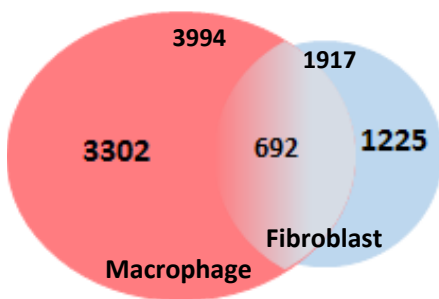
**Table 1**

**Fibroblasts**

GO Terms	p
response to wounding	4.91e-15
cell migration	8.3e-11
defense response	4.45e-10
extracellular matrix organization	8.86e-10
cell surface receptor linked signaling pathway	1.17e-09
programmed cell death	2.56e-09
response to cytokine stimulus	3.25e-09
inflammatory response	3.31e-09
apoptosis	3.49e-09
blood vessel development	3.66e-09

**Macrophages**

GO terms	p
translation	2.25E-27
ncRNA metabolic process	2.47E-15
immune response	9.23E-14
ribonucleoprotein complex biogenesis	1.47E-12
translational elongation	9.86E-12
ribosome biogenesis	1.55E-11
cellular respiration	3.54E-11
RNA processing	5.01E-11
generation of precursor metabolites and energy	1.79E-10
ncRNA processing	1.96E-08



**Biological Processes designated by individual GO categories elicited by LPS in fibroblasts and macrophages.**

**A.** Confluent skin fibroblasts and macrophages treated with LPS for 24h. At the end of incubation period, total RNA was harvested and processed for hybridizations to Illumina Human HT-12 microarray chips. The table showing the significantly enriched GO categories elicited by fibroblasts and macrophages. **B.** Venn diagram showing gene numbers.

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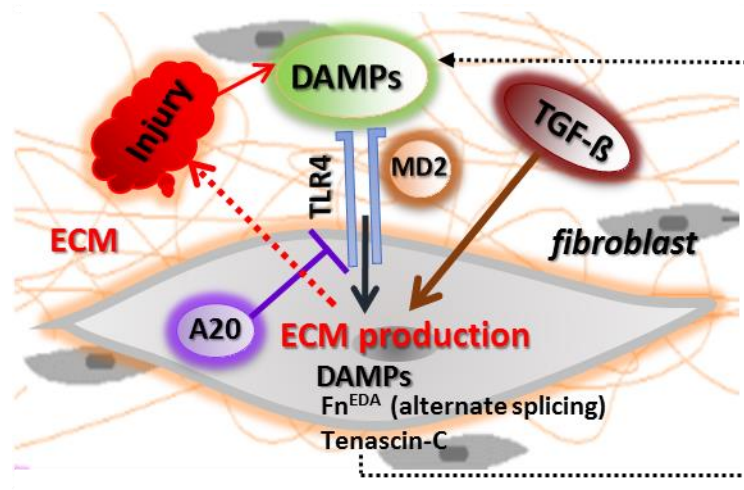
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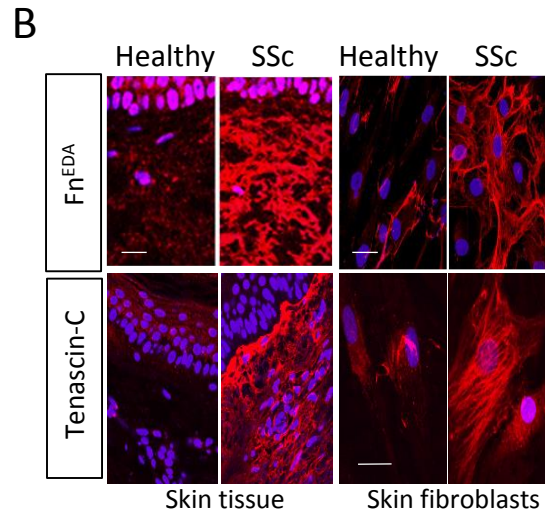
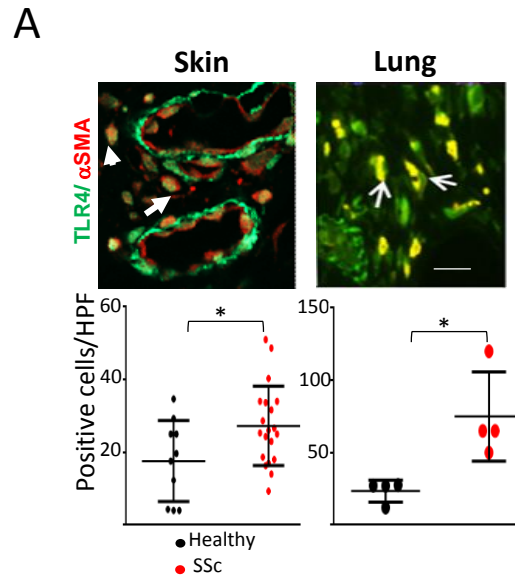
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Figure 1



**Vicious cycle of fibrosis.** Tissue damage occurs from chronic injury causes generation and accumulation of TLR4 DAMPs. DAMPs in turn activate fibroblast innate immune signaling via TLR4, enhancing fibrogenic responses and establishing a self-amplifying vicious cycle of fibrosis. Moreover, intrinsic protective signals (such as A20) that normally act as the brakes on fibroblast activation might be nonfunctional in SSc.

Figure 2

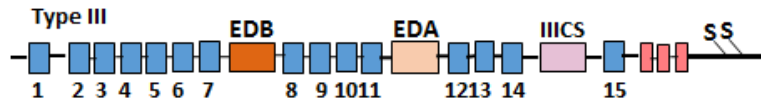


Elevated TLR4 and its DAMPs expression in SSc biopsies and fibroblasts. **A: Upper panels**, immunofluorescence of skin and lung biopsies from SSc patients with pulmonary fibrosis ( $n = 4$ ). Yellow, colocalization of the two antibodies (**white arrows**). Scale bars: 50  $\mu$ m. **Bottom panels**, quantitation of TLR4 staining. Each dot, number (mean) of immunopositive fibroblasts from four separate microscopic fields per biopsy. **B: Left panels**, immunofluorescence of SSc skin biopsies ( $n = 11$ ) and healthy controls ( $n=6$ ). **Right panels**, SSc fibroblasts ( $n = 3$ ) showed increased Fn<sup>EDA</sup> and tenascin-C compared to healthy controls ( $n=2$ ). Scale bars: 50  $\mu$ m.



Figure 3

A

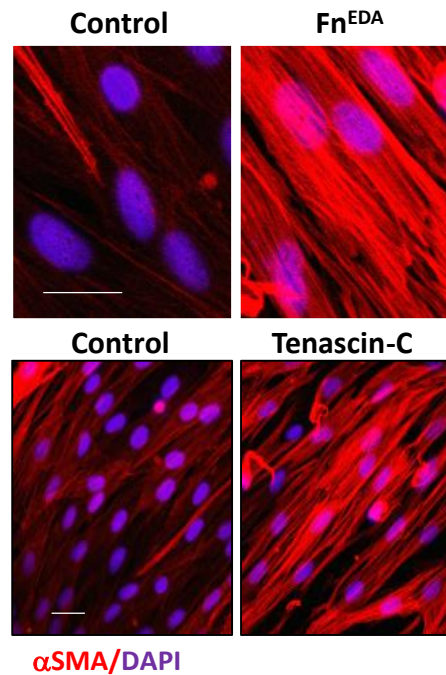


B



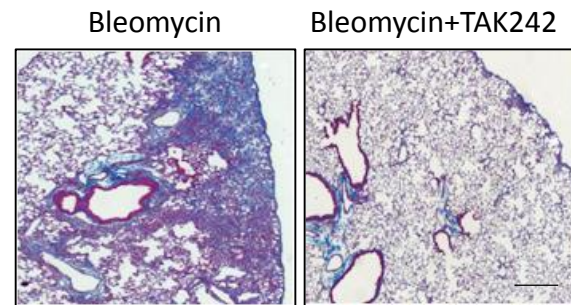
Schematic representation of fibronectin Type III module of fibronectin and tenascin-C. **A.** Fibronectin exists as a protein dimer. Three regions of variable splicing occur along the length of the fibronectin. One or both of the "extra" type III modules EDA (between 7 and 8) and EDB (between 11 and 12) may be present in cellular fibronectin ( $\text{Fn}^{\text{EDA}}$  and  $\text{Fn}^{\text{EDB}}$ ), and another connecting segment (CS, between the 14th and 15th type III module). EDA and EDB splicing is similar in all species, while that of the type III CS region is species-specific. **B.** Tenascin-C is a large hexameric extracellular glycoprotein. Tenascin C comprises several fibronectin type III homology repeats (TNIII) and a C-terminal fibrinogen-like globe (FBG). Alternative splicing in TNIII (between 5 and 6) can lead to small and large tenascin-C isoforms.

Figure 4



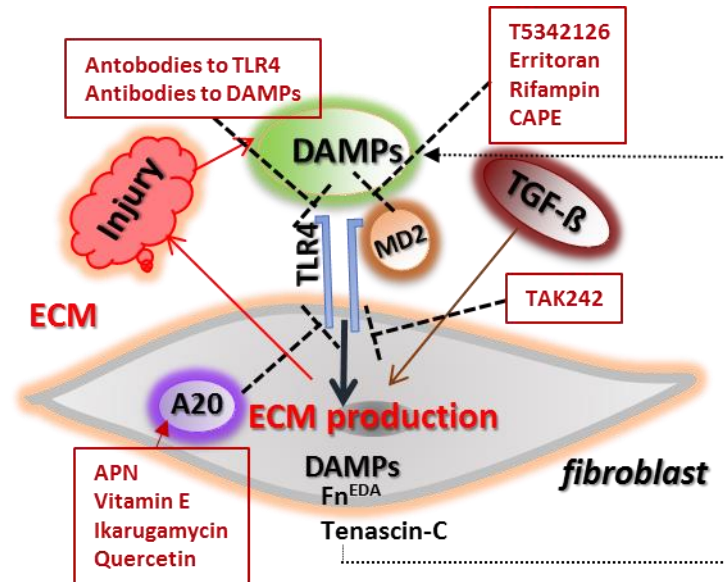
**TLR4 DAMPs promotes myofibroblasts differentiation in normal fibroblasts.** Human skin fibroblasts were incubated in medium with Fn<sup>EDA</sup> or tenascin-C for 72 hours. Immunofluorescence showed marked increase in myofibroblasts differentiation compared to untreated control. Scale bar, 50  $\mu$ m.

Figure 5



**TAK242 treatment ameliorates bleomycin-induced lung fibrosis.** C57/BL6 were administered bleomycin or PBS via s.c. injections for 14 days and lungs were collected 10 days following last injection. Trichrome stain for collagen showed reduced expression with TAK242 treatment. Scale bar, 10  $\mu$ m.

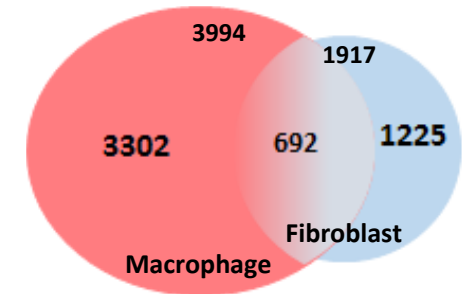
**Figure 6**



**Blockade of TLR4 signaling at different levels.** Antibodies to either TLR4 or TLR4 DAMPs directly inhibit TLR4 signaling. Endotoxin antagonists or small molecule inhibitors of TLR4/MD2 interaction are promising therapeutic TLR4 target. TAK242 via direct binding to TLR4 intracellular Cys747 residue blocks TLR4 signaling. On the other hand, APN, VitE and others can inhibit TLR4 signaling by inducing A20, a negative regulator of TLR4.

Table 1

Fibroblasts		Macrophages	
GO Terms	p	GO terms	p
response to wounding	4.91e-15	translation	2.25E-27
cell migration	8.3e-11	ncRNA metabolic process	2.47E-15
defense response	4.45e-10	immune response	9.23E-14
extracellular matrix organization	8.86e-10	ribonucleoprotein complex biogenesis	1.47E-12
cell surface receptor linked signaling pathway	1.17e-09	translational elongation	9.86E-12
programmed cell death	2.56e-09	ribosome biogenesis	1.55E-11
response to cytokine stimulus	3.25e-09	cellular respiration	3.54E-11
inflammatory response	3.31e-09	RNA processing	5.01E-11
apoptosis	3.49e-09	generation of precursor metabolites and energy	1.79E-10
blood vessel development	3.66e-09	ncRNA processing	1.96E-08



**Biological Processes designated by individual GO categories elicited by LPS in fibroblasts and macrophages.**

**A.** Confluent skin fibroblasts and macrophages treated with LPS for 24h. At the end of incubation period, total RNA was harvested and processed for hybridizations to Illumina Human HT-12 microarray chips. The table showing the significantly enriched GO categories elicited by fibroblasts and macrophages.

**B.** Venn diagram showing gene numbers.