Curr Rheumatol Rep (2015) 17: 2
DOI 10.1007/s11926-014-0474-z

SCLERODERMA (J VARGA, SECTION EDITOR)

Emerging Roles of Innate Immune Signaling and Toll-Like Receptors in Fibrosis and Systemic Sclerosis

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Published online: 22 January 2015

Abstract Pathological fibrosis is a distinguishing hallmark of systemic sclerosis (SSc) as well as a number of more common conditions. Fibrosis is a complex and dynamic process associated with immune dysregulation, vasculopathy, and uncontrolled extracellular matrix production leading to intractable scar formation in the skin and internal organs. Persistent or recurrent chemical, infectious, mechanical, or autoimmune injury in genetically predisposed individuals causes sustained fibroblasts activation. Innate immune signaling via toll-like receptors (TLRs) is increasingly recognized as a key player driving the persistent fibrotic response in SSc. In particular, expression of TLR4 as well as its endogenous ligands are elevated in lesional tissue from patients with SSc. Ligand-induced TLR4 activation elicits potent stimulatory effects on fibrotic gene expression and myofibroblast differentiation. Furthermore, TLR4 appears to sensitize fibroblasts to the profibrotic stimulatory effect of transforming growth factor-β. This review highlights recent advances and emerging paradigms for understanding the regulation, complex functional roles, and therapeutic potential of TLRs in SSc pathogenesis.

Keywords Toll-like receptor 4 · Fn-EDA · TGF-ß · Fibrosis · Systemic sclerosis · SSc · Scleroderma · Fibroblast

Introduction

Fibrosis is a prevalent medical concern and an unmet need. A hallmark of systemic sclerosis (SSc), fibrosis is characterized by excessive synthesis and accumulation of collagen and other extracellular matrix (ECM) molecules in skin, lungs, and other internal organs [1•]. Increased matrix stiffness, chronic oxidative stress, and extracellular accumulation of damage-associated molecular patterns (DAMPs) are recently recognized additional prominent features of fibrosis [1•, 2•, 3••, 4, 5]. Transforming growth factor-β (TGF-β) induces the full repertoire of fibrotic responses and plays a key role in pathogenesis [6]. Although multiple intracellular pathways are implicated in TGF-β-mediated fibrotic responses, cross-talk among these networks and the nature of their persistent deregulation in pathological inflammation and fibrosis remain poorly understood. Recent studies, discussed below, link innate immune signaling and toll-like receptors (TLRs) to TGF-β activity and persistent fibrotic responses.

Toll-like receptors are evolutionary conserved cellular sensors for both microbial (exogenous) pathogen-associated molecular patterns (PAMPs) and endogenous DAMPs [7]. Oxidative damage and tissue injury promote the local generation of DAMPs such as low-molecular-weight hyaluronan degradation products, alternately spliced fibronectin-extra domain A (Fn-EDA), tenascin C, and biglycan; or release of intracellular stress proteins such as high-mobility group protein-B1 (HMGB1) and heat shock protein 60 (Hsp60); and nucleic acids and immune complexes, each of which can induce cell activation via TLRs. Recent studies provide evidence for fundamental and previously unappreciated roles for TLRs and their endogenous ligands in SSc [8, 9]. Levels of both TLR4 and its endogenous ligands are elevated in SSc and elicit potent stimulatory effects on fibrotic gene expression [4, 3••]. Genetic targeting of TLR4 or its endogenous ligands ameliorates experimental fibrosis in mouse models of SSc. Mechanistic studies demonstrate that TLR4 dramatically enhanced the sensitivity of fibroblasts to the profibrotic stimulatory effect of TGF-β. Despite considerable recent progress, the pathological role of TLRs and its ligands in fibrosis and SSc still remains inadequately defined. We provide a brief

This article is part of the Topical Collection on *Scleroderma*

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overview of TLR structure, expression, and function, followed by recent insights and current understanding of the role of innate immune signaling and TLRs in fibrosis and SSc and discuss future research directions and potential therapeutic strategies targeting TLRs.

Toll-Like Receptors, Signaling, and Biological Outcomes

Toll-like receptors are transmembrane glycoproteins composed of an extracellular leucine-rich repeat extracellular domain and transmembrane and intracellular Toll/IL (interleukin)-1 receptor (TIR) domains [10, 11]. Leucine-rich repeats mediate ligand recognition and TIR domains mediate intracellular signal transduction. Toll-like receptor 4 (TLR4), an orthologue of Drosophila Toll, was the first TLR to be identified [12]. To date, 10 human and 12 mouse TLRs have been identified. While TLR1–TLR9 are conserved in both species, TLR10 is not functional in the mouse, whereas TLR11, TLR12, and TLR13 do not exist in the human genome. TLR1, TLR2, TLR4, TLR5, and TLR6 are expressed on the cell surface, whereas TLR3, TLR7/8, TLR9, and TLR11 are located in the endoplasmic reticulum (ER), endosomes, lysosomes, or endolysosomes [11].

Upon binding to exogenous PAMPs or endogenous DAMPs, TLRs dimerize and undergo the conformational changes necessary for downstream signal transduction. Activated TLRs engage two distinct intracellular pathways, myeloid differentiation factor 88 (MyD88)-dependent or the MyD88-independent. In general, all TLRs, except TLR3, recruit MyD88 through interaction with their respective TIR domains, resulting in the activation of IL-1R-associated kinases (IRAKs), IκB (inhibitor of NF-κB) kinase (IKK), and NF-κB, and secretion of inflammatory cytokines. TLR3 signals through TIR-domain-containing adaptor-inducing interferon (TRIF). TLR4 is the only TLR that engages both MyD88 and TRIF for signaling [11].

Ligands of TLRs

Both microbial PAMPs and endogenous DAMPs can serve as TLR ligands [13]. By recognizing the PAMPs (bacterial lipoprotein, lipopolysaccharide (LPS), and nucleic acid), TLRs play an essential role in host defense against microorganisms and viruses. TLR3 responds to double-stranded RNA produced during viral replication, whereas TLR7 and 8 recognize viral single-stranded RNA [11]. TLR13 in mice recognizes a conserved 23S ribosomal RNA sequence [14•]. Gram-negative bacterial LPS is recognized by TLR4. TLR9 acts as a receptor for unmethylated CpG motifs present in bacterial and viral DNA [13, 11]. The relevant ligands for TLR10 and TLR12 still remain unidentified.

The DAMPs are endogenous TLR ligands, released from cells upon necrosis or tissue injury, generated by stressed cells, or resulting from mechanical or biochemical fragmentation of ECM molecules, that serve as essential "danger signals" [7]. DAMPs enable organisms to sense, and mount a response to, tissue damage. Endogenous ligands for TLR4 include the heat shock proteins HSP60, HSP70, HSP22, and HSP72 and GP96 and HMGB1; fragmented or alternately spliced ECM molecules such as low-molecular-weight hyaluronic acid (LMW HA), fibronectin-EDA, tenascin C, and versican; and mitochondrial DNA released from dead or dying cell [3••, 4, 7]. Interestingly, the type III domain of fibronectin can be unfolded in response to mechanical forces or generated via extracellular proteolysis leading to TLR4-dependent inflammatory responses [15].

Negative Regulation of TLR Signaling

In order to prevent aberrant or sustained injurious innate immune responses, a number of "fail-safe" mechanisms evolved to negatively regulate TLR signaling [16•]. Alternately spliced TLR adaptors, ubiquitin ligases, deubiquitinases, transcriptional regulators, and microRNAs (miRNAs) [17, 13, 18] can all serve to dampen TLR signaling. The direct TLR target A20 (also known as TNFAIP3) acts as an E3 ubiquitin ligase to control NF-κB activation and TLR-mediated responses [19, 20]. In an arthritis model, A20 functions as a negative regulator of Nlrp3 inflammasome activation, and A20-KO mice develops Nlrp3-dependent spontaneous arthritis [21•]. Intriguingly, both A20/Tnfaip3 and Nlrp3 are susceptibility risk alleles for rheumatoid arthritis. The radio protective 105 gene product (RP105) negatively regulates MD2-dependent TLR4 signaling [22, 23]. RP105-deficient mice developed accelerated arthritis [24]. In contrast to murine RP105, little is known about the functional role of this molecule in humans. Other negative TLR regulators include single immunoglobulin IL-1R-related molecule (SIGGR/TIR8), ST2 (IL-33 receptor), soluble TLR9 (sTLR9) generated by cleavage of TLR9 [25], IRAK isoform IRAK-M, and suppressor of cytokine signaling-1 (SOCS-1) that suppresses IRAK activity and TLR4 signaling [16•]. The role of these negative TLR regulators in disease remains to be determined.

A Novel Role for TLRs in Organ Fibrosis Emerges

Persistence and progression of fibrosis involves feed-forward amplification loops that promote fibroblast activation and myofibroblast differentiation. As highlighted in the following section, recent studies implicate deregulated TLR expression and signaling as key factors underlying pathological fibrogenesis in liver, kidney, lungs, heart, skin, and SSc.

TLRs in Liver Fibrosis

Liver fibrosis due to hepatitis C virus (HCV) and alcoholic and nonalcoholic steatohepatitis (NASH) is mediated by the interactions between hepatic stellate cells (HSC) and immune cells, as well as by intracellular immune signaling pathways in HSCs. Innate immunity plays a key role in these processes.

In liver injury, HSC transformation into myofibroblasts and production of type 1 collagen underlie fibrogenesis. Activation of HSC can be elicited by LPS or by endogenous TLR4 ligands [26]. Elevated circulating LPS is prominent in chronic liver diseases [27]. Quiescent HSCs resist TGF-β activation due to constitutive overexpression of bone morphogenetic protein and activin membrane-bound inhibitor (BAMBI), a negative regulator of Smad-dependent TGF-β signaling [28, 29]. Upon TLR4 activation of HSC, BAMBI is downregulated, allowing unrestricted TGF-β signaling. TLR4-mutant mice are resistant to experimental hepatic fibrogenesis [29]. Mice with targeted deletion of TLR9 similarly showed reduced HSC activation and liver fibrosis [30]. Conversely, TLR3 appears to serve on anti-fibrotic function, and its deficiency in mice is associated with progression of alcohol-induced liver fibrosis [31]. These findings highlight the divergent functional roles of different TLRs in liver fibrosis.

TLRs in Pulmonary Fibrosis

In contrast to liver fibrosis, the role of TLR signaling in lung fibrosis is not well defined. Key pathological features of lung fibrosis include loss of alveolar epithelial cell integrity and interstitial fibrosis with distortion of normal tissue architecture [32•, 33]. Common etiologies include viral and bacterial infections, radiotherapy and chemotherapy, and graft versus host disease; however, in most cases, the etiology remains unidentified (idiopathic pulmonary fibrosis, IPF) [33]. A variety of recent studies implicate TLRs in the development of lung fibrosis. Functional polymorphisms in the TLR3 gene (L412F) with attenuated TLR3 function are associated with accelerated progression in IPF, consistent with an anti-fibrotic role [34•]. In contrast, elevated TLR9 expression and signaling in lung fibroblasts are associated with rapid progression of IPF [35, 36]. Furthermore, the TLR9 ligand CpG oligonucleotide (ODN) exacerbated lung fibrosis in a humanized mouse model [35]. However, other studies showed that TLR9 limited the lung fibrotic response induced by gamma herpes virus or by bleomycin [37, 38]. TLR4 plays a profibrotic role in the lung, and its downregulation by small hairpin RNA (shRNA) ameliorated LPS-induced pulmonary fibrosis in mice [39]. In contrast, TLR2/TLR4 double knockout mice showed enhanced radiation-induced lung fibrosis and reduced survival after acute lung injury [40, 41]. Another study showed that TLR4 was required for the resolution of lung inflammation and fibrosis [42]. The contributions of specific TLRs to lung fibrosis in mice and in humans merit better understanding.

Toll-Like Receptor 4 Implicated in Kidney Fibrosis

Glomerulosclerosis and tubulointerstitial fibrosis are hallmarks of chronic kidney disease [43]. Activation of TLR4 appears to play a critical role in renal fibrosis by augmenting TGF-β responses in tubular cells and in myofibroblasts via mechanisms involving downregulation of BAMBI [44]. Endogenous TLR4 ligands prominent in kidney injury include HMGB1, ECM components, and HSPs. Mice deficient in these TLR4 ligands, or in TLR4, were protected from renal fibrogenesis induced by a variety of experimental approaches [44–46]. For instance, mice deficient in TLR4, MyD88, or TRIF showed a significant reduction in fibrosis in chronic allograft nephropathy models [47].

TLRs in Myocardial Fibrosis

Cardiac injury, as well as aging, leads to remodeling, fibrosis, and stiffness that underlie ventricular dysfunction and heart failure [48]. Common triggers include ischemia, pressure overload, and viral infection. A role for innate immunity in cardiac fibrosis, particularly TLR2 and TLR4, is emerging. Recurrent exposure to LPS is, by itself, sufficient to induce cardiac fibrosis [49]. Mice with targeted deletion of TLR4 or TLR2 showed improved ventricular function, reduced remodeling, and collagen accumulation following myocardial infarction [50–53] In contrast, synthetic ligands of TLR9 (1668-thioate or 1612-thioate) were shown to attenuate cardiac hypertrophy and fibrosis following transverse aortic constriction [54]. Endogenous damage-associated TLR ligands potentially implicated in myocardial fibrosis include fibronectin-EDA, HSP60, HMGB1, tenascin C, galectin-3, S100A8, S100A9, and mitochondrial DNA [55].

TLRs in Skin Fibrosis

Skin fibrosis is a prominent feature of keloids, nephrogenic systemic fibrosis (NSF), morphea, and hypertrophic scars. Keloids are benign fibroproliferative tumors commonly occurring following trauma to the skin. A recent study indicated increased expression of TLR6, 7, and 8 in keloid lesions [56]. Nephrogenic systemic fibrosis occurs in individuals with advanced kidney failure exposed to gadolinium contrast agents (GdBCA). In human macrophages, GdBCA was shown to activate TLR4 and TLR7, resulting in production of proinflammatory and profibrotic cytokines and growth factors. These findings suggest that the environmental stimuli participate in the pathogenesis of NSF and other cutaneous fibrotic

disorders via TLRs signaling [57]. Hypertrophic scars occur following dermal damage by thermal injury or other forms of trauma. A recent study showed that levels of TLR4, MyD88, and proinflammatory cytokines were all elevated in fibroblasts from hypertrophic scars [58•].

Innate Immune Activation and TLRs in SSc

Genetic Polymorphisms in the TLR Signaling Axis in SSc

Genome-wide association studies (GWAS) in SSc reveal genetic associations with several innate immune signaling variants [59, 60•] (Table 1). A rare functional polymorphism in the TLR2 gene (Pro631His) showed robust association with both anti-topoisomerase antibody-positive SSc and pulmonary arterial hypertension [61]. Other studies identified genes for interferon regulatory factors (IRF5, IRF7, and IRF8) as risk factors for limited cutaneous SSc [62]. Of great interest, A20 or TNFAIP3, a negative regulator of TLR signaling, and (TNFAIP3)-interacting protein 1 (TNIP1) both showed strong association with SSc [63, 64]. Furthermore, expression of TNFAIP3 messenger RNA (mRNA) was significantly reduced in carriers of the rs117480515 allele compared to non-carriers of the allele among SSc patients. Levels of TNIP1 mRNA and protein were markedly reduced in lesional skin as well as in explanted fibroblasts from SSc patients. TNIP1 abrogated fibrotic responses in activated healthy and constitutively active SSc dermal fibroblasts [65]. The consistent genetic associations of TLR signaling variants with SSc, and with specific endophenotype, together with emerging functional studies, suggests that the TLR pathways may play a role in pathogenesis.

Emerging Role of TLRs in SSc Pathogenesis

Production of type I interferon (IFNα and β) is closely linked to TLR-mediated innate immune signaling [66, 67]. Dysregulated expression of type I IFN-inducible genes in peripheral blood cells is seen in SSc [68]. Moreover, sera from patients with anti-topo I-positive SSc patients induce IFN-α production in normal peripheral blood mononuclear cells in vitro [69]. Serum containing other SSc autoantibodies failed to induce this response. In response to TLR8 ligands, peripheral blood monocytes from SSc patients showed enhanced production of tissue inhibitor of metalloproteinase 1 (TIMP-1), a profibrotic factor [70]. Stimulation of dendritic cells from SSc patients showed increased sensitivity to TLR ligands compared to that from healthy controls [71].

Evaluation of SSc skin biopsies reveals elevated expression of TLR3 primarily localized to fibroblast-like dermal cells [72, 73]. In normal skin fibroblasts, TLR3 stimulation by poly(IC) induced dose- and time-dependent increase in IFN-β production and expression of IFN-regulated genes [73]. In contrast, the expression of fibrotic genes in these cells was inhibited. Viral infection of nonimmune cells might cause persistent tissue injury and chronic inflammation and fibrosis in SSc. A recent study described striking accumulation of Epstein-Barr virus (EBV) in dermal fibroblasts and endothelial cells of SSc skin biopsies [74•]. Infection of stromal cells with EBV in vitro induced activation of endosomal TLRs that was accompanied by fibroblast-myofibroblast transdifferentiation and increased profibrotic gene expressions [74•]. These findings suggest that chronic EBV infection or reactivation in SSc may trigger sustained fibroblasts activation and fibrosis. Chronic subcutaneous injection of a synthetic TLR3 ligand in mice induced dermal inflammation and expression of IFN-regulated and fibrotic genes [75].

Table 1 Genetic polymorphisms in the TLR pathways that are associated with SSc

	Chromosome	SNP associated with SSc	SSc subtype	Risk/protection	Reference
TLR2	4q32	rs5743704	Diffuse	Risk	[60•]
IRF5	7q32	rs4728142	Limited	Risk	[58•, 59]
		rs2004640			
		rs10488631			
IRF7	11p15	rs1131665	NA	Risk	[61]
		rs4963128			
		rs702966			
		rs2246614			
IRF8	16q24	rs11642873	Limited	Protective	[61]
		rs2280381			
TNFAIP3	6q23	rs5029939	Diffuse	Risk	[62]
TNIP1	5q33	rs4958881	NA	Risk	[63, 64]
		rs2233287			
		rs3792783			

Table 2 Endogenous TLR4 ligands expressed in SSc

Endogenous ligand	Expressed in SSc (skin/lungs)
Fn-EDA	+++
Tenascin C	++++
HA (low molecular weight)	++
Biglycan	+/−
GP96	−
HMGB1	−

− undetectable, + found, ++ strong, ++++ very strong expression

TLR4 Signaling in SSc Fibrosis

Recent studies further elucidate the role of TLR4 signaling in SSc. Expression of TLR4 was elevated in SSc skin and lung biopsies [4]. Strong TLR4 immunostaining was seen in a large percentage of fibroblasts and vascular cells within lesional tissues. Another report showed that both TLR4 along with its co-receptors MD2 and CD14 are overexpressed in lesional skin [76••]. Importantly, TLR4 levels correlate with progressive skin disease. Some patients with SSc were found to have circulating anti-fibroblast antibodies that were functional and could elicit TLR4-mediated inflammatory and profibrotic responses in vitro [77].

Other ligands of TLR4 also have profibrotic effects. In explanted skin fibroblasts, LPS induced TLR4-dependent increase in collagen synthesis and dramatically enhanced sensitivity to the stimulatory effects of TGF-β [4]. These responses involved downregulation of BAMBI, resulting in the augmented intensity of canonical Smad signaling. In addition, LPS also caused TLR4-dependent downregulation of antifibrotic miR-29. Therefore, multiple mechanisms in SSc including enhanced Smad signaling with downregulation of BAMBI and suppression of miR-29 might underlie these profibrotic effects of TLR4. In mouse, chronic LPS exposure augmented the production of proinflammatory chemokines and upregulation of TGF-β-regulated genes in the dermis [76••]. On the other hand, skin fibrosis induced by bleomycin was attenuated in mice harboring a nonfunctional mutant TLR4 [4].

Further evidence linking TLR4 with fibrosis in SSc comes from whole genome-wide transcriptional profiling studies. Previous gene expression studies analysis stratified SSc biopsies into four distinct intrinsic subsets (fibroproliferative, inflammatory, limited, and normal-like groups) [78]. An experimentally derived fibroblasts "LPS-regulated gene signature" was generated using skin fibroblasts incubated with LPS. This "LPS-regulated gene signature" is present in SSc biopsies clustering within inflammatory subset (Bhattacharyya S. and Varga J., unpublished data). Therefore, this SSc might be optimal responders to therapeutic strategies to block TLR4.

Alternately Spliced Fibronectins (Fn-EDA): a Damage-Associated Endogenous TLR4 Ligand With Key Roles in SSc

Using an immunohistochemistry-based survey, we found that several endogenous TLR4 ligands are elevated in SSc skin (Table 2). Most significant among these are low-molecular-weight hyaluronic acid (LMW HA), tenascin C, and, of special interest, fibronectin-EDA [4, 3••].

Fibronectins are high-molecular-weight glycoproteins present in soluble form in plasma or accumulating in tissue as insoluble ECM components [79]. Due to alternate splicing of the fibronectin gene, cellular fibronectin contains two extra domains A (EDA and EDB) which are excluded from plasma fibronectin [79]. Although there is little tissue fibronectin-EDA in adults, marked upregulation occurs during injury. Levels of fibronectin-EDA were found to be significantly elevated in both serum and lesional skin from SSc patients [3].

In vitro, Fn-EDA expression could be induced by treatment with TGF-β in normal fibroblasts in culture. In contrast, in SSc fibroblasts, synthesis of Fn-EDA was constitutively

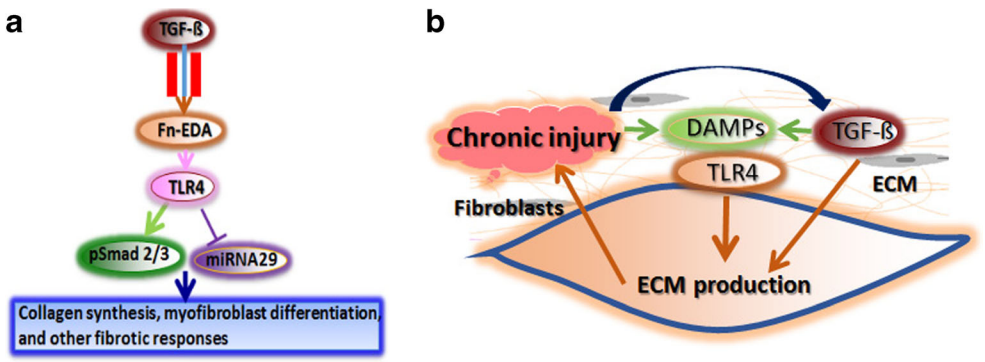

Fig. 1 **a** Fn-EDA-TLR4-mediated fibroblast activation. TGF-β generates extracellular accumulation of Fn-EDA that in turn triggers TLR4-dependent cellular signaling, resulting in profibrotic responses. **b** Vicious cycle of fibrogenesis. Toll-like receptor signaling switches self-limited repair into sustained fibrogenesis. Chronic injury leads to tissue damage, and generation and accumulation of endogenous DAMPs leading to ongoing activation of TLR4 in fibroblasts and progressive fibrosis

upregulated (Fig. 1a). Treatment of normal fibroblasts with Fn-EDA stimulated collagen production, myofibroblast differentiation, and wound healing. All these responses were abrogated by selective disruption of TLR4 signaling. Moreover, mice with targeted deletion of Fn-EDA showed attenuated skin fibrosis when challenged with bleomycin or with TGF-β [3••]. These observations therefore implicate an endogenous Fn-EDA-TLR4 signaling axis in fibroblasts in the pathogenesis of cutaneous fibrosis in SSc.

Summary and Perspectives

This review highlights emerging evidence implicating innate immune signaling in persistent fibroblast activation underlying fibrosis in a variety of chronic diseases. Based on these observations, we propose that TLR4 represents a molecular switch that, in response to endogenous damage-associated TLR ligands, convert a self-limited tissue repair process into persistent and progressive pathological fibrogenesis (Fig. 1b).

Although a growing body of evidence supports a key role for DAMPs and TLRs in driving pathological fibrosis, major questions remain. While GWAS provide evidence linking SSc with gene variants implicated in TLRs signaling, functional studies are necessary to elucidate the biological mechanisms by which associated variants contribute to the disease process. Even though multiple endogenous TLR ligands are elevated in SSc skin and lung and their deficiency in mice confers resistance to fibrosis, identification of specific TLR ligands that are most important in SSc, and the relevant target cell populations, is lacking. Moreover, the initial triggers eliciting prolonged injury, the unique and redundant profibrotic functions of the various TLRs and their co-receptors, and the regulation of their function by endogenous inhibitors similarly remain uncertain.

Nevertheless, in light of the central role that TLR4 appears to play in SSc, disrupting sustained TLR4 signaling at the level of ligand or receptor level represent novel potential strategies for therapy [80•]. As TLRs are in the frontline of anti-microbial innate immunity, the major challenge is to dampen overactive TLR-dependent immune signaling without impairing normal host defense. Promising approaches to target TLR4 include selectively blocking intracellular signaling or disrupting ligand-TLR4 receptor or co-receptor complex formation (Bhattacharyya S and Varga J, unpublished). As fibrosis in SSc currently has no effective therapy, intense investigation of the regulation, expression, and function of TLRs in fibroblasts and other cells may yield progress toward development of urgently needed therapies.

Compliance with Ethics Guidelines

Conflict of Interest Swati Bhattacharyya declares no conflict of interest.

John Varga reports the receipt of grants from the NIH and Takeda, as well as a pending patent on a TLR4 inhibitor.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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