Excessive tissue scarring or fibrosis is a common feature of the several chronic diseases [1]. Scleroderma is a multisystem disease clinically characterized by progressive fibrosis of the skin and internal organ, which accounts for the intractable nature and high mortality of this disease [2]. As the principal effector cells responsible for fibrosis, fibroblasts and myofibroblasts secrete collagen and other extracellular matrix (ECM) proteins and promote a profibrotic cytokine milieu. Transforming growth factor-β (TGF-β) stimulates collagen synthesis, myofibroblast differentiation, and epithelial-mesenchymal transition, and is implicated as a key initiating factor in both physiological and pathological tissue remodeling [3]. However, the mechanism responsible for the persistence of the fibrotic response associated with pathological repair in scleroderma remains poorly understood. Toll-like receptors (TLRs) are pattern recognition receptors that have evolved to recognize components of pathogens and also endogenous danger signals contributing to ‘sterile inflammation’. Systemic immune dysregulation via TLRs is evident in scleroderma leading to activation of fibroblast. Our recent studies identified fibronectin extra domain A (FnEDA), an alternative splice variant of the matricellular protein fibronectin, as an endogenous damage-associated ligand for toll-like receptor 4 (TLR4) as important drivers of maintaining persistent myofibroblast transdifferentiation and fibrosis [3]. In this report, we highlight the role of the FnEDA-TLR4 signaling axis in fibrosis, and the mechanisms involved in the activation of the pathway that can drive persistence of fibrosis in scleroderma.

**Profibrogenic signals elicited by TLR4**

As current studies implicate TLRs in the pathogenesis of...
fibrosis, we investigated the role of TLR4-driven fibroblast response in fibrogenesis. We have shown that TLR4 was constitutively expressed in lesional skin and lung tissues from patients with scleroderma [4]. Although the origin of fibrosis in injured adult tissues has been debated for decades, the majority of the results now implicate the myofibroblast/fibroblast as the primary fibrogenic cell [5]. Activation of TLR4 by microbial ligands (such as lipopolysaccharide) or endogenous ligands sensitizes fibroblasts to the effects of TGFβ, and thereby, promotes TGFβ-dependent stimulation of collagen and ECM production [4, 6, 7]. We showed that activation of TLR4 in fibroblasts augmented canonical Smad signaling by downregulating BAMBI. Moreover, the activation was accompanied by suppression of anti-fibrotic microRNA 29 expression. BAMBI is a negative regulator of TGF-β signaling that lacks an intracellular kinase domain required for downstream signaling. Therefore, in normal skin fibroblasts and in down-regulation of BAMBI by TLR4 allows unrestricted activation of TGF-β signaling [5, 6, 7].

Activation of TLR4 signaling increased collagen synthesis and enhanced the expression of tissue remodeling associated multiple genes. These profibrotic responses were abrogated by selective disruption of TLR4 signaling in vitro. Importantly, mice harboring a non-functional TLR4 were protected from the development of skin fibrosis. Similarly, mice lacking functional TLR4, MyD88 or TRIF showed reduced fibrosis upon bile duct ligation (BDL), or following unilateral ureteral obstruction (UUO) [6, 7].

**Figure 2. Persistent fibroblast TLR4 signaling triggered by endogenous TLR4 ligands underlies interminable fibrosis in scleroderma.** Recurrent tissue injury causes generation of “damage-associated molecular patterns” (DAMPs) that serve as danger signals. DAMPs are recognized as endogenous ligands for TLR4, such as ECM-derived molecules FnEDA and drive sustained TLR4 activation. TLR4 signaling in turn, by sensitizing fibroblasts to TGF-β and by down-regulating antifibrotic miRNA 29, forms a stimulatory feedback loop contributing to persistence and progression of fibrosis.

**Damage-associated endogenous TLR4 ligands**

The primary mechanism by which the innate immune system detects the presence of DAMPs is via a family of pattern recognition receptors (PRRs). Ligands for PRRs include not only molecules released by dying or damaged cells such as high mobility box 1 (HMGB1) and heat shock proteins (HSPs) s, self-DNA and RNA, ECM components generated through alternative splicing (8). However, the precise function of these protein isoforms is still unclear in most cases. Inclusion of the alternatively spliced regions is elevated during embryonic development and decreases with ageing. The ‘embryonic’ splicing pattern is re-established under certain circumstances in adults tissue repair, angiogenesis and fibrosis. Fibronectin is one of the best known protein generated by alternate splicing. Fibronectin has two main form: dimeric soluble plasma fibronectin (pFN) lacking EDA and EDB domain and multimeric cellular fibronectin (cFN) including EDA or EDB deposited in ECM. During skin wound healing, the inclusion of EDA and EDB domains is increased at the wound base [8].

**FnEDA, an important driver for TLR4 driven intractable fibrotic response**

As a possible endogenous TLR4 ligand we have undertaken a study to investigate the expression and regulation of FnEDA in scleroderma, and its role and mechanism of action in fibrosis. These studies showed that the alternately spliced variant of fibronectin FnEDA, but not FnEDB, was specifically elevated in the serum and in skin from patients with scleroderma, as well as in lesional tissues from mice with cutaneous fibrosis [3]. Furthermore, we found that TGF-β preferentially stimulated the production of FnEDA in normal fibroblasts via canonical Smad signaling pathway. In turn, exogenous FnEDA stimulated collagen synthesis and myofibroblasts differentiation in fibroblasts, and induced dermal sclerosis in 3D skin equivalents (Fig. 1). Serving as an endogenous TLR4 ligand, FnEDA elicited potent TLR4-dependent fibrotic responses in fibroblast. Moreover, in mice both genetic deletion of FnEDA as well as pharmacologic blockade of TLR4 alleviated cutaneous fibrosis.
Conclusions

The observations summarized here identify a novel role for the ECM-derived danger molecule FnEDA to activate fibroblast TLR4 signaling, resulting in augmented TGF-β sensitivity and consequent increased ECM production and intractable fibrosis (Fig. 2). Disrupting the ligand-TLR4 signaling axis, or blocking cellular TLR4 signaling using selective small molecule inhibitors, represent appealing novel strategies for breaking the self-amplifying cycle of fibroblasts activation and attenuating progressive fibrosis as treatment for scleroderma and other fibrotic diseases.

Conflicting interests

The authors have declared that no competing interests exist.

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