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Toll Like Receptors in systemic sclerosis: an emerging target

Short title: TLRs scleroderma

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Highlights

- TLRs are critical in systemic sclerosis
- Activation of TLRs can be mediated by danger signals from damaged cells
- DAMPs may mediate fibrosis via TLR-dependent release of TIMPs

Abstract

Pattern Recognition Receptors are critical receptors that elicit an immune response upon their activation that culminates in activation of NF- κ B and cytokine secretion. Key among these receptors are the Toll-Like Receptors (TLRs). These evolutionary conserved receptors form a key part in the defence against various pathogens and comprise a key part of the innate immune system. Systemic sclerosis is an autoimmune disease in which a breach of tolerance has occurred and leads to fulminant autoimmunity, dysregulated cytokines, pro-fibrotic mediators and activation of fibroblasts leading to fibrosis via collagen deposition. It has become apparent in recent years that the innate immune system and specifically TLRs are important in disease pathogenesis; responding to internal ligands to initiate an innate immune response ultimately leading to release of a variety of factors that initiate and perpetuate fibrosis. This review will examine the recent evidence of TLR signalling in systemic sclerosis and the internal danger associated molecules that may mediate the fibrotic cascade. Evaluation of their contribution to disease in systemic sclerosis and possible therapeutic targeting will be discussed.

Introduction

A major mechanism of innate immunity is the activation of a group of Pattern Recognition Receptors by molecules on specific pathogens. Toll-Like Receptors (TLRs) are a germline-encoded group of pattern recognition receptors that share high homology with the Toll gene in fruit flies and are found in eukaryotes and plants [1]. These TLRs recognise patterns on microbial species and internal damage associated molecular patterns that elicit an immune response without maintaining immunological memory. TLRs are widely and selectively expressed in multiple cell types including immune cells such as macrophages but also non immune cells such as epithelial cells and fibroblasts. They can respond to signalling via a variety of different cell types and a variety of different ligands either of endogenous origin, in the case of Danger Associated Molecular Patterns (DAMPs), or microbial origin. It is now widely acknowledged that as well as playing a role in multiple disease states TLRs play a primary role in fibrosis and systemic sclerosis. In terms of fibrosis, they can play a role via modulation through multiple cell types including macrophages. Understanding the pathogenesis of systemic sclerosis and the role that TLRs play in this has been difficult but recent advances in molecular immunological research and the advent of new molecular techniques has helped uncover their roles in the disease. What are the physiological and non-physiological ligands that mediate activation to TLRs in the context of fibrosis is a key question. The cellular component of skin fibrosis comprises fibroblasts, endothelial cells, pericytes and macrophages, all of which express PRRs [2] (Table 1).

Systemic Sclerosis (SSc) is an idiopathic autoimmune connective tissue disease that carries three hallmarks: vascular damage, inflammation and cytokine dysregulation and fibrosis [3]. The most obvious hallmark is skin fibrosis. Fibrosis is mainly in the skin but can also be present in the lungs and heart. To date the only disease modifying therapy found to regress the fibrosis is autologous stem cell transplant confirming the key role of the immune system in fibrosis, as there is an increased Tregulatory compartment after transplant and also alterations in the Th1/Th2 balance, with a skew toward a Th1 phenotype [4, 5] and a reduction post-transplant in autoantibodies [6]. Emerging evidence has suggested that the activation of the immune system in SSc may have an infectious basis or endogenous DAMPs [7]. Activation of the immune system and TLR triggering culminates in the release of pro-inflammatory and also pro-fibrotic mediators that ultimately results in the activation of a fibroblast to a myofibroblast with the release of copious amounts of Extracellular Matrix (ECM) and therefore fibrosis. Fibroblasts themselves also express TLRs, albeit at lower expression, and themselves are targets for TLR ligands. An explosion of recent interest in this area has identified molecular pathways that underpin TLR regulation and in SSc a variety of TLR ligands have been identified. The review aims to give an overview of the role of TLRs in SSc and propose areas for therapeutic intervention.

TLRs signalling

TLRs were first discovered in *Drosophila melanogaster* and "Toll" relates to a mutant gene encoding a receptor involved in embryonic development. Cells endowed with TLRs have an ability to respond to various ligands endogenous and microbial. TLRs are transmembrane proteins that contain an extracellular leucine rich repeat and a cytosolic Toll-IL-1 receptor domain (TIR) that is responsible for downstream signalling [8]. The activation of TLRs culminates in NF- κ B activation and pro-inflammatory cytokine release. TLRs recruit adaptor proteins that include MyD88, Mal, Trif and Tram [9]. MyD88 is utilized by all the TLRs with the exception of TLR3. TLRs are found primarily in the cellular membrane and some are found intracellularly upon endosomes making them uniquely placed to respond to their ligands. Negative regulation of TLR activation is important to restrain inappropriate inflammation and this occurs through microRNAs. MicroRNAs are epigenetic regulators of gene expression by negatively regulating gene expression by binding to the 3'UTR of

target mRNAs [10]. One of the most inducible microRNAs after LPS stimulation is microRNA155 that elevates to negatively regulate the immune response by targeting SHIP1 [11]. Mice with genetic ablation of microRNA155 have autoimmunity, indicating a critical role in negative regulation [11]. Of course other epigenetic modifiers could also negatively regulate TLRs such as histone modification. Negative regulation. Another negative regulator of TLR signalling is Suppressor of Cytokine Signalling 1 (SOCS1) [12]. SOCS1 is rapidly induced upon TLR4 stimulation to negatively regulate the TLR signalling and overexpression of SOCS1 results in attenuated NF-KB transcriptional activity [12] and SOCS1 gene deleted mice challenged with LPS have increased mortality [12]. Also AKT1 has been found to regulate the response to TLR agonists and endotoxin tolerance through its regulation of SOCS1 expression by reducing the expression of SOCS1 via its regulator microRNA miR155 [13].

Stranger Danger

The danger theory proposed by Matzinger states that the immune system responds to danger rather than just non-self [14]. Five years earlier Charles Janeway had suggested that the immune system is activated by conserved patterns in different microorganisms.

It is more than 20 years since Matzinger proposed the danger theory that explains why immune responses occur in organ transplants, tumours, trauma injuries and autoimmune disorders all without a microbial component. This model suggests that proteins normally not seen by the immune system "hidden" intracellularly are released upon damage or "stress" to the cells and evoke an immune response. This was for years a theoretical model until HMGB-1 was confirmed to be a DAMP [15]. Arguably HMGB-1 is the proto-typical DAMP. HMGB-1 is a nuclear protein that normally resides in the nucleus but can also be released upon which it can engage the LPS receptor TLR4 leading to inflammation [15]. This nascent field is growing rapidly with new DAMPs being described yearly and now included mitochondrial DAMPs released via trauma [16] This may not be as surprising as it first seems as mitochondria are endosymbionts derived from ancient bacteria engulfed by archezoan cells approximately 2 billion years ago. And like bacteria, have their own circular DNA that replicates independently of nuclear DNA. Indeed, they also have similar methylation to bacterial DNA suggesting one mechanism by which TLRs can sense mitochondrial DNA. Recently it was shown that hepatocyte mitochondrial oxidised DNA triggers TLR9 to mediate hepatic fibrosis.

What is "Danger" precisely? It is difficult to define danger in the context of inflammation, but DAMPs are generally recognised as intracellularly sequestered molecules hidden from the immune system under normal physiological conditions. However, under stressful conditions or tissue trauma these are actively released from the cell, exposed on stressed cells or passively released from these cells [17]. Here they then bind to cognate TLRs initiating an immune response. It is tacitly assumed that these molecules are only released from cells under adverse conditions. There are now multiple different DAMPs recognised and none of them seems to share any structural similarities. One of the prototypical DAMPs is High Mobility Group Box-1 protein (HMGB-1). HMGB-1 is a nuclear protein that can regulate gene expression but can be released extracellularly to elicit responses via TLRs and Receptor for advanced Glycation End Products. Interestingly HMGB-1 can be altered by redox status also and even monomethylation of HMGB-1 alters its location in the cells [18]. Research into many diseases has revealed the importance of DAMPs and their release under a variety of stressors including hypoxia for example. A central question is what cell type is the source of the DAMPs in SSc? Are these cell types also the source of the autoantigens in SSc? There is clear alterations in vascularity in SSc and it has been suggested that the earliest pathologic event is cell death of vascular endothelial cells. So an obvious source of DAMPs could be vascular endothelial cells that have become apoptotic but have not been cleared appropriately. This reduced ability to clear

apoptotic cells also occurs in lupus [19]. There is a clear reduced uptake of apoptotic cells by macrophages in lupus [20]. This host cell derived DNA inciting stimulation of TLRs requires further investigation as the source of DNA in SSc has not been uncovered.

DAMPs released during ECM remodelling

Danger molecules can be generated by the ECM. The ECM is comprised of multiple molecules including collagen, fibronectin and others. The ECM provides a physical framework between cells and tissues and can also act as signalling molecules. These can be altered to produce forms that are recognised the TLR system as 'danger' to initiate signalling cascades. Coercion of local fibroblasts by myofibroblasts activated by DAMPs may propagate the signal resulting in amplification.

TLR2 and systemic sclerosis

TLR2 is a membrane bound TLR that responds to gram positive bacterial cell wall components to provoke an immune response including lipoproteins of bacteria and mycoplasma. However, endogenous ligands such as serum amyloid A have also been identified for TLR2 [21, 22]. TLR2 recognises its ligands through the formation of a heterodimer with TLR1 or TLR6, these then recognise distinct ligands [23].

Serum amyloid A is an acute phase protein that is mainly synthesised in the liver and is induced after infection or trauma, which can be induced many fold above baseline [24].

Serum amyloid A has long been known to be associated with acute phase responses and inflammation, it is also elevated in SSc [25]. Its role in disease though remained unclear. Our group was the first to demonstrate that serum amyloid A activates TLR2 as incubation of recombinant protein in TLR4 reporter cells had no effect [26]. Making use of dominant negative NF-KB constructs transfected into the cells we could determine that serum amyloid A-mediated induction of Interleukin-6 was dependant on NF-KB. We could then show that the expression level of TLR2 was elevated in SSc patients fibroblasts and blockade with a specific TLR2 neutralising antibody compared to a isotype control antibody reduced IL-6 levels [26]. IL-6 is critical in mediating pro-fibrotic effects in dermal fibroblasts via a STAT3-dependant mechanism [27].

Recently a study has found elevated serum levels of serum amyloid A in SSc patients and confirmed our observations of IL-6 being induced in dermal fibroblasts with recombinant protein addition [28], interestingly, there was a strong association with high amyloid A levels and pulmonary involvement, indicating this could be used as a biomarker. Furthermore TLR2 stimulation leads to increased chemotaxis of monocytes via secretion of MCP-2 [29]. Rare genetic variant in TLR2 is associated with SSc and higher levels of IL-6 in dendritic cells, which is involved in the fibrotic signalling cascade and is associated with pulmonary hypertension.

Dendritic cells are the sentinals of the immune system sensing the local environment and responding appropriately. A rare variant in A20, a negative regulator of TLR signalling, is also associated with SSc [30]. S100A7 is also elevated in systemic sclerosis [31] and this is a TLR2 ligand. Indeed in bleomycin mediated lung fibrosis in pre-clinical studies deletion of TLR2 resulted in reduced inflammation and fibrosis [32]. Interestingly, targeting TLR2 with an anti-TLR2 neutralising antibody in the lung fibrosis bleomycin model also reduced fibrosis in the lungs with reduced collagen and inflammatory mediators but also reduced phosphorylated STAT3 levels [32], which is downstream of IL-6 and proved to activate fibroblasts to myofibroblasts [27, 33, 34]. TLR2 stimulation on B cells from SSc patients also leads to an upregulation of both pro-inflammatory (IL-6) and pro-fibrotic cytokines such as TGF- β 1 from these cells which was attenuated by CD19 deletion [35].

TLR4 and DAMPs

TLR4 is the best characterised TLR to date and is the receptor for LPS (endotoxin) a constituent component of all gram negative bacteria.

Whilst LPS is the main PAMP that binds TLR4 a number of endogenous danger signals can also bind TLR4 to initiate pro-inflammatory signals [36]. Although activated by a wide variety of ligands, many of which share no structural similarities, most studies use LPS to activate TLR4. Some of the activator of TLR4 signals are directly pro-fibrotic [17, 37]. The list of endogenous ligands for TLR4 is now growing rapidly and include heat shock proteins, S100s, high mobility-group box protein 1 (HMGB-1) [38, 39], hyaluron [40], fibronectin and tenascin-C [41] (Table 2). Tenascin-C has been shown to activate TLR4 in adipocytes and induce ECM remodelling [41].

Tenascin-C is an important mediator of arthritis as KO mice are protected from experimental arthritis and that it mediates its effects through TLR4 [42]. It is an extracellular matrix component that can be in many forms. Recent evidence has suggested that tenascin-C can activate hepatic cells to become hepatic stellate cells, the key cells responsible for liver fibrosis. Recently an elegant study confirmed a role for tenascin-C in systemic sclerosis with patients having highly elevated levels of the molecule compared to controls in both tissue biopsies and also serum [43]. Tenascin-C mediated an increased expression of collagen and alpha-smooth muscle actin and this was attenuated by MyD88 blockade and TLR4 KO cells, indicating it signals through this pathway as would be expected if TLR4 is the receptor. Finally they show that tenascin-C knockout mice are protected from skin and lung fibrosis in the bleomycin model with a reduction in lung altered mechanics in KO mice [43]. This activation of fibroblasts via tenascin-c and TLR4 appears to involve MyD88 but may also activate the Smad pathway indirectly as KO mice had reduced Smad signalling [43]. Smad signalling can be activated by TGF- β 1 which is induced by bleomycin instillation.

Fibronectin has also been identified as binding to TLR4, but only fibronectin that contains alternatively splice exons encoding type III repeat extra domain (EDA) [44]. Fibronectin in this form is recognised by the TLR and immune system. The splicing of fibronectin EDA domains is tightly regulated during embryogenesis with human adult tissue devoid of this EDA domain until tissue damage occurs. Thus it is used to regulate the generation of tissues and organs and under situations of damage is used as an instructive signal to help wound healing. Interestingly recipient-derived fibronectin promotes the fibrosis associated with chronic graft rejection and is suggested to be through the enhanced TLR-mediated alterations of T regulatory cells [45]. Elevated levels of fibronectin EDA has recently been described in SSc serum and also skin [46]. Incubation of fibroblasts with this fibronectin EDA resulted in formation of the pathogenic myofibroblast with increased collagen and ECM secretion [46]. Furthermore, this was markedly reduced in mice lacking fibronectin EDA or TLR4 thus showing the dependence of TLR4 for the fibronectin EDA-mediated increase in collagen. This was also blocked with a pharmacological agent blocking TLR4. It is suggested that the initial damage and wound response liberates fibronectin EDA this interacts with TLR4 to initiate downstream signalling resulting in a reduction of microRNA29a thus leading to derepression of its target: collagen. Stimulation of TLR4 induces fibrosis by augmenting TGF- β signalling [47]. In the bleomycin model of fibrosis it was demonstrated that mice without TLR4 compared to wild type mice had significantly less fibrosis and this was associated with reduced antibodies and IL-6 [48] and SSc tissue has higher levels of TLR4 expression (figure 1). Gene expression analysis after TLR4 stimulation in fibroblasts leads to global gene changes including a dominance of genes involved in wound healing and ECM regulation and as expected changes in inflammatory genes. It maybe suggested that spliced fibronectin helps resolve the wound after initial tissue damage by binding to expressed TLR4 but a failure of this to cease leads to persistent activation and fibrosis which is

perpetuated by the plethora of cytokines, chemokines and TIMPs that are released from activated myofibroblasts culminating in unrelenting fibrosis (figure 2). TLR4 stimulation in dendritic cells from SSc patients also leads to enhanced secretion of the CC chemokine ligand 18 (CCL18) [49]. Chemokines are clearly elevated in the sera of SSc patients which will aid leukocyte recruitment to the tissue [50]. Figure 3 gives an overview of the possible pathogenic mechanism.

Internal TLRs

Intracellular TLRs include TLR3, 7, 8 and 9. These intracellular TLRs are cytosolic sensors for nucleic acids normally of viral origin [51]. Such is the importance of intracellular TLRs in promoting inflammation to repel viruses that some pathogens have developed mechanisms to manipulate them. The localisation of the TLRs is important here in that viruses hijack the host cell and given the fact that nucleotides from the host could be potent agonists of these TLRs it is important that these are compartmentalised. After stimulation with ligands these TLRs are translocated from the ER to the endolysosomes mediated by the ER protein UNC93B1 [52]. However, UNC-93B1 is dispensable for ligand recognition and initial signal transduction [52]. Interestingly, mice with a mutation in UNC-931B suffer from systemic lethal inflammation due to a skewing toward TLR7 trafficking and activation and increased Th17 cells and IFN- γ [53].

It has been demonstrated that using poly IC, a synthetic dsRNA, to simulate TLR3 signalling in monocytes leads to increased siglec1 expression in SSc [54]. This is important in the binding of macrophages to other cell types. TLR3 activation also leads to upregulated endothelin-1; an important pro-fibrotic molecule [25]. Furthermore, a polymorphism in TLR3 has been found to be associated with idiopathic lung disease with a greater decline in lung function [55]. Also TLR3 KO mice has exaggerated lung fibrosis when challenged with bleomycin compared to wild type challenged mice suggesting a defect TLR3 signalling system enhances pulmonary fibrosis [55]. We have previously found that incubation of SSc monocytes with single stranded RNA leads to upregulation of TIMP-1 that ultimately leads to enhanced fibrosis [56]. We could show that incubation of patients sera which was eliciting a TLR8-dependant increase in TIMP-1, with an RNA degrading enzyme, reduced sera alone-mediated TIMP-1.

We went on to show that the single stranded RNA-mediated increase in TIMP-1 is dependent on MyD88 and also IRAK4 as we used cells from an IRAK-4-deficient patient [56]. It was also demonstrated that the TIMP-1 is functional. We postulate that the RNA species in the SSc patient's serum is complexed to autoantibodies that from an RNA-associated autoantibodies aggregate leading to TLR mediated fibrosis. Such changes in self RNA and or DNA being recognised by the immune system are common in other autoimmune diseases [57]. Interestingly, single stranded RNA is found in many types of virus, suggesting that a viral origin is responsible. We have now demonstrated that the single stranded RNA mediates TIMP-1 via TLR8 and depends on trimethylation of histone27 which alters the expression of the AP-1 transcription factor Fos-Related Antigen-2 (FRA2) [58]. This links possibly viral activation of TLR8 to activation of FRA2 via epigenetic modulation by histone methylation [58]. Reduction of FRA2 by small interfering RNA attenuated the increased TIMP-1 levels [58].

Epstein Barr Virus (EBV) is a herpes DNA virus and evidence has emerged that this may be associated with SSc by the fact of serological evidence of infection in SSc patients [59, 60]. It was recently found that EBV genes are upregulated in SSc skin biopsies [61]. Furthermore, infection of normal dermal fibroblasts or endothelial cells with EBV induced interferon regulatory factors, interferon genes and TGF- β , ultimately leading to expression of ECM proteins and alpha smooth muscle actin [61]. Thus EBV may be the initial trigger of SSc. Although direct evidence of EBV viral infection in SSc

pathogenesis is lacking it maybe that infection on fibroblasts with EBV enables the cell to become responsive to growth factors such as TGF- β enabling enhanced proliferation and endowing the cells with excessive ECM. Recent work is suggestive of a viral cause however, this is controversial and no direct link has been proven.

Interestingly, gadolinium, a contrast agent, that can cause nephrogenic systemic fibrosis, has been shown to activate TLR7 in monocytes ultimately resulting in the release of pro-inflammatory and pro-fibrotic mediators [62].

TLR9 is the receptor that recognises unmethylated CpG motifs in bacterial and viral DNA these motifs are prevalent in microbial but not vertebrate genomic DNA and can also recognise mitochondrial DNA. DNA II gene deleted mice which breaks down DNA selectively in macrophages have an polyarthritis associated with DNA mediated activation [63]. Activation of the TLR9 leads to intracellular signalling and an IFN response with IFN regulated genes increased. In IPF it has been shown that TLR9 expressing fibroblasts is more prevalent in rapidly progressive patients than slowly progressing patients [64] and fibroblasts derived from the rapid progresses were more responsive to the TLR9 agonist stimulation and could induce the differentiation of blood CD14⁺ monocytes to fibrocytes that secrete collagen and other ECM molecules [64]. It was also shown that the potent pro-fibrotic TGF- β 1 leads to upregulation of TLR9 in normal fibroblasts and differentiate into myofibroblasts with a persistent and strong expression of α -Sma [65]. These CpG stimulated cells both stably expressed α -Sma and PDGFR α , a key receptor for fibrosis generation and an increased resistance to apoptosis and reduced caspase-3 levels, possibly mediated through HIF-1 α expression [65]. In mouse fibroblast it was also shown that CpG stimulation leads to enhanced myofibroblasts and cytokine induction and using a wound healing model in mice it was shown that blocking the nucleic acid with a third-generation dendrimer reduced granulation tissue [66]. TLR9 gene deleted mice are protected from experimental liver fibrosis also suggesting a universal phenomenon [67].

Is the TLR system druggable?

Can we drug the TLR system to modify the pro-fibrotic response to ligands? Small molecule inhibitors are now attractive therapies for autoimmune diseases. This is because they are specific and generally well tolerated. A challenge has always been to limit unanticipated side effects due to inhibition of downstream signalling. Initial excitement for the use of P38 Mitogen-Activated Protein Kinase (MAPK) inhibitors was tempered due to unacceptable side effects. Because P38 α is such an important molecule in inflammation targeting this in archetypal inflammatory diseases such as arthritis seemed rationale but orally administered pamapimod in RA failed both to show efficacy compared to the comparator methotrexate, importantly also had significantly higher side effects including infections, skin disorders and elevated liver enzymes. However, the notion of targeting downstream signalling is not dead and many other signalling nodes can be targeted orally.

Multiple molecules have now been developed that can inhibit and suppress TLR signalling. Inhibiting this can occur by blocking the interaction with the DAMP and its receptor, prior to intracellular signalling or blocking the internal signalling (table 3).

There is now a TLR2 blocking antibody manufactured by Opsona therapeutics [68]. This molecule has been found to be safe and tolerable and may be of use in SSc where TLR2 activation plays a role in myofibroblasts generation [69]. Adaptor proteins MyD88 and Mal could also be therapeutic targets and these have been inhibited in rheumatoid arthritis synovial explants *in vitro* [70], however because MyD88 is critical in response to microbes it may be that targeting this globally carries an increased risk of infection. Recent evidence has also demonstrate that TLR2 mediated fibrosis can be

blocked in vivo in an animal model by specific ablation of the adaptor protein MyD88 in pericytes [71]. Pericytes are suggested with some controversy, to be the precursors to myofibroblasts. It is thought that these cells once activated can convert to myofibroblast and secrete ECM; it is interesting to note that these cells can be activated by histones; ascribed DAMPs [71]. Therefore, this suggests that targeting either TLR2 or MyD88 is effective in attenuating fibrosis. TLR3 has also been targeted with CNTO3157 which is a monoclonal antibody that binds TLR3 preventing the association of dsRNA and thus inhibits TLR3 mediated effects. CNTO3157 has been shown to attenuate the effects of viral challenge on lung function in asthmatic patients [72]

Emerging evidence suggests that blocking TLR8 can be useful in SSc [17, 37, 58]. Idera pharmaceuticals have a TLR7/9 antagonist in clinical trial for plaque psoriasis and also dermatomyositis (table 4). AZD1419 is a TLR9 agonist developed by Dynavax that targets the TLR9 system to modify the immune response in allergic asthma. Another possible therapeutic is to sequester the nucleic acids and this has already been done using a third generation dendrimer that scavengers nucleic acid and this reduced granulation tissue in an animal model [66].

An alternative approach is to target epigenetic modifications that are important in mediating TLR signalling. For instance multiple microRNAs, including miR155 [11] are important in negative feedback loops to repress signalling and thus altering these could repress TLR signalling and thus fibrosis. Use of microRNA mimics would be useful in this situation by binding to the 3'UTR of the target mRNAs of proximal effectors [73, 74]. Another approach may be to inhibit histone methylation through the use of histone methyltransferase inhibitors such as DZNep targeting Enhancer of zeste homologue 2 (Ezh2), which we have shown is effective [58]. EZH2 is a histone lysine methyltransferase enzyme mediating the trimethylation onto lysines in the histone H3 and thus promotes epigenetic gene silencing. EZH2 has recently been shown to be critical in the generation of Treg cells and their expansion and can govern the life and death of peripheral T cells. These data strongly support the idea that epigenetic modification can be targeted to alter TLR signalling. However, much like targeting the archetype NF- κ B globally, targeting broad TLR suppression, may lead to increased infectious disease. Few data are available on targeting histone modifications in TLR signalling and may yield more specific inhibitors. Targeting histone methyltransferases as opposed to 5'azacytidine targeting global methylation is likely to have much less side effects.

CRISPR-Cas9

Clustered, regularly interspaced, short palindromic repeat (CRISPR-Cas9) is a powerful genome editing technique. CRISPR is a bacterial adaptive immune mechanism, that can target specific sequences of DNA [75]. This consists of unique spacer sequences short repetitive palindromic sequences and sequences encoding Cas proteins. It is a type of immune mechanism present in bacteria to protect them from viral infection [76]. The system has been engineered so that a guideRNA (gRNA) with the Cas9 that specifically cleaves DNA activating double strand break repair machinery, this includes nonhomologous end-joining and homology directed repair. As an RNA-guided DNA endonuclease, Cas9 can easily be programmed to target new sites by altering its guide RNA sequence. This has made sequence specific gene editing much easier. This techniques has been used to genome edit in cells in vitro and in vivo in mice to genetically remove sequences [77]. This new technique with far reduced "off target" effects could be used to precisely genome edit the TLR system. This could be used to permanently ablate either the TLR or a downstream effector. CRISPR-Cas9 can also be used to introduce a mutation as opposed to deletion of the gene and this could for instance in the case of TLR4 induce tolerance thus reducing the immune response.

Conclusion

Recent literature has now well established the role of TLRs in systemic sclerosis pathogenesis through the activation of their receptors to downstream signalling and resulting release of pro-fibrotic molecules. Indeed, in normal fibroblasts activation of TLRs, normally expressed at a low level, can result in fibrosis independent of inflammatory cells. The accumulating data that support the involvement of these receptors in fibrosis are fuelling efforts to target these. The importance of their involvement in SSc pathogenesis suggests that they a tractable target and various molecules can inhibit these TLRs and appears successful in animal models. This now requires further study and areas such as long non coding RNAs that regulate TLR signalling by altering chromatin state [78] could be new therapeutic targets. New technologies enabling identification of epigenetic modifiers will expose these as regulators of TLR signalling and such epigenetic changes being rapid may explain tolerance induction to TLR ligands. Finally, a breakdown between the equilibrium between us and the microbiome may also be responsible for the autoimmunity.

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Figure legend

Figure 1 Elevated TLR4 in SSc skin biopsies. Mean levels of TLR4 in SSc skin biopsies measured by qPCR data is normalised to the house keeping gene 18S and shown as fold change compared to control skin biopsies. Data is the mean and standard deviation.

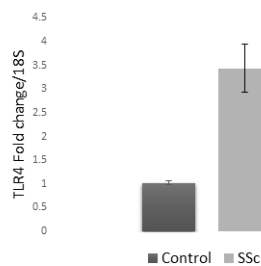


Figure 2 Possible pathway through which DAMPs trigger fibrosis via TLR4. TLR4 is activated by DAMPs which are released passively after tissue trauma this leads to activation of the signalling pathways leading to pro-fibrotic cytokine release including Interleukins, this activates the fibroblasts to transition to the myfibroblast; the chief cell in fibrosis.

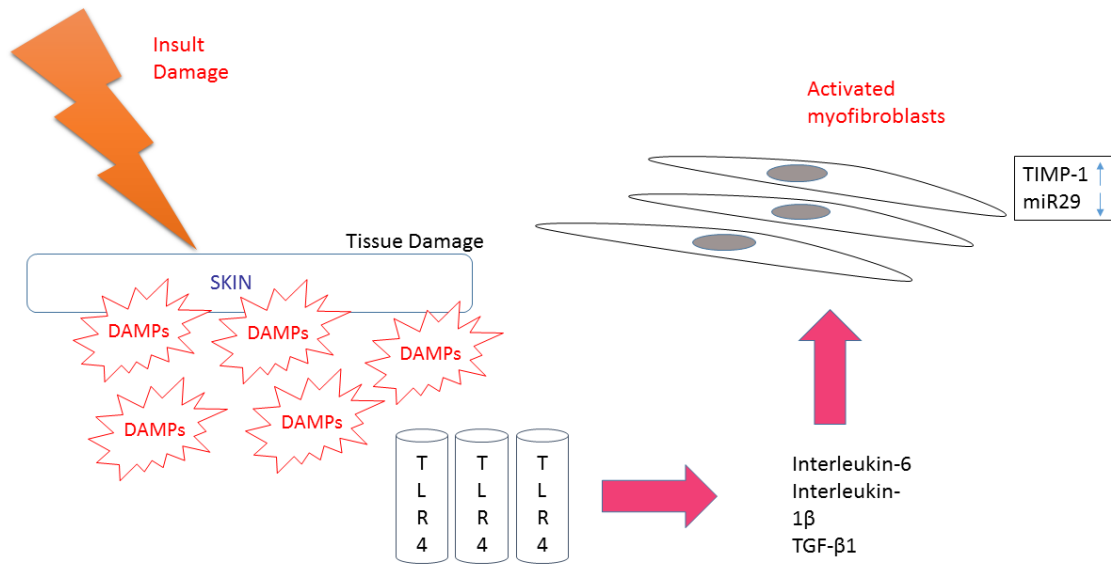


Figure 3 Possibly pro fibrotic pathways in SSc.

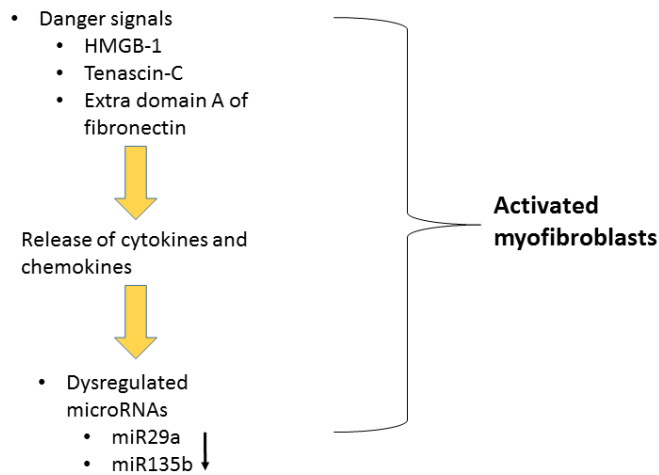


Table 1 Cell types in SSc skin

Cell Type	TLR expression
Fibroblasts	+
Pericytes	+
Macrophages	+++
Dendritic cells	++++
T cells	+

+= low expression

Table 2 TLRs and their known endogenous ligands

TLR	Endogenous ligand	
TLR2	HMGB-1	
	Serum amyloid-A	
	Snapin A	
TLR3	mRNA	
TLR4	HMGB-1	
	HSP-20, -60, -70, -96	
	Fibrinogen	
	Extra domain A of Fibronectin	
	Tenascin C	
	Surfactant protein-A S100A8/9, CD138	
TLR6/2	Soluble tuberculosis factor	
	HSP-60, -70, -96	
TLR7	ssRNA (immune complexes)	

TLR8	ssRNA (immune complexes), human cardiac myosin	
TLR9	DNA (immune complexes)	

Table 3 Ways of interfering with TLR signalling

Blocking exogenous ligand/TLR interaction
Blocking the TLR directly with an antibody
Inhibiting downstream signalling with small molecule inhibitors such as MyD88
Enhancing negative regulators of the TLR signalling pathway
Modifying epigenetic regulators of the TLR pathways such as microRNAs

Table 4 Current conditions with TLR7/8/9 antagonists under consideration

Plaque Psoriasis
Dermatomyositis
B-cell lymphoma