REVIEW ARTICLE

Toll-like receptors in mediating the pathogenesis in Systemic Sclerosis

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Short title: TLRs in systemic sclerosis pathogenesis

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Abbreviations: TLRs (Toll-like receptors); SSc (systemic sclerosis); lcSSc (limited cutaneous SSc); dcSSc (diffuse cutaneous SSc); DAMPs (danger associated molecular patterns); RP (Raynaud's phenomenon); pDCs (plasmacytoid dendritic cells); IFN-I (Type I interferon); PAMPs (pathogen associated molecular patterns); HMGB1 (high-mobility group protein 1); RAGE (receptor for advanced glycation-end-product); ROS (reactive oxygen species); ATP (adenosine tri-phosphate); ECM (extracellular matrix), ATA (anti-topoisomerase antibodies); ACA (anti-centromere antibodies); EDA (fibronectin extra domain A); MyD88 (myeloid differentiation primary response); dsRNA (double strand RNA); ssRNA (single strand RNA); dsDNA (double strand DNA); ssDNA (single strand DNA); PBMCs (peripheral blood mononuclear cells); ETosis (extracellular traps release); NETosis (neutrophil extracellular trap release); RT-qPCR (quantitative reverse transcription PCR); Poly I:C (Polyinosinic:polycytidylic acid); CXCL4 [chemokine (C-X-C motif) ligand 4]; mitoDNA (mitochondrial DNA); BDCA2 (Blood dendritic cell antigen 2); ODN2006 (oligo deoxynucleotide 2006); AMP (antimicrobial peptide); HBD- (human β-defensin-); IL- (interleukin-): B19 (Parvovirus B19), HCMV (Cytomegalovirus), EBV (Epstein–Barr virus); TNF-α,(tumor necrosis factor α):
TGF-β (transforming grow factor beta); SSc-Ig (SSc immune complex); Ig (immune-globulin); MMP- (matrix metallo-proteinase); TIMP-1 (tissue inhibitor of metalloproteinase 1). Hsp (heat shock proteins). LDL (low density lipoproteins). HBD (human β-defensin).

Summary
Toll-like receptors (TLRs) are evolutionary conserved receptors essential for the host defence against pathogens. Both immune and non-immune cells can express TLRs, although at different levels. Systemic sclerosis (SSc) is a chronic disease in which autoimmunity, dysregulated pro-fibrotic mediator release and activation of fibroblasts lead to dysregulated collagen deposition and fibrosis. There is now increasing knowledge that the innate immune system and, in particular, TLRs take a part in SSc pathogenesis. The list of endogenous ligands that can stimulate TLRs in SSc is growing: these ligands represent specific danger associated molecular patterns (DAMPs), involved either in the initiation or in the perpetuation of inflammation, and in the release of factors that sustain the fibrotic process or directly stimulate the cells that produce collagen and the endothelial cells. This review reports evidences concerning TLR signalling involvement in SSc. We report the new DAMPs, as well as the TLR-linked pathways involved in disease, with emphasis on Type I interferon signature in SSc, the role of plasmacytoid dendritic cells (pDCs) and platelets. The dissection of the contribution of all these pathways to disease, and their correlation with the disease status, as well as their values as prognostic tools, can help to plan timely intervention and design new drugs for more appropriate therapeutic strategies.

Keywords: Toll-like receptor, systemic sclerosis, Interferon signature, immune cells, fibrosis

Introduction
Systemic sclerosis (SSc) is an autoimmune and fibrotic disease with a high disease
burden and mortality rate (1). Three most important hallmarks characterise SSc: autoimmunity, fibrosis and vasculopathy. Autoimmunity is an important component, as autoreactive T cells and autoantibodies play a central role in SSc pathogenesis (1, 2). Fibrosis is the most lethal feature responsible for organ failure (1-3). Microvascular constriction and endothelial damage, clinically expressed by Raynaud’s phenomenon (RP), are the first manifestation of SSc in 90-98% of cases and precede the disease onset by years (4). According to the extension of the skin fibrosis, it is possible to define two major subsets of SSc: limited cutaneous SSc (lcSSc) and diffuse cutaneous SSc (dcSSc) (5). Currently, no effective drugs exist which can modify the disease course. Dysregulation of the innate immune system in genetically predisposed individuals plays a role in SSc and aberrant Toll-like receptor (TLR) activation seems central to pathogenesis (6, 7). TLRs are a germline-encoded group of pattern recognition receptors, which comprise 10 members (TLR1–TLR10) in humans and 12 members (TLR1–TLR9, TLR11–TLR13) in mice (8). They are key for recognition of invading pathogens. As transmembrane receptors, they localise either at the cell surface or in the endosomal compartment. Since they are involved in self- versus non-self-discrimination, TLRs have implication in various autoimmune and auto-inflammatory conditions (9). Non-immune cells, included fibroblasts, endothelial cells and platelets also express TLRs and respond to a wide array of microbial molecules (pathogen associated molecular patterns, PAMPs). However, TLRs also respond to endogenous non-microbial stimuli, referred to as danger associated molecular patterns (DAMPs) (10). Cellular stress and traumas induce DAMP release. The exact identification of the DAMPs involved in TLR stimulation in SSc is pivotal to the development of specific therapies (6).

**Danger molecules (DAMPs) in autoimmunity**

One of the first discovered DAMP is the high-mobility group protein 1 (HMGB1, 11). DAMPs are generally inside the cells, hidden to the immune system but traumas or stress induce their release. HMGB1, the prototype of DAMPs, is normally expressed in the nuclei, but if released can trigger, among other TLRs, the TLR4. By binding to damaged DNA, HMGB1 can also favour TLR9 stimulation (12). Activated platelets in SSc blood release micro-particles which contain HMGB1 (13). HGMB1 also binds receptor for advanced glycation-end-product (RAGE), an immunoglobulin superfamily member (11).
In general, DAMPs are heterogeneous molecules: apart from HMGB1, other DAMPs are Ca^{2+}, H_{2}O_{2}, reactive oxygen species (ROS), adenosine tri-phosphate (ATP), self-nucleic acids, heat shock protein, S100 proteins (alarmins), fragments of the extracellular matrix (ECM), uric acid, heparin sulphate. Released intracellular mitochondria also represent DAMPs (14). Several DAMPs in SSc can concur to diseases pathogenesis (6).

Membrane TLRs and their DAMPs in SSc
In humans, TLR1, 2, 4, 5, 6 and 10 localise on the cell surface of immune and non-immune cells. TLR4 recognises bacterial-derived lipopolysaccharides (LPS), a PAMP mainly expressed on the surface of Gram-negative bacteria, as well as other factors acting as DAMPs, among these, heat shock proteins (Hsps), taxol, fibronectin extracellular matrix components (ECM), fatty acids, LDL, fibrinogen. TLR2 recognises lipoproteins, peptidoglycans and lipopeptides, hyaluronic acid, Hsp 70 liparabinomannan from a variety of microorganisms and even HMGB1 (15,16). A role for TLR2 is likely in SSc pathogenesis (5, 6): TLR2 hyper-expressing SSc fibroblasts overproduce IL-6, a critical molecule in fibrosis (17). Serum amyloid A, which is high in SSc (18), stimulates TLR2 in fibroblasts. A rare polymorphism of TLR2 was associated with diffuse SSc and with anti-topoisomerase antibodies (ATA) and pulmonary arterial hypertension (19). TLR2 forms heterodimers with TLR1 or TLR6 (in a ligand specific manner) to recognize a variety of PAMPs such as peptidoglycans, lipotechoic acid, zymosan, and mannan. However, no information is available on the role TLR1 and TLR6 in SSc.

Bhattacharyya et al. demonstrated that the fibronectin extra domain A (EDA), an endogenous TLR4 binder, was elevated in the circulation and in the lesional skin biopsies of SSc patients, as well as in mice with experimentally induced cutaneous fibrosis (20). Disrupting TLR4-signalling abrogated the deleterious effect of EDA, in that TLR4 stimulation induced collagen production and myofibroblast differentiation. In mice, the blockade of TLR4 mitigated experimentally induced fibrosis. Thus, the example of TLR4 and EDA is paradigmatic of how a DAMP, when out of control, activates multiple unwanted pathways leading to disease (20). An additional endogenous ligand for TLR4 studied by the same authors is tenascin C, which also mediates fibrosis in SSc. Tenascin C is not expressed normally, but is expressed transiently during wound healing and
tissue remodelling. Tenascin C sustains fibrosis in a mouse model of SSc via TLR4 (21). Accordingly, an enhanced TLR4-responsive gene signature was present in SSc skin biopsies (22). Collectively these studies point to the involvement of TLR4 signalling in fibrosis in SSc.

With regard to other TLRs, one study addressed the expression of TLR5 and TLR10 (23) in SSc fibroblasts and found upregulation of both TLRs. TLR5 is possibly exerting a suppressive effect on collagen expression, likely as an attempt to regulate fibrosis. Indeed, TLR5 triggering by the PAMP flagellin could inhibit collagen deposition in *in vitro* cultured fibroblasts (23). However, it is probably unlikely that flagellin stimulate TLR5 in SSc. It is clear that TLR5 is primarily the receptor for the flagellin (24), but additional functional roles for TLR5 are likely to be revealed in SSc and other autoimmune diseases (25). Interestingly, a study showed that HMGB1 also acts as an agonist of TLR5 and induces a signalling that activates MyD88 and NF-κB in other settings (26). In the study mentioned, HMGB1-TLR5 interaction induces release of pro-inflammatory factors, which results in a higher sensitivity to pain in animals (26). However, the putative endogenous ligands for TLR5 in SSc is unknown. Since HGMB1 is upregulated in SSc during cell damage (13), the role of TLR5 may deserve further investigation. In rheumatoid arthritis (RA), monocytes expressed high levels of TLR5, and this expression correlated with disease activity and TNF-α production (27). The authors of the study suspected that endogenous ligands present in the RA-affected synovia could be responsible for TLR5 triggering, although the TLR5 specific DAMP in RA also remains elusive. Some other studies point towards other “suspects”, included members of the Hsps, a family of proteins produced in response to stressful conditions (28). For instance, a study that analysed tongue squamous carcinoma cells lines reported that Hsp27 is indeed a ligand for TLR5. Engagement of TLR5 by Hsp27 induced NF-kB activation in the cancer cells. Interestingly, by proteomic analysis and immunohistochemistry a study showed that Hsp27 is highly expressed in SSc skin (29) Thus, it will be worth exploring whether HMGB1 and Hsp27 can act as DAMPs for TLR5 in SSc and the effect of these interactions. TLR10 can form dimers with TLR2, and may exert an anti-inflammatory function. Indeed, human TLR10 is a functional receptor, expressed by B cells and plasmacytoid dendritic cells (pDCs), and activates gene transcription through MyD88 (30). This TLR is an interesting candidate for further studies in SSc, since a recent paper
showed that double strand RNA (dsRNA) is a ligand for TLR10. The authors proposed that TLR10 can regulate the IFN-I pathway by sequestering dsRNA from TLR3 (see below), to prevent TLR3 signalling in response to dsRNA (31).

**DNA/RNA-sensing TLR7/8/9 and their specific DAMPs in SSc**

TLR7, TLR8 and TLR9 have a high sequence homology and share dependency on the MyD88-pathway (6-8) (Fig. 1). TLR9 recognises double strand DNA (dsDNA) expressing un-methylated CpG motifs. TLR7 recognises single strand RNA (ssRNA), whereas TLR8 recognises RNA products generated by the lysosomal endoribonuclease RNase T2 (32). The release of nucleic acids during cell death, apoptosis, necrosis or extracellular traps release (ETosis) is a common event during acute infection/inflammation/traumas (33, 34). Thus, three levels of protection exist: the first relies on the endosomal localization of these TLRs, in that self-DNA and -RNA usually fail to enter into the cells, whereas DNA/RNA of endocellular bacteria or endocyted extracellular bacteria can more easily reach endosomal TLRs. A second level of protection relies on self-nucleic acids degradation by nucleases, to avoid persistence in the extracellular milieu. Third, pathogen-derived DNA, unlike human DNA is usually un-methylated, and contains more CpG islands. TLR9 is indeed more prone to recognise DNA with such characteristics. Of note, in some autoimmune diseases, included SSc, defect of methylation are present and more un-methylated self-DNA may be available to engage TLR9 (35).

Elkon’s group demonstrated that SSc sera containing autoantibodies induced high levels of IFN-α in HD peripheral blood mononuclear cells (PBMCs) in a pDC and RNA/DNA dependent manner (36). A paper by Eloranta et al. (37) also showed that sera from SSc patients, mixed with necrotic or apoptotic material, induced IFN-α production in pDCs, apparently activated by immunoglobulin (Ig)-immune complexes (ICs) formed by SSc autoantibodies. Thus, typical autoantibodies present in SSc even several years before overt disease manifestation are able to activate pDCs and the IFN-α pathways. The stimulation depends on endosomal TLR receptors as bafilomycin, which prevents the acidification of the endosomes, inhibited IFN-α production induced by IgG immune complexes. In our assays pDCs stimulated with SSc plasma produced IFN-α, and this secretion was significantly inhibited by addition of an anti-Fc receptor antibody,
indicating that antibodies were at least partially responsible for IFN-α production (38). Accordingly, western blot analysis of IgG that were immune-precipitated from SSc plasma, reveals that IgG had DNA attached. Thus, typical SSc antibodies, and possibly other autoantibodies with unknown specificity, could act as DAMPs also because they bind nucleic acids. Of note, SSc autoantibodies also stimulated fibroblasts to secrete pro-fibrotic mediators (39).

A study reported a significant elevation of TLR9 expression in SSc dermis compared to controls dermis, and detection of a TLR9 signature in SSc skin (40). In vitro treatment of normal cutaneous fibroblasts with the TLR9 ligand unmethylated-CpG induced a pro-fibrotic profile, involving autocrine TGF-β production. A recent paper detected a significant upregulation of TLR7 gene expression in PBMCs of a group of SSc patients compared to a non-SSc group by the RT-qPCR technique (41). It is worth mentioning here that several genes linked to autoimmunity are located on the X chromosome. Remarkably, among these genes are TLR8 and TLR7 (41). Many autoimmune disorders, SSc included, are markedly sex-biased and studies support a gene-dose effect of the X chromosome loci in systemic lupus erythematosus (SLE) predisposition and perhaps establishment (42). Guery’s group recently reported that B cells expressing TLR7 bi-allelically (due to lack of X chromosome inactivation) were more responsive than monoallelic cells during TLR7 driven B cell differentiation (43). It will be worth to analyse this aspect in SSc, in that no studies addressed this issue so far.

RNA sensing TLR3 and its DAMPs in SSc

Unlike the other endosomal nucleic acid sensors described above (TLR7, TLR8, TLR9), TLR3 is the only TLR to work in a MyD88-independent way. TLR3 associates with TRIF (Fig. 1), and signals through IRF3, a key factor involved in IFN-β production and described to increase in SSc skin fibroblasts. The role of TLR3 is partially controversial. TLR3 activation (by Polyinosinic: polycytidylic acid, Poly I: C) stimulated IFN-I by fibroblasts but this, in turn, reduced fibroblast ability to produce ECM components. On the other hand, Poly I: C stimulation promoted the expression of TGF-β by the same cells, thus contributing to fibrosis (15, 44, 45). However, TLR3 function in SSc fibroblasts and endothelial cells is not only linked to the IFN-I axis, but can be more complex. A paper by Farina et al. has shown that dsRNA induces endothelin 1 (EDN1) in endothelial cells and
fibroblasts from SSc patients. EDN1 has a role in vascular complications in SSc, pulmonary hypertension and ulcers (46). Thus, dsRNA can be important in SSc to promote fibrosis and not only for inducing IFN-I.

The role of TLR and pDCs

The most recent literature pointed to the pDCs as very important player in SSc. Indeed pDCs are the strongest producers of IFN-I, via TLR7/8/9 stimulation, and an IFN-I signature is present in half of SSc patients (37, 47-49). A role for pDCs in SSc was already postulated by the fact that, as mentioned, SSc specific autoantibodies (ACA and ATA), were able to stimulate PBMCs of HD to produce IFN-I. Targeting pDCs, by anti-BDCA2 antibodies, blocked IFN-α secretion (36, 37). Most recently, besides IFN-I release, pDCs have been shown to secrete, in SSc, chemokine (C-X-C motif) ligand 4 (CXCL4), a molecule originally identified as a chemokine but clearly exerting a plethora of different functions (50-52). A multicentre study indicated CXCL4 as an important SSc biomarker, which predicts a poor prognosis and correlates with lung fibrosis and pulmonary arterial hypertension (48).

Both Radstake’s group and Barrat’s group (48, 51) observed that pDCs infiltrate the SSc-involved skin, and both demonstrated that SSc pDCs release CXCL4. We also observed pDCs in SSc skin, where they appeared chronically activated, since they expressed Mx1 (38), an IFN-activated gene, as well as CXCL4. SSc pDCs over-releasing CXCL4 produced much higher IFN-α upon CpG (ODN2006, CpGb) challenge (48, 51). Barrat’s group went on showing that SSc pDCs over-produce CXCL4 because they have an aberrant TLR8 expression, as compared to HD or SLE pDCs. Stimulation of TLR8 is indeed the key event, which mediates CXCL4 secretion by SSc pDCs. Both pDC-derived CXCL4 and IFN-α release are mediated by the phosphatidylinositol 3-kinase d (PI3Kd), and the specific inhibition of this pathway can block the secretion of both CXCL4 and IFN-α, without affecting IL-6 release. This is important because suggests that it could be possible to inhibit the chronic activation of pDCs in SSc by acting on this pathway (51). The physiological ligands that potentiate TLR8 signalling in SSc pDCs and the reason why TLR8 expression is
upregulated in these cells remain elusive. *In vivo* depletion experiments further support a role for pDCs in SSc pathogenesis, as pDC depletion prevented disease in mouse models of scleroderma and could revert fibrosis as well (51, 52). Thus, using depleting antibodies or targeting pDC function could be novel approaches to treat SSc patients. Despite it was clearly shown that CXCL4 amplifies CpGb-driven responses and responses elicited by artificial ligands for TLR7 and TLR8, the exact molecular mechanism of CXCL4 contribution to the IFN-I signature remained elusive.

**CXCL4-DNA complexes as new DAMPs in SSc**

The oligonucleotide CpGb and CXCL4 amplified pDC-release of IFN-α *in vitro*, (48, 51). However, CpGb contains a phosphorothioate backbone, which makes the molecule resistant to the enzymatic degradation. Using natural DNA, which, unlike CpGb, is sensitive to enzymatic degradation, we uncovered the underlined mechanistic link between CXCL4 and IFN-α production by pDCs: CXCL4 forms nano-crystalline complexes with DNA, and this enables otherwise non-stimulatory natural DNA to induce immune amplification *via* TLR9-activation. Notably, we demonstrated that CXCL4-DNA complexes are detectable and measurable in SSc plasma, and correlate with circulating IFN-α (38). Crucial abilities relevant for CXCL4 to function as DAMP are: a) capacity to bind the DNA, b) favour DNA internalization in immune cells and c) protect DNA from degradation, as depicted in the cartoon in Figure 1, which also reports the most relevant DAMPs in SSc. However, the emerging new paradigm is that the characteristics listed above are necessary but not sufficient to render a DNA/RNA binding molecule an efficient DAMP. It is crucial that the nucleic acids-binding molecules organise the nucleic acid fragments into a molecular complex characterized by distance between the DNA/RNA ligands (inter-nucleic acids spacing) optimal for the efficient triggering of the specific TLRs (53). We provide a scheme in Figure 2 (54). Thus, not all the DNA-binding proteins possess the requirements to amplify TLR signalling. In this context, we may also hypothesize that some nucleic acids-binding molecules could even interfere with TLR stimulation by sequestering released nucleic acids, thus working as decoy factors (and perhaps become tools for inhibitory strategies). This hypothesis may deserve investigation as a way to block the effects of CXCL4-nucleic acids complexes *in vivo*. 
Given that self-RNA can be a crucial DAMP for both TLR7/8 and TLR3, we anticipate that CXCL4 could amplify RNA stimulation of these TLRs as well, as our preliminary studies indicate that CXCL4 can also condense human and bacterial RNA (Lande and Frasca, unpublished). Up to now, we observed that pDCs secrete IFN-α also when challenged with CXCL4-RNA complexes, but not when cultured with CXCL4 alone or RNA alone (Fig. 3A).

The role of platelets in SSc
The role of platelets in SSc is established (44). In SSc there is a relatively high incidence of anti-platelets antibodies, which mediate platelet activation (55). Platelets express at least functional TLR1/2/4 and TLR3/7/9 (56). The triggering of TLR7 leads to the cell surface exposure and release of CD40L (also known as CD154), which is a costimulatory molecule located in platelets’ alpha granules (57). CD40L interacts with CD40 expressed by leukocytes (B-cells, monocytes, dendritic cells, neutrophils) and endothelial cells. The CD40-CD40L interactions is central in immunity, as it mediates costimulation, implements DC maturation, induces survival of B-cells after antigen recognition by the B-cell receptor (58-60). Interestingly, CD40L is commonly upregulated in SSc blood (61). Accumulating evidences are pointing to a pathogenic role of the aberrantly activated platelets in the process of tissue damage and general inflammation in several autoimmune diseases (62-64). Platelets are likely involved in primary and/or secondary RP, as platelet activation markers are detectable during RP (65, 66). Platelets can also favour neutrophil-extracellular trap release (NET) (13), the process that leads to release of huge amounts of DNA and autoantigens in the tissues, fuelling a harmful loop. It is also worth mentioning here the important role of platelets in SSc also with respect to CXCL4 upregulation: platelets are indeed the major source of released CXCL4 upon infections or traumas (50). Indeed, platelets release huge amounts of CXCL4 after activation. Of interest, some studies uncovered that the lungs are sites of thrombopoiesis, and reservoirs for platelets (67). Platelets regulate pulmonary vascular permeability of alveolar capillaries and have specialized activities in lung repair. Since CXCL4 elevation in SSc associates mostly with lung fibrosis and pulmonary arterial hypertension, it may be worth to understand the exact CXCL4
expression in the SSc lung tissue. For instance, it can be of interest to clarify whether platelets or pDCs are the most important CXCL4 producers in the lung, and which ligand/receptor pairs are responsible for platelet activation and CXCL4 release, if occurring, in the lung.

Interestingly, although anucleated, platelets contain mitochondrial DNA (mitoDNA), thus they can be a source of mitoDNA-CXCL4 complexes. We detected platelets in SSc skin, where they appeared to form aggregates and co-localised with CXCL4 staining (Lande and Frasca, unpublished observations). Interestingly, Boudreau et al. demonstrated that upon activation, platelets release mitochondria in the extracellular milieu (68). Of interest, we observed that mitoDNA, which is more similar to bacterial DNA, when complexed with CXCL4, was able to activate pDCs even better than huDNA (Fig. 3B).

Other possible DNA/RNA DAMPs in SSc

Two studies demonstrated that the antimicrobial peptide (AMP) cathelicidin LL37, a molecule amplifying TLR7/8/9 signalling and widely studied (69, 70), is overexpressed in SSc skin, especially in the dermis (71, 72). LL37 was the first antimicrobial peptide shown to bind DNA and activate IFN-α release in pDCs via TLR9, as a mechanism explaining the IFN-α signature in psoriasis skin (69).

Later, other cationic proteins (73-75) and the AMP HBD2 and HBD3 (human β-defensins) were shown to perform similar functions. HBD2 and HBD3 mRNA levels were higher in lesional skin of localised scleroderma patients, compared to unaffected skin and skin from healthy volunteers (76). We have also analysed LL37, HBD2 and HBD3 expression in SSc skin by immunohistochemistry (Frasca, unpublished work). Occasionally we found HBD2 or HBD3 expression but more often, we detected LL37 upregulation, especially in the dermis, and this coincided with IFN-induced gene expression (Mx1), in accordance with Sato’s group experiments (Frasca, unpublished, 72). LL37 may be a putative TLR7/TLR8 stimulator in SSc, as it stimulates TLR7/8 in myeloid DCs, after forming complexes with human-RNA, leading to TNF-α and IL-6 production (70).

TLRs and the IFN-I signature in SSc

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As already mentioned, almost 50% of SSc patients exhibit an IFN-I gene signature in blood and tissues. Moreover, a few polymorphisms of IFN-regulatory genes can be associated with SSc (49). In some cases the type I interferons have been found beneficial in SSc animal models, for instance in the bleomycin model, where IFN-β attenuated the disease. However, many other papers indicate IFN-I as deleterious in SSc (49) and a trial administering IFN-α failed to cure SSc and was instead harmful to the patients (77). In addition, IFN-α treatment for other pathological conditions can induce SSc (78, 79). Although an IFN-I signature can be detected both in early and long-lasting SSc, it appears that this signature can be present even at very early SSc stages (49, 80).

We would like to emphasize here that, according to the literature, activation of the IFN-I pathway as an early event in SSc is associated, together with high CXCL4, with more severe disease manifestation and poor prognosis (36, 37, 49, 80). This is in agreement with our observation that the presence in SSc plasma of CXCL4-DNA complexes correlated with the circulating IFN-I signature in particular in a group of patients with early active SSc (38). Thus, disrupting CXCL4-nucleic acid interaction, or blocking excess of CXCL4, can be a possible intervention in early SSc. Of course, also at later stages a correlation between plasma CXCL4-DNA complexes and circulating IFN-α is present (38), suggesting that CXCL4 contributes to the IFN-I signature at any disease stages, thus also long lasting patients can benefit from anti-IFN-I treatment.

We guess that treatment with drugs able to contrast DNA sensing TLR activation at very early stages, especially in those patients that show immediately a significant IFN-I signature, could be of help. SSc patients could benefit from treatment with old and, therefore less expensive drugs, such as chloroquine, already used for SLE (as a repositioning drug strategy). Moreover, chloroquine can also block TLR3 signalling (81).

In the context of the activation of the IFN-I signature in SSc, a very recent paper demonstrated that IRF7, a master regulator of the IFN-I signature, is upregulated in SSc skin (82), and can represent a link between the prominent IFN-I signature and fibrosis. Indeed the authors demonstrated that IRF7 associated with Smad3 in SSc fibroblasts and that absence of IRF7 in the bleomycin mouse model attenuated fibrosis of the dermis.

**Pathogens as activators of TLRs in SSc**
The interplay between pathogenic viruses or bacteria and the immune system may contribute to autoimmune diseases (83, 84). Indications that Herpes virus infections play a role in SLE or multiple sclerosis are present in the literature (85, 86). One mechanism by which pathogens favour autoimmunity is the phenomenon of “molecular mimicry” between self- and pathogen-derived molecules, which can confound the immune system self-non self-discrimination (87). As a second mechanism, the inability to clear the pathogens favours persistent infections and therefore continuous stimulation of innate immune cells, via TLRs. Some infectious agents have been proposed as possible SSc triggering factors and these are Parvovirus B19, Cytomegalovirus, Epstein–Barr virus (EBV), Retroviruses (88).

Latent infections by EBV, which replicates in primary human monocytes, could trigger SSc. Induction of EBV viral lytic genes induced TLR8 expression in both HD and SSc monocytes infected with EBV (89). EBV can infect fibroblasts and endothelial cells of SSc skin and this can lead to an aberrant TLR stimulation in these cells. Such stimulation could induce well-known markers of fibrosis, including TGF-β and EDN1, and conversion of fibroblasts into myofibroblasts (90).

The cytomegalovirus (HCMV) infections are also candidate for SSc induction, in that SSc can manifest shortly after an acute episode of infection with HCMV (91). Products of HCMV are involved in the induction of a fibrotic program in human dermal fibroblasts and cause vasculopathy similar to that observed in SSc. However, a clear activation of TLRs in immune and non-immune cells by HCMV is lacking (92). On the other hand, the B19 Virus infection of monocytes from patients with SSc was found to induce TNF-α, more frequently than in HD monocyte cultures (92). The B19 Virus-induced production of TNF-α positively correlated with the amount of viral DNA detected at the end of incubation time, suggesting a role for viral DNA in cytokine production, likely through TLR9 activation (93).

**Conclusions**

SSc is a disease in which innate immune cells play a role and likely support adaptive immune cell licensing and activation, favouring autoimmunity establishment. TLR-engagement may activate immune cells. However, several studies report that non-immune cells, namely fibroblasts and endothelial cells, cell types important in SSc...
pathogenesis, can express basal low levels of the same TLRs. Such expression can increase under inflammatory conditions. Therefore, also these cells can experience an aberrant TLR-driven stimulation (94-98). Of note, endothelial cells dysfunctions and dysregulation of wound repair are indeed typical of SSc. Several DAMPs have been identified in SSc, which can trigger various TLRs, among which TLR4, TLR7, TLR8 and TLR9 ligands are probably the most important, although also TLR3-signaling can play a role in SSc pathology. The IFN-I activation pathway also plays a crucial role in SSc. The IFN-I signature at onset or in early stages, as mentioned above, forecasts a poor prognosis, and early IFN-I block can be of help. Newly discovered molecules, such as CXCL4, can represent a link between pDCs, the IFN-I axis and the fibrotic process. We need more in vivo and in vitro studies to confirm the importance of the new players. New therapeutic agents should be tested in parallel with old treatments (for instance some of those currently used in SLE), for capacity to block the IFN-I pathways, CXCL4, and pDCs.

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Author contributions
LF organized and wrote the manuscript, and prepared the figures with the help of RL. Both LF and RL planned and performed the experiments reported in the review.

Disclosures
The authors declare no conflicts of interest

References
27. Charmberlain ND, Vila OM, Volin MV, Volkov S, Pope RM, Swedler W, Mandelin II AM, Shahrara S. TLR5, a novel and unidentified inflammatory mediator in Rheumatoid


42. Souyris M, Mejia JE, Chaumeil J, Guery JC. Female predisposition to TLR7-driven autoimmunity: gene dosage and the escape from X chromosome inactivation. Semin Immunopathol 2018; 41:153-64.


Figures and Figure Legends

Figure 1. Endogenous TLR ligands identified in SSc. Binding of DAMPs (released by injured tissues) to TLR4 and TLR1/2 triggers the production of inflammatory cytokines (IL-6, IL-8, TNFα, TGF-β), as well as factors involved in the ECM deposition, such as the Tissue Inhibitor of Metalloproteinases (TIMP-1), and Collagen 1. Whether TLR5 is triggered by endogenous ligand in SSc is unknown (possible candidates may be HMGB1 or HSPs, according to studies in other settings (see text). SSc immune-complexes (SSc-IC) which include autoantibodies ATA, ACA etc., as well as nanocristalline particles of platelet- and pDC-derived CXCL4 bound to nucleic acids (DNA and RNA) can stimulate endosomal nucleic-acid sensing TLRs TLR7, TLR8, TLR9 and TLR3, once internalized. SSc-IC also induce IL-6, IL-8, MMP-2, MCP-1, TGF-β1 and pro-collagenα1 by fibroblasts.

Figure 2. TLR-stimulation ability depends on the polycation-DNA-complex structure, which influences the packaging of the DNA and the inter-DNA spacing of contiguous DNA molecules. A) Schematic representation of a structural type of DNA-polycation complex: cationic molecules bind and organise DNA chains (blue cylinders) into a columnar structure with a short-ranged order. B) Hypothetical structure of TLR9 and its interaction with dsDNA. C) The optimal geometric spacing (inter-DNA distance)
between ordered dsDNA molecules bound to CXCL4 (or other polycations, such as LL37 or HBD3) is in the range between 3 and 4 nm (d). This amplitude almost matches the steric size of TLR9 and allows activate multiple TLR9 at the same time leading to the optimal IFN-α production. Outside this range (d smaller or larger than 3-4 nm), stimulation results in a modest or no IFN-α production.

**Figure 3. CXCL4 in complex with human nucleic acids of various origin stimulates IFN-α release by pDCs.** A) Purified pDCs (175x10³/ml) from 5 different healthy donor (HD) PBMCs were treated with CXCL4 alone (1 μM), human RNA alone (huRNA, 20 μg/ml), or CXCL4 pre-complexed with huRNA (at the same concentrations), overnight. The release of IFN-α in the culture supernatants was tested by ELISA, as described (29). B) PDCs (175x10³/ml) from 7 different HD PBMCs were stimulated overnight with CXCL4 alone (1 μM), human DNA alone (huDNA, 10 μg/ml), mitochondrial DNA (mitoDNA, 10 μg/ml), or CXCL4 pre-complexed with the two DNA types. IFN-α in the culture supernatants was tested by ELISA. Horizontal bars are the means, vertical bars are standard errors of the mean, P-values by Wilcoxon signed-rank test.
**A**

Packed DNA molecules

*After:* Schmidt et al. Nat Mat, 2015

**B**

**Endosome**

90° rotation

**C**

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<th>Packed dsDNA</th>
<th>Packed dsDNA interacting with TLR9</th>
</tr>
</thead>
<tbody>
<tr>
<td>d&gt;4nm</td>
<td>d&gt;4nm: no/low IFNα</td>
</tr>
<tr>
<td>3&lt;d&lt;4</td>
<td>3&gt;d&lt;4: high IFNα</td>
</tr>
<tr>
<td>d&lt;3</td>
<td>d&lt;3: no/low IFNα</td>
</tr>
</tbody>
</table>