

Toll-like receptors in mediating pathogenesis in systemic sclerosis

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Introduction

Systemic sclerosis (SSc) is an autoimmune and fibrotic disease with a high disease burden and mortality rate [1]. Three most important hallmarks characterize 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 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.

Summary

Toll-like receptors (TLRs) are evolutionarily 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 profibrotic 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 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: fibrosis, immune cells, interferon signature, systemic sclerosis, Toll-like receptor

Dysregulation of the innate immune system in genetically predisposed individuals plays a role in SSc, and aberrant Toll-like receptors (TLRs) activation seems central to pathogenesis [6,7]. TLRs are a germline-encoded group of pattern recognition receptors, which comprise 10 members (TLR-1–TLR-10) in humans and 12 members (TLR-1–TLR-9 and TLR-11–TLR-13) in mice [8]. They are key for recognition of invading pathogens. As transmembrane receptors, they localize either at the cell surface or in the endosomal compartment. As they are involved in self-*versus* non-self-discrimination, TLRs have implications in various autoimmune and autoinflammatory conditions [9]. Non-immune cells, including 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].

DAMPs in autoimmunity

One of the first-discovered DAMPs 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, TLR-4. By binding to damaged DNA, HMGB1 can also favour TLR-9 stimulation [12]. Activated platelets in SSc blood release micro-particles which contain HMGB1 [13]. HMGB1 also binds receptors for advanced glycation-end-product (RAGE), an immunoglobulin superfamily member [11].

In general, DAMPs are heterogeneous molecules: apart from HMGB1, other DAMPs comprise Ca^{2+} , H_2O_2 , reactive oxygen species (ROS), adenosine triphosphate (ATP), self-nucleic acids, heat shock proteins (Hsps), S100 proteins (alarmins), fragments of the extracellular matrix (ECM), uric acid and heparin sulphate. Released intracellular mitochondria also represent DAMPs [14]. Several DAMPs in SSc can contribute to disease pathogenesis [6].

Membrane TLRs and their DAMPs in SSc

In humans, TLR-1, -2, -4, -5, -6 and -10 localize on the cell surface of immune and non-immune cells. TLR-4 recognizes 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 are Hsps, taxol, fibronectin extracellular matrix components (ECM), fatty acids, low-density lipoprotein (LDL) and fibrinogen. TLR-2 recognizes lipoproteins, peptidoglycans and lipopeptides, hyaluronic acid, Hsp 70 lipoarabinomannan from a variety of microorganisms, and even HMGB1 [15,16].

A role for TLR-2 is possible in SSc pathogenesis [5,6]: TLR-2 hyperexpressing SSc fibroblasts overproduce interleukin (IL)-6, a critical molecule in fibrosis [17]. Serum amyloid A, which is high in SSc [18], stimulates TLR-2 in fibroblasts. A rare polymorphism of TLR-2 was associated with diffuse SSc and with anti-topoisomerase antibodies (ATA) and pulmonary arterial hypertension [19]. TLR-2 forms heterodimers with TLR-1 or TLR-6 (in a ligand-specific manner) to recognize a variety of PAMPs, such as peptidoglycans, lipoteichoic acid, zymosan, and mannan. However, no information is available on the roles of TLR-1 and TLR-6 in SSc.

Bhattacharyya *et al.* demonstrated that the fibronectin extra domain A (EDA), an endogenous TLR-4 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 TLR-4 signalling abrogated the deleterious effect of EDA, in that TLR-4 stimulation induced collagen production and myofibroblast differentiation. In mice, the blockade of TLR-4 mitigated experimentally induced fibrosis. Thus, the example of TLR-4 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 TLR-4 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 TLR-4 [21]. Accordingly, an enhanced TLR-4-responsive gene signature was present in SSc skin biopsies [22]. Collectively, these studies point to the involvement of TLR-4 signalling in fibrosis in SSc.

With regard to other TLRs, one study addressed the expression of TLR-5 and TLR-10 [23] in SSc fibroblasts and found up-regulation of both TLRs. TLR-5 possibly exerts a suppressive effect on collagen expression, perhaps as an attempt to regulate fibrosis. Indeed, TLR-5 triggering by the PAMP flagellin could inhibit collagen deposition in *in-vitro* cultured fibroblasts [23]. However, it is probably unlikely that flagellin stimulate TLR-5 in SSc. It is clear that TLR-5 is primarily the receptor for the flagellin [24], but additional functional roles for TLR-5 are likely to be revealed in SSc and other autoimmune diseases [25]. Interestingly, a study showed that HMGB1 also acts as an agonist of TLR-5 and induces signalling that activates myeloid differentiation primary response protein (MyD88) and nuclear factor kappa B (NF- κ B) in other settings [26]. In the study mentioned, HMGB1-TLR-5 interaction induces the release of proinflammatory factors, which results in a higher sensitivity to pain in animals [26]. However, the putative endogenous ligands for TLR-5 in SSc is unknown. As HMGB1 is up-regulated in SSc during cell damage [13], the role of TLR-5 may deserve further investigation. In rheumatoid arthritis (RA), monocytes expressed high levels of TLR-5 and this expression correlated with disease activity and tumour necrosis factor (TNF)- α production [27]. The authors of the study suspected that endogenous ligands present in the RA-affected synovia could be responsible for TLR-5 triggering, although the TLR-5-specific DAMP in RA also remains elusive. Some other studies point towards other 'suspects', including members of the Hsps, a family of proteins produced in response to stressful conditions [28]. For instance, a study that analysed tongue squamous carcinoma cell lines reported that Hsp27 is indeed a ligand for TLR-5. Engagement of TLR-5 by Hsp27 induced NF- κ B activation in cancer cells. Interestingly, using 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 TLR-5 in SSc and the effect of these interactions. TLR-10 can form dimers with TLR-2, and may exert an anti-inflammatory function. Human TLR-10 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, as a recent paper has shown that double-strand RNA (dsRNA) is a ligand for TLR-10. The authors proposed that TLR-10 can regulate the interferon (IFN)-I pathway by sequestering dsRNA from TLR-3 (see below) to prevent TLR-3 signalling in response to dsRNA [31].

DNA/RNA-sensing TLR-7, -8 and -9 and their specific DAMPs in SSc

TLR-7, -8 and -9 have a high sequence homology and share dependency on the MyD88 pathway [6–8] (Fig. 1). TLR-9 recognizes dsDNA expressing unmethylated cytosine-phosphate-guanosine (CpG) motifs. TLR-7

recognizes single-strand RNA (ssRNA), whereas TLR-8 recognizes 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 self-RNA usually fail to enter into the cells, whereas DNA/RNA of endocellular bacteria or endocytosed extracellular bacteria can reach endosomal TLRs more easily. A second level of protection relies on self-nucleic acid degradation by nucleases to avoid persistence in the extracellular milieu. Thirdly, pathogen-derived DNA, unlike human DNA, is usually unmethylated and contains more CpG islands; TLR-9 is more prone to recognize DNA with such characteristics. Of note, in some autoimmune diseases, including SSc, defects of methylation are present and more unmethylated self-DNA may be available to engage TLR-9 [35].

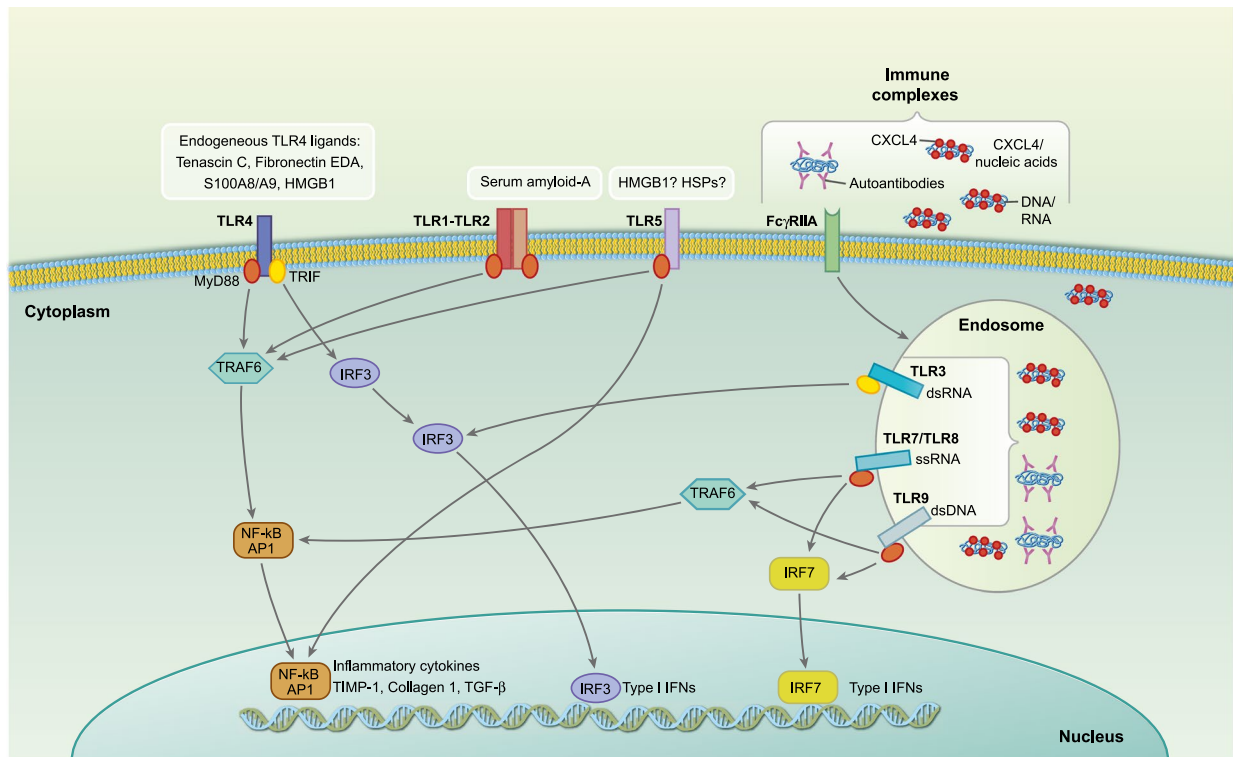


Fig. 1. Endogenous Toll-like receptor (TLR) ligands identified in systemic sclerosis (SSc). Binding of danger-associated molecular patterns (DAMPs) (released by injured tissues) to TLR-4 and TLR-1 and -2 triggers the production of inflammatory cytokines [interleukin (IL)-6, IL-8, tumour necrosis factor (TNF)- α , transforming growth factor (TGF)- β], as well as factors involved in the extracellular matrix (ECM) deposition, such as the tissue inhibitor of metalloproteinases (TIMP-1) and collagen 1. Whether TLR-5 is triggered by endogenous ligand in SSc is unknown (possible candidates may be high-mobility group protein 1 (HMGB1) or heat shock proteins (HSPs)), according to studies in other settings (see text). SSc-immune complexes (SSc-IC), which include autoantibodies anti-topoisomerase antibodies (ATA) and anti-centromere antibodies (ACA), etc. as well as nanocrystalline particles of platelet- and platelet dendritic cell (pDC)-derived chemokine C-X-C ligand motif (CXCL4) bound to nucleic acids (DNA and RNA) can stimulate endosomal nucleic-acid sensing TLRs TLR-7, TLR-8, TLR-9 and TLR-3, once internalized. SSc-IC also induces IL-6, IL-8, matrix metalloproteinase (MMP)-2, monocyte chemoattractant protein 1 (MCP-1), TGF- β 1 and pro-collagen α 1 by fibroblasts.

Kim *et al.*'s group demonstrated that SSc sera containing autoantibodies induced high levels of IFN- α in healthy donor (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. Stimulation depends on endosomal TLR receptors because bafilomycin, which prevents the acidification of the endosomes, inhibits 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 was immune-precipitated from SSc plasma revealed that IgG had DNA attached. Thus, typical SSc antibodies, and possibly other autoantibodies with unknown specificity, could also act as DAMPs because they bind nucleic acids. Of note, SSc autoantibodies also stimulated fibroblasts to secrete profibrotic mediators [39].

A study by Fang *et al.* reported a significant elevation of TLR-9 expression in SSc dermis compared to control dermis and detection of a TLR-9 signature in SSc skin [40]. *In-vitro* treatment of normal cutaneous fibroblasts with the TLR-9 ligand unmethylated CpG induced a profibrotic profile, involving autocrine TGF- β production. A recent paper detected a significant up-regulation of *TLR7* gene expression in PBMCs of a group of SSc patients compared to a non-SSc group using the reverse transcription-quantitative polymerase chain reaction (RT-qPCR) technique [41]. It is worth mention that several genes linked to autoimmunity are located on the X chromosome. Remarkably, among these genes are TLR-8 and TLR-7 [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]. Souyris *et al.*'s group recently reported that B cells expressing TLR-7 bi-allelically (due to lack of X chromosome inactivation) were more responsive than monoallelic cells during TLR-7-driven B cell differentiation [43]. It will be worthwhile to analyse this aspect in SSc, in that no studies have addressed this issue so far.

RNA sensing TLR-3 and its DAMPs in SSc

Unlike the other endosomal nucleic acid sensors described above (TLR-7, TLR-8, TLR-9), TLR-3 is the only TLR to

work in a MyD88-independent manner. TLR-3 associates with TIR-domain-containing adapter-inducing IFN- β (TRIF) (Fig. 1) and signals through interferon regulatory factor 3 (IRF-3), a key factor involved in IFN- β production and described to increase in SSc skin fibroblasts. The role of TLR-3 is partly controversial. TLR-3 activation (by polyinosinic:polycytidylic acid, Poly I:C)-stimulated IFN-I by fibroblasts but this, in turn, reduced fibroblast ability to produce ECM components. Conversely, Poly I:C stimulation promoted the expression of TGF- β by the same cells, thus contributing to fibrosis [15,44,45]. However, TLR-3 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 has indicated the pDCs as an extremely important player in SSc. pDCs are the strongest producers of IFN-I via TLR-7/-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, as well as IFN-I release, pDCs have been shown to secrete, in SSc, CXCL4, a molecule originally identified as a chemokine but clearly exerting a plethora of different functions [50-52]. A multi-centre study indicated CXCL4 as an important SSc biomarker, which predicts a poor prognosis and correlates with lung fibrosis and pulmonary arterial hypertension [48].

Both van Bon *et al.* [48] and Ah Kioon *et al.* [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, as 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]. Ah Kioon *et al.* went on to show that SSc pDCs overproduce CXCL4 because they have an aberrant TLR-8 expression compared to HD or SLE pDCs. Stimulation of TLR-8 is 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 δ (PI3K δ), 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 it 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 TLR-8 signalling in SSc pDCs and the reason why TLR-8 expression is up-regulated 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 also revert fibrosis [51,52]. Thus, using depleting antibodies or targeting pDC function could be novel approaches to treat SSc patients. Although it was clearly shown that CXCL4 amplifies CpGb-driven responses and responses elicited by artificial ligands for TLR-7 and TLR-8, the exact molecular mechanism of CXCL4's contribution to the IFN-I signature remains 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 TLR-9-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 Fig. 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 acid-binding molecules organize the nucleic acid fragments into a molecular complex characterized by distance between the DNA/RNA ligands (internucleic acids spacing) optimal for the efficient triggering of the specific TLRs [53]. We provide a scheme in Fig. 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 acid-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 TLR-7/-8 and TLR-3, we anticipate that CXCL4 could also amplify RNA stimulation of these TLRs, as our preliminary studies indicate that CXCL4 can also condense human and bacterial RNA (Lande and Frasca, unpublished). Up to the present time, we have observed that pDCs also secrete IFN- α 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-platelet antibodies, which mediate platelet activation [55]. Platelets express at least functional TLR-1/-2/-4 and TLR-3/-7/-9 [56]. The triggering of TLR-7 leads to cell surface exposure and release of CD40L (also known as CD154), which is a co-stimulatory molecule located in platelet alpha granules [57]. CD40L interacts with CD40 expressed by leucocytes (B cells, monocytes, DCs, neutrophils) and endothelial cells. The CD40–CD40L interactions are central in immunity as they mediate co-stimulation, implement DC maturation and induce survival of B cells after antigen recognition by the B cell receptor [58–60]. Interestingly, CD40L is commonly up-regulated in SSc blood [61]. Accumulating evidence points 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 probably 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.

The important role of platelets in SSc is also worth mention with respect to CXCL4 up-regulation: platelets are 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. Because CXCL4 elevation in SSc associates mainly with lung fibrosis and pulmonary arterial hypertension, it may be worthwhile 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.

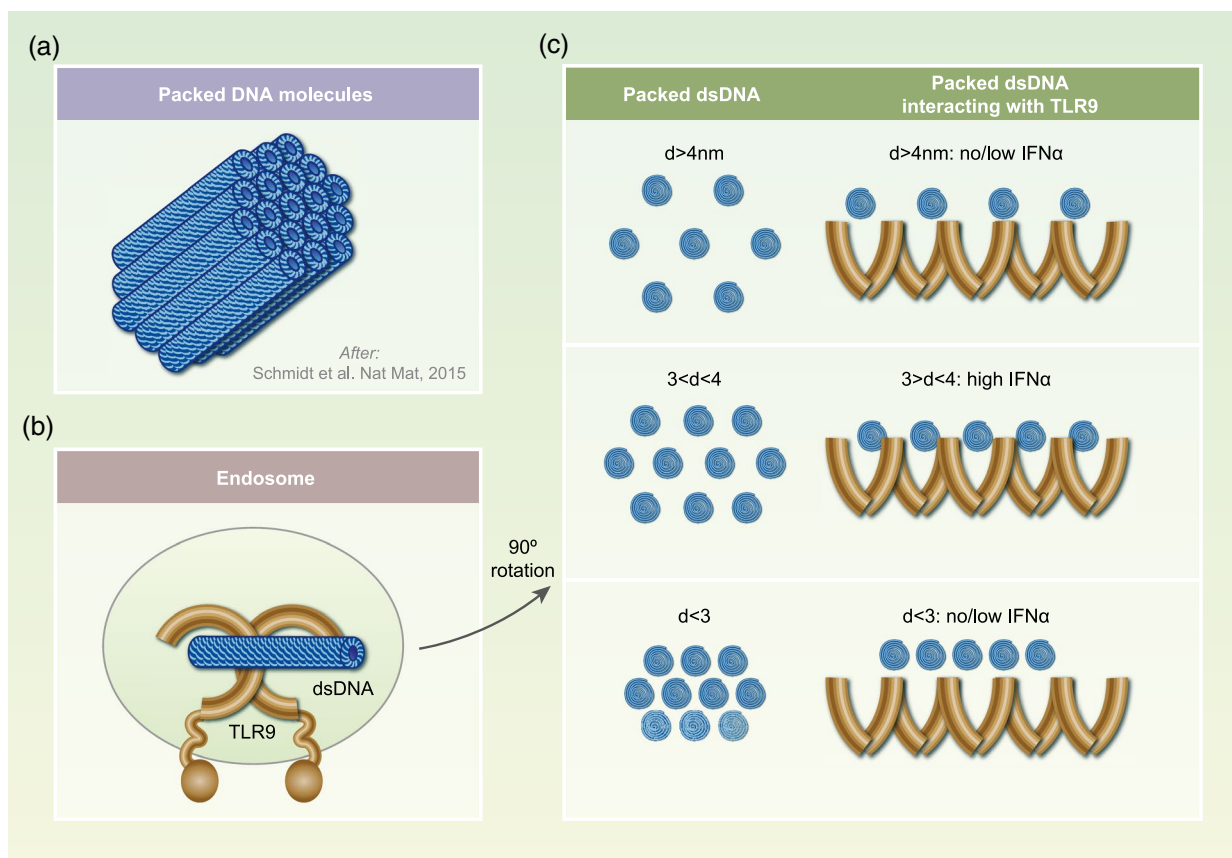


Fig. 2. Toll-like receptor (TLR)-stimulation ability depends on the polycation–DNA complex structure, which influences the packaging of the DNA and the inter-DNA spacing of contiguous DNA molecules. (a) Schematic representation of a structural type of DNA–polycation complex: cationic molecules bind and organize DNA chains (blue cylinders) into a columnar structure with a short-ranged order. (b) Hypothetical structure of TLR-9 and its interaction with dsDNA. (c) The optimal geometric spacing (inter-DNA distance) between ordered dsDNA molecules bound to chemokine C-X-C ligand motif (CXCL4) [or other polycations, such as LL-37 or human β -defensin (HBD3)] is in the range between 3 and 4 nm (d). This amplitude almost matches the steric size of TLR-9 and allows activate multiple TLR-9 at the same time leading to the optimal interferon (IFN)- α production. Outside this range (d smaller or larger than 3–4 nm), stimulation results in a modest or no IFN- α production.

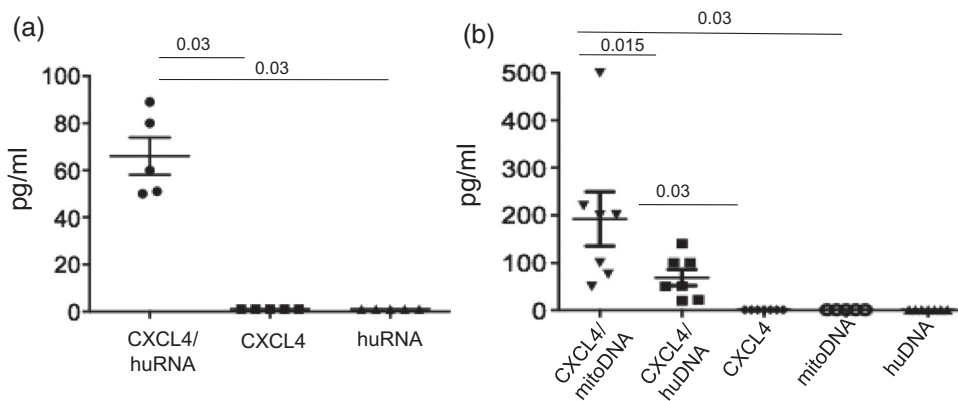


Fig. 3. Chemokine C-X-C ligand 4 motif (CXCL4) in complex with human nucleic acids of various origin stimulates interferon (IFN)- α release by platelet dendritic cells (pDCs). (a) Purified pDCs (175×10^3 /ml) from five different healthy donor (HD) peripheral blood mononuclear cells (PBMCs) were treated with CXCL4 alone (1 μ M), human RNA alone (huRNA, 20 μ g/ml) or CXCL4 pre-complexed with huRNA (at the same concentrations), overnight. The release of IFN- α in the culture supernatants was tested by enzyme-linked immunosorbent assay (ELISA), as described [29]. (b) pDCs (175×10^3 /ml) from seven different HD PBMCs were stimulated overnight with CXCL4 alone (1 μ M), human DNA alone (huDNA, 10 μ g/ml), mitochondrial DNA (mitoDNA, 10 μ g/ml) or CXCL4 precomplexed 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’s signed-rank test.

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-localized with CXCL4 staining (Lande and Frasca, unpublished observations). 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 more effectively than huDNA (Fig. 3b).

Other possible DNA/RNA DAMPs in SSc

Two studies have demonstrated that the anti-microbial peptide (AMP) cathelicidin LL-37, a widely studied molecule amplifying TLR-7/-8/-9 signalling [69,70], is over-expressed in SSc skin, especially in the dermis [71,72]. LL-37 was the first anti-microbial peptide shown to bind DNA and activate IFN- α release in pDCs via TLR-9 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 localized scleroderma patients compared to unaffected skin and skin from healthy volunteers [76]. We have also analysed LL-37, HBD2 and HBD3 expression in SSc skin by immunohistochemistry (Frasca, unpublished work). Occasionally we found HBD2 or HBD3 expression but, more often, we detected LL-37 up-regulation, especially in the dermis, and this coincided with IFN-induced gene expression (Mx1) in accordance with Takahashi *et al.*'s [72] experiments (Frasca, unpublished). LL-37 may be a putative TLR-7/-8 stimulator in SSc, as it stimulates TLR-7/-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

As mentioned previously, 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 to be beneficial in SSc animal models; for instance, in the bleomycin model, where IFN- β attenuated the disease. However, many other papers indicate IFN-I to be deleterious in SSc [49]; a trial administering IFN- α failed to cure SSc and was instead harmful to 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. At later stages a correlation between plasma CXCL4–DNA complexes and circulating IFN- α is also present [38], suggesting that CXCL4 contributes to the IFN-I signature at any disease stages, thus long-lasting patients can also benefit from anti-IFN-I treatment.

We estimate that treatment with drugs able to contrast DNA-sensing TLR activation at very early stages, especially in those patients who immediately show 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 TLR-3 signalling [81].

In the context of the activation of the IFN-I signature in SSc, a very recent paper demonstrated that interferon regulatory factor 7 (IRF7), a master regulator of the IFN-I signature, is up-regulated in SSc skin [82], and can represent a link between the prominent IFN-I signature and fibrosis. 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 herpesvirus 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, such as parvovirus B19, cytomegalovirus, Epstein–Barr virus (EBV) and retroviruses [88].

Latent infections by EBV, which replicates in primary human monocytes, could trigger SSc. Induction of EBV viral lytic genes induced TLR-8 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].

Cytomegalovirus (HCMV) infections are also candidates 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 programme 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]. Conversely, B19 virus infection of monocytes from patients with SSc was found to induce TNF- α , more frequently than in HD monocyte cultures [92]. 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, probably through TLR-9 activation [93].

Conclusions

SSc is a disease in which innate immune cells play a role and probably 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, these cells can also experience an aberrant TLR-driven stimulation [94–98]. Of note, endothelial cell dysfunctions and dysregulation of wound repair are typical of SSc. Several DAMPs have been identified in SSc which can trigger various TLRs, among which TLR-4, TLR-7, TLR-8 and TLR-9 ligands are probably the most important, although TLR-3-signalling can also 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 the capacity to block the IFN-I pathways, CXCL4 and pDCs.

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Disclosures

The authors declare no conflicts of interest

Author contributions

L. F. organized and wrote the manuscript and prepared the figures with the help of R. L. Both L. F. and R. L. planned and performed the experiments reported in the review.

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