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Role of Metabolism in Fibrosis: Translational Implications (S Bhattacharyya, Section Editor)

Mitochondrial DNA-Sensing Pathogen Recognition Receptors in Systemic Sclerosis-Associated Interstitial Lung Disease: a Review

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Abstract

Purpose of the Review Systemic sclerosis (SSc) is a condition of dermal and visceral scar formation characterized by immune dysregulation and inflammatory fibrosis. Approximately 90% of SSc patients develop interstitial lung disease (ILD), and it is the leading cause of morbidity and mortality. Further understanding of immune-mediated fibroproliferative mechanisms has the potential to catalyze novel treatment approaches in this difficult-to-treat disease.

Recent Findings Recent advances have demonstrated the critical role of aberrant innate immune activation mediated by mitochondrial DNA (mtDNA) through interactions with toll-like receptor 9 (TLR9) and cytosolic cyclic guanosine monophosphate-adenosine monophosphate synthase (cGAS).
Summary In this review, we will discuss how the nature of the mtDNA, whether oxidized or mutated, and its mechanism of release, either intracellularly or extracellularly, can amplify fibrogenesis by activating TLR9 and cGAS, and the novel insights gained by interrogating these signaling pathways. Because the scope of this review is intended to generate hypotheses for future research, we conclude our discussion with several important unanswered questions.

Introduction

Systemic sclerosis (SSc) is a chronic, multisystem autoimmune disorder characterized by microvascular damage, immune dysfunction, and fibrotic remodeling of the skin and internal organs. Interstitial lung disease (ILD) affects approximately 90% of patients with this disorder [1] and is the leading cause of SSc-related mortality [2•]. Current treatment strategies include non-specific immunomodulatory agents that exhibit variable efficacy in improving or stabilizing lung function [3•], and anti-fibrotic drugs that delay disease progression in a subset of patients [4]. Thus, improved treatment options for this difficult-to-treat and, at times, devastating disease represent an unmet clinical need.

The mechanism(s) through which the loss of self-tolerance engenders tissue fibrosis are not well understood. Perturbations in adaptive immunity and tissue-resident fibroblast biology [5] are strongly implicated, whereas innate immune processes remain far less studied [6•]. Innate immune activation is stimulated by pattern recognition receptors (PRRs) that recognize broadly conserved microbial epitopes termed "pathogen-associated molecular patterns" (PAMPs) derived from microbes and cell- or tissue-derived endogenous ligands termed "danger-associated molecular patterns" (DAMPs) [7]. Given the well-established immune etiology of SSc-ILD [1], studies of innate immunity may illuminate intervenable disease mechanisms that can be targeted for the development of PRR-specific therapies.

Mitochondria are increasingly recognized as contributors to immune activation through their ability to function as DAMPs [8•]. In response to cellular stress, several mitochondria-associated molecules are potentially released into the extracellular compartment—known as mitochondrial DAMPS—that have been shown to activate various immune receptors (summarized in Table 1) and initiate proinflammatory signaling pathways implicated in the pathogenesis of several diseases [9, 10], including SSc-ILD [11, 12]. Among the mitochondrial DAMPs that have been described, the fibroproliferative contribution of mitochondrial DNA (mtDNA) has been an active area of investigation; this review will focus specifically on mtDNA-induced PRR activation in the setting of SSc-ILD.

In addition to encoding mitochondrial genes, DNA derived from mitochondria functions as a potent DAMP for DNA-sensing PRRs [8•]. The mitochondrial genome is unique in both its circular structure [13] and its low to undetectable level of CpG methylation [14]. It is particularly prone to injury [15] and is sufficiently distinct from nuclear DNA to elicit "non-self" responses from cytosolic DNA sensors [8•]. Intracellular or "endogenous" sources of mtDNA include free DNA that is herniated into the cytosol from damaged mitochondria [16], while extracellular or "exogenous" mtDNA is generated by at least two known mechanisms: either via regulated secretion of extracellular vesicles (EVs) containing intact mitochondria that may represent a form of defective mitophagy [17] or via the release of cell-free mtDNA during necroptosis [18]. Both intracellular [16] and extracellular [19] sources of mtDNA potently activate innate immunity through interactions with PRRs such as toll-like receptor 9 (TLR9) [20] and cytosolic cyclic guanosine

Table 1. Mitochondrial DAMPs and associated immune receptor(s)

Mitochondrial DAMP	Receptor
mtDNA	TLR9
	cGAS
	NLRP3
	AIM2
ATP	P2XR
	P2YR
	NLRP3 (via P2X7R)
TFAM	RAGE
N-formyl peptides	FPR
Succinate	SUCNR1
Cardiolipin	NLRP3

AIM2, absent in melanoma 2; *ATP*, adenosine triphosphate; *DAMP*, damage-associated molecular pattern; *FPR*, formyl peptide receptor; *P2XR*, purine receptor subtype X; *P2YR*, purine receptor subtype Y; *PRR*, pattern recognition receptor; *mtDNA*, mitochondrial DNA; *NLRP3*, NOD-like receptor protein 3; *RAGE*, receptor for advanced glycation end products; *SUCNR1*, succinate receptor 1; *TFAM*, mitochondrial transcription factor A; *TLR9*, toll-like receptor 9

monophosphate-adenosine monophosphate synthase (cGAS) [19]. Activation of these cytosolic DNA sensors by mtDNA stimulates pro-inflammatory responses driven by a multitude of soluble mediators such as interleukin 1-beta (IL-1β), IL-6, IL-8, tumor necrosis factor-alpha (TNF-α), and type I interferons (IFNs) that have been previously reviewed elsewhere [21••, 22••]. This review synthesizes recent work characterizing the functional role of mtDNA as a DAMP for TLR9 and cGAS and the therapeutic potential of these interactions in the context of SSc-ILD.

Damage and Repair of mtDNA

First identified in 1963, mtDNA is located in the mitochondrial matrix and is comprised of a double-stranded circular DNA molecule containing 37 genes encoding 13 proteins involved in oxidative phosphorylation, 2 rRNAs, and 22 tRNAs [23]. The mitochondrial genome is particularly vulnerable to damage due to (1) its lack of protective histones, (2) limited number of DNA repair enzymes, (3) lipid-rich membranes that increase susceptibility to peroxidation chain byproducts, and (4) proximity to endogenous and exogenous reactive oxygen species (ROS) located in the inner mitochondrial membrane [23]. These processes are not only injurious to mtDNA, but also alter mitochondrial function in a manner that promotes fibroproliferative processes in the lung. For example, myofibroblast activation has been associated with impaired mitochondrial redox homeostasis via transforming growth factor beta (TGFβ)-mediated NADPH oxidase 4 (NOX4) expression [24, 25] and aberrant induction of mitochondrial biogenesis and aerobic glycolysis through a TGFβ-dependent mechanism [26, 27]. Additionally, imbalances in mitochondrial oxidation [28, 29] and metabolism [30] have been linked to recruitment of pro-fibrotic macrophages. While the contribution

of mitochondrial dysfunction in the pathogenesis of the sclerodermatous lung warrants its own dedicated review, our group [31] and others [32] have shown that aberrant mitochondrial function is reflected in mtDNA; thus, the remainder of this review will focus on the immunopathogenic role of mtDNA in the setting of SSc-ILD.

Given the propensity of the mitochondria to accumulate excessive quantities of ROS, recent efforts have endeavored to connect this phenomenon with the SSc disease state. Studies have suggested a novel association between ROS-mediated mtDNA damage and SSc by showing that the skin [33] and lungs [34] of these patients are enriched for ROS. This proposed connection is supported by work demonstrating potential for a feedforward signaling loop where lung fibroblasts derived from SSc-ILD patients treated with exogenous TGFβ resulted in mitochondrial ROS production which, in turn, was found to be indispensable for TGFβ-mediated production of pro-fibrotic mediators such as alpha smooth muscle actin (αSMA), NOX4, and connective tissue growth factor (CTGF) [34]. Given the presence of fibrogenic ROS in the SSc-ILD lung, it is not surprising that the resultant DNA oxidation, particularly nucleotides rich in 8-oxo-7,8-dihydroguanine (8-oxoG) lesions, amplifies TGFβ1 signaling [35]. ROS-mediated mutations in mtDNA-encoded respiratory chain enzymes have also demonstrated a significant association with TGFβ1 [36•], which may account for augmented TGFβ1 signaling observed in SSc-ILD lung fibroblasts [37]. Overall, these findings suggest a previously unknown connection between ROS, mtDNA, and SSc-ILD that might be leveraged for therapeutic benefit.

The mitochondrial genome's exquisite sensitivity to injury is mitigated by unique homeostatic processes. For example, heteroplasmy [38••] and high copy numbers of mtDNA [39] limit genomic damage and its associated dysfunction. Furthermore, the opposing mitochondrial processes of fusion (merging of two mitochondria into one larger organelle) and fission (the division of one mitochondrion into two new organelles) support mtDNA integrity [40]: fusion redistributes healthy mtDNA, while fission sequesters damaged mitochondria [41]. An additional mechanism involves mitophagy, an evolutionarily conserved catabolic process that eliminates excessive or damaged mitochondria [42]. While each of these mechanisms may contribute to autoimmune lung conditions such as SSc-ILD [43], most pertinent to this review is another maintenance process—the generation of mitochondrial DAMPS via extrusion of heavily damaged mtDNA into the cytosol or extracellular space [44].

Mechanisms of mtDNA Release

The release of mtDNA from the mitochondrial matrix has considerable immunologic consequences, one of which involves its DAMP-related function [8•, 19] that stems from its genome being rich in hypomethylated CpG motifs and prone to oxidative damage [45] and/or mutagenesis [36•]. First described as an inflammatory mediator in 2004 [46], mtDNA has been framed as an endogenous ligand for DNA-sensing PRRs, with TLR9 and cGAS being the most extensively studied. Cytosolic and extracellular mtDNA can

both function as agonists for these PRRs, where their contributions to inflammatory pathology have been extensively studied.

The presence of cytosolic mtDNA is a recently described and highly significant phenomenon. The release of damaged mtDNA directly into the cytosol is believed to occur via mitochondrial outer membrane openings including the mitochondrial permeability transition pore (MPTP) [47], macropores formed by activation of BAX and BAK proteins [16], and/or via voltage-dependent anion channel (VDAC) oligomers [48]. Although largely unknown as to whether these mechanisms work in parallel or in concert, they are integral in facilitating the cytosolic release of both mutated [49] and oxidized [50] mtDNA. While various heteroplasmic mtDNA mutations have been shown to initiate pro-inflammatory responses in circulating monocytes [51], the role of oxidized mtDNA as an endogenous DAMP has been described in several conditions characterized by autoimmunity and inflammatory remodeling. For example, oxidized mtDNA containing an abundance of 8-oxoG lesions exhibited immune-activating properties when compared to mtDNA devoid of such oxidized lesions [52], demonstrating robust activation of TLR9 [20] and cGAS [19] in autoimmune conditions such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) [8•]. While similar observations have yet to made in SSc-ILD, it is intriguing to speculate whether perturbation(s) in TLR9 [11, 12] and cGAS [11, 53] activation seen in this condition are derived from the presence of cytosolic mtDNA, a possible convergent molecular pathway linking divergent clinical states.

Mirroring its role as an intracellular DAMP, the immunopathogenic contributions of mtDNA circulating in the extracellular compartment are equally compelling. Extracellular mtDNA has been broadly classified as cell-free or encapsulated within EVs [54]. While other forms of extracellular mtDNA have been identified, such as the mtDNA contained within cell-free mitochondria [18, 55] or neutrophil extracellular traps (NETs) [56], because the majority of work in this context has focused on mtDNA in its cell-free or EV-laden forms, the immunopathogenic potential of these entities will be discussed below.

Cell-free mtDNA (cf-mtDNA) accumulation has been archetypally attributed to necrosis-induced exocytosis of cellular contents into the extracellular space, but can also be associated with hypoxic, inflammatory, and oxidative stress [54, 55, 57]. Early studies describing this process in platelets [55, 57] have been complemented by studies of tissue trauma [54], where cf-mtDNA was shown to mediate inflammation by acting as an endogenous DAMP for TLR9 [58] and cGAS [59]. In studies conducted by our group [11, 31, 60] and others [12, 61], circulating cf-mtDNA predicted poor clinical outcomes in such diverse conditions as idiopathic pulmonary fibrosis (IPF), sarcoidosis, and, relevant to this review, SSc-ILD. In some settings, cf-mtDNA may be oxidized [45] or mutated [62]. Oxidized cf-mtDNA has been shown to be a potent endogenous ligand for TLR9 and cGAS in a variety of inflammatory lung diseases, including SSc-ILD, by amplifying TGFβ signaling and pro-fibrotic responses [63]. Far less is known about whether mutations in cf-mtDNA are associated with SSc-ILD, as reports of cf-mtDNA mutations are largely restricted to the oncology literature [64] where varying degrees of heteroplasmy have hampered identification of disease-specific gene variants [65] and their functional implications. Better understanding of the connection

between mtDNA oxidation, mutation, and DNA-sensing PRR activation can potentially elucidate novel molecular mechanisms of fibroproliferative disorders.

Extracellular mtDNA can also be cargoed within EVs released by cells at homeostasis and in response to various forms of stress [66]. Size-classified as exosomes (< 100 nm), microvesicles (100–1000 nm), or apoptotic bodies (> 1000 nm) [66], EVs are viewed as facilitating cellular communication through the intercellular transport of nucleic acids and proteins. EVs have also been shown to contain mitochondrial components such as intact organelles, mtDNA, and mitochondrial proteins, all of which may function as inflammatory stimulants [66]. Of relevance to this review, our own work identified a previously unrecognized connection between EV-mtDNA and cytosolic DNA sensor activation in the context of SSc-ILD [11]. Specifically, in a study of a longitudinal patient cohort, we found that SSc-ILD plasma is enriched for EV-mtDNA that exhibited TLR9 and cGAS activating potential while also predicting the progression of lung disease reflected by a > 10% relative decline in forced vital capacity [11]. Although in our study EV-mtDNA did not exhibit aberrant oxidation, reports in related conditions show that oxidized EV-mtDNA potently activates TLR9 [67] and cGAS [68]. Depending on the cell of origin, these immunopathogenic functions may accompany context-dependent homeostatic roles. For example, bone marrow-derived mesenchymal stem cells extrude partially depolarized mitochondria via EVs as an oxidative stress induced survival response to repress TLR signaling [69], and injured renal tubular epithelial cells release EVs rich in mitochondria to initiate repair programs [70]. While similar processes remain unknown in SSc-ILD, further study of EV-mtDNA and its biological functions are needed to delineate its pleiotropic function in mediating both disease and physiologic states.

Cytosolic DNA Sensors

The nature of the mtDNA and the mechanism of its release, either intracellular or extracellular, directly impact its proinflammatory functions. However, therapies that would be expected to suppress oxidant induced mtDNA release, such as vitamins A and E, penicillamine, N-Acetyl cysteine, and Coenzyme Q_{10}, have shown only limited efficacy in treating various clinical manifestations of SSc, including ILD [71]. Because extracellular mtDNA was not an endpoint in these studies, it is difficult to know whether the lack of clinical benefit relates to its DAMP effects. Nevertheless, the studies described thus far suggest that interventions aimed at mitigating the immunologic consequences of mtDNA represent a better treatment approach. In the next portion of this review, we will discuss recent advances in the understanding of TLR9 and cGAS, which belong to a family of cytosolic DNA sensors that can be activated by mtDNA, and how these findings may be translated into novel therapeutic strategies in the context of SSc-ILD.

Toll-like Receptor 9

As a member of the well-studied family of toll-like PRRs [72•], TLR9 was first identified in 2000 [73] and recognizes unmethylated CpG dinucleotides present in certain bacteria and host-derived substances. In the resting state, TLR9 resides in the endoplasmic reticulum (ER), and upon encountering unmethylated CpG dinucleotides, TLR9 undergoes UNC93B1-mediated endosome trafficking [74], proteolytic cleavage of its ectodomain [75], acidification-induced CpG binding [76], and conformational changes [77] that result in recruitment of the adapter protein myeloid differentiation primary response gene-88 (MyD88) [74]. MyD88 interacts with interleukin-1 receptor-associated kinase 4 (IRAK-4) and IRAK-1 [78], to recruit tumor necrosis factor receptor-associated factor 6 (TRAF6) and activate TGFβ associated kinase 1 (TAK1) [79]. TAK1 phosphorylates IκB kinase (IKK) complex, which results in nuclear factor-kappa B (NF-κB) activation and subsequent transcription of proinflammatory cytokines such as IL-6, IL-12, and TNF-α [21••, 80]. TLR9's function has been most well described in the context of PAMPs; however, because SSc-ILD is not known to result from invading microbes, TLR9's interactions with DAMPs may be more relevant in this setting.

A role for TLR9 has been proposed in models of sterile inflammation [81], tissue fibrosis [82] and, more recently, human SSc [63]. For example, Fang et al. described TLR9 enrichment in SSc skin biopsies that was accompanied by a transcriptional signature reflective of chronic TLR9 activation [63]. A possible contribution to disease pathogenesis was demonstrated when CpG-stimulated dermal fibroblasts from these patients developed TGFβ-dependent TLR9 activation [63]. Analogous results were observed in SSc-ILD, a disease characterized in part by innate immune overactivation [83], where our own work found that normal human lung fibroblasts stimulated with TGFβ1, and CpG-DNA displayed an activation state characterized by αSMA expression and mtDNA release [11]. These responses were suppressed by hydroxychloroquine (HCQ) [11], which inhibits endosomal acidification and subsequent cleavage induced activation of TLR9 [84]. SSc-ILD lung fibroblasts showed a similar phenotype [11], framing TLR9's potential as a cross-organ mediator of skin and lung fibrosis.

The studies shown above focused largely on TLR9's fibrosis-promoting functions in TGFβ1 stimulated fibroblasts. An alternate but equally compelling process is suggested by studies of SSc patients in which TLR9-expressing T- and B-cell populations displayed potential association with skin thickness as measured by the modified Rodnan skin score (MRSS) [85]. While the expression of this PRR by lymphocyte populations defined by their role in adaptive immunity may seem at first conflicting, it adds to an emerging literature regarding the expanding, and at times unexpected, functions of TLR9. While it remains to be seen whether TLR9-expressing lymphocytes are pathogenic or protective in this setting, because TLR9 appears to restrict auto reactive B-cell activation in other conditions, TLR9 may promote host tolerance. This interpretation is supported by loss- and gain-of-function studies in animal models, where TLR9-deficient mice developed exacerbations of experimentally induced lung fibrosis [86] and granuloma formation [87], and in wild type mice in which experimental administration of synthetic

TLR9 ligands appeared protective in the bleomycin lung fibrosis model [86]. In contrast, a pathogenic role for TLR9 was shown in a novel mouse model of fibrosis caused by the administration of fibrotic lung fibroblasts derived from humans [82]. When viewed in combination, these data support a paradigm wherein TLR9 exerts alternatively protective or pathogenic roles in fibrosis that depends on cell-specific input and temporal cues.

While this complex biology may hinder the development of pathway-specific therapies, the availability of safe and well-tolerated TLR9 antagonists has facilitated leveraging this biology for clinical benefit in autoimmune disorders such as SSc [88]. The most extensively studied agent is HCQ, a member of the quinolone family. Its proposed biological effects include inhibition of endosomal acidification [89], which would be expected to oppose cleavage-induced activation of TLR9. Initially developed as an antimalarial agent, HCQ is routinely used in the management of SLE [90], RA [91], and, most recently, SSc-related inflammatory arthritis [92]. While benefit in SSc-ILD has yet to be explored, its safety and efficacy are currently being evaluated in pediatric ILD [93], and results of this work could provide scientific premise for its indication in SSc-ILD.

The potential of HCQ is complemented by studies of Bortezomib, a proteasome inhibitor whose properties include inhibition of intracellular TLR9 trafficking [94]. Currently approved for the treatment of relapsed refractory multiple myeloma [95], Bortezomib has demonstrated anti-fibrotic properties in animal models of SSc-like skin [96] and lung fibrosis [97], and its use for SSc-ILD has been tested in a phase II clinical trial for which results are currently not available [98•]. Nonetheless, studies to repurpose FDA-approved drugs such as Bortezomib and the aforementioned HCQ for SSc-ILD could catalyze new therapeutic approaches for this disease.

While the therapies described above suppress processes associated with TLR9 activation, directly targeting TLR9 itself may be another option. For example, the synthetic oligonucleotide IMO-8400 is a TLR9 inhibitor, whose preclinical efficacy [99, 100], has yet to be studied in SSc. Another potential approach involves E6446, an orally available small-molecule non-oligonucleotide inhibitor that sequesters TLR9-activating DNA ligands to attenuate receptor activation [101–103]. TLR antagonistic compound 5 (TAC5) and its derivative TAC5a are small molecule direct inhibitors of endosomal TLRs, including TLR9, that reduce NF-κB, TNF-α, and IL-6 production and prevent disease progression in mouse models of psoriasis and SLE [104]. SM934, a synthetic derivative of the anti-malarial agent artemisinin, has been shown to be effective in several murine models of SLE through mechanisms involving suppression of TLR9-dependent MyD88-dependent signaling [105–107]. Although these agents have not been evaluated in SSc-ILD, they highlight the possibility of repurposing TLR9 targeted interventions for this disease.

cGAS-STING

Since its discovery in 2013 [108], cGAS has been increasingly acknowledged for its recognition of cytosolic DNA. The biology of this interaction is complex, as binding of cytosolic double-stranded (ds) DNA to cGAS

occurs in a DNA-sequence independent manner with activation being length dependent [109]. Cytosolic dsDNA sequences exceeding 45 base-pairs stimulate formation of stable ladder-like networks of 2:2 DNA-cGAS oligomer complexes that are critical for activation [109]. The cGAS proteins then undergo a conformational change that catalyzes binding of adenosine triphosphate (ATP) and guanosine triphosphate (GTP), inducing synthesis of 2′3′-cGAMP [110], a second messenger protein detected by the cyclic-dinucleotide sensor STING [108], and a 40-kDa dimeric transmembrane protein located in the ER [111]. This form of STING translocates to the Golgi, where it undergoes post-translational modifications such as palmitoylation [112], and activates TANK-binding kinase 1 (TBK1) through autophosphorylation and phosphorylation of STING and interferon regulatory factor 3 (IRF3) [111]. IRF3 phosphorylation leads to its dimerization and subsequent translocation to the nucleus [111], where it triggers the production of type I IFN and proinflammatory cytokines such as IL-1β, IL-6, and TNF-α [22••, 108]. In a relatively short period of time, significant progress has been made elucidating the mechanism of this important PRR.

Given the above work accomplished in characterizing this signaling pathway, a connection to SSc and ILD has been an area of ongoing investigation. Work completed by our group showed that SSc-ILD patients have elevations in plasma mtDNA which correlates significantly with cGAS-STING activating capacity and production of type I IFNs and IL-6, suggesting a potential immunopathogenic connection [11]. This interpretation is supported by a report in which mutations in the mitochondrial membrane protein ATAD3A augmented IFN signaling through cGAS-STING, resulting in a clinical syndrome suggestive of SSc [49]. However, because this study did not measure mtDNA, it is possible that cGAS-STING activation arose through alternative mechanisms, such as the recently described contribution of centromere alterations and chromosome instability observed in SSc dermal fibroblasts [113]. Further investigation linking the accumulation of extracellular mtDNA, cGAS-STING activation, and SSc-ILD has the potential to shed novel insight into the innate immune mechanisms mediating this process.

While the preponderance of human data suggests fibrogenic interactions between mtDNA and cGAS-STING, results of animal modeling have yielded conflicting results. For example, STING activation appears to drive inflammatory fibrosis in mouse models of silicosis [114] and in a gain of function mutations that mimic the development of STING-associated vasculopathy with onset in infancy (SAVI), a pediatric autoinflammatory syndrome associated with pulmonary fibrosis [115]. However, the converse has been found in the widely used bleomycin model of lung fibrosis, where constitutive and ubiquitous STING deletion exacerbated neutrophilic infiltration and fibrosis in a type I IFN-independent manner [116]. Similar to the TLR9 data described above, these findings suggest context-dependent roles for cGAS-STING activation that would benefit from additional investigation.

Despite the contradictory in vivo results, cGAS antagonism has demonstrated promise as a potential therapeutic strategy in preclinical studies. A variety of medications such as acetylsalicylic acid, quinolones, and

sulfonic acid exhibit unexpected cGAS inhibition [117–119] that could be tested in SSc-ILD, though the potential for off-target effects may dampen enthusiasm for their use. Alternatives would include specific approaches such as the small molecules CU-32 and CU-76, which selectively inhibit cGAS by targeting the protein-protein interface of cGAS for IRF3 activation and IFN induction [120] without observable off-target effects on other DNA-sensing PRRs such as TLR9 [120]. Species-specific effects may be an issue to overcome, as another small molecule, RU.521, was successful in binding to the catalytic site of cGAS and reduced binding affinity to ATP/GTP in cells derived from mice [121], but not humans [122]. Superior efficacy was observed with the small molecule G140 and the monoclonal antibody PF-07043030 [123, 124], which raises the exciting possibility of testing these or related approaches for cGAS targeted treatment of SSc-ILD.

In addition, studies of STING inhibition have also been pursued for this purpose. H-151 is a small molecule that covalently binds to a cysteine residue on the STING protein, preventing its activation in the Golgi [125]. While this agent has yet to be studied in SSc-ILD, it has shown to be efficacious in pre-clinical models of cisplatin-induced acute kidney injury and psoriasis [126, 127]. SN-011 is another small molecule STING inhibitor that functions by competing for the cGMP binding site required for activation [128]. Other agents under investigation include nitro-fatty acid derivatives that interrupt STING palmitoylation [129], the cyclin-dependent protein kinase (CDK) inhibitor Palbociclib that disrupts STING dimerization [130], and Astin C, which blocks IRF3 recruitment [131]. Despite these encouraging results, studies with STING antagonism remain in the pre-clinical phase and have yet to be investigated for SSc. Nevertheless, such work holds promise for the future management of rheumatic diseases such as SSc-ILD.

Conclusions

Recent work has demonstrated an emerging association between mtDNA-mediated activation of TLR9 and cGAS and SSc-ILD, suggesting a potential immunopathogenic contribution to the development and/or progression of disease that has catalyzed novel therapeutic approaches in treating this condition (Fig. 1A–B). However, doing so will require further understanding of this biology and as shown in Box 1, additional questions require answers. For example, the source and significance of extracellular mtDNA require determination, as does the functional distinction between cf-mtDNA and EV-mtDNA. The inconsistencies between human and mouse studies warrant additional investigation and highlight the need for improved modeling systems. Finally, further understanding of the mechanisms through which interactions between mtDNA and TLR9 and cGAS stimulate pathologic lung remodeling will be required to fully leverage this pathway for treatment of SSc-ILD. Better understanding of these questions will undoubtedly lead to new and effective treatments for this complex condition.

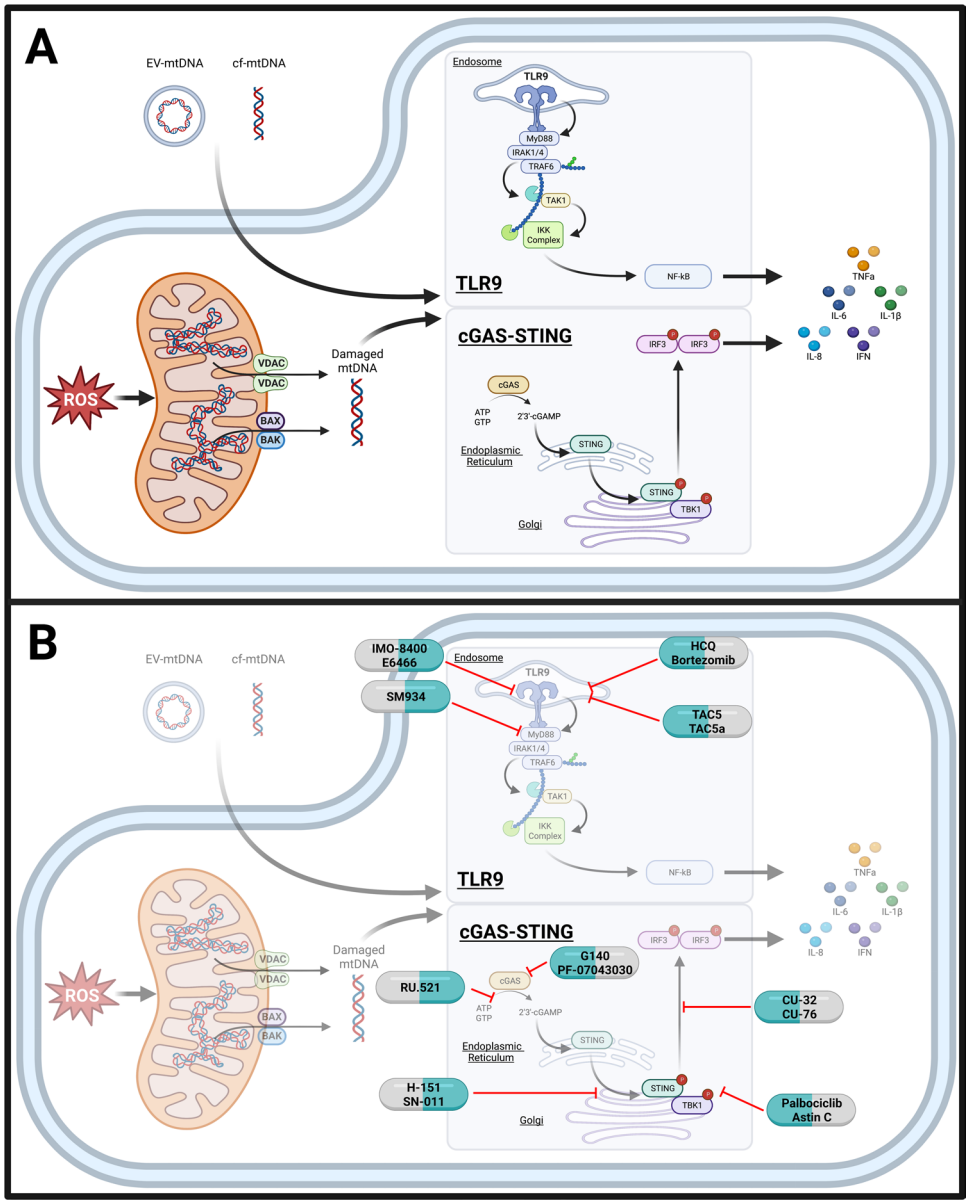

Fig. 1 Proposed model of mtDNA-mediated innate immune activation via TLR9 and cGAS-STING. **A** Both intracellular and extracellular sources of mtDNA mediate activation of cytosolic DNA-sensing pathogen recognition receptors (PRRs), toll-like receptor 9 (TLR9), and/or cytosolic cyclic guanosine monophosphate-adenosine monophosphate synthase (cGAS). Mutated or oxidized mtDNA is released into the cytosol through either the mitochondrial permeability transition pore (MPTP), macropores formed by activation of BAX and BAK proteins, and/or via voltage-dependent anion channel (VDAC) oligomers, where it functions as a damaged associated molecular pattern (DAMP) to activate either TLR9 or cGAS. Alternatively, mtDNA can be released into the extracellular compartment, as either cell-free (cf-mtDNA) or encapsulated within extracellular vesicles (EV-mtDNA) to mediate activation of these PRRs. **B** Both direct and indirect antagonisms of TLR9 and cGAS-STING have been explored as potential therapeutic options in various interstitial lung diseases, including scleroderma. Created using BioRender.com.

Box 1 Unanswered questions

1. What is the source and significance of extracellular mtDNA in SSc-ILD?
2. What is the relationship between mitochondrial dysfunction, abnormalities in mtDNA, and DNA-sensing PRR activation in SSc-ILD?
3. Are cf-mtDNA and EV-mtDNA equally able to induce activation of TLR9 and/or cGAS-STING?
4. Does the worsening of experimentally induced fibrosis in animal models reflect experimental limitations or pathway-specific effects?
5. What are the best models to study interactions between mtDNA and cytosolic DNA sensors in SSc-ILD?
6. Can treatments targeting TLR9 and/or cGAS-STING mitigate disease progression in SSc-ILD?

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Compliance with Ethical Standards

Conflict of Interest

Alexander Ghincea declares that he has no conflict of interest. Samuel Woo declares that he has no conflict of interest. Sheeline Yu declares that she has no conflict of interest. Taylor Pivarnik declares that she has no conflict of interest. Vitoria Fiorini declares that she has no conflict of interest. Erica L. Herzog declares that she has no conflict of interest. Changwan Ryu declares that he has no conflict of interest.

Human and Animal Rights and Informed Consent

This article does not contain any studies with human or animal subjects performed by any of the authors.

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- •• Of major importance

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