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Review

Role of RP105 and A20 in negative regulation of toll-like receptor activity in fibrosis: potential targets for therapeutic intervention

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Abstract: Toll-like receptors (TLRs) are essential defensive mediators implicated in immune diseases. Tight regulation of TLR function is indispensable to avoid the damaging effects of chronic signaling. Several endogenous molecules have emerged as negative regulators of TLR signaling. In this review, we highlighted the structure, regulation, and function of RP105 and A20 in negatively modulating TLR-dependent inflammatory diseases, and in fibrosis and potential therapeutic approaches.

Keywords: TLR; RP105; A20; inflammation; SSc; fibrosis

1. Introduction

Toll-like receptors are evolutionarily conserved pattern recognition receptors (PRRs) that recognize and respond to both microbial pathogen-associated molecular patterns (PAMPs) and endogenous damage-associated molecular patterns (DAMPs) or so-called "danger signals" [1]. Upon sensing PAMPs or DAMPs, these promiscuously expressed cellular receptors trigger NF-κB activation, leading to the secretion of proinflammatory cytokines and promoting a vigorous inflammatory response. DAMPs include extracellular matrix components such as alternately spliced fibronectin (Fn-EDA), tenascin-C, low-molecular-weight hyaluronan degradation products, and biglycan; or intracellular stress proteins such as high-mobility group protein-B1 (HMGB1) and heat shock protein 60 (Hsp60) released from damaged cells; and nucleic acids and immune complexes, each of which can induce cell activation via TLRs [2–4]. Uncontrolled TLR activation can lead to unchecked production of inflammatory mediators culminating in diseases [5–7]. Limiting the

duration and amplitude of TLR signaling by negative regulation is therefore essential to inhibit unchecked inflammation. There is a multitude of negative regulators of TLR signaling, including alternative splicing of TLR adaptors (e.g. MyD88s), the cell surface molecule radioprotective 105 (RP105), the ubiquitin-editing enzyme A20 that modulate the activity of key TLR signaling intermediates, transcriptional regulators, and microRNAs (microRNA-19a, -34a, -146a, and -146b) [8–15].

Recent studies implicate DAMPs and their TLR-dependent cellular responses as key factors underlying pathological fibrosis in the liver, kidney, lungs, heart, keloids, and systemic sclerosis (SSc) [16–20]. Levels of TLR4 and its cognate DAMPs, alternatively spliced Fn-EDA and tenascin-C, are elevated in SSc and elicit potent stimulatory effects on fibrotic gene expression [21]. Genetic targeting of TLR4 and its DAMPs or selective TLR4 inhibitor in mice ameliorates experimental fibrosis in models of SSc and explanted SSc fibroblasts. Alternatively, impaired negative regulation of TLR signaling might result in unchecked TLR activation and TLR-dependent fibrotic responses, thus contributing to fibrotic diseases. Therefore, restoring or boosting endogenous expression or function of the TLR inhibitors such as A20 or RP105 might hold promise for effective anti-fibrotic therapies. Speculated RP105 and A20 mediated regulation of TLR driven fibrotic responses were described in Figure 1. This review highlights recent insights and current understanding of the basic structure and function of negative regulators of TLR signaling in inflammation and fibrosis and discuss about the potential therapeutic strategies targeting TLR negative regulators.

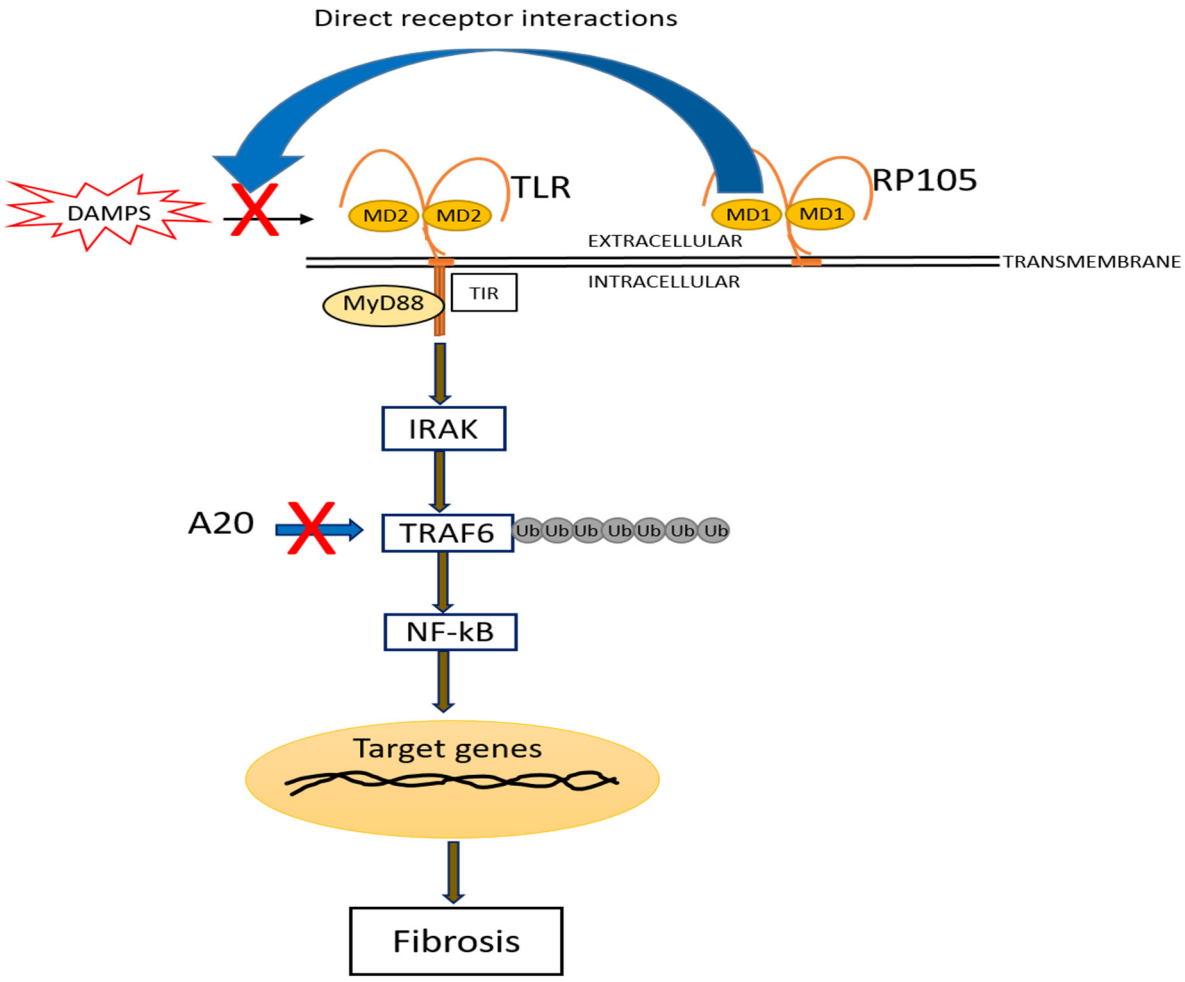

Figure 1. Regulation of TLR mediated fibrotic responses by RP105 and A20. TLR receptor upon ligand (DAMP) recognition, interacts with its adapter proteins to trigger downstream signaling via IRAK/TRAF6/NF-κB signaling cascade and in association with TGF-β results in fibrosis. RP105, a TLR homolog inhibits the DAMP recognition of TLR and block TLR signaling. A20 inhibits the polyubiquitination and activation of TRAF6 and thus block TLR-mediated fibrotic events.

2. Toll-like receptors (TLRs) and signaling pathway

2.1. TLRs

Toll-like receptors are type 1 integral membrane proteins that consist of leucine-rich repeats in their ectodomain for ligand recognition, a transmembrane domain, and toll/interleukin 1 receptor (TIR) in the cytoplasmic domain to activate downstream signaling cascade [22,23]. Broadly, there are 13 murine and 10 human TLRs, that can be sub-classified into two types based on their localization. TLRs-1, -2, -4, -5, -6 and -10 are confined to cell surface, while TLR-3, -7, -8, -9, -11, -12, and -13 are located on intracellular components [24–26]. The expression of TLRs is not restricted to immune cells but is promiscuous on non-immune cells including fibroblasts and epithelial cells [27–29].

2.2. TLR signaling

Adapters for TLR signaling include myeloid differentiation factor 88 (MyD88), TIR-domain-containing adaptor protein-inducing IFN-β (TRIF), TIR-associated protein (TIRAP), and TRIF-related adaptor molecule (TRAM), that initiate TLR signaling upon ligand binding and subsequent TLR dimerization [30–32]. These adapter proteins are ligand-specific, and either interact mutually or with TIR in response to individual or combination of TLRs. MyD88 is a central adapter protein that signals all TLR responses excluding TLR3 and triggers activation of IL-1R associated kinases (IRAKs) and TNF receptor-associated factor 6 (TRAF6)-mediated NF-κB activation [33,34]. TLR3 signals through TRIF. Notably, TLR4 is the only TLR that engages both MyD88 and TRIF for signaling, while TLR2 mediated signaling is associated only through MyD88 interaction. All TLR signaling downstream effectors converge in the activation of NF-κB, which regulates the expression of various inflammatory cytokines [35]. The activation of NF-κB is mediated by MyD88 dependent TLR signaling including phosphorylation of IRAK, and their subsequent dissociation from MyD88, which then interacts with TRAF6, which in turn triggers polyubiquitination of TRAF6 and NEMO. Ubiquitinated TRAF6 and NEMO recruit TAK1 and regulates signaling pathways involving the IKK complex, resulting in NF-κB activation [23,36–38].

3. Negative regulation of TLR signaling

To forestall sustained and deleterious TLR signaling, a variety of negative regulators evolved to dampen the magnitude and duration of the TLR signaling generated by endogenous DAMPs [39]. In this context, the present review summarizes (Table 1) and highlights recent insights into these negative regulators of TLR. Most of the TLR negative regulatory mechanisms comprise ubiquitination and epigenetic mechanisms. E3 ubiquitin ligases including TRIAD3A and Nrdp1, regulate K48-mediated ubiquitination of critical TLR adapters, TRIF and MyD88 respectively [40,41]. As further described below, A20 is an important ubiquitin-editing negative regulator of TLR signaling which by deubiquitinating K63-linked ubiquitin chains from TRAF molecules inhibits TLR signaling [40]. Furthermore, downstream of TLR signaling upon ligand stimulation is mediated via key adapter molecules of TLR. Disassociation of adapter complexes (MyD88, TRIF, TRAF6, etc) is therefore important in the negative regulation of TLR signaling. For instance, flightless I homolog (Fliih) is identified as one of the not previously recognized proteins to interact with MyD88 [42]. Further, this study also demonstrated that Fliih could suppress TLR4-MyD88 mediated NF-κB activation, by disassociating the interaction of MyD88 and TLR4 signaling complex. The findings of this study formed a new dimension for TLR signaling pathway [42]. Similarly, very recently identified TLR negative regulators affecting the adapter complex include S100A10, SPOP, and Tob2 (Table 1). While these intriguing observations implicate these negative regulators of TLR signaling in inflammatory pathways, further investigation of their potential role in the pathogenesis of fibrosis is warranted.

Table 1. Insights on recent key negative regulators of TLR activity.

Negative regulator	Class/family	Function	*In vitro*	*In vivo*	Receptor	Outcome	Reference
β-arrestin 2	GPCR regulator	Facilitates SHP-1 to Tir and K63-dependent ubiquitination by TRAF6 and TAK1	HEK293 T cells, RAW264.7 cells, and mouse primary macrophages	β-arrestin 2-deficient mice	TLR4	Regulates Tir-mediated immune evasion	[43]
IRAK-M	Inactive kinase of IRAK family	Formed IRAK-TRAF6 complexes by limiting the disassociation of IRAK with MyD88	Primary bone marrow-derived macrophages (IRAK-$M^{-/-}$)	IRAK-$M^{-/-}$ mice challenged with bacteria	TLR4; TLR9	Promoting endotoxin tolerance	[44,45]
TLR10	The orphan receptor of TLR family	Suppression of MyD88- and TRIF-inducing IFN-β-mediated signaling pathway	Human myelomonocytic U937 cells	TLR10 transgenic mice—LPS-induced septic shock model	TLR2; TLR2/6; TLR3; TLR4	Decreased production of cytokines (IL-6, IL-8, type I IFN, IFN-β, TNF-α)	[46]
Prolactin	Neuroendocrine hormone	Inhibits LPS mediated elevated TLR4 expression and phosphorylation of NF-κB	Cotyledon explant culture	-	TLR4	Reduction of LPS induced TNF-α, IL-1β and IL-6 production	[47]
RING finger protein 182 (RNF182)	RNF family	K48-dependent polyubiquitination of p65	Primary peritoneal macrophages	-	TLR3, 4, and 9	Silencing of RNF182 triggered the production of inflammatory cytokines including IL-6 and TNF-α, but not type I IFN, and enhanced NF-κB luciferase activity	[48]

Continued on next page

Negative regulator	Class/family	Function	*In vitro*	*In vivo*	Receptor	Outcome	Reference
Cullin B4 (CULB4)	Cullin4B-Ring E3 ligase complex	CULB4 deficiency upregulated Phosphatase and tensin homolog (PTEN), thereby activating GSK3β signaling mediated inflammatory responses	Myeloid cells	CULB4 deficient mice	TLR2/3 and 4	Silencing of CULB4 upregulated the expression of cytokines including IL-6, IL-1β, and TNF-α; reduced the expression of IL-10	[49]
S100A10	S100 family of intracellular calcium-binding protein	Interacts with the TIR domain of TLR competitively and consequently inhibits the association of TLR adapters with either MyD88 or TRIF	Macrophages from S100A10-deficient mice	S100A10-deficient mice	TLR-2, -3, -4	Elevated expression of TNF-α, IL-6, IL-12, and IFN-β mRNA in S100a10$^{-/-}$ macrophages	[50]
Speckle-type POZ protein (SPOP)	Representative substrate-recognition subunit of the cullin-RING E3 ligase	Associates with TLR adapter, MyD88, and suppresses the MyD88-dependent TLR4 signaling	Bone marrow cells were isolated from the tibias and femurs of wild-type C57BL/6 mice, SPOP-deficient THP-1 cells	-	TLR-2, -4, -7, -9	SPOP inhibited LPS-induced expression of cytokines, including TNF-α, IL-1β and IL-6, at both the mRNA and protein levels	[51]
Transducer of ErbB2.2 (Tob2)	Tob/BTG1 antiproliferative family of proteins	Downregulation of MyD88 and TRAF6 associated NF-κB activity	Tob2 depleted murine peritoneal macrophages, HEK293T cells	Tob2-defective C57BL/6 mice	TLR-4, -7/8	Depleted macrophages resulted in elevated production of inflammatory cytokines including TNF-α and IL-6	[52]

3.1. TLR signaling negatively regulated by TLR homolog: radioprotective 105 (RP105)

3.1.1. Structure of RP105

RP105, also named CD180, is an unconventional TLR homolog. Structurally, it consists of an extracellular leucine-rich repeat domain as well as the transmembrane domain but lacks a TIR signaling domain unlike other homologs of TLR. Expression of RP105 was initially thought to be limited to B cells [53]. However, RP105 was also found on human and mouse monocytes and dendritic cells. In HEK 293 and dendritic cells, RP105 functions as a negative regulator of TLR4 signaling, where RP105 co-expressed with MD1 to form a complex and associates with TLR4/MD2 to inhibit lipopolysaccharide (LPS)-mediated TLR4 responses [54]. MD1 is a secreted molecule without a membrane-spanning domain. It is an essential adaptor molecule of RP105 and is required for trafficking of RP105 to the cell surface [55]. MD1 alone does not act as a signaling molecule. However, in response to ligands MD1 associates with RP105 to form RP105/MD-1 complex to initiate signal transduction. Subsequent studies performed using $RP105^{-/-}$ and $MD1^{-/-}$ mice linked the physiological association of RP105 with MD1, wherein both mice exhibited similar B cell phenotypes [56,57]. Lyn (protein kinase of Src-family), NF-κB, and various kinases including, protein kinase C, mitogen-activated protein kinase (MAPK), PI3K, and Bruton type kinase are found to be some of the down regulatory pathways of RP105 regulated TLR4 signaling [58–60].

3.1.2. Functions of RP105

The physiological role of RP105 in TLR-mediated inflammation is variable and is determined by its expression on various cell types. For example, RP105 suppresses TLR4 activation when expressed in myeloid cells, while it mediates TLR4 signaling in B cells [53,61]. In light of this concept, findings from a study performed in $RP105^{-/-}$ mice demonstrated elevated levels of basal serum B-cell activating factor (BAFF) as compared to wild type controls; which was exacerbated upon LPS stimulation, implicating the possible role of TLR signaling in B cell activation [62]. Moreover, LPS-challenged RP105-deficient mice exhibited elevated cytokine production and endotoxicity in bone marrow-derived dendritic cells (TNF, IL-12p70, IL-6 and IP-10) and peritoneal macrophages (TNF), confirming the role of RP105 as a negative regulator of TLR activation in both dendritic cells and macrophages [8]. The role of RP105 on smooth muscle cells was explored in an elegant study based on vascular modeling during neointima formation in $RP105^{-/-}$ mice. Increased restenosis was observed in RP105 knockout mice, and lack of expression of RP105 on vascular smooth muscle cells both *in vitro* and *in vivo* resulted in enhanced neointima [63]. On the other hand, reduction of atherosclerotic burden in lethally irradiated mice treated with $RP105^{-/-}$ bone marrow was demonstrated. $RP105^{-/-}$ chimeras exhibited altered expression of inflammatory B2 circulating cells, leaving B1 circulating cells unaffected and the reduction of plaque burden is related to reduced B cell activation in those mice suggesting that deficiency of RP105 can ameliorate atherosclerotic lesions [64].

In a consequent study, the effect of RP105 deficiency was evaluated in LPS-treated mice lacking both low-density lipoprotein receptor and RP105 ($LDLr^{-/-}/RP105^{-/-}$) *in vivo* and in monocytes *in vitro*. LPS stimulation affected monocyte migration into the vessel wall and thereby reduced early atherosclerosis by 40% compared to $LDLr^{-/-}$ controls [65]. The same team reported 90% aggravation

of vein graft lesions as well as intraplaque hemorrhage in RP105$^{-/-}$ smooth muscle cells and mast cells. Altogether these findings indicated RP105 as a potent endogenous TLR4 inhibitor in smooth muscle and mast cells, while promoting TLR4 function in B cells [66]. RP105 functions in different cell types and detailed mechanisms of regulating immune-mediated inflammation and other related diseases are warranted.

3.1.3. RP105 and TLR4 signaling

Although the role of RP105 depends on the cell type, it functions as a negative regulator of TLR4 dependent immune signaling in various diseases. For instance, in an experimental model of myocardial infarction, apparent cardiac dilatation and alteration in cardiac parameters including systolic pressure, heart rate, were evident in RP105$^{-/-}$ mice as compared to wild type mice [67]. In a rat model of myocardial ischemic reperfusion (I/R) injury, RP105 adenovirus decreased myocardial apoptosis by downregulating TLR4 mediated intracellular signaling of P38 MAPK and transcription factor activator protein 1 (AP-1) [68]. Moreover, RP105 exerted anti-inflammatory effects by suppressing TLR-MyD88 signaling and reduced the expression of inflammatory cytokines including TNF-α and IL-6, and transcription factor, NF-κB [69]. Furthermore, constitutive expression of MD-1, an essential adaptor for RP105, resulted in cardio-protective effects against hypertrophy and fibrosis by inhibiting NF-κB signaling, while MD-1 KO mice demonstrated detrimental results [70]. Overexpression of RP105 in a rat model of myocardial ischemia also resulted in attenuation of TLR4 dependent inflammation, apoptosis, and autophagy [69,71]. Furthermore, hypoxic cardiac microvascular endothelial cells with minimal RP105 expression offered no protection against hypoxic injury where overexpression of adenovirus-RP105 rendered protection from hypoxia and reduced hypoxia-mediated inflammation and apoptosis by inhibiting TLR4/MAPK/NF-κB signaling [72].

RP105 was shown to be a miR-327 target. A three-fold increased expression of miR-327 was found during ischemia-induced myocardial damage, which was correlated with RP105 down-regulation. Further, inhibition of miR 327 using adenovirus transfection resulted in significantly elevated expression of RP105 that in turn down-regulated TLR4/MyD88-NF-κB signaling cascade and ameliorated myocardial inflammation [73].

Rat cardiomyocyte cells exposed to hypoxic conditions led to downregulation of RP105 with a concomitant increase of miR-141-3p. On the other hand, inhibition of miR-141-3p triggered RP105 stimulation and thus contributed to anti-hypoxic effects via modulating PI3K/AKT signaling [74]. Likewise, RP105 exerted neuroprotective effects in PC12 cells *in vitro* where RP105 reduced neuronal injuries stimulated by oxygen-glucose deprivation (OGD)/reoxygenation via activating PI3K/AKT signaling, suggesting PI3K as one of the pivotal downstream regulators of RP105 [75]. RP105 involves a catalytic subunit p110δ of PI3K in triggering innate immune responses by releasing inflammatory cytokine TNF-α from macrophages during mycobacterial infection [76].

CD19, a B cell specific transmembrane signaling protein is one of the fundamental regulators in orchestrating immune signal transduction of RP105, mediated through Lyn/CD19/Vav cascade. In response to LPS, RP105 triggers Lyn activation, followed by CD19 phosphorylation, Lyn kinase activation, interaction with adapter molecule Vav, and eventually JNK activation of RP105 [77]. A clinical study, using monocytes from patients with primary biliary cirrhosis and chronic viral hepatitis revealed reduced expression of RP105 resulting in hypersensitivity to LPS induction in

cirrhosis patients. Further, the elevated expression of TLR4 and MyD88 was observed in cirrhosis patients, correlating the significance of RP105 in regulating TLR4 signaling with pre-clinical findings [78]. Tracing endogenous ligands of RP105 and understanding their mechanism in modulating functions of RP105 would be a significant therapeutic strategy in inflammatory diseases. Towards this context, cell–cell adhesion molecule named CEACAM1, was found to be a negative regulator of RP105 and suppressed RP105 activated secretion of IL-6 mediated fever responses in murine monocytes [79]. Future investigations on downstream effectors of RP105 in eliciting physiological responses and the development of novel therapeutic small molecules of RP105 agonists are warranted.

3.1.4. RP105 in autoimmune inflammatory diseases

RP105 negative B-lymphocytes were reported in peripheral blood in rheumatic diseases including systemic lupus erythematosus (SLE), Sjogren's syndrome, IgG4 related disease, and dermato-myositis, suggesting key contribution in disease pathophysiology [80–83]. The expression of RP105 in various rheumatic diseases is mentioned in Figure 2. Intriguingly, RP105 negative B cells were no longer characterized as B cells but were found to exhibit a specific phenotype of CD95+CD86+CD38+IgD-IgMlo [84]. In SLE patients, loss of RP105 is associated with B cell activation and increased disease activity. The disease activity is evaluated by SLE Disease Activity Index (SLEDAI) and Systematic Lupus Activity Measure (SLAM) [80,85]. Moreover, RP105 expression was also reduced in peripheral blood B cells of patients with diffuse cutaneous systemic sclerosis (dcSSc) patients. RP105 triggered the production of natural antibodies from non-switched B cell subset, and its deficiency in SSc patients, therefore, resulted in differences amongst pathological and natural antibodies production [86]. As most of these mentioned diseases are featured by B-cell activation, examining the role of RP105 in other cell types will provide significant insights into disease pathogenesis. Recent unpublished data from our laboratory provide evidence for the role of RP105 in negative regulation of fibrosis thereby identifying a distinct new function. RP105 attenuated TLR4 signaling in human foreskin fibroblasts and mouse skin and lung fibroblasts *in vitro*. Findings from *in vivo* study revealed bleomycin administered RP105 null mice showed exaggerated skin fibrosis, compared to bleomycin administered wild type mice. It will be of great interest to determine the role of RP105 in other fibrosis models and other forms of fibrosis as well and the possibility that, targeting (inducing) RP105 will have a protective role in fibrosis.

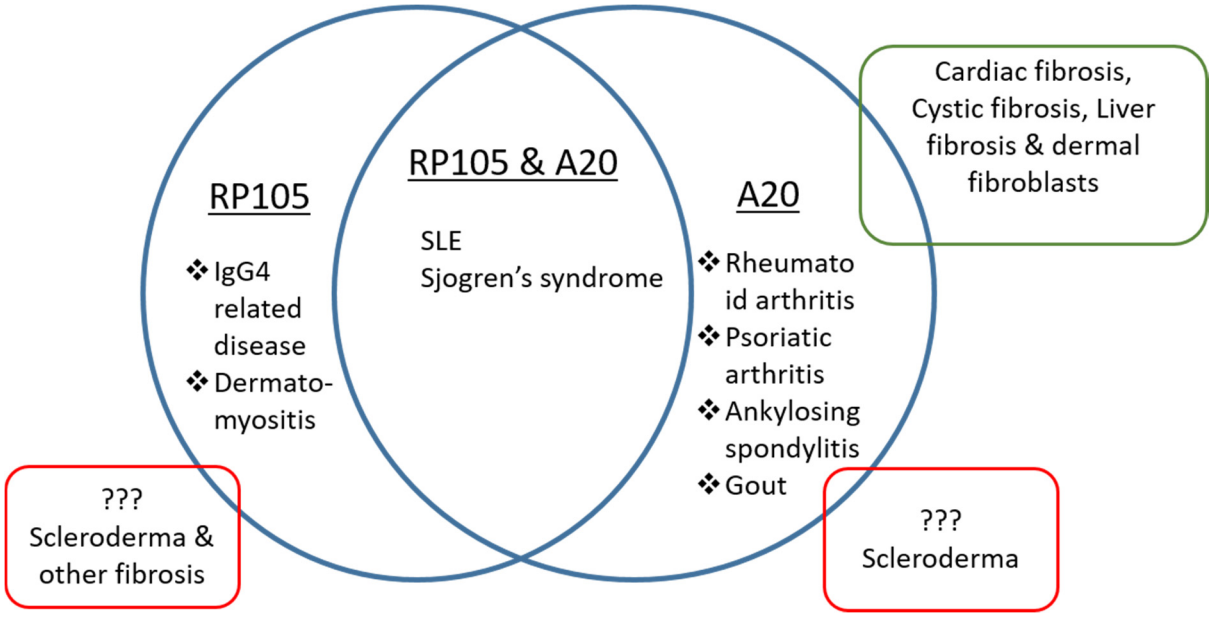

Figure 2. Expression of RP105 and A20 in rheumatic and fibrotic diseases.

3.2. A20 negatively regulates TLR signaling pathway by ubiquitination

Numerous proteases exhibit deubiquitinase activity with varied specificity for ubiquitin (Ub) linkages. Some of the deubiquitinases that function as negative regulators of NF-κB signaling pathway include A20 (TNF-α inducible protein 3 (TNFAIP3)), CYLD, Cezanne, and OTULIN [87]. Linear ubiquitin chain assembly complex (LUBAC) mediated ubiquitination is crucial in the regulation of immune responses. OTULIN (also termed as gumby, FAM105B) and A20 are the deubiquitinase enzymes, that counter regulates the ubiquitination, by cleaving linear (Met1) and Lys63 (K63) associated Ub chains, respectively from target molecules such as RIPK1 and IKKγ, thereby functioning as negative regulators of the canonical NF-κB pathway [88,89]. Among the aforementioned several deubiquitinases, A20 seems to play a significant role in the pathogenesis of fibrosis. Hence, we will discuss the role of A20 in inflammation and fibrosis will be discussed elaborately in the following sections.

Ubiquitin-editing enzyme A20 was discovered 30 years ago as a gene expressed upon TNF stimulation on human endothelial cells from the umbilical vein, primarily to render protection from cell death induced by TNF [90]. Later, A20 was primarily recognized as a negative regulator of TLR4-induced immune responses [91–93]. In most cells at rest, expression of A20 is minimal but is upregulated transiently in inflammatory conditions through NF-κB activation [94]. Subsequently, A20 was found to play a critical inhibitory role in inflammatory and immune responses, especially TNF-induced NF-κB activation and NF-κB signaling triggered by pattern recognition receptors, T and B cell receptors, interleukin-1 receptors, and NOD-like receptors, induced by a wide spectrum of stimuli [95,96]. Mice lacking A20 showed severe inflammation in response to sublethal doses of LPS through persistent activation of NF-κB and IKK and died prematurely. This study established the importance of A20 as a negative regulator of NF-κB driven inflammation and suggested its crucial role for immune homeostasis [97].

3.2.1. Structure and signal transduction of A20

The A20 protein is comprised of two functional domains, an N-terminal ovarian tumor domain (OUT) with deubiquitinating activity, and seven zinc finger domains at a C-terminal with E3 ubiquitin ligase activity both of which facilitate inhibition of NF-κB signaling [98]. This prototypic activity of A20 as a negative regulator of NF-κB-pathway can be attributed to the presence of these two domains in the promoter region of A20 [94]. The C103 catalytic cysteine site of the N-terminal OTU domain exhibits deubiquitinating enzyme (DUB) activity and is known to be responsible for K63 deubiquitination of TRAF6. ZnF4 of C-terminal had E3 ubiquitin ligase activity which is responsible for the degradation of the target proteins by K48 ubiquitination [99,100].

Following TNF stimulation on most cell types, TNF binds to TNF receptor 1 (TNFR1) triggering receptor trimerization. TNFR1 recruit adaptor proteins receptor-associated death domain protein (TRADD) and receptor-interacting protein 1 (RIPK1). TRADD recruits TRAF2/5 and E3 ubiquitin ligase cIAP1 and 2. Further, cIAP1 and 2 conjugate to K63-linked polyubiquitin chains in addition to conjugation with RIPK1, which facilitates IKK-activating kinase TAK1 through its K63 ubiquitin-binding subunit TAB2/3 and M1-specific E3 ligase Linear Ub chain assembly complex (LUBAC). LUBAC conjugates RIPK1 and NEMO with M1 linked linear Ub chains, allowing recruitment of IKK complexes. After it's activation by TAK1, IKK is released from TNFR1 signaling complex I followed by oligomerization by NEMO mediated M1-ubiquitin binding. This activity promotes phosphorylation of IKK and late phase activation, nuclear translocation, and expression of NF-κB response genes. A20 is recruited/induced by NF-κB to TNFR1 signaling complex I through M1-ubiquitin binding ZnF7. A20 bound to M1-linked ubiquitin competes with other ubiquitin-binding proteins and protects from deubiquitinase-mediated cleavage and thus prevents downstream signaling by preventing degradation of M1-linked chains. Also, ZnF7 of A20 binds to the IKK complex and prevents its prolonged activation post its release from TNFR1 complex I. A20 exhibits anti-inflammatory activity by deubiquitinating subunit (DUB) which removes K63-linked polyubiquitin from RIPK1 and NEMO. Additionally, A20 also facilitates the proteasomal degradation of RIPK1 and TNFR1 through ZnF4 E3 ligase activity. This activity of A20 is extremely crucial for regulating the production of pro-inflammatory cytokines and also components of NLRP3 inflammasome signaling. In addition, A20 has been shown to act as a DUB that removes K63-linked polyubiquitin from different target proteins, including RIPK1 and NEMO. Furthermore, A20 has been shown to target RIPK1 and TNFR1 for proteasomal degradation through its ZnF4 E3 ligase activity [99–101].

TNF receptor-associated factors (TRAFs) are the adaptor proteins that regulate the responses of TNFR-family members. TRAF family comprises seven proteins, including TRAF 1–7. Besides regulating TNFR responses, TRAF6 also mediates IL-1R, IL-18R, and Toll-like receptors (TLR) signaling. The rest of the TRAF family proteins other than TRAF1, consist of the RING-finger domain (RING) and exhibit E3 ligase activity [102]. TRAF is one of the targets, by which A20 exerts its biological effects [103]. Among various TRAFs, ubiquitin ligase TRAF6 mediates TLR and IL-1β induced NF-κB signaling, but has no role in TNF-α regulated inflammatory signaling and RIP ubiquitination [104]. While TRAF2 plays a significant role in A20 mediated TNF-α dependent NF-κB activation [97]. TRAF6 stimulates NF-κB activation employing K63 dependent ubiquitination. OTU domain of A20 facilitates the cleavage of ubiquitin chains from TRAF6, regulates polyubiquitination of TRAF6, and thus blocks TLR/TRAF6 dependent NF-κB

activation [105]. OTU domain-mediated TRAF6 regulation of A20 was studied in Tnfaip3$^{Otu/Otu}$ mice (elimination of A20 deubiquitinase activity through mutations). These mice, when challenged with LPS showed aberrant kinase and NF-κB inflammatory activation demonstrating catalytic C103 OUT domain of A20 as a prerequisite in inhibiting LPS stimulated inflammation by cleaving ubiquitin chains from TRAF6 [100]. Another important deubiquitinase enzyme that restricts TRAF6 mediated NF-κB signaling is CYLD [106]. Interestingly, TRIP6, an LPA2 receptor-interacting adaptor protein was found to inhibit the interaction of A20 and CYLD enzymes to TRAF6 and promote NF-κB signaling [107]. TRIP6 inhibitors might be useful to block this positive regulation of TRAF6 mediated NF-κB activation.

3.2.2. Functions of A20: *in vitro* evidence

The role of A20 in regulating inflammation was demonstrated by showing that A20 inhibited the TLR3-induced dimerization of IRF3 through modulating NF-κB/TRAF pathway. Interestingly, A20 knockdown reversed the effect [108]. A20 has a role in promoting apoptosis. In keratinocytes, A20 triggered TNF-induced cell death by activating NF-κB and its downstream targets including cIAP1/2 and TRAF1, which stabilizes NF-κB-inducing kinase, NIK leading to apoptosis. This study implicates A20 as a crucial player in mediating NF-κB-dependent apoptosis in skin diseases including psoriasis [109]. Moreover, dynamic expression of A20 attenuated TNF-α induced TAK1 mediated vascular endothelial injury, noted in human umbilical vein endothelial cells (HUVEC). Overexpression of A20 in these cells caused downregulation of p38 MAPK, while either A20 inhibition or TAK1 stimulation stemmed in inverse outcomes [110]. The role of A20 in gingival keratinocytes was also explored using human telomerase immortalized gingival keratinocytes. A20 depletion resulted in excess production of cytokines including IL-6, IL-8, and increased apoptosis as evident from DNA fragmentation and generation of cleaved caspase 3 compared to A20 competent cells, while A20 overexpression reversed the effects. Further evidence implicating A20 signaling in periodontal inflammation was evident from enhanced NF-κB activation and cytokine production in A20 depleted THP-1 (macrophage-like cells) and bone marrow macrophages, emphasizing the anti-inflammatory role of A20 in periodontal inflammation [111]. These findings suggest the role of A20 in maintaining oral mucosa homeostasis and provide a scope to develop targeted therapies to ameliorate periodontal inflammation [112].

3.2.3. Functions of A20: *in vivo* evidence

An exploratory study revealed anti-inflammatory and cytoprotective properties of A20 by generating mice with point mutations in ZnF7 and ZnF4 ubiquitin-binding domains of A20. A20$^{ZnF7/ZnF7}$ knock-in mice demonstrated spontaneous inflammatory phenotype characterized by severe arthritis with paw swelling, nail loss, joint inflammation, splenomegaly, lymphadenopathy, apoptotic cells in the liver, and elevated inflammatory cytokines. Double mutant A20$^{ZnF4ZnF7/ZnF4ZnF7}$ mice showed severe early systemic inflammation. In this study, A20 acted primarily by ubiquitin-binding protein through ZnF7 and ZnF4 to restrict pro-inflammatory signaling [113].

Another interesting contribution of A20 is to preserve endothelial barrier integrity by maintaining vascular endothelial cadherin expression. LPS challenged endothelial cell restricted A20 knockout mice (A20$^{\Delta EC}$) showed attenuated vascular injury. The subsequent mechanistic study

implicated the association of interleukin-1 receptor-associated kinase M (IRAK-M) in inducing A20 expression, thereby maintaining endothelial barrier integrity [114]. Interestingly, microglia A20-deficient ($A20^{\Delta mg}$) mice, developed spontaneous neuro inflammation due to CD8+ T cells infiltration rendering mice susceptible to viral infections [115]. These cell-specific functions of A20 associated with distinct phenotypes were outlined in Figure 3.

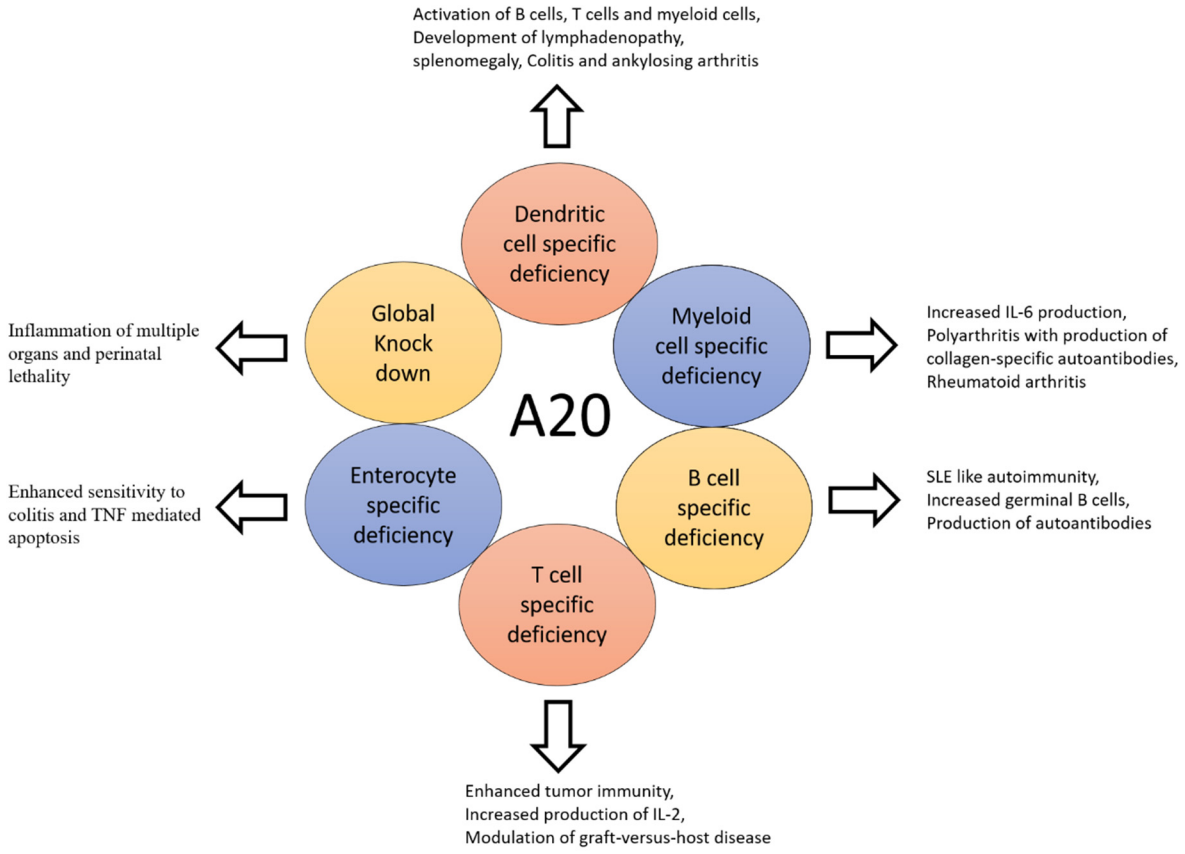

Figure 3. Cell specific functions of A20 associated with distinct phenotypes. Global knock down of A20 resulted in perinatal lethality. Various functions of A20 with controlled deletions specific to immune cells are represented along with phenotypes and disease involvement. Complete knock down of A20 as well as specific deficiency in cell types including DCs, myeloid cells, B cells, T cells and enterocytes are presented.

Mouse with global knockdown of A20 ($A20^{-/-}$) were generated to unveil the potential mechanism in regulation of TNF mediated signaling by A20 *in vivo*. However, $A20^{-/-}$ pups died due to multiple organ inflammation including liver, kidneys, intestine, joints, and bone marrow [97]. Further, this type of premature death was observed similarly in double mutant $A20^{-/-}$ recombinase-activating gene-1 deficient ($RAG\text{-}1^{-/-}$) mice. $A20^{-/-}$ mice challenged with LPS (5 mg/kg) and TNF (0.1 mg/kg), died within 2 hrs, while $A20^{+/-}$ and $A20^{+/+}$ mice survived [97]. Lethality of A20-deficient mice limits understanding the physiological relevance of A20. $Tnfaip3^{flox/flox}$ mice were then used to generate cell type-specific deletion of A20 in dendritic cells, myeloid cells, B cells, T cells, macrophages, and fibroblasts [116,117].

B cell-specific A20 deficient mice ($A20^{flox/flox}$ crossed with CD19-cre mice) had a phenotype indicated with excess B cell proliferation with a production of immunoglobulins [118]. B cell-specific A20 null mice also showed SLE-like autoimmunity and increased germinal B cells [119,120]. While, A20 deficient mice specific to DCs showed no lethality, but developed colitis, ankylosing arthritis, lymphadenopathy, and splenomegaly suggesting the essential role of A20 in DCs [121]. Myeloid cell-specific A20 deletion in macrophages and granulocytes ($Tnfaip3^{flox/flox}$ LysM-Cre mice) was associated with increased production of IL-6 as well as collagen-specific autoantibodies. These mice developed spontaneous polyarthritis [122]. Furthermore, A20 deletion in intestinal epithelial cells ($A20^{IEC-KO}$) was associated with colitis and TNF mediated apoptosis [123].

3.2.4. A20 in diseases

Aberrant expression of A20 contributes to immune pathologies of malignancies, systemic lupus erythematosus, rheumatoid arthritis, psoriasis, diabetes, fibrosis, cardiac and neurological disorders [124–127]. Figure 2 shows the expression of A20 in various rheumatic and fibrotic diseases. Genetic studies identified A20 single nucleotide polymorphisms (SNPs) in association with inflammatory and autoimmune diseases [128–130]. A European Caucasian cohort found an association of TNFAIP3 rs5029939 with susceptibility to SSc and other autoimmune diseases [131]. Also, a candidate gene study found that all the tested variants predispose to autoimmune phenotype of SSc [132].

Aberrant A20 expression is associated with a variety of diseases. In a study of spondyloarthritis, reduced A20 expression was associated with excess production of TNF-α [133]. In contrast, a dramatic increase of A20 expression was evident from peripheral blood mononuclear cells from patients with chronic Hepatitis B compared to healthy subjects. The mRNA expression of A20 was positively correlated to TNF-α, while anti-correlated with TLR4 expression. Increased A20 induction in hepatitis B was anticipated either to overcome the TLR mediated immune responses or to protect liver injury from sustained inflammation was not illustrated [134]. Subsequent findings from another study supported enhanced A20 levels in hepatitis B patients as well as *in vitro* and *in vivo* models of hepatitis. Upregulated A20 thus offered hepato-protection with reduced inflammatory responses by inhibiting NF-κB [135]. Further, A20 was proposed to be a biomarker in depressive disorders. Antidepressants augmented A20 expression and improved anti-inflammatory effects in patients with major depression [136].

3.2.5. A20 in fibrotic diseases

As global A20 loss caused severe spontaneous inflammation, fibroblast-specific deletion of A20 mice has been considered to study the effect of A20 in fibrosis. Huang et al. explored the role of A20 in cardiac fibrosis using neonatal rat cardiac myocytes in *in-vitro* and A20 transgenic mice, *in vivo.* The results revealed that overexpressed A20 reduced hypertrophic responses, inflammation, and fibrosis by interrupting TAK1-JNK/p38 signaling and R-Smad activations. In addition, ectopic A20 inhibited aortic banding-induced fibrosis as well as reduced collagen synthesis in cardiac fibroblasts [137]. Another study documented attenuation of obesity induced cardiac inflammation by A20 [138]. Jung et al implicated A20 as a negative regulator of the non-canonical TGF-β1 pathway in mouse liver cells, primary hepatocytes, and in A20 KO mice, *in vivo*. The study demonstrated that

Smad6 recruited A20 and inhibited the Lys-63-dependent polyubiquitination of TRAF6, a central player in TGF-β1 induced activation of TAK1 and its downregulating kinases including p38 MAPK and JNK [139].

A very recent study looked into the role of A20 in lung fibrosis. The authors reported that diminished A20 levels resulted in accumulation of C/EBPβ in alveolar macrophages triggering pulmonary fibrosis. Using genetically targeted Tnfaip3$^{+/-}$Lyz2-cre and Tnfaip3$^{F/+}$Lyz2-cre mice, A20 reduced k63 dependent ubiquitination and degradation of C/EBPβ. The authors linked these effects with glycogen synthase kinase-3β (GSK-3β), which phosphorylates A20 in response to injury. Elevated A20 expression caused degradation of C/EBPβ to mitigate fibrosis. These results provide some mechanistic insights into the anti-fibrotic role of A20 in pulmonary fibrosis [140]. Another recent study revealed that A20 expression was significantly upregulated in patients with hepatic fibrosis, as well as in mouse models of hepatic fibrosis, due to augmented inflammatory responses. A20 overexpression in hepatic stellate cells resulted in attenuation of liver fibrosis, suggesting anti-fibrotic potential of A20 [141].

Enhancing A20 expression via pharmacological stimulation might result in attenuation of profibrotic responses. A recent study showed that Gibberellin induced A20 and reduced NF-κB activity in LPS stimulated airway epithelial cells [142]. A connectivity map using gene expression data was used to identify A20 inducing drugs. Bronchial and primary nasal epithelial cells were treated with ikarugamycin and quercetin, A20 inducing drugs selected from a database. The results showed A20 induction to a lesser extent in cells derived from patients with cystic fibrosis compared to non-fibrotic controls [143]. Adiponectin, an adipocyte-derived cytokine was shown to induce A20, thereby downregulating fibrotic responses [144]. Adiponectin regulation of A20 expression was previously reported in adipose tissue macrophages where A20 expression was positively correlated with that of adiponectin [145]. Bariatric surgery was associated with heightened levels of adiponectin, enhanced A20 expression in white adipose tissue with improved metabolic and inflammatory markers [145]. We recently implicated A20 as an intrinsic negative regulator of Smad-dependent canonical TGF-β1 signaling in fibroblasts. Our unpublished data explored the contribution of A20 in SSc pathogenesis in murine model of bleomycin induced scleroderma using A20$^{+/-}$ mice and A20$^{fl/fl}$ mice. A20$^{+/-}$ mice showed exacerbated dermal and lung fibrosis, compared to A20$^{fl/fl}$ mice. Bleomycin induced A20$^{+/-}$ mice demonstrated a notable rise in increased dermal thickness and elevated expression of collagen mRNA extracted from skin, as compared to bleomycin induced A20$^{fl/fl}$ mice. Further, exacerbated lung distortion along with enhanced myofibroblasts positive cells and collagen accumulation was evident in A20$^{+/-}$ mice compare to A20$^{fl/fl}$ mice, suggesting A20 induction as a therapeutic approach for fibrosis.

3.2.6. A20 regulation by repressing its transcriptional repressor DREAM

The transcriptional repressor downstream regulatory element antagonist modulator (DREAM) is widely expressed in immune cells and multiple organs including brain, heart, thymus, testis, and thyroid gland, while minimally expressed in lungs [146–148]. DREAM is a Ca^{2+}-binding protein family member containing 4 Ca^{2+} binding motifs ("EF-hands") that interact with downstream regulatory elements (DRE) to inhibit transcription [146]. DREAM was recently shown to be a transcriptional repressor of A20 [149]. DREAM binds to DRE in the promoter region of A20 to inhibit A20 gene transcription, thereby inhibiting NF-κB signaling. On the other hand, upstream

stimulatory factor 1 (USF1) binds to E-box domain of DRE to upregulate A20 expression suggesting a reciprocal relationship between DREAM and USF1 in regulating A20 expression. DREAM$^{-/-}$ mice showed augmented A20 expression in a variety of tissues and LPS mediated anti-inflammatory responses [149].

Levels of A20 and DREAM were anti-correlated in our study performed using DREAM KO mice (unpublished). Expression of A20 was increased in bleomycin administered DREAM KO mice, with significant amelioration of skin and lung fibrotic responses as compared to bleomycin induced wild type mice. Enhanced A20 expression is associated with reduced dermal thickness in skin, as well as reduced collagen accumulation in lungs. These intriguing findings suggest that targeting DREAM might be a potential therapeutic strategy in inflammatory and fibrotic diseases.

In this regard, antidiabetic drug repaglinide was reported to inhibit DREAM. Repaglinide competitively blocked the interaction of DREAM and Activating transcription factor 6 (ATF6), a transmembrane receptor whose activation is reduced in Huntington disease. Treatment with Repaglinide stimulated ATF6 signaling and enhanced neuroprotection in Huntington disease [150]. Towards this end, two novel DREAM ligands, IQM-PC330 and IQM-PC332 were identified using target structure-based design approach that functions by blocking the interaction of DREAM and ATF6. Interestingly, IQM-PC330 was found to be more potent and exerted sustained neuroprotective effects in both *in vitro* and *in vivo* experimental models of Huntington disease, compared to Repaglinide [151]. These findings suggest that small molecule pharmacological inhibitors of DREAM might enhance A20 activity and might represent a potential anti-fibrotic therapeutic strategy.

4. Conclusions and future perspectives

In this review, we have highlighted primarily two negative regulators of TLR signaling, A20, and RP105, and have discussed the implications of each of them in the pathogenesis of autoimmune diseases focusing primarily on fibrosis in SSc and strategies for therapeutic intervention. Although a comprehensive discussion of all negative regulators of TLR signaling lies outside of the scope of the current review, they also play an important part in TLR regulation. Evolving insights from *in vitro* experiments, transgenic animal models, and clinical information suggest a pathogenic contribution of DAMP-TLR4 signaling in progressive fibrosis in SSc. TLR4 signaling in fibroblast by DAMPs appears to convert a self-limited tissue repair process into pathological fibrosis. Interestingly, several drugs targeting the TLR4 signaling, including eritoran and TAK-242, failed in sepsis clinical trials, suggesting an urgent need for novel treatment strategies. These agents might also be considered as anti-fibrotic approaches in a drug "repurposing" strategy. Intriguingly, our findings using small molecule TLR4 inhibitor T5342126 and TAK242 display potent anti-fibrotic activity in animal models as well as in SSc fibroblasts [7,152]. On the other hand, restoring the expression of endogenous inhibitors of TLR signaling such as A20 or RP105 by pharmacologic agents might be other approaches for anti-fibrotic therapies. We found that levels of A20 were significantly reduced in fibrotic SSc biopsies, while DREAM, a negative regulator of A20, was elevated (unpublished data). Therefore, an inverse correlation between A20 and its repressor DREAM exists in SSc patients where TLR4 signaling is persistently elevated. Analysis in other studies showed an inverse correlation of A20 expression with the severity of depression, suggesting modulating A20 expression to rebalance TLR-mediated inflammatory signaling as a potential therapeutic strategy [136].

Interestingly, both adiponectin and a small molecule adiponectin receptor agonist AdipoRon with potent anti-fibrotic activity can induce A20 expression in skin fibroblasts and skin tissue sections from a mouse model of skin fibrosis [153]. Selectively ablating DREAM activity using anti-diabetic drug repaglinide might be an alternative approach to boost A20 function.

Given the various reports on RP105 as a negative regulator of TLR signaling, we speculate its probable role in controlling TLR mediated fibrosis. No reports are available exploring RP105 as a negative regulator in the context of fibrotic disorders. Preliminary findings from our laboratory (unpublished) indicated that RP105 by modulating TLR4 signaling inhibits fibrosis. Inducing RP105 expression or function seems to be a promising targeting approach that merits further investigation. In summary, this review highlighted negative regulators of TLR signaling and underlined opportunities for restoring or boosting the expression of A20 or RP105 by pharmacological agents, which might hold promise for effective anti-fibrotic therapies.

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Conflict of interest

All authors declare no conflicts of interest in this paper.

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