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Autoreactive B cell responses targeting nuclear antigens in systemic sclerosis: Implications for disease pathogenesis

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A B S T R A C T

A hallmark of disease pathogenesis of systemic sclerosis (SSc) is the presence of autoreactive B cell responses targeting nuclear proteins. Almost all SSc-patients harbour circulating antinuclear autoantibodies of which anti-topoisomerase 1, anti-centromere protein, anti-RNA polymerase III and anti-fibrillarin autoantibodies (ATA, ACA, ARA and AFA, respectively) are the most common and specific for SSc. In clinical practice, autoantibodies serve as diagnostic biomarkers and can aid in the identification of clinical phenotypes of the disease. However, factors driving disease progression in SSc are still poorly understood, and it is difficult to predict disease trajectories in individual patients. Moreover, treatment decisions remain rather empirical, with variable response rates in clinical trials due to patient heterogeneity. Current evidence has indicated that certain patients may benefit from B cell targeting therapies. Hence, it is important to understand the contribution of the antinuclear autoantibodies and their underlying B cell response to the disease pathogenesis of SSc.

Introduction

Systemic Sclerosis (SSc) is a rheumatic autoimmune disease affecting the skin and internal organs. The pathophysiology of SSc is characterized by a triad of vasculopathy, autoimmunity and fibrosis [1]. Although these three pathophysiological features are thought to be strongly connected, their interplay is not fully understood.

Dysregulation of the immune system in SSc-patients is hallmarked by a break of B cell tolerance towards nuclear antigens [2]. Over 95% of SSc-patients have detectable antinuclear antibodies [3]. The break of B cell tolerance involves the escape of several central and peripheral immune checkpoints by autoreactive B cells (as reviewed in detail elsewhere: [4–6]). These immune checkpoints do, thereby, normally prevent the generation of autoreactive B cell responses. The break of B cell tolerance towards nuclear antigens is an early marker of immune dysregulation in SSc, since antinuclear autoantibodies can already be present years before disease onset [7]. At least 9 different nuclear antigens have been described for SSc [3,8]. The B cell responses targeting topoisomerase 1 (TOP1), centromere proteins (CENP) and RNA polymerase III (Pol III) are most commonly observed in SSc-patients [9,10] (Table 1), and are part of the ACR/EULAR 2013 SSc-criteria [11] and VEDOSS criteria for suspected very early SSc [12].

Autoreactive B cell responses can contribute to the pathogenesis of autoimmune diseases in several ways, for example by their ability to produce autoantibodies. Autoantibodies can be directly pathogenic, as evidenced in idiopathic thrombocytopenic purpura [13] and myasthenia gravis [14]. In other conditions, they are thought to act as indirect contributors to disease pathogenesis through immune complex formation, like RA [15], or, even as passive bystanders useful as biomarkers but without an otherwise defined function in the respective disease process, like celiac disease [16] and diabetes mellitus type 1 [17].

Besides the production of autoantibodies, B cells can also exert pro-inflammatory effects by their ability to produce cytokines, present antigens to and co-stimulate T cells [4]. In SSc, B cells are considered overactive and thought to play a pro-inflammatory role by upregulating T cell co-stimulatory molecules CD80 and CD86, producing

Abbreviations: ACA, anti-centromere protein autoantibodies; ACR, American College of Rheumatology; ANA, anti-nuclear autoantibodies; ATA, anti-topoisomerase I autoantibodies; ARA, anti-RNA polymerase III autoantibodies; AFA, anti-fibrillarin autoantibodies; CCISS, Comprehensive Care in SSc (Leiden cohort); CENP, centromere protein; CTD, connective tissue disease; dcSSc, diffuse cutaneous SSc; EULAR, European Alliance of Associations for Rheumatology; Fib, fibrillarin; HLA, human leukocyte antigen; ICD, international classification of disease; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; IVIG, intravenous immunoglobulin; lcSSc, limited cutaneous SSc; mRSS, modified Rodnan skin score; Pol III, RNA polymerase III; RA, rheumatoid arthritis; RP, Raynaud's phenomenon; RTX, rituximab; SLE, systematic lupus erythematosus; SSc, systemic sclerosis; TOP1, topoisomerase I; VEDOSS, very early diagnosis of systemic sclerosis.

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Table 1
Overview of SSc-specific autoantibodies.

Autoantibody	Autoantigens	Prevalence	Specificity
Anti-centromere	Centromere protein (e.g. A, B and C)	20–57.8%	93% [10,159]
Anti-topoisomerase I	Topoisomerase I	14–71%	99.6% [66]
Anti-RNA polymerase III	RNA polymerase III	4–20%	97.5% [10]
Anti-fibrillarin	Fibrillarin	2–18%	Exact number not reported

proinflammatory cytokines (e.g. IL-6) and profibrotic cytokines (e.g. TGF-β). More specifically, autoreactive B cells that underly autoantibody responses might play an important role in the pathogenesis of autoimmune diseases. In RA, autoreactive B cells that recognize citrullinated proteins are highly activated and have a proinflammatory phenotype [18]. The favourable effects of B cell targeting therapies in several autoimmune disease underline the importance of the pro-inflammatory effects of B cells. This is underlined by the notion that autoantibody levels are not necessarily affected by effective B cell targeting treatment [19].

Due to the heterogeneity of SSc and lack of knowledge about factors driving progression, it remains difficult to predict disease courses of individual patients. Despite recent therapeutic advances, which improved clinical outcomes and quality of life of SSc-patients, SSc still has the highest mortality and morbidity of all rheumatic diseases [20]. Elucidating the contribution of disease-specific autoantibodies and their underlying B cell response in the processes underlying SSc might help to understand whether these antibodies or B cell responses drive disease pathogenesis. Eventually, this will be crucial to understand disease progression, improve disease classification, and aid development of targeted therapeutic interventions. Therefore, in this review we summarize the current knowledge on the contribution of SSc-specific autoantibody and B cell responses to SSc-pathogenesis. To structure this review, we divided the results into three parts: clinical studies, insights from animal models and *in vitro* studies. Thereby, we identify gaps in knowledge and formulate directions for future research.

Study design

Study design and search strategy

This study is a scoping review. We searched on Pubmed for literature on: (1)Are B cell responses targeting nuclear antigens implicated in the pathogenesis of SSc?, and (2)What is the effect of therapies that target the adaptive immune system including autoreactive B cells?. We used a search strategy composed of a combination of the following search terms: “systemic sclerosis”, “autoantibodies”, “anti-topoisomerase I”, “anti-Scl-70”, “anti-centromere”, “anti-RNA polymerase III”, “anti-fibrillarin”, “anti-U3RNP”, and “autoreactive B cell response”. For the first question, no specific inclusion criteria were applied, and for the second, we selected studies that: (1)included ≥5 SSc-patients, (2)were written in English or Dutch, and (3)were (randomized) clinical trials, observational studies or case-series. Moreover, in included articles the snowball method was used to search for more relevant articles.

Autoantibodies

Autoantibodies included in this review were selected based on (1) presence in ≥5% of the SSc-population, and (2)a specificity of ≥90%.

Results

SSc-specific autoantibodies

Based on our pre-formulated criteria, we focused on autoantibodies and B cell responses targeting topoisomerase I (TOP1), centromere proteins (CENPs), RNA polymerase III (Pol III) and fibrillarin (fib). These responses are present in ≥5% of SSc-patients and highly specific, as they are found in <10% in other autoimmune diseases (Table 1) [9,10]. Other autoantibodies in SSc are either not disease specific or ≤5% observed [21,22].

A commonality between TOP1, CENP, Pol III and fib is their nuclear localization in cells in physiological conditions. ATA were originally termed anti-Scl-70 antibodies, as they were initially found to react with a 70kDa protein by immunoprecipitation of nuclear protein extracts with sera from SSc-patients [23]. Later, it was recognized that TOP1 is the antigen of ATA and that the 70kDa protein was a degradation product of the native 100kDa TOP1 protein [24,25]. TOP1 is a regulator of the topology and transcription of DNA. The major antigen of ACA is centromere protein B (CENP-B) [26,27]. CENP-B is a DNA-binding protein facilitating the formation of centromeres. ACA-positive sera can, to a lesser extent, also recognize other centromere proteins, like centromere protein A and C. The ARA-response targets Pol III which is an enzyme responsible for transcription of non-coding RNA [28,29]. The AFA response, also known as anti-U3-RNP, targets fibrillarin. Fibrillarin, which should not be wrongly confused with the extracellular matrix protein fibrilline, is 34-kDa protein component of a nucleolar ribonucleoprotein involved in pre-ribosomal RNA processing that is localized in the fibrillar region of the nucleus of cells [30,31].

Several studies have investigated the epitopes on the targeted antigens which are recognized by the antinuclear autoantibodies. These studies have tested the reactivity of circulating antibody towards fragments or peptides of the protein. For TOP1, epitopes bound by ATA are thought to be spread over the entire protein [32–37]. Some fragments of TOP1 are thought to contain immunodominant epitopes, for example the region of TOP1 between amino acids 484 and 560 and between 653 and 704. ACA also reacts with multiple epitopes of CENP-B, although the C-terminal domain is thought to be more immunodominant and can be bound by most patient sera [38–41]. Interestingly, several papers have found immunodominant sequences on CENP-A which is bound by ACA. This sequence can also be found on CENP-B and, therefore, might indicate cross-reactivity of ACA towards multiple proteins [42–45]. Although studied in less detail, ARA and AFA are also thought to be able to bind multiple epitopes on pol III and fib, respectively [46–50]. A major caveat to the studies discussed above is that they do not consider the conformation of the protein, as the fragments or peptides might not have the same conformation as they would have in the context of the whole protein. Therefore, epitope mapping studies that do consider the conformation of the targeted antigens are of crucial importance to determine the epitopes bound by antinuclear autoantibodies.

HLA-associations with B cell responses targeting nuclear proteins

The establishment of long-lasting B cell memory is dependent on T helper cells. T helper cells become activated upon recognition of processed antigens presented by antigen-presenting cells on human leukocyte antigen (HLA) class II molecules. Genetic HLA-variations determine the binding affinity for specific antigens and the efficient presentation of these antigens to T cells. Several genetic variations in HLA class II loci have been identified that associate with SSc and, in particular, with specific autoreactive B cell responses in SSc, as reviewed in detail by Broen et al. [51]. *HLA-DRB1*08:01* and *HLA-DRB1*07:01*, for example, were the classical alleles independently and exclusively associated with ACA presence, while *HLA-DPA1*02:01* and *HLA-DQB1*03:01* associate with ATA presence, *HLA-DRB1*11:04* with ARA presence [52] and DQB1*06:04 with AFA presence [30]. HLA-associations with specific B

cell responses indicate that these responses develop in a T cell dependent context. It also indicates that these autoreactive B cell responses develop as separate responses that are favoured by distinct genetic backgrounds, each of which confers risk to develop a distinct clinical SSc-phenotype.

Associations between B cell response targeting nuclear antigens and clinical characteristics of SSc

Strong association between B cell responses targeting nuclear proteins and presence of SSc

In SSc, B cell responses targeting nuclear proteins strongly associate with disease. Individual, SSc-specific autoantibodies show high disease specificity. ARA and AFA are rarely observed in other diseases, including systematic lupus erythematosus (SLE), primary biliary cirrhosis and Sjogren Syndrome [53,54]. ATA can also be seen in SLE [55]. A meta-analysis showed that ATA was detected in 4.1% of SLE patients [55]. One study found that 25% of their SLE patients were positive for ATA [56], but serum ATA titers in SLE patients were significantly lower compared to SSc patients [55,56]. Pulmonary hypertension has been observed to be more common among ATA positive than ATA negative SLE patients [55,56], which raises the question of overlapping features between these disease groups based on autoantibody expression. ACA also occur in SLE (2–4%) [57,58], Sjogren syndrome (4%) [59,60] and RA (3%) [58]. Nonetheless, most ACA-positive patients in these clinical disease groups do present with scleroderma features including Raynaud's phenomenon, sclerodactyly and puffy fingers [59–61].

To understand the role of antinuclear B cell responses in disease initiation of SSc, it is helpful to define stages before disease onset. In other autoimmune diseases, like SLE and RA [62,63], important insights have been gained from separating the disease course into different phases that are defined by clinical and immunological features. Although a clear separation of these phases might not be present and may oversimplify disease complexity, this can help to understand underlying disease processes. Recently, similar concepts were applied to SSc by separating very early SSc from more advanced stages, but these definitions are mainly based on clinical features, as immunological aspects are still less-well defined.

In the 1980s, the first classification criteria for SSc mainly focused on skin fibrosis [64,65]. Building upon previous criteria, the ACR/EULAR 2013 criteria added emphasis to the vascular manifestations, which improved the classification of individuals with early, mild and the limited subtype of disease [66]. *"Very early SSc"* was identified as a condition characterized by RP, puffy fingers, disease-specific autoantibodies, and microvascular alterations detected by capillaroscopy [12]. In 2014, the very early systemic sclerosis criteria (VEDOSS) were formulated with a minimum of 2 and maximum of 3 of the items above [67].

Nonetheless, the basic immunological steps involved in disease development may be similar among autoimmune diseases. (1)In the first phase: autoimmunity develops in genetic-susceptible persons by a "first hit", (2)a subclinical phase ending with the clinical onset of the disease leading to a (3)third phase characterized by clinical symptoms, the diagnosis and disease chronicity.

Several studies have investigated the presence of antinuclear autoantibodies before disease onset. Burbelo et al. performed a retrospective case-control study to assess presence of autoantibodies (ATA, anti-CENP-A, ARA) before SSc-diagnosis and/or development of renal crisis using sera collected from the U.S. Armed Forces [7]. By searching on codes of the international classification for diseases 9 (ICD-9) for SSc, which is a limitation of the study, records were reviewed to identify 16 patients coded as SSc and renal crises with sera available. These were matched to 30 ICD-9 SSc-code identified patients without renal crisis [7]. Half of the patients had detectable autoantibodies before SSc-diagnosis. Intriguingly, specifically for ATA, a rise in titer could be observed towards time of initial diagnosis. As this study included a small number of mainly male black SSc patients, the extrapolation of the data to other populations is hampered. Similar, a study on ACA positive individuals found the lowest ACA titers in patients without apparent connective tissue diseases [68]. A more recent study on ACA characteristics showed that very early SSc-patients had the lowest levels of ACA-IgG and ACA-IgM, while definite SSc-patients with organ involvement had the highest ACA- levels. Of 138 very early SSc-patients with follow-up, 42% progressed to definite SSc during a median follow-up period of 2 years (range:1-4), and ACA-IgG levels at baseline (=first visit in cohort entry) were significantly associated with progression to definite SSc [69]. This study suggests that higher ACA-levels are a risk for the 'second phase': progression to pathogenic autoimmunity. In line with these observations, studies in isolated RP or suspected SSc have shown that SSc-specific autoantibodies are strong predictors of progression to definite SSc [70–73]. AFA was not evaluated in these studies, probably because AFA are not part of the ACR/EULAR 2013 criteria.

It is noteworthy that co-existence of the SSc-specific autoantibodies is extremely rare [74]. A meta-analysis study found that 0.52% of patients with SSc or SSc-associated symptoms had expression of both ACA and ATA (ARA and AFA were not assessed) [75]. Likewise, a study from the EUSTAR-cohort found that 29/4687 (0.6%) SSc-patients were positive for ACA and ATA. Data on coexistence with ARA was not reported, and AFA was not assessed. In 8 of 14 available sera, the presence of both autoantibodies was confirmed and ARA was undetectable [76]. In our cohort (Comprehensive Care in SSc [CCISS] at Leiden University Medical Center [77]), similar observations are made: of 708 SSc-patients fulfilling ACR/EULAR 2013 criteria, 3 patients (0.4%) are ATA- and ACA-positive, none are ATA- and ARA-positive nor ACA- and ARA-positive. A recent report by Clark et al. confirmed these observations [78]. For AFA, specifically, three studies report no coexistence with other SSc-specific autoantibodies [79], but one study from 1992 of 27 AFA-positive patients found that 19% also had ATA [30]. This discrepancy is perhaps caused by different assay techniques.

Additionally, the SSc-specific autoantibodies strongly cluster with clinically distinct phenotypes: ATA strongly associates with the diffuse cutaneous (dcSSc) subset, whereas ACA is more frequently observed in limited SSc (lcSSc) [80–83].

Furthermore, ATA are associated with severe digital vasculopathy [84] and interstitial lung disease (ILD) [22,85]. In contrast, presence of ACA is associated with higher prevalence of calcinoses and gastro-intestinal involvement [3,86,87], and the lowest incidence of pulmonary fibrosis, scleroderma renal crisis (SRC) and cardiac involvement [85]. Presence of ACA generally carries a better prognosis than most other SSc-autoantibodies with respect to survival [85,86,88]. ARA is one of the strongest risk factors for SRC and gastric antral vascular ectasia [85,89]. AFA is associated with diffuse cutaneous SSc, pulmonary hypertension and renal disease [30,79,90,91]

Taken together, the most reliable biomarker to predict the pattern of organ involvement in SSc is provided by the specificity of SSc-specific autoantibodies [22] which is supported by the specific associations for clinical phenotype. The autoantibody reactivity allows patients to be stratified early in the disease course which facilitates a tailored approach and management [66,85,92]. When discussing the prognostic significance of especially ATA, it is important to take into account the variability in ATA results due to differences in testing methods [93,94]. The strong association with SSc and the role as biomarkers might suggest a potential pathogenicity of these autoreactive B cell responses, similarly to what is reported for SLE [95], anti-phospholipid syndrome [96], and Sjogren's syndrome [97].

Correlation of autoantibody levels and isotypes and autoreactive B cells with disease activity

In case of a direct pathogenic effect of autoantibodies, autoantibody levels can be highly informative as they may correlate with disease activity. For example, measuring PR3- and MPO-ANCA-titers in ANCA-associated vasculitis is useful to predict relapses [98].

In the context of SSc, three studies evaluating antibody titers and

Table 2
Isotypes and titers of SSc-specific autoantibodies.

Year	Author	Patients	Results
1984	Tramposh [99]	15 ACA +	15 ACA + patients of whom seven classified as progressive SSc. ACA were IgG only, with no IgM positive sera at any time during the follow up time. No consistent change in titer over time was found.
1990	Hildebrandt [29]	20 ACA + and 17 ATA + SSc pt	Isotype determination by ELISA and immunoblotting showed a high prevalence of ACA-IgG, IgA-ACA, whereas IgM-ACA seemed to be rare. The same was found for ATA with a high frequency of IgG-ATA, IgA-ATA, and lower frequency of IgM-ATA. The three isotypes of ACA and ATA could not be detected in health age and sex matched subjects thus indicating the high specificity of all three isotypes.
1995	Vazquez-Abad [100]	13 ACA + and 6 ATA+	All ACA had IgG, and 3 had IgA and/or IgM. Three patients developed ACA IgG during the study. Of the 6 ATA +, 3 had IgG and IgA and 3 had IgG, IgA and IgM. One patient developed ATA IgA during the study. Fluctuating levels occurred over time, but there was no association between clinical features and change in autoantibody isotype.
2000	Kuwana [101]	28 ATA +	In 20% of ATA + patients ATA became undetectable during disease course; these patients presented with less extensive skin and lung involvement and better survival rates than patients with persistently elevated ATA.
2003	Hu [102]	59 ATA +	The titers of IgG-ATA and IgA-ATA were positively correlated with the total skin score. Changes in the levels of both IgG and IgA paralleled changes in the total skin score. In three patients, an increasing IgG ATA levels preceded an increase in the total skin score. Patients with very active disease had a higher mean IgG and IgA titers than those with inactive disease.
2005	Kuwana [108]	90 ARA+	32 had a high level of ARA (>=15.2 units) and 58 with a low level (<15.2). All but 1 patient with a high level of ARA had dcSSc which was significantly higher than in the low level group. The maximum total skin score and frequency of tendon friction rubs were significantly increased in the high level antibody group compared to the low level group. In 6 patients levels were serially evaluated and in 4 patients, the levels increased early in the disease course and then decreased, correlating closely with the total skin score.
2007	Perera [103]	212 ATA+	Positive correlation between the IgG ATA levels and the modified Rodnan skin thickness score. The median IgG ATA levels were significantly higher in the diffuse cutaneous patients compared with patients in limited cutaneous subgroups.
2009	Nihtyanova [109]	64 ARA +	There was considerable inter- and intra-patient variability in ARA levels (11-210 U/ml). there was no correlation between absolute ARA levels (at baseline or throughout the disease course) and outcome. Change in ARA levels correlated with change in skin score.
2013	Hasegawa [33]	53 ATA +	Significantly positive correlations between ATA antibody levels and mRSS and skin thickness progression rate, and a significantly negative correlation with disease duration.
2020	Boonstra [105]	103 ATA +	SSc-patients with disease progression were significantly more often anti-topo I IgM-positive than those who did not experience disease progression (21 [91%] of 23 versus 33 [57%] of 58; P < 0.01) which was confirmed in the independent validation samples. ATA IgG-positive SSc-patients who are also positive for anti-topo I IgM more often experience disease progression compared to anti-topo I IgG-positive patients who are negative for anti-topo I IgM. ATA IgG-positive patients are almost always positive for anti-topo IgA.
2020	Van Leeuwen [84]	129 ACA+ and 102 ATA+	Patients that solely expressed ACA-IgG showed a trend towards less severe microangiopathy compared to patients expressing also ACA-IgM and/or IgA. Levels of ACA-IgG and ATA IgM were associated with the severity of microangiopathy.
2021	Van Leeuwen [69]	624 ACA IgG +	Definite SSc-patients with organ involvement express higher ACA-IgG and higher-ACA IgM levels compared to very early SSc-patients. Within the group of definite SSc-patients ACA-IgG levels and ACA IgM levels associated with future disease progression. In very early SSc, higher levels of ACA-IgG associate with progression to definite SSc within two years.

disease activity were conducted in the nineties (Table 2): the first evaluated ACA-titer in the disease course [99], the second ACA-isotypes [27], and the third fluctuation of ACA- and ATA-isotypes over time [100]. The results were inconclusive. These studies had limited sample sizes and used invalidated outcome measurements. In the early 2000s, multiple small studies in SSc have shown that the ATA-IgA and -IgG levels correlated with the severity of skin disease [101–104].

Recently, our research group has assessed ACA- and ATA-isotypes and levels [84,105]. We found that ATA-IgG positive SSc-patients with disease progression were significantly more often ATA-IgM positive than those without disease progression (91% vs. 57%; p<0.01) [105]. IgM antibodies are particularly produced during the early phase of a B cell response upon activation [106]. Upon this initial phase, the levels of IgM drop as a consequence of class switching by B cells and due to the short half-life of IgM antibodies [106,107]. (Therefore, IgM antibodies are indicative for recent B cell activation.) The presence of ATA-IgM in patients with progression might, thus, indicate a recent activation of the ATA B cell response in these patients. Additionally, ACA-IgG and ATA-IgM levels were associated with the severity of microangiopathy and ATA-positive with ILD, respectively [84].

In a study with 90 ARA-positive SSc-patients, 97% of the patients with high ARA-levels (>=15.2 units) had dcSSc, versus 81% of patients with low ARA-levels (<15.2 units). The maximum total skin score and frequency of tendon friction rubs were significantly increased in the high ARA-level group compared to the low level group. A longitudinal analysis of ARA-levels in 10 patients showed that ARA-levels increased early in the disease course and then decreased, correlating closely with the total skin score [108]. Another study showed that ARA-levels at

baseline or during the disease course did not predict organ complications in 64 ARA-positive SSc-patients, although they correlated with the severity of skin involvement [109].

By assessing B cell populations containing TOP1-reactive B cells, Fukasawa et al. [110] recently reported a positive correlation between the affinity of ATA-expressing B cells for TOP1 and production of pro-inflammatory cytokines. The results of this study not only imply a pro-inflammatory and pro-fibrotic role of TOP1-reactive B cells in the disease pathogenesis of SSc, but also that these effects mediated by TOP1-reactive B cells are dependent on their affinity for the antigen [110]. However, these results have to be interpreted with caution and independent replication is needed. For example, in this study, the affinity of B cells for TOP1 was not directly determined. Instead, a non-validated assay was used to determine the reactivity of ATA towards TOP1. Consequently, single-cell sorting of the TOP1-reactive B cells using fluorescently labelled antigen might have influenced the analysis in an affinity-dependent manner.

When evaluating the presence of multiple isotypes in patients, generally speaking, studies find a high frequency of ATA-IgA and ACA-IgA, and a lesser frequency of ATA-IgM and ACA-IgM [27,69,84,101,105]. Of importance when interpreting these results is that often only patients positive for IgG are included in these studies. It is not possible to completely exclude the possibility that patients who were positive only for IgM or IgA were missed.

To summarize, only recently a few large international studies have assessed ATA- and ACA-isotypes and titers and their associations with disease phenotype in larger sample sets. These studies show more frequent disease progression and worse disease activity when more isotypes are present or if the autoantibody levels are higher. This could indicate a pathogenic role of ATA and ACA or of their underlying B cell responses. In ARA, higher levels were seen more often in dcSSc-patients and seem to associate with skin score, but large studies are lacking making it difficult to draw definitive conclusions. No studies on levels and isotypes of AFA were found.

Effect of most frequently used immunosuppressive therapies in SSc on autoantibody B cell responses

The efficacy of immune interventions targeting individual components of the immune system (e.g. B cells) can be indicative for the contribution of the targeted component of the immune system to disease pathogenesis. For the most commonly used immunosuppressive therapies in SSc [111,112], no studies have evaluated the effect of cyclophosphamide or mycophenolate mofetil on the autoreactive B cell response targeting nuclear proteins. For methotrexate, one case report described the disappearance of ARA after treatment with corticosteroids and methotrexate [113], but no other studies have evaluated methotrexate alone.

For autologous hematopoietic stem cell transplantation (HSCT), multiple studies have investigated its effect on the ATA B cell response, but not for ACA, ARA and AFA. The exact mechanism of action in HSCT is unknown. The rationale is that high-dose immunosuppression eradicates autoreactive T and B cells and that the infused autologous hematopoietic stem cells reconstitute a naïve and self-tolerant immune system. Indeed, one study showed that the phenotype of B cells upon HSCT is less proinflammatory and that B cells post-HSCT more often have regulatory capacities [114].

Two studies categorized SSc-patients treated with HSCT in clinical responders and no-responders. One study showed that peripheral B cell counts were significantly higher in the 3 clinical no-responders than in the 4 clinical responders after HSCT [115]. Another study showed that the absolute number of B cells was lower in the responders than in healthy controls, but comparable to the non-responders [116]. Moreover, 4/5 responders and 3/4 non-responders seropositive for ATA before HSCT became negative after HSCT [116].

In an earlier study, serum ATA-levels were found to decrease after HSCT. A significant, although limited correlation ($r=0.52, p<0.05$) was observed between the change in mRSS and change in serum ATA-levels at 36 months after HSCT [117]. Subsequently, one study assessed the effect of HSCT on ATA-levels in 18 SSc-patients of whom 15 were ATA-IgG positive and 5 ATA-IgM positive [118]. After HSCT, ATA-IgG prevalence did not change significantly, whereas all patients no longer had detectable serum ATA-IgM. Although ATA-levels decreased in most of the patients after HSCT, they still remained strongly positive in most, and there was no correlation with clinical response [118].

Immunoadsorption, therapeutic plasma exchange and plasmapheresis are more specific immunosuppressive therapies targeting the antinuclear B cell response, but to the best of our knowledge no studies in SSc have been published.

Tocilizumab and rituximab (RTX) are two more recent immunosuppressive therapies. Of these, tocilizumab has a broader mechanism of action by indirectly and directly targeting B cells in SSc via the inhibition of IL-6 function. So far, no studies have been published with regard to the effect of tocilizumab specifically on the antinuclear B cell response in SSc. RTX is a CD20-specific monoclonal antibody applied for autoimmune diseases including RA and ANCA-associated vasculitis [119]. CD20 is a molecule expressed throughout the development and differentiation of B cells and only downregulated when B cells differentiate into plasmablasts and plasma cells. RTX can affect antibody titers through targeting of cells that can differentiate towards autoantibody producing cells. A study of 12 ATA positive dcSSc patients undergoing RTX monotherapy for up to 5 years showed a decrease of the ATA/total IgG ratio during RTX treatment [120]. This ratio, however, is difficult to interpret because RTX decreases total IgG. In one study, only modest and variable changes in autoantibody titers after RTX was found in 15 dcSSc-patients receiving two intravenous doses of 1000mg RTX [121]. Bosello et al. found that ATA levels did not change significantly over time in 14 ATA positive dcSSc patients treated with RTX [122]. Similar, a recent RCT in Japan showed no significant difference in ATA titers between the RTX and placebo groups in the study period of 24 weeks [123]. This may indicate that SSc IgG autoantibodies are largely derived from long-lived plasma cells, a population that remains unaffected by RTX.

Conclusions on associations between B cell response targeting nuclear antigens and clinical characteristics of SSc

In conclusion, the coincidence of ATA, ACA, ARA and AFA is extremely rare. In very early disease, the SSc-specific autoantibodies are the most important predictors for progression to definite SSc. Moreover, ATA, ACA and ARA occur preferentially on distinct genetic background (HLA), are associated with distinctive clinical subsets, specific patterns of organ involvement, and levels of certain isotypes have been associated with disease progression and severity of skin involvement. HSCT seems to lower ATA-titers in a subgroup of patients, whereas RTX does not affect autoantibody titers. So far, most research has focused on the presence of B cell responses targeting nuclear antigens, but a more in-depth analysis of antigen-specific B cell responses and of the autoantibodies in SSc is missing. Importantly, most studies have small sample sizes and often methodological shortcomings. The clinical evidence presented so far shows that SSc-specific autoantibodies are important diagnostic and prognostic biomarkers, and gives some evidence for a possible pathogenic role of disease-relevant processes.

Lessons learned from animal models on SSc-specific antinuclear B cell responses

Autoreactive B cells responses and their corresponding autoantibodies have been studied in detail in *in vivo* animal models. The strongest evidence for autoantibody pathogenicity is usually obtained by passively transferring human serum or isolated autoantibodies into mice, and evaluating if this leads to disease manifestations compatible with the human disease. As far as we know, there are no studies directly transferring ATA, ACA ARA or AFA autoantibodies into animals.

Alternatively, autoreactive B cell responses can also be evoked in animals by immunization with the autoantigen. Several studies have investigated the ATA B cell response upon administration of TOP1 to mice. Hu et al. initially found that an ATA-response can be induced by the immunization of BALB/c, SJL, NOD and C57/BL6 mice with TOP1, but no signs of inflammation or fibrosis using this model were observed [124]. However, administration of TOP1 with a strong adjuvant induced inflammation and fibrosis of skin and lungs in C57BL/6 mice [125,126], suggesting that additional immune activators may be required for the development of SSc-related disease manifestations in mice with ATA-autoantibodies. Notably, Mehta et al. reported that an ATA-response and fibrosis could be induced upon immunization of Balb/c mice with TOP1-loaded dendritic cells [127]. However, ATA-titers were only marginally increased in mice immunized with TOP1-loaded dendritic cells compared to mice immunized with unpulsed dendritic cells and fibrosis was observed before the ATA detection. This makes it difficult to draw conclusions on the role of the ATA B cell response within this model [127]. Studies inducing an ACA, ARA or AFA B cell responses *in vivo* upon administration of CENP-B, Pol III or fib are lacking, or do not report murine outcomes [128].

Yue et al. [129] studied autoreactive B cell responses in immunodeficient Rag2$^{-/-}$ IL2rg$^{-/-}$ upon adoptively transferring peripheral blood mononuclear cells of SSc-patients. Inflammation of lung, kidney and muscles characterized by B cell dominated cellular infiltrates was observed in the recipient mice engrafted with peripheral blood mononuclear cells from SSc-patients, but not in mice that received cells from healthy donors or SSc-patients treated with RTX [129]. Moreover, autoantibodies matching the ANA-pattern (determined by immunofluorescence staining of human HEp-2 cells) of the donor were observed in 2/6 mice [129]. This study indicates that B cells from SSc-patients are strongly pro-inflammatory. However, the exact contribution of the autoreactive B cells within this model remains to be determined.

SSc-specific autoantibodies can also be observed in animal models in which SSc-associated disease manifestations develop spontaneously as a consequence of genetic modifications or are induced by injection of chemical or biological agents. In several spontaneous models, like Tsk1, Tsk2 and Fli-1 mice, the development of ATA-, ACA- and ARA-responses have been reported [130–133]. One study even found a correlation between ATA-levels and fibrosis and skin thickness in Tsk1-mice [134]. Besides these spontaneous models, an ATA-response could also be observed in mice in which SSc-characterizing disease manifestations were induced by bleomycin and hypochlorous acid [135,136]. Interestingly, an AFA B cell response can be induced in mice by mercury which can modify the antigenic properties of fib [31,137,138]. Whether chemical agents can also induce or contribute to the development of antinuclear autoreactive B cell responses in human is not clear.

Conclusions based on the studies on animal models on SSc-specific antinuclear B cell responses

So far, there are no studies describing the direct transfer of ATA, ACA, ARA or AFA in experimental animals. However, TOP1 immunization can induce SSc-like disease and an ATA-response in mice, indicating that the ATA B cell response might be a decent contributor to disease pathogenesis. Nevertheless, from these ATA mouse models it is not yet clear by which effector mechanisms (e.g. antibody production, cytokine production, co-stimulation of T cells) the ATA B cell response exerts its effects.

In vitro indications for a contribution of antinuclear B cell responses in SSc disease pathogenesis

Whereas clinical studies and studies on animal models are particularly helpful in understanding whether autoantibodies are pathogenic, *in vitro* studies are important to elucidate how the pathogenic autoantibodies contribute to the disease pathogenesis.

Antigens targeted by ATA, ACA, ARA and AFA are normally sequestered in the nucleus and, thereby, inaccessible for autoantibodies. However, when cells go into apoptosis, nuclear antigens can be released or become accessible on apoptotic vesicles [139,140]. For example, TOP1 and CENP-B can be found in the supernatant of apoptotic cells [141,142]. In addition, defects in the clearance of apoptotic cell remnants is implied in the pathogenesis of systemic sclerosis and could increase the amount of antigen available for the activation of B cells [143,144].

An interesting hypothesis regarding the pathogenicity of ATA was presented by Henault et al. who showed that purified ATA from SSc-patients could bind to fibroblasts after binding of soluble TOP1 to fibroblasts [142,145]. This binding surface could induce adhesion and activation of co-cultured monocytes, indicating that the binding of ATA-TOP1 to fibroblasts had a direct functional effect on fibroblasts [142]. Additionally, a profibrotic effect of ATA-positive patient serum on fibroblasts was observed by Corallo et al. [146]. The interaction between TOP1 and fibroblasts needed for the subsequent interaction with ATA is thought to be mediated by a proteoglycan expressed on the membrane of fibroblast that contains a heparin sulfate group, as heparin sulfate and biosimilars block the interaction between TOP1 and fibroblasts [147]. However, the specific interactor with TOP1 on the surface of fibroblasts and the mechanism by which the fibroblast is activated remain to be identified.

SSc-associated antibodies might contribute to pathogenesis of SSc through formation of nucleic acid-containing immune complexes. ATA, ACA, ARA and AFA interact with antigens that bind nucleic acids. Nucleic acid-containing immune complexes can exert various pro-inflammatory effects by activating nucleic acid-sensing TLR receptors. Kim et al. initially found that ATA-containing but not ACA-containing sera of SSc-patients could form immune complexes that were able to induce IFN-α production by plasmacytoid dendritic cells [148]. However, the induction of IFN-α in this study might have been caused by antibodies with other specificities, as Eloranta et al. showed that the effect observed was dependent on RNA-binding, rather than DNA-binding antibodies, as SSc-sera containing ATA or ACA but without autoantibodies targeting RNA-binding proteins were not able to induce IFN-α production by plasmacytoid dendritic cells [149].

Finally, Raschi et al. found that immune complexes obtained from ATA-, ACA- and ARA-positive serum of SSc-patients were able to activate endothelial cells and induce the production of pro-fibrotic and pro-inflammatory mediators by fibroblasts [150,151]. However, it remains unclear how these immune complexes affected endothelial cells and fibroblasts, in particular because fibroblasts and endothelial cells lack expression of Fcy receptors which are generally thought to be required to mediate the internalization of immune complexes [151,152].

Conclusions on in vitro indications for a contribution of antinuclear B cell responses in SSc disease pathogenesis. Besides the suggested direct effect of ATA on fibroblasts, ATA, ACA, ARA and AFA might contribute to disease pathogenesis by forming nucleic acid-containing immune complexes. However, the studies which hypothesize these possible pathogenic effects do not pose exact mechanisms. Moreover, the actual contribution of the measured effects of the autoantibodies in vitro to disease pathogenesis *in vivo* is unclear.

Discussion

This review summarized available evidence on the role of SSc-specific antinuclear B cell responses against TOP1, CENPs, Pol III and fib in the development and progression of SSc. Understanding the contribution of these antinuclear B cell responses to disease pathophysiology is important in a time of therapeutic progress with focus on personalized medicine and the development of more targeted pharmacological approaches.

Intriguingly, presence of specific antinuclear B cells responses in SSc can be associated with distinct clinical disease subsets, and different

Box 1
Highlights regarding the role of the B cell responses targeting nuclear antigens in the disease pathogenesis in SSc.

Clinical:

- There is a strong association between B cell response targeting nuclear antigens and diagnosis/presence of SSc.
- Clinical phenotypes of SSc and several disease manifestations are associated with specific antinuclear B cells responses.
- An in-depth analysis of the B cell response targeting nuclear antigens in SSc is still largely missing.
- The precise effect of immunosuppressive therapy on the autoreactive antinuclear B cell responses has not been studied.

Animal models:

- Direct transfer of ATA/ACA/ARA/AFA into animal models has not been described.
- Immunization with TOP1 can induce SSc-like disease and an ATA response in mice, but it is not clear to what extend the induced ATA B cell response contributes to the development of SSc-like features in these mice.

In vitro experiments:

- Nuclear autoantigens targeted by ATA and ACA can become accessible when cells die.
- A direct proinflammatory and profibrotic effect of ATA on fibroblasts is suggested, however a clear mechanisms underlying these effects is missing.
- ACA/ATA/ARA/AFA might form immunogenic nucleic-acid containing immune complexes, however whether these immune complexes are formed in SSc *in vivo* is unclear.

prognostic features. Moreover, ATA-, ACA-, ARA- and AFA-levels in SSc-patients have been associated with progression of the disease [69,101-105,108,109], which might be indicative of a contribution of the SSc-specific antinuclear B cell responses to the pathogenesis of SSc. The rare co-existence of the B cell responses targeting TOP1, CENPs, Pol III and fib [74-76,79] might be in line with this concept, as this indicates that the development of autoreactive antinuclear B cell responses is not simply the consequence of certain disease processes in SSc, like fibrosis.

Nonetheless, the available evidence on ATA, ACA, ARA and AFA is not in favor of a direct pathogenic role of these autoantibodies. This is particularly indicated by the disconnection between the beneficial effects of RTX and a reduction in autoantibodies levels [121,123,153-155]. Antibody transfer of ATA/ACA/ARA/AFA resulting in SSc-like phenotypes has not been described in animal models. Moreover, there is a lack in hypotheses on how the antinuclear autoantibodies might lead to disease manifestations.

However, it cannot be excluded that autoantibodies contribute to disease pathogenesis. Autoantibodies might possibly aggravate inflammation once this is initiated, especially since autoantigens can become accessible for autoantibodies when cells die [139–142]. Alternatively, it could be possible that only part of the autoantibodies that have a specific feature could be pathogenic. Such autoantibody diversity is, for example, also observed for anti-myeloperoxidase antibodies in ANCA-associated vasculitis in which antibodies against specific epitopes of myeloperoxidase had pathogenic properties [156]. In line with this notion: a subgroup of ATA-positive patients shows a milder disease course with hardly any fibrotic complications. This could point to a certain degree of heterogeneity of the ATA/ACA/ARA/AFA-autoantibody responses, which needs further study to be fully understood.

Besides a role for the autoantibodies, the close association between SSc and specific autoantibody responses might be caused by features of the underlying B cell responses, which by themselves contribute to disease pathogenesis. The favorable outcomes observed by B cell targeting therapies in SSc support this concept. Eventually, it will be important to characterize TOP1-reactive, CENP-reactive, Pol III-reactive and fib-reactive B cells to SSc-pathogenesis in an antigen-specific manner to enable the characterization of function and phenotype of these cells. This will allow to determine their T cell co-stimulatory potential, for example, and the ability to produce pro-inflammatory cytokines or pro-fibrotic mediators.

In light of the above, we conclude that several aspects should be addressed in future studies, particularly because the majority of the data presented in this review is derived from studies with small sample sizes or methodological limitations. Firstly, the initiation and development of the autoantibody responses in genetically susceptible individuals and individuals that progress from pre-disease to disease is poorly understood. An evolution of the autoantibody response just before disease onset has been reported in other diseases, like SLE and RA. The ACPA-response in RA patients, for example, matures before disease onset, as indicated by rising ACPA-titers, an increase of citrullinated antigens recognized and an expanded use of immunoglobulin isotypes. In SSc, however, only one study has so far investigated autoantibody profiles before clinical disease onset and found that half of the SSc-patients had detectable autoantibodies before diagnosis [7]. Such a link between disease and evolution of autoreactive B cell responses might be indicative for a considerable role of these responses in disease pathogenesis. Topics to investigate include the course of autoantibodies titers, class switching, affinity maturation and epitope spreading of antinuclear antibodies. Furthermore, to be able to understand how autoreactive antinuclear B cell responses are initiated and driven, it would be important to determine the source and nature of the antigen activating the B cells. There are various hypothesis on the origin of the antigen, for example studies in mice have shown that various environmental toxins and pollutants can initiate SSc-specific autoreactive B cell responses [31,135,136]. In addition, the presence of nuclear autoantibodies of the IgA isotype and involvement of various mucosal tissues in SSc patients might indicate a mucosal origin of the autoreactive B cell responses [105,157]. Another interesting observation is the co-occurrence of malignancies in SSc patients with particular antinuclear B cell responses, like the anti-pol III B cell response [158]. This indicates that malignancies might also be a source of antigen. The aforementioned hypotheses might include antigens which are structurally altered or have structural similarities in comparison with the originally described antigen.

In summary, although the autoreactive B cell responses targeting nuclear proteins are closely linked to the SSc-pathogenesis, their exact role remains to be fully determined (Box 1). This overview shows that a better understanding of the role of autoreactive B cells in SSc is relevant

Box 2
Open questions on autoreactive B cells targeting nuclear antigens in SSc.

- What triggers the break of B cell tolerance against nuclear antigens in SSc?
  - Is it specific for SSc?
- How does the autoreactive B cell response against nuclear antigens develop over time?
  - Is it present before disease onset?
  - Does it evolve before disease onset?
- What are the specific features of the autoantibody response targeting nuclear antigens in SSc patients?
- What is the contribution of autoreactive B cells to disease (progression/inflammation/onset?)?

Research Question: What is the contribution of the autoreactive B cell responses targeting nuclear antigens to disease pathogenesis of SSc?

for better elucidation of the SSc-pathogenesis and consequently personalized treatment options. Future research should particularly focus on a more in-depth analysis of the B cell response targeting nuclear antigens and characteristics of the autoantibodies in SSc (Box 2).

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