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Pathogenic role of anti-nuclear autoantibodies in systemic sclerosis: Insights from other rheumatic diseases

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Summary

Systemic sclerosis (SSc) is a severe autoimmune disease characterized by vasculopathy, fibrosis, and dysregulated immunity, with hallmark autoantibodies targeting nuclear antigens such as centromere protein (ACA) and topoisomerase I (ATA). These autoantibodies are highly prevalent and disease-specific, rarely coexisting, thus serving as crucial biomarkers for SSc diagnosis. Despite their diagnostic value, their roles in SSc pathogenesis remain unclear. This review summarizes current literature on ACA and ATA in SSc, comparing them to autoantibodies in other rheumatic diseases to elucidate their potential pathogenic roles. Similarities are drawn with anti-citrullinated protein antibodies (ACPA) in rheumatoid arthritis, particularly regarding disease specificity and minimal pathogenic impact of antigen binding. In addition, differences between ANA and ACPA in therapeutic responses and Fab glycosylation patterns are reviewed. While ACA and ATA are valuable for disease stratification and monitoring activity, understanding their origins and the associated B cell responses is critical for advancing therapeutic strategies for SSc.

KEYWORDS
anti-centromere antibodies, anti-topoisomerase I antibodies, glycosylation, rheumatic diseases, systemic sclerosis

1 | INTRODUCTION

Rheumatic autoimmune diseases, including rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and anti-neutrophil cytoplasmic antibodies (ANCA)-associated vasculitis (AAV), are characterized by various autoantibodies. Over the years, multiple studies have summarized these autoantibody characteristics into comprehensive overviews.[1–3] However, systemic sclerosis (SSc), another rheumatic disease with autoantibodies, is often overlooked due to its rarity and heterogeneity. Therefore, in the scope of this review, we aim to answer the following question: do disease-specific autoantibodies contribute to the disease pathophysiology of SSc? We will address published literature about autoantibodies in SSc together with knowledge obtained from other rheumatic diseases to discuss the potential pathogenic role of these autoantibodies in SSc.

1.1 | Systemic sclerosis

SSc is a severe autoimmune disease characterized by a triad of microangiopathy, immune dysregulation, and fibrosis.[4] Early clinical manifestations are varied but often include Raynaud's phenomenon (RP), a common yet non-specific sign of SSc, in which blood flow to the extremities is restricted due to endothelial cell dysregulation and

exaggerated vasoconstriction.[5] Additionally, some patients exhibit puffy and swollen fingers as well as gastro-esophageal reflux.[6] As the disease progresses, multiple organ systems are affected, leading to widespread fibrosis of vital organs and severe skin thickening.[7] Other manifestations include pulmonary hypertension (PH), interstitial lung disease (ILD), and renal crisis.[4] Despite the severity of SSc, the complex interplay between its pathophysiological features remains poorly understood, presenting a significant challenge for both patients and physicians, and highlighting a high unmet medical need.[8]

1.2 | Prevalence of autoantibodies in SSc

One of the hallmarks of SSc is the aberrant activation of immune cells, particularly the loss of tolerance of B cells towards nuclear antigens, which plays a crucial role in the disease's pathogenesis.[9] These autoreactive B lymphocytes can proliferate into autoantibody-producing plasmablasts and plasma cells. Indeed, up to 95% of patients with SSc test positive for anti-nuclear antibodies (ANA), with seropositivity detectable years before disease onset.[10] Among these autoantibodies are antibodies recognizing topoisomerase I (ATA) and anti-centromere antibodies (ACA), the two most prevalent disease-specific ANAs which rarely coexist (<2%).[11] Additionally, ACA and ATA are associated with distinct clinical phenotypes, making them valuable biomarkers for disease stratification.[12] ACA positivity correlates with limited skin involvement (limited cutaneous SSc (lcSSc)), gastrointestinal complications, and slower disease progression, while ATA is linked to widespread skin involvement (diffuse cutaneous SSc (dcSSc)), higher mortality rates, and ILD.[13–15] Other ANAs include anti-RNA polymerase I–III antibodies, anti-fibrillarin antibodies, and anti-Th/To antibodies[13]; however these ANAs have a much lower prevalence in SSc. Therefore, this review focuses primarily on the potential pathogenic roles of ACA and ATA. Furthermore, besides break of tolerance against nuclear antigens, antibodies against platelet-derived growth factor receptor, endothelial cells, and vascular receptors are reported.[16] Since these autoantibodies are not disease-specific and can also occur in healthy individuals, they are beyond the scope of this review.

1.3 | Characteristics of anti-topoisomerase-I antibodies

In contrast to anti-citrullinated protein antibodies (ACPA) in RA, ATA and ACA target whole proteins instead of a single post-translational modification (PTM). The protein human topoisomerase I (TOP1) is a 100 kDa enzyme located in the cell nucleus that facilitates relaxation of supercoiled DNA.[17] This DNA relaxation is essential not only for DNA replication and transcription but also for repair of strand breakage during recombination. Human TOP1 is composed of four subdomains with varying levels of sequence conservation. The N-terminal domain is highly charged and unstructured, exhibiting low sequence homology among species, whereas the DNA-binding core domain and the C-terminal domain are highly conserved in mammals. Furthermore, although TOP1 from viruses and bacteria are relatively small (~36 kDa), core and C domains of *Saccharomyces cerevisiae* TOP1 are 58% and 62% identical to the human counterparts.[18,19] In humans, initial reactivity of ATA was described against a 70 kDa breakdown product of full-length TOP1, known as Scl-70, which lacks the N-terminal domain, indicating the immunodominance of the core and C-terminal domains in full length TOP1.[20]

Epitope-mapping studies provide further insights into the specific regions of antigens that antibodies target, helping to understand the molecular basis of interactions between antibodies and their antigens. Several studies have explored ATA reactivity towards fragments or peptides of TOP1, revealing that ATA epitopes are distributed throughout the entire protein, with immunodominant regions identified specifically between amino acids 484–560 and 653–704.[21–24] However, using these fragments or peptides, conformational epitopes might be missed.[8] Indeed, deletion studies and competitive inhibition assays identified a region of ≥52 amino acids (512–563), which is essential for ATA recognition.[25] This fragment is highly conformational in nature and is located in the conserved core domain.[17] Therefore, further epitope-mapping studies that preserve the conformation of TOP1 are necessary to fully characterize the immunodominant regions recognized by ATA.

Another approach to gain deeper insight into the immunodominant regions of TOP1 is the use of monoclonal antibodies (mAbs) derived from SSc patients. Using these mAbs and epitope mapping techniques such as shotgun mutagenesis, the bindings sites of ATA mAbs on TOP1 can be identified.[26] Furthermore, by engineering the sequence for the cell receptor (BCR) into a Ramos B cell, these mAbs and their corresponding BCR could provide insight into the TOP1-specific B cell response. This approach has been shown successful in studying anti-modified protein antibodies including ACPA. These mAbs were highly cross-reactive towards multiple PTMs and Ramos B cells with these BCRs were activated on stimulation with multiple PTM-antigens.[27] Lastly, mAbs derived from different patients could provide insight into the diversity of the autoantibody repertoire. However, to our knowledge, only one study to date has reported mAbs against TOP1 derived from SSc patients.[28] This study described the binding characteristics of three mAbs generated from SSc patients to different TOP1 fusion proteins, but detailed information regarding the identification and generation of these antibodies was not provided. Therefore, to obtain comprehensive information about epitopes bound by ATA, well-validated mAbs derived from SSc patients are essential.

1.4 | Characteristics of anti-centromere antibodies

Most ACA reactivity in sera is directed towards centromere protein B (CENP-B), although recognition of centromere protein A and C has also been reported, to a lesser extent.[29] Similar to TOP1, CENP-B is localized in the cell nucleus under physiological conditions. This

80 kDa protein binds specifically to a 17 bp sequence known as the CENP-B box.[30] During mitosis and meiosis, the binding of CENP-B to this box facilitates correct segregation of the chromosomes into daughter cells. The high level of sequence homology between human and mouse CENP-B indicates that it is a conserved mammalian gene.[31] Human CENP-B also structurally resembles autonomously replicating sequence-binding protein 1 (Abp1p) found in fission yeast *Schizosaccharomyces pombe*.[32]

ACA epitopes are found in multiple regions of CENP-B, but the C-terminal region is recognized by most patient sera and is therefore considered to be more immunodominant.[33–36] However, in a small sample population of SSc patients from North Africa, the N-terminal domain of CENP-B contained a major epitope for ACA.[37] Interestingly, epitope-mapping studies of ACA reactivity towards centromere A protein identified immunodominant sequences overlapping with CENP-B and Epstein–Barr nuclear antigen 1 (EBNA-1), suggesting possible cross-reactivity of ACA with other proteins.[38–40] Complementary to ATA and in contrast to ACPA in RA, no mAbs derived from SSc patients against CENP-B have been reported yet, hindering further studies into ACA epitopes on CENP-B.

2 | AUTOANTIBODY LEVELS AND DISEASE MANIFESTATIONS

In rheumatic diseases, autoantibody levels can correlate with specific clinical manifestations and with disease activity, suggesting a potential pathogenic function.[41] Indeed, the transfer of anti-myeloperoxidase (MPO) antibodies from patients with AAV to mice has a direct pathogenic effect.[42] On the other hand, elevated autoantibody levels can also indicate recent B cell activation, without necessarily having a direct pathogenic role. Over the years, the pathogenicity of ACPA in RA has been subject of debate, as some studies suggest a protective effect of ACPA,[43–47] raising the question whether autoantibodies merely reflect disease activity instead of being pathogenic.

2.1 | Correlation between disease activity, autoantibody levels, and treatment

A recent review summarized the reported correlations between antibody titers and disease activity in SSc, concluding that disease activity correlates with autoantibody levels and the presence of multiple isotypes is often associated with disease progression.[8] In studies from our department, SSc patients with progressive disease were more ATA-IgM$^+$ (91%, 21/23) compared to patients without disease progression (57%, 33/58). Additionally, patients with dual positivity for ATA-IgG and ATA-IgM more often experienced disease progression than patients with solely ATA-IgG positivity.[48] Similar trends were observed for ACA$^+$ SSc patients, with ACA-IgG$^+$ patients showing less severe microangiopathy compared to patients with positivity for ACA-IgM and/or ACA-IgA. Furthermore, the levels of ACA-IgG and ATA-IgM were associated with the severity of microangiopathy, with ATA positivity showing a stronger correlation.[49] Lastly, in patients with very early SSc, ACA-IgG and ACA-IgM levels correlated with future disease progression.[50] Similarly, other studies have observed that higher ATA levels were associated with extensive skin tightness and early onset of cardiac involvement and ILD.[51,52] However, as the exact mechanism contributing to this correlation has not been reported, these findings underscore the importance of understanding the relationship between autoantibody levels and disease activity in SSc, which could have significant implications for prognosis and treatment strategies.

2.2 | Therapy

Investigating the interplay between B cell-targeting therapies, ANA levels, and subsequent disease manifestations can elucidate the contributions of each component to disease pathogenesis. Although no definitive cure for SSc exists, various treatment options are available to help reduce disease burden. Some of these therapies specifically target disease-associated symptoms whereas other treatments focus on the underlying immune response.

Disease-modifying anti-rheumatic drugs (DMARDs) prescribed to patients with rheumatic diseases include immunosuppressive therapies such as mycophenolate mofetil, cyclophosphamide (CTX), and methotrexate (MTX). MTX treatment has been shown to decrease ACPA titers in patients with early (rheumatoid) arthritis and is associated with reduced disease activity.[53–55] In patients with AAV, 41% of MPO-positive patients became seronegative after 6 months of CTX treatment, which correlated with an improved quality of life compared to baseline.[56] For SSc, these therapies have demonstrated efficacy in improving the modified Rodnan skin score (mRSS) in early diffuse SSc (MTX) and in treating SSc-ILD (CTX and MTX).[57] However, to date, the effects of these therapies on ACA or ATA levels have not been reported. Given the beneficial effects on autoantibody titers and disease activity observed in RA and AAV, similar effects on ACA and ATA levels in SSc might be expected. Nonetheless, detailed studies on changes in these specific titers upon treatment are necessary to understand the correlation between ACA andATA titers and therapeutic outcomes.

Other treatment strategies specifically target the potential pathogenic functions of (autoreactive) B cells and their secreted (auto)antibodies. These therapies include rituximab (RTX), tocilizumab (TCZ), and autologous hematopoietic stem cell transplantation (AHSCT).

2.3 | Monoclonal antibodies

RTX, a chimeric mAb, targets B cells through their CD20 expression.[58] CD20 is a transmembrane protein expressed on B cells from late pre-lymphocytes until differentiation into plasma cells and plasmablasts.[59] Since CD20 expression is downregulated on

antibody-producing B cells, RTX primarily affects only those B cells that have not yet differentiated into plasmablasts. In both RA and AAV, RTX treatment has been shown to be effective, with reduced autoantibody titers.[56,60,61] However, in AAV, a strong delayed B cell repopulation (>1 year) with decreased Ig levels was observed, resulting in immunocompromised patients.[62] In SSc, anti-CD20 B cell depletion has been shown to effectively improve skin involvement. However, for ATA-positive SSc patients, autoantibody levels did not change significantly over time, although the exact time range was not further specified.[63] Similarly, in a double-blind, placebo-controlled trial with 56 patients, no significant difference in ATA titers between RTX and placebo treatments was observed.[64] Despite multiple clinical trials reporting ATA- and ACA-positivity at baseline, follow-up data regarding ACA or ATA levels are often lacking, making it difficult to interpret the effectiveness of anti-CD20 therapy on autoantibody levels. Furthermore, in other rheumatic diseases, tissue-resident B cells have been observed to escape RTX treatment, possibly due to limited penetration of RTX into the tissues.[65] Therefore, the beneficial effect of RTX on the autoantibody levels in SSc might be limited to CD20-expressing cells in the circulation, although no studies have investigated the effect of RTX treatment on the diversity of the ACA and ATA B cell repertoire.

Another mAb therapy, TCZ, does not directly target B cells, but competitively inhibits IL-6 binding to lymphocytes by targeting the IL-6 receptor.[66] Since IL-6 plays a crucial role in the final differentiation of B cells into antibody-producing cells, TCZ may be an effective therapy for targeting their transition into plasmablasts. Indeed, over the years TCZ has been approved for multiple rheumatic diseases, including RA, where it improved signs and symptoms of disease.[67–70] Unfortunately, effect of TCZ on ACPA titers is not widely assessed, and only a limitedimpact of autoantibody seropositivity on TCZ effectiveness has been observed among multiple studies.[71] Furthermore, another study reported similar effectiveness of TCZ in both seronegative and seropositive RA patients, raising the question whether seronegative RA shares the similar pathogenetic pathways as seropositive RA. Therefore, the beneficial effect of TCZ, and also RTX, in seropositive RA are suggested to reflect a more immune cell-driven form of the disease, rather than a direct pathogenic role of ACPA.[71,72] Similarly, in a small study with 14 SLE patients, TCZ treatment led to significant decline in anti-double stranded DNA (dsDNA) antibodies, but this was accompanied by a decrease in total IgG levels and circulating plasma cells.[73] In a randomized, placebo-controlled trial, TCZ stabilized lung function in patients with SSc-ILD.[74] However, ACA and ATA levels were not measured during follow-up, so the effect of this treatment on autoantibody levels cannot be assessed. Results from a phase 2 ($n=87$) and a phase 3 ($n=210$) trial with predominantly ATA-positive SSc patients (42%-52%) reported contradictory outcomes regarding changes in skin thickness between participants treated with TCZ and those treated with placebo after 48 weeks, although it was suggested that TCZ could preserve lung function in early diffuse SSc.[75,76] Any effects on autoantibody titers or B cells were not assessed, but based on observed effects in other rheumatic diseases, TCZ's beneficial effects are likely due to inhibiting IL-6-mediated activation on various immune cells rather than at the autoantibody-specific level.

2.4 | Stem cell transplantation

Although the exact mechanism of AHSCT remains unknown, it is hypothesized that combination of high dose immunosuppressants and autologous stem cell infusion eliminates autoreactive B cells and resets the immune system towards a more naïve state. For a time, AHSCT was the only therapy to achieve long-term, drug-free, and symptom-free remission in several refractory autoimmune diseases.[77] Since this therapy is highly invasive with several risks, only severely ill patients for whom other conventional therapies, including DMARDs, have failed are eligible for AHSCT.[77,78] Therefore, most studies have small sample sizes and only include patients with active and progressive refractory disease. Nevertheless, for RA and SLE, AHSCT resulted in sustained clinical improvement with reduced autoantibody levels, although some patients still had active disease or experienced relapse during follow-up.[79–84]

In a study involving 22 SSc patients, naïve B cell frequencies increased from 60 to 360 days after AHSCT compared to baseline, while memory B cell numbers decreased during this same period.[85] Similarly, another study analyzing the peripheral B cell compartment, showed an increase in naïve B cells and higher levels of immune regulatory cytokine IL-10 post-AHSCT. Moreover, mean mRSS improved from 21.8 ± 11.3 to 11.5 ± 7.3, and mean FVC remained stable.[86] Regarding seropositivity for ATA post-AHSCT, a study distinguishing between clinical responders ($n=5$) and non-responders ($n=4$) found that patients in both groups became seronegative for ATA after more than 5 years post-AHSCT.[87] Conversely, another study involving 18 ATA-positive SSc patients who underwent AHSCT showed strong ATA positivity post-treatment, with no statistically significant decrease in ATA-IgG levels between responders and non-responders.[88] Although a possible explanation for this difference was not identified, it raises the question whether ATA seropositivity correlates with clinical outcome after AHSCT. Results from another clinical trial favor AHSCT over CTX as treatment for patients with dcSSc.[89] Detailed studies into longitudinal autoantibody repertoire after treatment revealed temporary variations in serum autoantibody profiles post-ASHCT in only eight out of 32 subjects, with such variations not observed after CTX treatment. Interestingly, changes in ATA titers did not correlate with clinical endpoints, suggesting that autoantibodies against TOP1 are not pathogenic but rather serve as diagnostic biomarkers. Additionally, these data might also indicate that ATA- or ACA-producing plasma cells are spared during AHSCT. Notably, since only dcSSc patients, who are typically more ATA-positive than ACA-positive, were included, conclusions regarding ACA should be drawn with caution. Another study that performed bulk RNA sequencing on PBMCs from the same clinical trial demonstrated that the IGH repertoire was reset to a more naïve state, predominantly comprising the IgM isotype.[90] However, no data were obtained regarding autoreactive B cells targeting TOP1 or CENP-B,

or their impact on ACA and ATA levels. Furthermore, some patients with ATA-positivity at baseline seroconverted to ACA-positive post-AHSCT.[89] Therefore, the effect of AHSCT on autoreactive B cells and autoantibody positivity requires further elucidation.

2.5 | CAR T cell therapy

Chimeric antigen receptor (CAR) T cell therapy, which targets CD19-expressing B cells, has been successful in treating refractory B-cell lymphoma by effectively depleting B cells.[91,92] This approach could also be beneficial for rheumatic diseases, particularly for patients who have failed DMARDs or are ineligible for AHSCT. In contrast to CD20, which is downregulated on antibody-secreting cells, plasmablasts express high levels of CD19.[93] Thus, CAR T cell therapy could deplete CD19-positive B cells within lymphatic organs and inflamed tissues, in which autoreactive B cells could be a potential source of autoantibodies.

The first application of CAR T cell therapy was reported in a severely ill 20-year-old woman with refractory SLE.[94] Following preparatory lymphodepletion with fludarabine and CTX, CD19 CAR T cells were administered, resulting to complete and sustained depletion of circulating B cells within 7 days. Interestingly, while IgG levels remained stable (~5 g/L), anti-dsDNA autoantibody level decreased rapidly (50004 U/mL) within five weeks, suggesting that CD19-positive plasmablasts are the primary source of these autoantibodies. Lastly, disease activity also decreased significantly, with no activity observed 44 days post-therapy. Recently, first time treatment of a SSc patient with CD19 CAR T cells was reported.[95] In this initial study, six months post-treatment, full B-cell depletion was achieved, with IgG levels remaining above 700 mg/dL. Comparable to the SLE study, ANA titers decreased below detection levels, and heart, joint and skin manifestations improved rapidly, although severe disease persisted. Similarly, in another ATA-positive SSc patient treated with CD19 CAR-T cells, lung function improved, and ATA levels dropped from approximately 60 to 6.4 U/mL.[96] However, despite a decrease in mRSS, a relatively high mRSS of approximately 11 persisted post-therapy. Interestingly, Fcγ-receptor-activating immune complexes, thought to contribute to pathology in ATA-positive SSc,[97] disappeared. In a larger study involving four SSc patients, eight SLE patients, and three patients with idiopathic inflammatory myositis, no significant effect of plasmablast percentage in SSc patients was observed post-CAR T cell treatment, as these percentages were low at both baseline and follow-up.[98] Furthermore, ATA levels only slightly decreased post-treatment, and while the mRSS declined, ATA-levels remained high. Given that only severely ill patients with anti-RNP-III antibodies or ATA were included, effect of CAR T cell therapy on ACA levels cannot be assessed.

While CD19 CAR T cell therapy appears to be effective in severely ill ATA-positive SSc patients, its impact on ATA levels has varied across studies. Additionally, this therapy depletes all CD19-expressing cells, not just autoreactive B cells, and may spare potential autoantibody-producing plasma cells. Therefore, CAR T cell therapy might be most effective on autoantibody levels in cases of predominantly autoantibody-producing plasmablasts. An ideal therapy would selectively target antigen-specific B cells, while leaving the total B-cell repertoire unaffected. This could also provide insights into the pathogenic role of these autoreactive B cells and their produced autoantibodies. Chimeric autoantibody receptor (CAAR) therapy offers such specificity by targeting autoreactive B cells based on their specificity for the autoantigen B cell receptor.[99] However, studies on CAAR therapy for rheumatic diseases have not yet been conducted, leaving the exact contribution of ACA- or ATA-specific B cells to SSc pathogenesis unexplored. Further research is necessary to clarify these associations and optimize therapeutic strategies for SSc.

2.6 | Summary: Autoantibody levels and disease manifestations

Over the years, various treatment strategies have been employed to manage rheumatic diseases, including SSc. These therapies range from DMARDs to B-cell depletion like AHSCT and CD19 CAR T cell therapy. When comparing ACA and ATA to autoantibodies in other rheumatic diseases with regard to disease activity and response to therapy, these autoantibodies seem to show similar characteristics as ACPA in RA. Higher levels are associated with disease activity and treatment with DMARDs improved clinical manifestations. However, in contrast to ACPA, response to RTX is limited and autoantibody titers remained stable post-treatment, suggesting a potential different source of autoantibody-producing B cells in SSc. Despite extensive research, the exact mechanisms driving SSc pathogenesis remain unclear. However, a significant body of evidence suggests a pathogenic contribution of (autoreactive) B cells rather than pathogenic autoantibodies, as many therapies show clinical effectiveness while autoantibodies are still detectable. Still, these therapies predominantly target the overall B cell response, leaving the role of ATA- and ACA-specific B cells less understood. While ATA has been extensively studied, the role of ACA is less clear. This discrepancy is largely due to the fact that most studies focus on ATA-positive SSc patients, resulting in a lack of comprehensive knowledge about ACA's potential pathogenicity. Further research is needed to elucidate the distinct contributions of these autoantibodies and the specific B cell subsets involved in SSc pathogenesis.

3 | PATHOGENICITY OF AUTOANTIBODIES IN RHEUMATIC DISEASES

A pathogenic autoantibody is typically defined by two key criteria: first, autoantibody must bind to a relevant antigen at the site of tissue damage, and second, lesions attributed to the autoantibody should be replicable in an experimental setting.[100] Evidence for autoantibody pathogenicity is often obtained in vivo

by transferring isolated autoantibodies or serum from diseased humans to animal models, predominantly mice, and assessing whether the transfer induces symptoms akin to those observed in human disease. Additionally, in vitro studies can be employed to determine if an autoantibody causes apoptosis or alters the function of target cells.

3.1 | In vivo studies

Over the years, several studies have addressed the pathogenicity of autoantibodies in rheumatic diseases by transferring isolated antigen-specific antibodies or patient-derived mAbs to mice. Although some studies initially reported ACPA-mediated joint pain, osteoclast activation, and bone loss in mice,[101–103] these articles were later retracted or corrected because the used mAbs in these studies lacked specificity for citrullinated proteins.[104–106] Recently, some studies have described a protective effect of a subset of ACPA mAbs in mice, suggesting that ACPA could be a potential therapy.[44–46] On the other hand, passive transfer of human IgG fractions containing anti-MPO or anti-proteinase 3 (PR3) antibodies to mice or rats resulted in antibody-mediated vasculitis, thereby suggesting a direct pathogenic effect of these autoantibodies.[107,108] To date, no studies have directly transferred isolated ACA or ATA from patients with SSc into in vivo animal models. Additionally, the specific conditions required for these autoantibodies to be pathogenic might not be replicated in unmanipulated mice. Several studies have used immunization strategies in mice to elicit immune responses against human TOP1 or CENP-B. Two studies immunized different mouse strains with TOP1 mixed in Freund's incomplete or complete adjuvant to evaluate whether an autoreactive antibody response could be directly pathogenic.[109,110] While immunization with both types of adjuvants generated an ATA response, only immunization with TOP1 in complete Freund's adjuvant induced skin and lung fibrosis. This fibrosis was associated with higher levels of IL-6, TGF-β, and IL-17, and decreased IL-10 production, suggesting a robust adjuvant is required to provoke SSc-related manifestations in ATA-positive mice. Another study explored the effects of immunizing Balb/c mice with TOP1-loaded dendritic cells to assess the autoantibody response and fibrotic outcomes.[111] This approach resulted in an ATA response towards one of the TOP1 peptides; however, the difference in OD values between TOP1-immunized mice and controls was minimal. Furthermore, fibrosis was present prior to immunization, complicating the assessment of the specific contribution of ATA to disease manifestations in this model.

Regarding ACA, the production of these autoantibodies following immunization with human CENP-B has been less explored. Only one study from 1992 reports immunization of mice with recombinant CENP-B. However, this study primarily focused on identifying human-specific antigenic epitopes, and outcomes in mice were not reported.[112]

In addition to actively immunizing mice with human nuclear antigens, researchers have explored the effect of SSc patient-derived PBMC transfer into animal models. A humanized mouse model was developed by transplanting PBMCs from SSc patients into severely immunocompromised ($Rag2^{-/-}$/$IL2rg^{-/-}$) mice.[113] Mice engrafted with PBMCs from HD or SSc patients treated with RTX were included as controls. Mice receiving SSc PBMCs exhibited inflammatory manifestations in muscles, lungs, and kidneys, whereas mice engrafted with RTX-treated or HD PBMCs showed no SSc-related pathologies. Interestingly, while five out of six SSc patients showed positive ANA patterns as determined by IF staining of human HEp-2 cells, only two out of six mice displayed comparable ANA patterns, despite exhibiting similar SSc-related manifestations. Notably, none of the mice engrafted with RTX-treated PBMCs had detectable ANA levels, which contrasts with the hypothesis that RTX spares antibody-producing plasmablast and plasma cells. However, since only one out of three RTX-treated patients had detectable ANA, this could explain the absence of ANA in the mice. Although this study highlighted the strong proinflammatory role of PBMCs in SSc pathogenesis, the exact contribution of each component and the specific role of autoantibody-producing B cells still need to be determined. Two additional studies have examined the role of B cells in mice engrafted with SSc patient-derived PBMCs, yet neither assessed ACA or ATA production.[114,115] Lastly, in a study evaluating the combined effects of PBMCs and skin grafts from HD or SSc patients in mice, ANA was exclusively detected in mice receiving both SSc skin grafts and autologous SSc PBMCs. However, these mice did not reproduce the inflammatory events originally found in the SSc skin biopsies, thereby showing that solely ANA positivity did not induce disease.[116]

While ACA and ATA are specific autoantibodies associated with SSc in humans, various mouse models have been developed to spontaneously generate ACA and ATA responses through genetic modifications or upon administration of biological or chemical agents. Early studies using tight skin mice demonstrated the production of both ACA and ATA, with observed correlations between fibrosis, skin thickness, and elevated ATA levels.[117,118] Additionally, in a bleomycin-induced fibrosis model, bleomycin-treated mice exhibited lung fibrosis, and their sera tested positive for ANA, although the specific reactivity was not further characterized.[119] Nevertheless, these mouse models do not fully replicate the complex pathophysiological features of SSc observed in humans, limiting their utility in studying the intricate interactions between vasculopathy, fibrosis, dysregulated immunity, and subsequent autoantibody production.

In summary, to date, no suitable model specifically addressing the role of purified ACA and ATA upon passive transfer to mice has been established. Therefore, it is difficult to assess the pathogenicity of these autoantibodies in vivo.

3.2 | In vitro studies

Autoantibodies can potentially mediate disease through direct antigen binding via their fragment antibody-binding (Fab) domains.[120] The mechanisms behind this autoantibody targeting can be elucidated using in vitro studies. For ACPA, some studies hint towards

an agonistic role of these autoantibodies in RA. For instance, in an osteoclast lineage, ACPA targeting citrullinated vimentin enhanced bone resorption.[121] On the other hand, ACPA display a high level of cross-reactivity together with low avidity compared to recall antigens, thereby questioning how ACPA could potentially directly activate cellular receptors.[122,123] For SLE and AAV, direct pathogenicity of autoantibodies targeting dsDNA, MPO, and PR3 was observed,[124,125] suggesting differences in in vitro pathogenicity of autoantibodies in rheumatic diseases.

In SSc pathogenesis, in vitro studies could give more insight into the mechanisms by which disease-specific autoantibodies may exert pathogenic effects. For both ACA and ATA, presence of their respective antigens is normally restricted to the nucleus under physiological conditions, making them inaccessible for autoantibody binding. Therefore, as production of pathogenic autoantibodies implies a breach of tolerance to self-antigens, there must be a source of self-antigens or neoantigens outside the nucleus that evokes this immune response.[126]

One potential source of nuclear antigens outside the nucleus is apoptotic blebs, which have been identified as an antigen source in skin lesions and fibroblasts of SSc patients.[127,128] However, the mere presence of an antigen source does not directly imply a pathogenic role; evidence of an effect mediated by autoantibodies is crucial.

A direct pathogenic role for ATA has been described for fibroblasts, where soluble TOP1 released by apoptotic cells bound to fibroblasts and was subsequently recognized by ATA.[129,130] This ATA-TOP1 complex induced monocyte adhesion and activation, highlighting a direct functional effect of ATA on fibroblasts, potentially contributing to the inflammatory processes observed in SSc. Similarly, research by Arcand et al. demonstrated that TOP1 can stimulate the migration of healthy fibroblasts through activation of several signaling pathways, including PLCγ1, c-Raf, ERK-1/2, and p38 MAPK.[131] Additionally, ATA from SSc sera enhanced binding of TOP1 to heparan sulfate proteoglycans on fibroblast surface, potentially contributing to fibrosis.[132] In studies using dermal fibroblasts from SSc patients, both commercially acquired ACA and ATA were shown to induce apoptosis upon treatment with these polyclonal antibodies, with unaffected fibroblasts from lcSSc patients showing greater susceptibility compared to affected fibroblasts from dcSSc and lcSSc patients.[133] While these studies proposed intriguing mechanisms by which ATA could contribute to fibroblast activation, future research should aim to elucidate the exact interactions and mechanisms involving TOP1 and ATA. Moreover, the use of both healthy and affected fibroblasts complicates the interpretation, as it is challenging to discern between disease-specific intrinsic fibroblast characteristics and effects induced by ATA.

Besides apoptosis, other sources of antigens for ACA and ATA binding may originate from PTMs, similar to citrullination for ACPA in RA, or processes such as molecular mimicry and epitope spreading. For instance, in a Balb/c mouse model, oxidation of TOP1 resulted in elevated levels of ATA in sera, although this was induced using potent oxidative reagents.[134] Similarly, as mentioned earlier, ACA has been shown to react with EBNA1, suggesting a potential origin from molecular mimicry and subsequent epitope spreading rather than direct recognition of the self-antigen. Computational approaches have identified microbial pathogens sharing epitopes with both TOP1 and CENP-B.[135] However, since these matches were generated *in silico*, there is no evidence of actual cross-reactivity.

Although a pathogenic role for ACA and ATA seems plausible, given that their antigens are present in apoptotic conditions, detailed studies into the exact mechanisms by which the Fab domains of these autoantibodies contribute to disease are still lacking. Therefore, further research is necessary to investigate the pathogenicity of ACA and ATA Fab binding in well-controlled experimental settings that are relevant to SSc pathogenesis.

3.3 | Immune complexes

In addition to their direct pathogenic effects through antigen binding via their Fab domains, autoantibodies can interact with soluble target antigens, resulting in immune complex (IC) formation. These formed ICs could then interact with the complement system, bind Fc receptors (FcRs) or activate toll-like receptors (TLRs), leading to various pro-inflammatory effects. For instance, in RA, ACPA-containing ICs have been shown to activate inflammatory effector mechanisms on macrophages via FcγRIIa.[136] Similar effects were observed in mast cells, which were activated by ACPA-IgG ICs.[137] Additionally, ACPA could activate both the classical and alternative pathway of the complement system in vitro.[136] Moreover, incorporation of rheumatoid factor into ACPA-containing ICs had a strong FcR-mediated and complement-dependent pathogenic effect.[138] Additionally, data from SLE patients showed defective clearance of ICs which could also activate the complement system.[139]

Since both ACA and ATA target proteins that bind to nucleic acids, these autoantibodies may contribute to SSc pathogenesis through the formation of nucleic acid-containing ICs. Incubation with purified nucleic-acid containing ICs from ATA- and ACA-positive serum has been shown to result in EC activation and a pro-fibrotic and pro-inflammatory signature in fibroblasts, mediated by TLRs.[140,141] However, because TLR expression in these cell types is restricted to endosomes,[142] endocytosis of ICs is required. Yet, both fibroblasts and endothelial cells lack Fcγ receptor expression required for IC internalization, questioning the proposed pathway in this study. Some cell types, such as B cells and dendritic cells, express TLRs on their cell membrane, making the receptors accessible for nucleic-acid containing ICs.[143] Although TLR activation on B cells by nucleic-acid ICs has not been demonstrated in SSc, one study found that ICs from serum with ATA, but not ACA, resulted in IFN-α production by plasmacytoid dendritic cells.[144] However, later studies showed that IFN-α production was dependent on RNA binding, as this production was only observed for ATA- and ACA-positive serum that also contained RNA-binding autoantibodies.[145] While some studies support the pathogenicity of ATA- and ACA-ICs, the precise mechanisms by which these nucleic-acid containing ICs contribute to SSc pathogenesis remain unclear.

3.4 | Summary: Pathogenicity of autoantibodies in rheumatic diseases and

Autoantibodies can exert direct pathogenic effects by targeting their antigen with the Fab domain. However, when soluble antigens are bound, formed ICs can activate various pro-inflammatory pathways via their Fc part. Although evidence for ACA and ATA is not very clear, for other rheumatic diseases, including RA, pathogenicity of the autoantibody might predominantly arise from this IC formation, leading to the activation of pro-inflammatory effector mechanisms. More studies using isolated ACA and ATA in vitro could determine if this is also applicable for these autoantibodies in SSc.

4 | FC GLYCOSYLATION OF AUTOANTIBODIES IN RHEUMATIC DISEASES

Besides binding of their antigens with the Fab domains, antibodies can mediate immune responses through their non-specific Fc domain, leading to outcomes such as complement-dependent cytotoxicity (CDC), antibody-dependent cellular toxicity (ADCC), and antibody-dependent phagocytosis (ADCP). These responses depend on the strength of interactions between the Fc domain and various proteins, including FcRs and complement protein C1q. PTMs, such as glycosylation, can influence these binding affinities and specificities.[146] Proteins can undergo N-linked glycosylation, where glycans are attached to an asparagine (Asn or N) within a N-glycosylation consensus sequence. This sequence consists of asparagine-X-serine/threonine (N-X-S/T) where 'X' can be any amino acids except for proline.[147] Additionally, N-glycosylation can also occur at an asparagine-X-cysteine (N-X-C) motif, although this is less common.[148] All Ig isotypes have evolutionary conserved N-glycosylation sites in their Fc constant domains.[149] IgG, the most abundant immunoglobulin in circulation, has one conserved glycosylation motif located on N297 in the CH2 chain (Figure 1). This glycosylation site is fully N-glycosylated in healthy IgG. Most IgG Fc N-glycans have complex structures, typically containing a di-antennary arrangement with varying levels of antennary galactose, and a core fucose.[150] Even minor changes in glycan composition can significantly affect the conformation of the Fc region. These conformational changes can alter how the Fc regions interacts with receptor proteins, thereby modulating the antibody's effector functions, including ADCC and CDC.[151,152]

4.1 | Fc glycosylation rheumatic diseases

In rheumatic diseases, low galactosylation levels of total IgG Fc glycans are frequently associated with inflammation and disease progression.[150] For example, reduced terminal galactose has been observed in Fc glycans of total serum IgG from patients with RA compared to healthy donor.[153,154] Similarly, during pregnancy — a period associated with temporary disease remission — galactosylation levels of total IgG increase, followed by a rapid decrease after birth.[155] Interestingly, no protective effect of Fc glycosylation was observed for IgA during pregnancy, as no associations with disease activity were found.[156] Contrarily, reduced disease activity correlated with higher levels of IgG Fc galactosylation and sialylation in patients with RA.[157] Notably, these studies were performed using

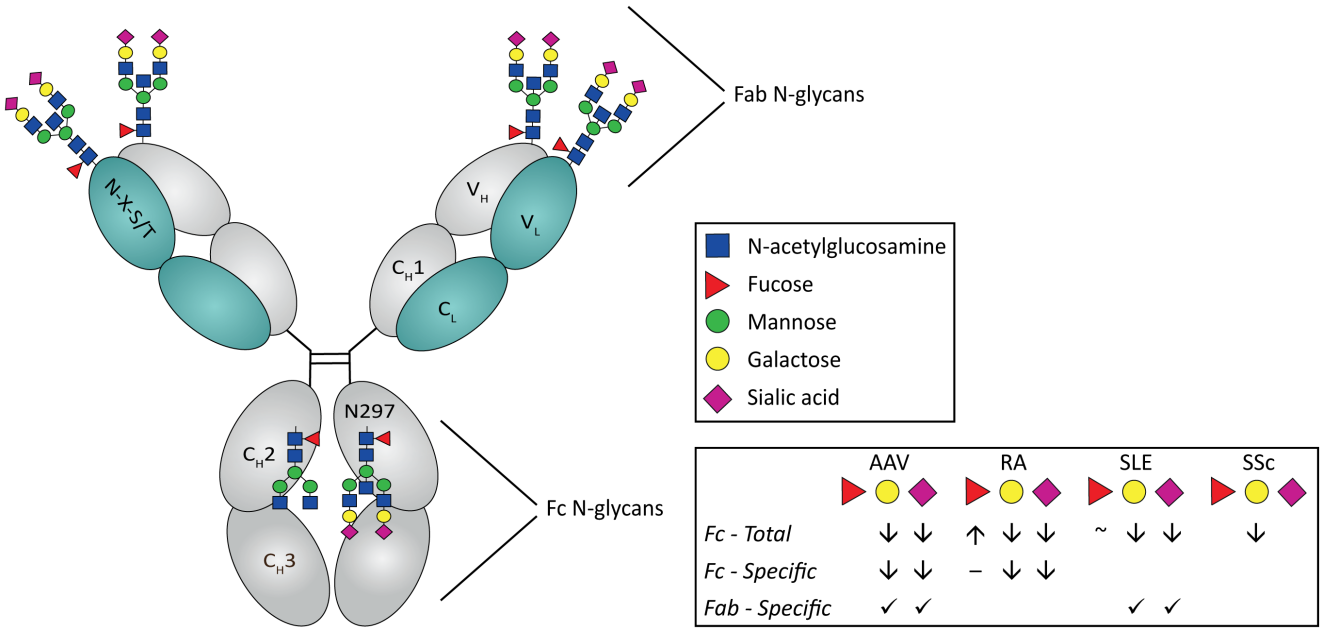

FIGURE 1 Schematic representation of an IgG molecule. The Fc domain is fully N-glycosylated at N297, while theFab domain is N-glycosylated at N-X-S/T consensus motifs in 15%–25% of healthy IgG. Table depicts changes in IgG-Fc fucosylation, galactosylation, and sialylation and presence of IgG-Fab glycans in rheumatic diseases.

lectin-based assays. Therefore, as no specific Fc glycan analysis was performed, these results could have been affected by N-glycans in the Fab domain. In patients with AAV, lower levels of galactosylation and sialyation on total IgG have been associated with impending relapse.[158] Another study reported lower levels of galactosylated and higher levels of agalactosylated IgG for MPO-positive and PR3-positive AAV patients. However, as total glycans were released with glycoamidases from almonds with no subsequent specific IgG Fc N-glycan analysis, contributions of Fab N-glycans cannot be excluded.[159] In a study of women with SLE ($n=188$), patients with lupus nephritis had lower levels of sialylated, galactosylated, and fucosylated glycans compared to those without this inflammatory complication, while bisected N-acetylglucosamine (GlcNAc) glycans were increased in SLE patients with lupus nephritis.[160] However, similar to the AAV study, glycans were released using PNGase F, a glycoamidase that removes N-glycans from both Fc and Fab domain.[161] Therefore, the findings can be affected by N-glycans attached to the Fab domain.

Only a few studies have investigated Fc glycosylation in SSc. In a bleomycin mouse model, reduced levels of IgG2 sialylation and IgG3 galactosylation levels were observed,[162] which is consistent with the reduced IgG Fc sialylation seen in mouse models of RA.[163] In humans, significant lower IgG galactosylation has been observed in SSc patients ($n=298$) compared to healthy controls ($n=436$) and patients with localized scleroderma ($n=93$).[164] Low IgG galactosylation was significantly correlated with the dcSSc subset, mRSS, and erythrocyte sedimentation rate. This suggests that the IgG galactosylation ratio of total IgG molecules might reflect inflammation as well as the degree and severity of skin fibrosis in SSc. Unfortunately, the study did not address IgG subclass specific IgG galactosylation nor extended the analyses to other glycan traits, such as Fc sialylation. Additionally, while ANA-specific IgG galactosylation was not analyzed, no clear correlations were observed between IgG galactosylation and the presence of ACA or ATA. Further studies specifically measuring Fc N-glycans are necessary to gain more insight into the glycosylation patterns of total IgG in SSc.

4.2 | Fc glycosylation patterns antigen-specific IgG

While detailed analyses on Fc glycans in total IgG (and to a lesser extent IgA) are available, only a few studies have addressed the Fc glycan profile for antigen-specific autoantibodies. To date, no published articles specifically address the Fc glycosylation traits of autoantibodies in SSc. The only available data comes from a poster presentation that reported the purification of ACA from sera of 24 patients with rheumatic diseases.[165] This study compared the glycosylation traits of total IgG1 and centromere-specific IgG1. It reported significant decreases in both sialylation and bisecting galactosylation for centromere A-specific IgG. However, the study included only five patients with SSc, and detailed information is lacking.

Although there is no data on Fc glycosylation traits for ACA and ATA in SSc, research on other autoimmune diseases, such as RA and SLE, provides valuable insights. In ACPA-positive arthralgia patients, antigen-specific IgG1 glycosylation patterns were comparable to total IgG1.[166] However, prior to RA onset, ACPA-IgG1 displayed lower galactosylation levels compared to patients who did not progress to RA. In established RA, ACPA-IgG1 exhibited a specific Fc glycan profile distinct from total IgG1 in the same patients.[167] Interestingly, in a small study with 18 RA patients, ACPA-IgG4 glycosylation patterns differed from ACPA-IgG1 patterns, with higher levels of sialylated glycans.[168]

In patients with AAV, while Fc N-glycans of MPO-specific IgG did not differ from total IgG of AAV patients, galactosylation levels were lower compared to total IgG of healthy controls.[169] Different outcomes have been reported for Fc N-glycan glycosylation pattens for PR3-specific IgG. Espy et al. found a correlation between vasculitis development and low sialylation levels of anti-PR3 IgG, whereas Wuhrer et al. did not observe this negative correlation between disease activity and anti-PR3 IgG sialylation, though anti-PR3 IgG1 Fc galactosylation, sialylation, and bisection were reduced compared to total IgG1 from the same patients.[170,171] This discrepancy may be due to the use of Sambucus nigra agglutinin (SNA) lectin in the initial study, which preferentially binds sialic residues in the Fab region over those in the Fc domain. Lastly, low galactosylation and sialyation levels of total IgG1, but not PR3-ANCA IgG1, predicted disease relapse, indicating a complex interplay between total and antigen-specific IgG that also depends on the disease state.[172]

In SLE, anti-histone IgG was found to be less sialylated compared to total IgG.[173] The authors suggested that this undersialylation might contribute to inflammation by enhancing the engagement of polymorphonuclear cells in the clearance of apoptotic cells. However, enrichment of Fc-sialylated IgG was performed using SNA lectin, a method as mentioned earlier preferentially binding Fab-sialylated IgG over Fc-sialylated IgG.[174–177] Consequently, using SNA lectin purification to assess Fc sialylation may lead to a skewed autoantibody pattern and not accurately reflect Fc sialylation levels. Another study explored the effects of EndoS treatment in BSBX mice, which spontaneously develop SLE-like disease, including the production of nuclear autoantibodies.[178] EndoS is an enzyme that cleaves the sugar moiety of IgG after the first GlcNAc, thereby interfering with IgG-FcγR interactions. The results showed a significant improvement in survival rates for mice treated with EndoS, with 40% survival after 55 weeks compared to only 20% survival in control mice after 20 weeks. As these studies were conducted in mice, there may be differences in disease manifestation and immune responses compared to humans. A study involving treatment-naïve SLE patients ($n=101$) positive for anti-dsDNA antibodies utilized cluster analysis on UPLC-MS to reveal distinct glycosylation patterns between anti-dsDNA IgG and total IgG.[179] Interestingly, a correlation was found between disease activity and increased levels of galactosylation, and sialylation of anti-dsDNA antibodies, despite

the general association of decreased galactosylation and sialylation with pro-inflammatory effects.[167,180] However, the authors employed machine learning on a relatively small sample size and categorized IgG samples based on disease activity scores on beforehand, which is not a common method in Fc glycopeptide analysis and could influence reported outcomes.

Although these studies provide insights into IgG Fc glycosylation patterns, examining these glycans in purified ACA and ATA would offer the most valuable information for understanding their roles in SSc.

4.3 | Effect of glycosylation on Fc effector functions

As mentioned earlier, antibodies can mediate a wide range of immune responses via their Fc domain, but these responses depend on the strength of interactions between the Fc domain and the different receptors. Glycosylation patterns in an antibody's Fc region can modulate these interactions, as afucosylated IgG1 is associated with improved binding to FcRγIIIa and ADCC, while sialylation reduced FcRγIII-mediated ADCC (Fig. 1).[181,182] Conversely, IgG1 with terminal galactosylation promotes CDC through enhanced binding to C1q.[183] Higher levels of galactosylation and sialylation are also linked to enhanced affinity for FcRγIIa, resulting in enhanced ADCP.[184] Recently, comprehensive overviews of glycosylation effects on effector functions in general have been published elsewhere.[146,150,185] Although detailed studies regarding the effect of glycosylation on Fc effector function in rheumatic diseases are limited, one study reported that agalactosylated ACPA-IgG significantly enhanced TNF-α release in an FcγRI-dependent manner compared to healthy IgG. However, these findings should be interpreted with caution as mechanistic details are lacking.[186] Other studies suggest a role of asialylated anti-PR3 and ACPA by type I FcγR-mediated cellular activation, but these assumptions have not been thoroughly evaluated.[167,171]

4.4 | Summary: Fc glycosylation of autoantibodies in rheumatic diseases

Over the years, multiple studies have investigated the role of Fc glycosylation in rheumatic diseases. While insights into total Ig(G) Fc glycosylation have been valuable, examining glycan profiles of disease-specific autoantibodies could provide a deeper understanding of how aberrant Fc glycosylation contributes to disease pathogenesis. Currently, only a limited number of rheumatic diseases have had their antigen-specific IgG Fc N-glycosylation profiles revealed. Additionally, most studies primarily speculate on the effects of autoantibody Fc glycosylation on effector functions. Therefore, future research should focus on elucidating the impact of autoantibody glycosylation patterns, including those of ACA and ATA, on the pathogenesis of rheumatic diseases.

5 | FAB GLYCOSYLATION OF AUTOANTIBODIES IN RHEUMATIC DISEASES

In addition to the conserved N-glycosylation sequence in the Fc region, approximately 15%–25% of circulating human IgG contains N-glycans in their Fab domain (Fig. 1).[187] Among the IgG subclasses, IgG4 exhibits the highest percentage of Fab glycosylation (44%), whereas the other IgG subclasses are less Fab glycosylated (IgG1: 12%, IgG2: 11%, IgG3: 15%).[188] These Fab glycans generally exhibit higher levels of bisection, galactosylation, and sialylation compared to their Fc counterparts, while fucosylation is reduced.[189,190] Interestingly, whereas Fc glycosylation locations are evolutionarily conserved, only five germline-encoded alleles (IGHV1-8, IGHV4-34, IGHV5-10-1, IGLV3-12, and IGLV5-37) possess N-linked glycosylation sites in the Fab domain.[187] Consequently, the naïve B-cell repertoire typically lacks these sites, with most Fab N-glycosylation sites being introduced during antigen-specific immune responses following somatic hypermutation.[188,191] These sites are predominantly located near antigen-binding pockets in both heavy and light chains. Similar to Fc glycans, Fab glycans influence the structural composition and subsequent stability of antibodies. Additionally, introducing an N-glycosylation motif in monoclonal antibody sequences has been shown to affect the formation of antibody aggregates and ICs, suggesting potential modulation of IgG effector functions.[192–195]

5.1 | Fab glycosylation in rheumatic diseases

In contrast to the Fab glycosylation levels observed in healthy individuals, initial studies using size-exclusion chromatography (SEC) demonstrated that ACPA-IgG has a higher molecular mass compared to total IgG, indicating a greater abundance of N-linked glycans on the Fab domain of these ACPA.[196] Furthermore, these Fab glycans were highly sialylated and present on more than 90% of ACPA-IgG, which was five times higher than the level found on total IgG from the same patient.[197] Additionally, autoantibodies in synovial fluid, sampled from the site of inflammation, exhibited a Fab N-glycan prevalence of more than 100%, suggesting that multiple glycans are attached to a single ACPA-IgG molecule. The prevalence of ACPA Fab glycans is linked to disease manifestations: while ACPA Fab N-glycans are abundant at disease onset, lower levels are associated with an increased likelihood of drug-free remission.[198,199]

Similar findings were reported for AAV, where affinity-purified anti-MPO IgG showed higher levels of Fab N glycosylation than total IgG. These results were further confirmed by mass-spectrometry.[169,200] Likewise, SNA-binding anti-PR3 IgG induced higher levels of reactive oxygen species produced by neutrophils compared to non-SNA binding fractions, suggesting Fab glycans affect autoantibody binding to neutrophils.[200] In a larger study encompassing multiple autoimmune diseases including RA, AAV, and SLE, increased levels of Fab glycosylation were observed for anti-CCP2 (86%), anti-PR3 (31%), and anti-dsDNA (26%) antibodies compared to total IgG Fab glycosylation from the same patients.[201]

Interestingly, in addition to anti-dsDNA antibodies, higher levels of Fab glycans were noted for anti-Smith antibodies in SLE patients, whereas anti-Ro52 antibodies did not show increased Fab glycosylation compared to total IgG.

5.2 | Fab glycosylation in SSc

Although multiple studies have investigated Fab glycosylation of antigen-specific IgG for autoimmune diseases,[169,200–202] there are no published reports on the Fab glycosylation of SSc-specific autoantibodies. To address this gap, a pilot experiment was conducted to determine if IgG and IgA specific for TOP1 or CENP-B harbor Fab glycans, using previously described SEC. Since glycans add approximately ~2.4 kDa to an antibody's molecular mass,[202] this typically results in earlier elution for Fab glycosylated antibodies. In this pilot experiment, neither ACA-IgG nor ACA-IgA exhibited earlier elution compared to total IgG or IgA of the same patient (Figure 2A,B). Similarly, TOP1-specific IgG and IgA showed comparable elution profiles to total IgG and IgA (Figure 2C,D). This contrasts with ACPA-IgG, which is known to be highly Fab glycosylated (~90%),[196,197] resulting in a higher molecular mass and earlier elution compared to total IgG (Figure 2E). Overall, these results did not provide evidence for Fab glycosylation of IgG and IgA specific for TOP1 or CENP-B in the tested samples. Sequences from patient-derived ACA or ATA mAbs could offer more insight in potential Fab glycosylation; however, no such sequences have been published to date.

5.3 | Role of Fab glycosylation in rheumatic diseases

Fab glycans can significantly alter the structure of the antigen-binding domain, thereby potentially affecting antigen affinity. Indeed, Fab glycans on ACPA mAbs have been shown to reduce binding to low-affinity antigens while maintaining binding to high-affinity antigens, compared to ACPA mAbs without Fab glycans.[203] This effect is likely due to the close proximity of Fab glycans to the antigen-binding pocket. However, it is important to note that this conclusion is based on crystal structures of only two Fab fragments, which limits the generalizability of these findings.

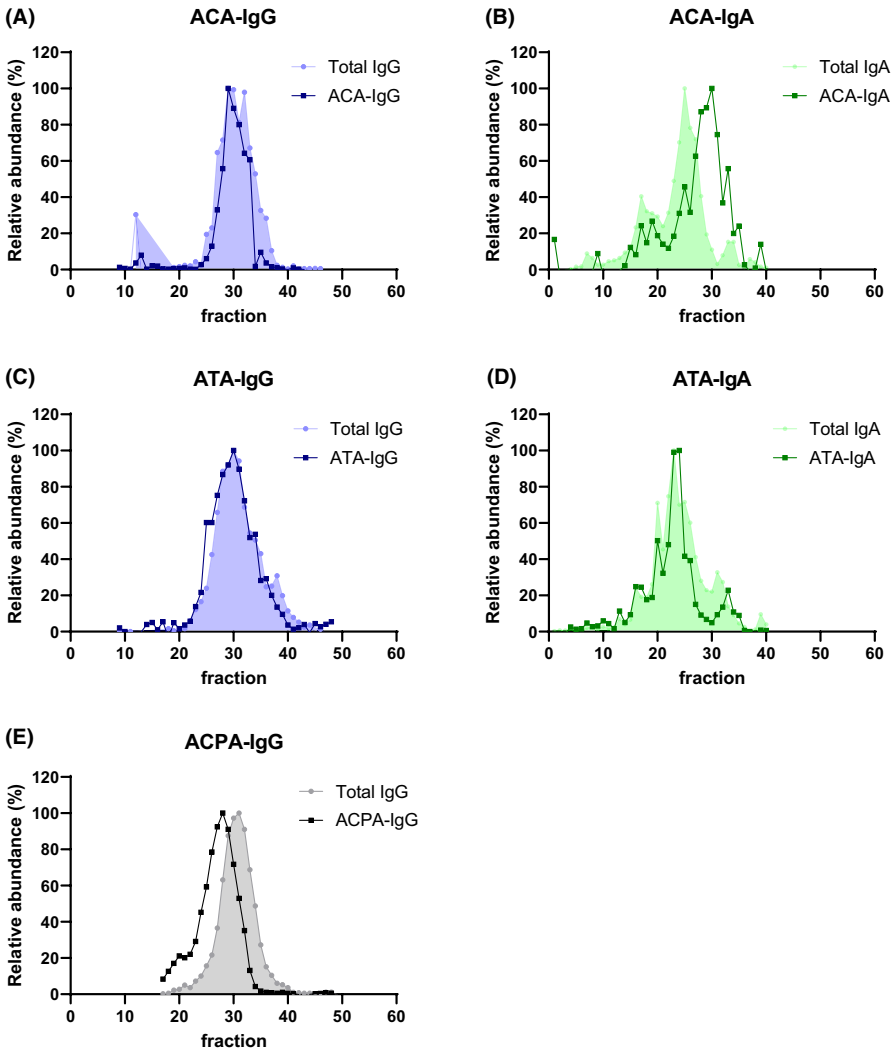

FIGURE 2 ACA and ATA do not exhibit a higher molecular weight compared to total IgG and IGA, providing no indication for the presence of Fab glycans. ACA$^+$ SSc patient serum fractionation by size exclusion chromatography, followed by ELISA detection, shows that ACA-IgG (A; anti-centromere B IgG antibodies) and ACA-IgA (B; anti-centromere B IgA antibodies) do not elute earlier than total IgG or IgA molecules, respectively. Results of a representative patient are shown ($n = 3$). The earlier elution could also not be observed for ATA-IgG (C; anti-topoisomerase I IgG antibodies) and ATA-IgA (D; anti-topoisomerase I IgA antibodies) compared to total IgG or IgA antibodies, respectively. Results of a representative ATA$^+$ SSc patient are shown ($n = 6$). Serum of an anti-citrullinated protein antibodies (ACPA)-IgG positive RA patient was measured as positive control. ACPA-IgG (anti-CCP2 IgG antibodies) elute earlier than total IgG, which is an indication for the presence of Fab glycans (E).

Detailed studies into the location of N-glycosylation motifs in B cell receptor (BCR) sequences of ACPA-specific B cells revealed that the introduction of N-glycosylation sites in ACPA-IgG often requires multiple mutations and is predominantly found in specific positions.[204] The authors hypothesized that these mutations arise from affinity-independent, B cell-driven mechanisms. This suggests that autoreactive B cells with Fab glycans in their BCRs have a competitive advantage over B cells lacking such glycans, thereby linking Fab glycosylation of autoantibodies to the autoreactive B cell response.

5.4 | Summary: Fab glycosylation rheumatic diseases

Although antigen-specific autoantibodies in some rheumatic diseases, including RA and AAV, have been shown to possess N-glycans in their Fab domains, preliminary data for ACA and ATA did not indicate similar findings. Additionally, while ACPA is known to exhibit high levels of Fab N-glycans, the precise role of this Fab glycosylation in disease pathogenesis remains unclear. Current research suggests that Fab glycosylation may be more influenced by B cell-driven mechanisms rather than by affinity-dependent processes.

6 | CONCLUSION

SSc is a severe autoimmune disease characterized by a high prevalence of autoantibodies against TOP1 and CENP-B. Despite the disease-specific nature of these autoantibodies and their mutually exclusive coexistence, the mechanisms behind their development and their potential contributions to diseases pathogenesis remain unclear. This review summarized the existing literature on the association of ACA and ATA with disease activity and therapy, their potential direct pathogenicity, and their antibody characteristics including glycosylation. Additionally, relevant literature from other rheumatic diseases was discussed to provide context for the findings related to ACA and ATA.

Comparing ACA and ATA to autoantibodies from other rheumatic diseases reveals similarities to ACPA in terms to disease specificity, correlation with disease activity, and the seemingly minimal pathogenic role of antigen-specific Fab binding. However, differences emerge regarding responses to therapy and Fab glycosylation patterns. Notably, the role of autoreactive B cells in the pathogenesis of both diseases has become a focus of increasing interest. While ACA and ATA serve as valuable biomarkers for disease stratification and activity, further research into their origins and the associated B cell is response is crucial.

CONFLICT OF INTEREST STATEMENT

All authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data supporting the findings of Figure 2 in this study are available from the corresponding author upon reasonable request. For all other sections no data sharing is applicable.

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