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Emerging molecular mechanisms and therapeutics of autoimmune liver or kidney diseases

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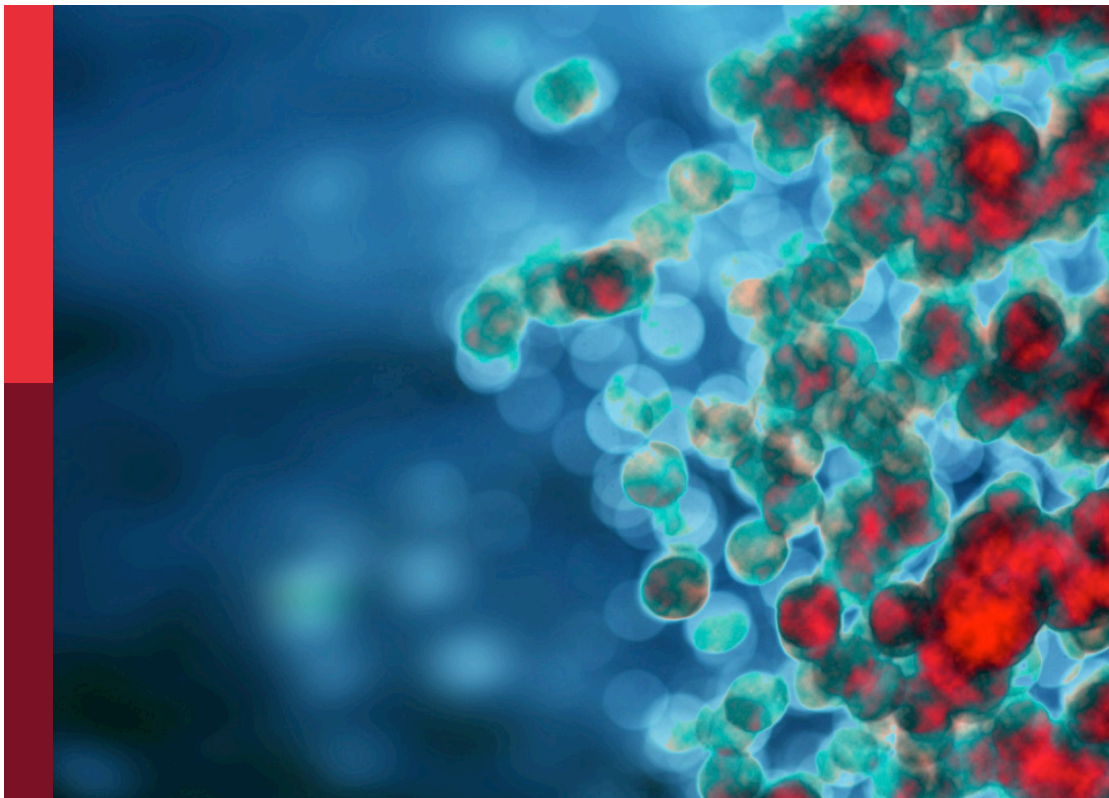

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Emerging molecular mechanisms and therapeutics of autoimmune liver or kidney diseases

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Telitacicept for autoimmune nephropathy

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B cells and the humoral immunity are important players in the pathogenesis of autoimmune diseases. BAFF (also known as BLYS) and a proliferation-inducing ligand APRIL are required for the maintenance of the B-cell pool and humoral immunity. BAFF and APRIL can promote B-cell differentiation, maturation, and plasma cell antibody secretion. BAFF/APRIL overexpression has been identified in several autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus, IgA nephropathy, etc. Telitacicept, a novel fully human TACI-Fc fusion protein that binds both BAFF and APRIL, was approved in China in March 2021 for the treatment of systemic lupus erythematosus at a recommended dose of 160 mg/w subcutaneously and is in clinical trials for the treatment of multiple indications in other autoimmune diseases. In this review, we explored telitacicept's mechanism of action and clinical data. In addition, the immune features of autoimmune nephropathy were discussed, emphasizing lupus nephritis, IgA nephropathy, and membranous nephropathy.

KEYWORDS

B-cell activating factor BAFF, a proliferation-inducing ligand APRIL, telitacicept, lupus nephritis, IgA nephropathy

1 Introduction

According to a national cross-sectional survey conducted in 2018-2019, the prevalence of chronic kidney disease in China is 8.2%, affecting 82 million people, which is down from 10.8% a decade ago, but the epidemiological situation remains grim (1, 2). Although the proportion of metabolically related secondary kidney diseases such as diabetes and hypertension is increasing (3), the proportion of immune-related kidney diseases such as lupus nephritis, IgA nephropathy, and membranous nephropathy remains high (4). Autoimmune abnormalities play a vital role in the development and progression of autoimmune nephropathies, and immunomodulation is an important strategy for the treatment of these diseases. In the past, treating autoimmune nephropathy mainly relied on hormones and immunosuppressants. However, with the in-depth exploration of the pathogenesis of autoimmune nephropathy and the rapid development of biomedical research, monoclonal antibodies, such as rituximab and belimumab, have been increasingly applied to kidney disease. As a new biological agent, telitacicept has preliminarily shown good therapeutic effect in the clinical studies in the fields of

systemic lupus erythematosus, IgA nephropathy, myasthenia gravis, rheumatoid arthritis, Sjogren's syndrome, etc. This review summarized and analyzed the pharmacological mechanism, metabolic characteristics, and clinical application of telitacicept. It also comprehensively presented the application of telitacicept in autoimmune nephropathy and its future application prospects.

2 Autoimmune nephropathy

Many renal diseases have been linked to autoimmune damage, such as lupus nephritis, IgA nephropathy, autoimmune membranous nephropathy, anti-neutrophil cytoplasmic antibody-associated glomerulonephritis, anti-glomerular basal-membrane glomerulonephritis, and C3 nephropathy. B cells play a critical role in the initiation and progression of autoimmune nephropathy. They can differentiate into plasma cells and secrete autoantibodies that act specifically or non-specifically on kidney antigens to form immune complexes and then cause kidney damage (5). B cells undergo several developmental stages in the bone marrow, such as progenitor B cells, pre-B cells, immature B cells, and mature B cells (Figure 1). Mature B cells, also known as initial B cells, reach the B-cell region of peripheral immune organs and settle down, where they receive the stimulation of foreign antigens, activate, proliferate, and further differentiate and mature into plasma cells and memory B cells (7). Plasma cells include short-lived and long-lived plasma cells. Short-lived plasma cells secrete a large number of autoantibodies, which contribute to the outbreak of autoimmune diseases. Long-lived plasma cells (also known as autoreactive plasma cells) reside in the bone marrow and inflammatory tissues. They can continuously secrete autoantibodies to maintain the chronic inflammatory process without relying on antigenic stimulation or the assistance of B and T cells. Long-lived plasma cells are resistant to traditional immunosuppressive agents and biologics that target CD20 B cells and are associated with difficulties in treating autoimmune diseases (8). B cells can be divided into B1 and B2 cell lines according to whether they play innate or adaptive immune functions. B1 cells belong to innate immune cells, while B2 cells are the primary cells that secrete antibodies and participate in the humoral immune response (7). A number of cytokines are involved in the development and differentiation of B cells, of which BAFF and APRIL are key factors. Both BAFF and APRIL are members of the tumor necrosis factor ligand superfamily. BAFF and APRIL have two receptors: TACI (transmembrane activator and calmodulin cyclin ligand interaction factor) and BCMA (B-cell maturation antigen). In addition, BAFF can bind to a third receptor, BAFF-R (also called BR3). These receptors are usually expressed by immune cells of the B cell lineage (9). BAFF is a transitional and mature B-cell survival factor that explicitly binds B lymphocytes, co-stimulates their proliferation, and promotes the survival of splenic B cells *in vitro* (10, 11). APRIL mediates IgA class transformation and recombination and maintains the survival of plasma cells (including long-lived plasma cells) (12, 13). High expression of BAFF and APRIL in patients with autoimmune diseases favors the survival of plasma cells, which leads to sustained and enhanced production of autoantibodies that can result in kidney and other tissue damage (14). The pathogenesis of lupus nephritis, IgA nephropathy, membranous nephropathy, and BAFF and APRIL's role in developing the disease are discussed below.

2.1 Lupus nephritis

Systemic lupus erythematosus (SLE) is an autoimmune disease that can affect multiple systems throughout the body, with approximately 60% of cases involving the kidneys, known as lupus nephritis (LN) (15). The pathogenesis of lupus nephritis is multifaceted and incompletely defined, and it is currently believed that sex hormones and environmental exposures can lead to immune system dysfunction in genetically susceptible individuals, such as overreaction of B and T cells, loss of immune tolerance to

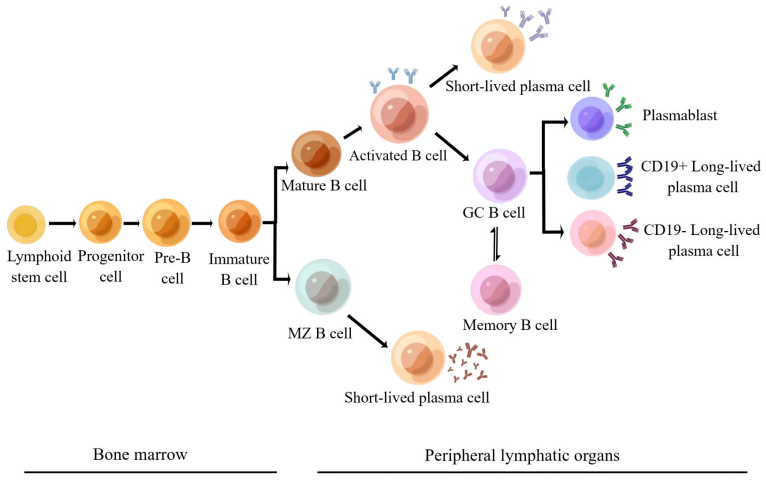

FIGURE 1
Development and differentiation of B2 cell lines (6) (Adapted from Schrezenmeier et al. By Figdraw).

autoantigens, deficiencies in antibody production and clearance, circulation and tissue deposition of immune complexes, activation of complement and cytokines, and kidney damage (16). Michelle and DC et al. found that serum BAFF levels were higher in SLE patients than in healthy controls and that BAFF levels were positively correlated with CD19+ B cell percentage and the MEX-SLEDAI disease activity score (the Mexican version of the lupus disease activity classification standard) in SLE patients. Serum APRIL levels were also higher in SLE patients than in healthy controls and positively correlated with MEX-SLEDAI and SLICC (International Clinical Collaboration classification criteria) scores (17, 18). The above results are consistent with previous findings of elevated BAFF and APRIL levels in lupus-susceptible mice with SLE (19–23). Matthias et al. discovered that APRIL and BAFF mRNA levels were significantly increased (12-fold and 30-fold, respectively) in the glomeruli of patients with proliferative lupus nephritis, as were tubule interstitial expressions of APRIL, BAFF, BCMA, and TACI (24). Bertrand et al. showed that, compared with control mice, mice treated with the selective APRIL inhibitory antibody Apophe had significantly reduced proteinuria, glomerular cell reduction, and PAS-positive substance deposition at the age of 6 months. There was no significant difference in morbidity and mortality between Apophe-treated mice and control mice 8 weeks after the treatment was stopped (25). Furthermore, studies showed reduced mortality and lower serum IgM, IgG, and anti-DNA antibody levels in APRIL-deficient lupus nephritis mice. Therefore, targeting BAFF/APRIL is expected to be a new strategy for treating lupus nephritis (26). BAFF's monoclonal antibody, belimumab, specifically neutralizes BAFF. Several large, randomized, controlled phase III clinical trials compared the safety and efficacy of belimumab to standard treatment protocols, demonstrating significantly superior efficacy while maintaining a similar safety profile. This suggests that using targeted BAFF to treat lupus nephritis is a good idea (27–29).

2.2 IgA nephropathy

The most common primary glomerular disease worldwide is IgA nephropathy (IgAN), which is characterized by mesangial proliferation and IgA deposition in the glomeruli (30). Although the pathogenesis of IgAN has not been fully elucidated, the theory of multiple attacks is widely accepted. Genetic predisposition factors and abnormal intestinal mucosal immunity lead to increased IgA1 (Gd-IgA1) levels of abnormal glycosylation in individual circulation. In addition, the body produces anti-glycan antibodies that can recognize Gd-IgA1. Abnormally elevated serum Gd-IgA1 and specific anti-glycan antibodies form immune complexes and deposit in renal tissue, leading to proliferation of mesangial cells and extracellular matrix, secretion of cytokines and chemokines, and activation of the local complement bypass pathway, resulting in renal injury (31). Therefore, Gd-IgA1 production is at the core of the pathogenesis. However, the mechanism of Gd-IgA1 production is not fully elucidated. It is believed that innate immune activation mediated by toll-like receptor 9 (TLR9) is involved in the production of Gd-IgA1 (32, 33). McCarthy et al. reported elevated serum and intestinal lamina propria IgA levels in BAFF-overexpressing transgenic mice, and IgA deposition was found in the glomerular mesangium (34). W. Li et al. found that serum BAFF levels were positively correlated with IgA1 levels and mesangial IgA deposition density in IgAN patients (35). Xin et al. revealed that serum BAFF levels were increased in IgAN patients and were associated with clinical and pathologic features of the disease (36). The expression of the APRIL gene in the tonsil germatogenesis center of IgAN patients was increased and correlated with serum Gd-IgA1 level and disease severity. These findings imply that both BAFF and APRIL may be responsible for the creation of Gd-IgA1 (37). Makita et al. looked into the relationship between BAFF/APRIL and TLR9 activation and discovered that APRIL is important in TLR9-induced nephritis-induced IgA overproduction and IgG-IgA IC formation. TLR9 activation increased APRIL gene expression and serum levels. In spleen cells, serum abnormal glycosylated IgA levels were correlated with BAFF and APRIL expression levels, while serum IgG-IgA IC levels were matched with APRIL expression levels but not with BAFF expression levels (38). As a result, targeting BAFF/APRIL could become a new therapeutic strategy for IgAN.

2.3 Idiopathic membranous nephropathy

Membranous nephropathy is the most common pathological type of adult nephrotic syndrome, affecting approximately 30% of nephrotic syndrome patients (39, 40). Idiopathic membranous nephropathy (IMN) is responsible for at least 80% of all cases of membranous nephropathy (5). Idiopathic membranous nephropathy is an autoimmune glomerular disease caused by circulating autoantibodies against glomerular podocyte antigens (M-type phospholipase A2 receptor PLA2R and type 1 thrombospondin domain-containing 7A THSD7A). It is characterized by the deposition of large amounts of immune complexes on the epithelial side of glomerular capillary loops. PLA2R antibodies are found in approximately 70% of adult IMN patients, while THSD7A antibodies are found in approximately 2% of adult IMN patients. The sensitivity and specificity of PLA2R antibodies for the diagnosis of IMN are 0.78 and 0.99, respectively. Since antibodies are derived from plasma cells that are differentiated by B cells, B cells play a key role in the pathogenesis of idiopathic membranous nephropathy (41). BAFF and APRIL are involved in the differentiation and survival of B cells and in the conversion of immunoglobulin classes. Their overexpression is involved in various autoimmune diseases, however, the role of BAFF and APRIL in the pathogenesis of IMN and their association with the prognosis of IMN have not been clarified (23, 42, 43). Seung et al. discovered that plasma BAFF levels in IMN patients were higher than in healthy controls, while APRIL levels were comparable. Furthermore, BAFF levels were higher in relapse patients than in the control group, while APRIL levels were higher in non-remission patients. BAFF and APRIL expression levels, like those of other autoimmune diseases, are linked to renal prognosis (44). Rituximab has emerged as a new treatment option for refractory IMN in recent years. Ruggenenti et al. discovered that approximately 30% of IMN patients did not respond significantly to rituximab, which may be due to long-lived memory plasma cells that do not express CD20 (45). Telitacicept inhibits APRIL binding to long-lived plasma cells lacking CD20 expression as well as antibody

production. This opens up a new avenue for telitacicept in the treatment of refractory IMN.

3 A BAFF/APRIL dual inhibitor - telitacicept

3.1 Pharmacology

It is well known that B lymphocytes play a crucial part in the complex pathophysiology of autoimmune nephropathy. Therefore, inhibiting the production of pathogenic antibodies by B cells has become a therapeutic strategy for treating autoimmune nephropathy. BAFF is more involved in the development and maturation of B cells, while APRIL is mainly involved in activating mature B cells and the process by which plasma cells produce antibodies. Anti-BAFF monoclonal antibodies and recombinant fusion proteins (immunoglobulin Fc+TACI) are currently available as targeted therapies against BAFF and APRIL (21, 46). Telitacicept is a new full-human TACI-FC fusion protein prepared by using recombinant DNA technology to connect the extracellular segment of the receptor TACI on the surface of B cells and the Fc segment of IgG1 (Figure 2). It can bind BAFF and APRIL, effectively blocking their binding to the receptor (47). The immature B cells can be prevented from continuing to develop and mature by blocking BAFF, which is useful for preventing the recurrence of the condition. Blocking APRIL can prevent mature B cells from differentiating into plasma cells and impact the release of autoantibodies by autoreactive plasma cells, which can effectively manage the symptoms of the disease.

3.2 Pharmacokinetics

Currently, studies on the pharmacokinetics of telitacicept have been completed in patients with rheumatoid arthritis (RA), stable systemic lupus erythematosus, and healthy volunteers.

3.2.1 Pharmacokinetic profile of a single ascending dose of telitacicept in Chinese patients with rheumatoid arthritis

Linear

After normalizing the dose of telitacicept by body weight, the serum exposure total telitacicept and free telitacicept (i.e., the area AUClast and plasma peak concentration Cmax under the drug-time curve from time zero to the last quantifiable point) were linearly correlated with the weighted normalized dose of telitacicept. Combined with the elimination half-life, apparent clearance (total telitacicept 9.33~11.58 L/d) and apparent volume of distribution (total telitacicept 199.9~274.9 L) were observed in patients taking 180~540 mg telitacicept. In this dose range, total telitacicept and free telitacicept exhibited linear pharmacokinetics.

Absorption

The total serum telitacicept increased rapidly after single subcutaneous administration, and the median time for reaching the peak was 1 to 2 days in all dose groups. For example, when the dose was 180 mg, the mean serum peak concentration of free telitacicept was 929.9 ng/mL, and the mean peak time was 1.1 days.

Elimination

The mean terminal half-life of total telitacicept increased from 13.3~14.4 d at a low dose to 17.0~32.8 d at 180~540 mg. The BAFF-telitacicept complex elimination half-life increased with increasing telitacicept dose, indicating a shift from targeted clearance in the 1.2-18 mg telitacicept dose group to non-specific clearance in the 60-540 mg telitacicept dose group (48).

3.2.2 Pharmacokinetic characteristics of three different administration regimens in patients with RA

Serum total telitacicept and free telitacicept concentrations peaked within 1~1.5 days after each dose. The plasma peak concentration (Cmax) of the BAFF-telitacicept complex was 28987 IU/mL, 29329 IU/mL, and 65,919 IU/mL, respectively, for the three administration schemes (180 mg BIW, 180 mg QW, and 360 mg QW). The times to peak (Tmax) were 45.5 d, 56 d, and 40 d,

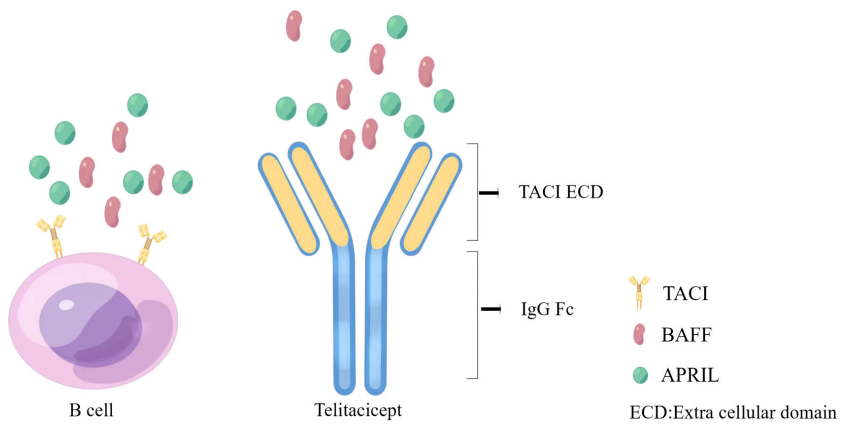

FIGURE 2
Mechanism of action for telitacicept (By Figdraw).

respectively. The BAFF-telitacicept complex was cleared by zero-order pharmacokinetics (49).

3.2.3 Pharmacokinetics of multiple doses of telitacicept in patients with SLE

Following multiple doses of telitacicept, total and free telitacicept reached its maximum serum concentration (Cmax) within 1 to 2 days. The mean elimination half-lives of total telitacicept and free telitacicept are 11.4~26.4 and 2.4~26.5 days, respectively (50).

3.2.4 Pharmacokinetic characteristics of telitacicept in healthy Chinese subjects

The median time for total telitacicept concentration to peak was 0.5~1 d in the 80~240 mg dose group, and the peak time was earlier in the low-dose group. The elimination half-lives of the three doses were roughly the same, ranging from 10.9 to 11.9 days. The median time for free telitacicept concentrations to peak was 1 day, and the clearance half-life increased slightly with increasing dose. The BAFF-telitacicept complex had a median peak time of 1557 days, and the peak time increased significantly with increasing dose. In the 160-240 mg dose range, free telitacicept demonstrated linear pharmacokinetics (51).

3.3 Comparison with other biological agents

Direct targeting of B cells – targeting CD20 receptors, such as the type I antibody rituximab and the type II antibody obinutuzumab, can largely eliminate peripheral B cells, including memory B cells, but not CD20- pre-B cells and plasma cells (short- and long-lived plasma cells). Indirect targeting of B cells – targeting B cell survival factors such as BAFF, as with belimumab, can directly inhibit B cell maturation and indirectly inhibit plasma cell maturation (short-lived plasma cells), but has no inhibitory effect on long-lived plasma cells. Simultaneous targeting of the B cell survival factors BAFF and APRIL, such as atacicept and telitacicept, can inhibit the transformation of immature B cells into mature B cells and mature B cells into plasma cells and promote the apoptosis of plasma cells (including long-lived plasma cells) (Table 1).

4 Application of telitacicept in autoimmune renal disease

4.1 Systemic lupus erythematosus

12 patients with mild systemic lupus erythematosus were randomly assigned to receive 180 mg of telitacicept or a placebo subcutaneous injection on days 0, 7, 14, and 21 in the phase I exploratory investigation. Considering the limited sample size, there was no discernible difference between the telitacicept and placebo groups in terms of SLEDAI scores. Telitacicept, however, reduced peripheral blood lymphocyte counts (CD19+ B cells and IgD+ B cells) and serum immunoglobulin levels in SLE patients (48).

In a phase IIb clinical trial, 249 patients with active systemic lupus erythematosus were randomly assigned to receive subcutaneous telitacicept 80 mg, 160 mg, 240 mg, or placebo (1:1:1:1) once weekly for 48 weeks in addition to standard of care. Telitacicept-treated patients had a significantly higher SLE Response Index (SRI4) response rate than placebo patients at week 48 (71.0% in the 80 mg group, 68.3% in the 160 mg group, and 75.8% in the 240 mg group, $p<0.0001$). Furthermore, the treatment group reduced the SELENA-SLEDAI score by 4 points more than the placebo group (50.0%) (75.8% in the 80 mg group, 77.8% in the 160 mg group, and 79.0% in the 240 mg group, $p<0.001$). At week 48, the occurrence of adverse events was comparable across all groups (57).

A self-controlled retrospective study assessed telitacicept's efficacy and safety in the treatment of children with refractory systemic lupus erythematosus (cSLE). After 5 to 26 weeks (80 or 160 mg per week) of telitacicept, the response rate of SRI4 in 15 refractory cSLE patients was 66.7% (10 cases). In 12 patients, the median hormone dose was reduced from 40 mg/d to 17.5 mg/d. 8 renal impaired patients with urine protein >0.5 g at baseline 24 hours before treatment showed a decrease in urine protein 24 hours after treatment. In 8 cases, 2 urine proteins turned negative, and 5 plasma albumin increased to normal. In addition, 3 of the 8 patients with renal impairment improved renal function to varying degrees (eGFR ml/min·1.73m2, from 17.4 to 26.6, 40.7 to 48.2, and 63.2 to 146.0, respectively) (58).

Preliminary data from a domestic phase III confirmatory study of telitacicept for the treatment of SLE are now available. In combination with standard therapy, 335 patients with SLE were randomly assigned to the telitacicept (160 mg) or placebo groups *via* subcutaneous injection once weekly for 52 weeks. The primary endpoint was reached at week 52, with a significantly higher proportion of patients in the telitacicept 160 mg group achieving SRI4 remission compared to the placebo group (82.6% vs. 38.1%, $p<0.001$) (59). The global multicenter Phase III clinical trial was approved by the European Union and the National Medicines Administration on September 26 and 28, 2022, respectively (60).

4.2 IgA nephropathy

Telitacicept's IgA nephropathy indication was approved by the US Food and Drug Administration (FDA), which exempted the Phase I clinical trial in the US and conducted the Phase II clinical trial directly. The first patient was enrolled and administered in November 2021. On November 18, 2022, the FDA approved a Phase III clinical trial of Telitacicept in the United States for the IgA nephropathy indication (61). The efficacy and safety of telitacicept in treating IgA nephropathy were initially evaluated in phase II domestic clinical trials. The data showed that after 24 weeks of treatment, urine protein levels were significantly reduced in subjects in the 240 mg group compared to baseline. In addition, the average 24-hour urine protein level was reduced by 49% compared to baseline, which was statistically significant compared to the

TABLE 1 Comparison of biologics directly and indirectly targeting.

	Molecular structure and mechanism of action	Disease in reference	Effectiveness and Safety	Applicable disease
rituximab	It is a human-mouse chimeric monoclonal CD20 type I antibody that specifically binds to the CD20 antigen on the surface of pre-B and mature B lymphocytes and initiates an immune response that mediates B-cell lysis (massive depletion of peripheral B cells, including peripheral blood memory B cells) (6).	proliferative lupus nephritis (52)	In a phase III clinical trial of lupus nephritis patients, the overall renal response rate at 52 weeks was greater in the Rituximab group than in the placebo group, although the difference was not statistically significant. Additionally, there was no change in the patient's clinical prognosis despite receiving Rituximab treatment for a full year. Adverse reactions include infusion adverse reactions, infection, etc (52).	Off-label for the treatment of SLE, MN, micropathological nephropathy, etc
obinutuzumab	It is a human-derived type II CD20 monoclonal antibody that targets the CD20 antigen expressed on the surface of pre-B lymphocytes and mature B lymphocytes and mediates B cell lysis. Obinutuzumab induces direct cell death with greater activity and affinity for the FcγRIII receptor protein than rituximab.	proliferative lupus nephritis (53)	When obinutuzumab was added to standard therapy alone, it resulted in a significantly higher rate of complete renal remission at week 52 than standard therapy alone. The most common adverse event was an infection, with an incidence that was similar to that seen in the control group (53).	Off-label for lupus nephritis
belimumab	It is a human lgG1λ monoclonal antibody specific for soluble human BAFF, which inhibits B cell survival (including autoreactive B cells) and B cell differentiation.	active lupus nephritis (29)	The remission rate of the urine protein-creatinine ratio and glomerular filtration rate at 104 weeks was higher in the belimumab group compared to the placebo group. Infection-related deaths occurred at a similar rate in both the belimumab group and the placebo group (29).	For active, autoantibody-positive systemic lupus erythematosus (SLE) patients 5 years of age and older with high disease activity (e.g., positive anti-dsDNA antibody and low complement, SELENA-SLEDAI score ≥ 8) despite conventional therapy
atacicept	TACI-FC fusion protein	active lupus nephritis (54)	The Phase IIb clinical trial of atacicept did not meet its primary endpoint, although there was a trend toward increased the SLE responder index 4 (SRI4) remission rates at week 24 in the 75 mg and 150 mg atacicept groups. Adverse reactions in the atacicept group were no higher than in the placebo group (54).	Clinical trials of Atacicept for RA, SLE, and others are ongoing (55)
telitacicept	TACI-FC fusion protein	SLE, IgA nephropathy, and others	As mentioned above, stage III SLE and stage IIb IgA nephropathy all showed effectiveness, the adverse reactions were within a controllable range, and symptomatic treatment was required.	Based on conventional treatment, there is still high disease activity and autoantibody-positive systemic lupus erythematosus; clinical trials for systemic myasthenia gravis are ongoing (56).

placebo group ($p<0.05$). Therefore, it reduces proteinuria in high-risk IgA nephropathy patients and may effectively reduce the risk of progression of IgA nephropathy (62).

4.3 Ongoing clinical trials

Seven autoimmune disease indications (including SLE, neuromyelitis optica spectrum disease, rheumatoid arthritis, IgA nephropathy, Sjogren's syndrome, multiple sclerosis, and myasthenia gravis) are in commercialization or clinical trials (63).

5 Telitacicept tolerability

A total of 89 adverse events, mostly mild or moderate, were reported in 12 patients in a phase I trial of multiple subcutaneous injections of telitacicept (180 mg QW*4) in SLE patients, with 14 likely to be related to telitacicept. Musculoskeletal and connective tissue diseases and infections were the most frequent side events, which were significantly higher in the telitacicept group than in the placebo group (7[77.78%] vs. 1[33.33%]) and slightly higher in the telitacicept group than in the placebo group (7[77.78%] vs. 2 [66.67%]). The majority of infections occurred within two weeks of the last dose, when the patient's immunoglobulin level had reached a trough, or after the addition of other immunosuppressive agents to treat SLE exacerbations. One patient in the telitacicept group experienced severe adverse events and reactions, including systemic lupus erythematosus activity index elevation and cholecystitis. Endotoxin shock occurred in one patient. Miliary tuberculosis and traumatic arthritis occurred in one patient. Epiglottitis occurred in one patient. However, none of them resulted in the experiment being terminated prematurely (50). In a phase IIb trial of multiple subcutaneous injections of telitacicept (80 mg, 160 mg, and 240 mg

QW*48) in SLE patients, the most common adverse events were upper respiratory tract infections and injection-site adverse reactions. The telitacicept and placebo groups had comparable rates of adverse and severe adverse events ($p > 0.05$). The study drug was not thought to be responsible for one death in the telitacicept 240 mg group (57). In the domestic phase III confirmatory SLE study, the incidence of adverse events and those leading to trial termination in the telitacicept group was similar to that in the placebo group (153 [91.6%]) vs. 142 [84.5%]. 8 [4.8%]) vs. 9 [5.4%]). However, the rate of serious adverse events was lower in the telitacicept group than in the placebo group (12 [7.2%] vs.24 [14.3%]). The most common adverse events were upper respiratory tract infection, decreased blood IgG and IgM, injection site adverse reactions, and urinary tract infections (59).

In a Phase I trial of a single subcutaneous injection of telitacicept in healthy volunteers, 36 subjects were randomly assigned to the telitacicept 80, 160, and 240 mg groups. A total of 42 mild or moderate adverse events were reported by 36 subjects. Elevated blood triglyceride levels, positive urinary white blood cells, and upper respiratory tract infections were all common side effects. Subcutaneous administration was well tolerated, with no unexpected side effects observed (51).

A total of 140 adverse events, all mild or moderate, were reported in a Phase I trial of a single subcutaneous injection of telitacicept (1.2, 6, 18, 60, 180, 360, and 540 mg) in 28 patients with RA. The most common adverse event was upper respiratory tract infection, and the telitacicept group was significantly higher than the placebo group (15 [71.43%]) vs. 3 [42.86%]) (48). In a trial of multiple subcutaneous injections of telitacicept (180 mg QW* 3,180 mg BIW* 8,360 mg QW*5) in RA patients, a total of 146 adverse events, all mild or moderate, were reported in 21 patients. The most frequent adverse effects were injection site reactions and upper respiratory tract infections. The telitacicept group outperformed the placebo group by a large margin (11 [78.6%] vs. 2 [28.6%]). 10 [71.43%]) vs.0 [0%]) (49).

In general, the adverse reactions of telitacicept mainly included infection, musculoskeletal and connective tissue diseases, injection site adverse reactions, etc. Yet, no significant adverse events occurred before the experiment came to an end, and effective symptom management was possible. In addition, a good safety profile was also shown in Phase II trials of telitacicept in IgA nephropathy, primary Sjogren's Syndrome, and myasthenia gravis (62, 64, 65).

6 Conclusion and prospect

The traditional treatment of autoimmune nephropathy mainly includes corticosteroids, cyclophosphamide, mycophenolate mofetil, and other immunosuppressive agents, which can non-specifically inhibit B cells and short-lived plasma cells. However, the efficacy has significant limitations, such as a low complete response rate, a long treatment cycle, and a significantly increased risk of infection and osteoporosis. Therefore, there is an urgent need to develop targeted drugs with better efficacy and safety. Telitacicept inhibits the B-cell survival factors BAFF and APRIL, preserving autoimmunity while exerting therapeutic effects. BAFF/APRIL overexpression is a common characteristic of several autoimmune nephropathies and other autoimmune diseases. So far, telitacicept is effective in clinical trials of SLE, Neuromyelitis optical spectrum disease, rheumatoid arthritis, IgA nephropathy, primary Sjogren's syndrome, relapsing-remitting multiple sclerosis, and systemic myasthenia gravis (56, 58, 63, 66). It is theoretically possible to demonstrate efficacy in idiopathic membranous nephropathy with abnormal BAFF/APRIL expression in autoimmune nephropathy. However, additional basic and clinical trials are required to confirm this. In addition, searching for appropriate specific treatment individuals (such as abnormal expression of BAFF/APRIL) is also a problem that must be solved before the current application of telitacicept so that patients can benefit more.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Oxidative stress and inflammation in diabetic nephropathy: role of polyphenols

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Diabetic nephropathy (DN) often leads to end-stage renal disease. Oxidative stress demonstrates a crucial act in the onset and progression of DN, which triggers various pathological processes while promoting the activation of inflammation and forming a vicious oxidative stress-inflammation cycle that induces podocyte injury, extracellular matrix accumulation, glomerulosclerosis, epithelial-mesenchymal transition, renal tubular atrophy, and proteinuria. Conventional treatments for DN have limited efficacy. Polyphenols, as antioxidants, are widely used in DN with multiple targets and fewer adverse effects. This review reveals the oxidative stress and oxidative stress-associated inflammation in DN that led to pathological damage to renal cells, including podocytes, endothelial cells, mesangial cells, and renal tubular epithelial cells. It demonstrates the potent antioxidant and anti-inflammatory properties by targeting Nrf2, SIRT1, HMGB1, NF-κB, and NLRP3 of polyphenols, including quercetin, resveratrol, curcumin, and phenolic acid. However, there remains a long way to a comprehensive understanding of molecular mechanisms and applications for the clinical therapy of polyphenols.

KEYWORDS

polyphenols, quercetin, resveratrol, curcumin, diabetic nephropathy, oxidative stress, inflammation

1 Introduction

Diabetes mellitus (DM) is a complicated chronic disease described by glucose dysregulation that is caused by absolute or relative defects, including type 1 diabetes (T1D) and type 2 diabetes (T2D). The global prevalence indicates that the number of DM is below half a billion individuals and is projected to increase by 25% and 51% in 2030 and 2045, respectively, putting enormous pressure on healthcare systems worldwide (1). As a complication of DM, diabetic nephropathy (DN), also called diabetic kidney disease

(DKD), is the primary reason for chronic kidney disease (CKD) and even end-stage renal disease (ESRD), which is related to increased morbidity and mortality in patients with diabetes. Moreover, approximately 30%-40% of diabetic patients with DN (2). Patients with DN require maintenance dialysis or a kidney transplant. However, these therapy approaches bring a considerable economic and psychological burden and consume substantial medical resources. Therefore, promoting the amelioration of DN is of vital clinical significance (3).

The pathogenesis of DN is complicated and still needs to be determined. It has been proven that rigorous management of blood pressure and blood glucose cannot prevent the progression of DN to ESRD, nor can it prevent DN-related deaths. Developing the understanding and research of DN's pathogenesis is crucial for expanding new methods for DN (4). Multiple pathways and mediators, including oxidative stress, inflammation, and angiotensin II (Ang-II), are involved in the incidence and progression of DN, of which oxidative stress is the most prominent (5, 6). Chronic hyperglycemia induces oxidative stress, promotes excess reactive oxygen species (ROS) production, reduces antioxidant capacity, induces the damage of oxidative stress in DNA and proteins, and stimulates the immune system to release inflammatory mediators and cytokines that affect glomerular capillaries and changes in renal tubular structure and function, thereby exacerbating renal and systemic damage. High glucose (HG)-induced overproduction of ROS is the primary initiator of cell damage in diabetes and its complications. For DN, although several therapies have been developed (7), such as Sodium-glucose cotransporter 2 (SGLT2), which effectively reduces inflammation and oxidative stress and has been shown to reduce the risk of major adverse events and progression of renal disease in patients with T2D, the ultimate treatment effect is not satisfactory (8).

Natural products, primarily from herbal sources, have long been explored as sources of drugs to treat a variety of major diseases. To date, significant efforts have been made to support and validate the potential effectiveness of natural and synthetic products in experimental studies and clinical applications, and indicating the antioxidant effects for kidney (9, 10). In preclinical studies, many natural products have recently been reported to alleviate kidney disease by modulating oxidative stress and inflammation (11). As a natural product, polyphenols are widely distributed in most plants and classified as phenolic acids, flavonoids, stilbenes, and lignans according to their structural properties (12).Nuclear factor E2-related factor 2 (Nrf2) is a significant regulator of antioxidant enzymes that protect the body from oxidative stress and inflammation, and Nrf2/antioxidant response element (ARE) signaling has been suggested as a promising target against oxidative stress-mediated diseases, such as diabetes and fibrosis. Dietary polyphenols, such as resveratrol, curcumin, and quercetin, can modulate Nrf2 signaling by mediating various kinases upstream of Nrf2 and also directly activate the expression of Nrf2 as well as downstream targets, such as heme oxygenase 1(HO-1) superoxide dismutase(SOD), and catalase (CAT), to inhibit oxidative stress and regulate inflammatory mediators (13–15).

Polyphenols exhibit anti-diabetic potential by reducing intestinal glucose absorption, increasing insulin secretion from pancreatic cells, and modulating gut microbiota and its metabolites (16–19). Natural polyphenols have wide-ranging pharmacological activities, particularly antioxidant activity and free radical-scavenging ability; moreover, they have been gradually used in the research of DN in recent years (20). This review provides a comprehensive summary of oxidative stress and oxidative stress-induced inflammation in DN and polyphenols targeting oxidative stress and inflammation to delay the progression of DN, which provides new insights into polyphenols as promising drug candidates for DN.

2 Oxidative stress and inflammation in DN

2.1 Role of oxidative stress

Oxidative stress is defined as an excessive accumulation of ROS caused by an imbalance between oxidants and antioxidants, resulting in oxidative damage to the body. Moderate ROS-mediated damage can usually be reversed, but excessive ROS production beyond the self-regulatory process often leads to irreversible damage to cellular function or death (21).ROS includes superoxide anion, hydrogen peroxide (H_2O_2), nitric oxide (NO), hydroxyl radical, and peroxynitrite (22). Mitochondria induce the production of ROS through the mitochondrial respiratory chain, which is the primary source of ROS. Other sources include nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX), changes in glucose metabolism, including polyol pathway flux, and changes in hemodynamics, advanced glycation end products (AGEs), and protein kinase C (PKC) (23, 24). Basal ROS levels are critical for maintaining various cellular tissue functions, such as gene expression, molecular transcription, and signaling transduction (25). However, excessive ROS accelerates pathological states, including the progression of DN, whereas the antioxidant defense system is activated to eliminate ROS generated from varied sources. Antioxidant enzymes include SOD, glutathione peroxidase (GSH-Px), CAT, glutathione reductase (GR), and paraoxonase (26). As markers of oxidative damage, malondialdehyde (MDA) and protein carbonyl can exacerbate oxidative damage in the body. Oxidative stress significantly affects the cause, onset, and process of DN, and hyperglycemia triggers the activation of the polyol pathway, AGEs, the receptor for advanced glycation end products (RAGE), and PKC. AGEs-RAGE signaling pathway activation potentiates the action of NOX and stimulates the production of ROS (27). Subsequently, ROS further interacted with NOX, aggravating the production of ROS (28). DN is associated with hypoxia, the generation of Ang II, and the production of ROS, leading to actin cytoskeleton reorganization of the podocyte, which induces podocyte injury, disrupts the glomerular filtration barrier and triggers proteinuria (29). Moreover, Ang II interferes with the formulation of ROS *via* the PKC/NADPH oxidase pathway, and ROS production is inhibited following the knockdown of PKC (30).

2.2 Oxidative stress and inflammation

Factors such as hyperglycemia and hypoxia induce oxidative stress and inflammatory responses. Additionally, along with

inducing tissue oxidative stress damage, the release of ROS triggers the aggregation of inflammatory cells and the formulation of inflammatory cytokines, growth factors, and transcription factors related to the pathological process of DN (31). The infiltration of inflammatory cells, such as lymphocytes, neutrophils, and macrophages, contributes to kidney injury in DN (32). The recruitment and differentiation of immune-inflammatory cells are regulated by numerous inflammatory cytokines, such as nuclear factor-kappaB (NF-κB), NOD-like receptor family pyrin domain containing 3 (NLRP3), interleukin-1β (IL-1β), IL-6, tumor necrosis factor-α (TNF-α), monocyte chemoattractant protein-1 (MCP-1), intercellular adhesion molecule-1 (ICAM-1) and transforming growth factor-β (TGF-β). These factors are expressed in renal vascular endothelial cells (ECs), podocytes, mesangial cells (MCs), fibroblasts, monocytes, macrophages, and renal tubular epithelial cells (RTECs) (33). NF-κB, a key class of nuclear transcription factors, is involved in immune-inflammatory responses, oxidative stress, and apoptosis (34). NF-κB typically exists in the cytoplasm in an inactive form, such as a heterodimer. It is transported to the nucleus when stimulated by various factors, such as ROS. It binds to the NF-κB binding site to activate the transcription of NF-κB and promote the release of adhesion molecules and pro-inflammatory factors, including MCP-1, ICAM-1, TGF-β1, IL-1β, IL-6, and TNF-α, which can also regulate ROS level (35). HG induces the expression of receptor activator of NF-κB in podocytes, which increases the expression of NOX4 and P22phox and mediates the progression of DN (36).

NLRP3 inflammasome is a multimeric protein complex that induces physiological and pathological inflammatory responses by sensing pathogens, mediates cell pyroptosis, and is a critical downstream factor of NF-κB. Moreover, NLRP3 inflammasome can be triggered by ROS, aggravating the inflammatory cascade and cell damage. Additionally, it mediates the release of inflammatory factors, triggers mitochondrial dysfunction, and promotes ROS formation (37). The inhibition of NLRP3 inflammasome improves renal function by markedly inhibiting HG-induced activation of NF-κB p65, the production of mitochondrial ROS (38). In *in vivo* and *in vitro* studies, HG induces infiltration of inflammatory cells, including macrophages; stimulates the formulation of inflammatory factors, including IL-1β, IL-6, MCP-1, and TNF-α; triggers oxidative stress; induces MDA expression; inhibits Nrf2 pathway activation; and diminishes the production of downstream enzymes, such as HO-1, NADPH quinone oxidoreductase-1 (NQO-1), GSH-Px, SOD and CAT (39, 40).

High mobility group box 1(HMGB1), a typical intranuclear non-histone protein, reaches the nucleus by both active secretion and passive release. Once transported to the cytoplasm, it is involved in the immune response. In contrast, when released outside the cell, it can act as a potent inflammatory mediator, either alone or as part of a pro-inflammatory cascade response to stimulate the immune system (41). Interestingly, HMGB1 is regulated by ROS and NLRP3, translocates from the nucleus to the cytoplasm, binds toll-like receptor (TLR)4, activates NF-κB, and ultimately induces an inflammatory response (42).Relatively, HMGB1 induces mitochondrial dysfunction, such as increased ROS production and mitochondrial fission (43, 44).Conversely, H2O2 in the mitochondria and nucleus induces the formation and secretion of HMGB1, which promotes inflammatory responses (45).

Oxidative stress and inflammation interact in the pathological mechanism of DN, disrupting the structure and function of the kidney. The foot processes (FP) of podocytes are lost or fused, which leads to podocyte hypertrophy and reduced levels of podocyte-related proteins, such as nephrin and podocin (46) adhesion and platelet thrombosis on ECs, which triggers endothelial dysfunction (47) production of fibronectin (FN), and collagen IV; and accumulation of extracellular matrix (ECM) in MCs, which leads to glomerulosclerosis (48). The inhibition of NF-κB translocation improves mesangial cell fibrosis and subsequently improves ECM accumulation (49). Moreover, Epithelial-mesenchymal transition (EMT) is induced by TGF-β, which is closely related to tubulointerstitial fibrosis (50, 51). Many studies support that oxidative stress and inflammation are interdependent and interconnected processes that coexist in an inflammatory environment. Inflammatory cells release large amounts of ROS at the site of inflammation, leading to increased oxidative damage. In addition, many ROS and oxidative stress products enhance the pro-inflammatory response. Inhibition of inflammatory response and oxidative stress, reduction in the number of renal collagen fibers and glomerulosclerosis, restoration of glomerular barrier function, persistent reduction in proteinuria (52), and reduction in the generation of serum creatinine (Scr) and blood urea nitrogen (BUN), recover renal function and ameliorate the process of DN to ESRD (53, 54).

2.3 Renal impairment

Oxidative stress-induced kidney damage includes direct and indirect mechanisms. ROS may damage the DNA, proteins, and lipids of tissue cells, directly leading to pathological changes in the glomeruli, renal tubules, and renal interstitium, including cellular components, such as decreased levels of nephrin, podocin, and podocyte-related proteins (55), fusion, and disappearance of FP, hypertrophy, and apoptosis of podocyte (56). Oxidative stress exacerbates the release of oxidative stress markers and inflammatory mediators of vascular ECs, such as MDA, TNF-α, and IL-6 (57). These factors reduce NO and endothelial NO synthase expression levels, activating the endothelin-1 signaling pathway (58) and exacerbating vascular smooth muscle cell aging while inducing vascular calcification (59). ROS promotes the expression of collagen IV, FN, and laminin and the accumulation of ECM in HG-induced MCs (60, 61). Oxidative stress affects autophagy (62) and promotes lipid accumulation (63), EMT, and apoptosis in RTECs (64). Ultimately, oxidative stress exacerbates progressive glomerular damage, tubular atrophy, and interstitial fibrosis, leading to decreased renal function and renal failure (65). As the primary source of ROS, the mitochondria are also the organelles that supply energy to the body. The accumulation of ROS can damage the mitochondria, including an imbalance of mitochondrial fission and mitochondrial fusion, mitochondrial mitophagy, downregulation of respiratory chain complexes,

insufficient ATP synthesis, interruption of mitochondrial membrane potential, the discharge of cytochrome-c and the generation of caspase-3, leading to mitochondrial damage (66), which are considered a significant factor in glomerular and tubular necrosis and apoptosis (67, 68).

Indirectly, diversified signalling pathways, such as AGE-RAGE (69), Kelch-like ECH-associated protein(Keap1)-Nrf2 (70), AMP-activated protein kinase (AMPK)/Sirtuin-1 (Sirt1) (71), SIRT1-forkhead transcription factor O (FOXO) (72), Sirt1 (73), NF-κB (74) and HMGB1 could be induced by oxidative stress. These pathways not only lead to oxidative stress injury but also stimulate the release of inflammatory and apoptotic factors, which induce inflammation and apoptosis. Primarily, oxidative stress is linked with variations in renal hemodynamics and metabolism, and chronic hyperglycemia-induced oxidative stress can induce elevated levels of Ang-II and activation of PKC (75), glycolipid disorders (76) and insulin resistance (77), which are important stimulants that promote oxidative stress. Ang-II activates NOX, which produces superoxide, thereby promoting renal vascular remodeling and increasing preglomerular resistance (78).PKC promotes significant upregulation of NOX4 and enhances the generation of ROS (79). Additionally, PKC can lead to insulin resistance and exacerbate insulin dysfunction (80). Podocytes contain insulin receptors, maintain insulin signal transduction and podocyte function, specifically knock out insulin receptors on podocytes, decrease autophagy-related proteins, such as Beclin1 and light chain 3 (LC3), cause autophagy disorders and podocyte injury (81). HG can upregulate the expression of AGEs and RAGE; induce the activities of PKCα, PKCβ, and NOX4; promote the production of ROS; destroy the mitochondrial function of human renal MCs, and affect the renal function of db/db mice (79). ROS induces the accumulation of excess lipids and the formulation of growth factors, including TGF-β, vascular endothelial growth factor (VEGF), and inflammatory factors, which are essential factors that lead to renal dysfunction and subsequent nephropathy.ROS mediates HMGB1/mitochondrial DNA signaling, promotes TLRs activation and inflammatory responses, and exacerbates EMT in proximal renal tubular epithelial cells (82) (Figure 1).

3 Overview of polyphenols

3.1 Chemistry of polyphenols

Polyphenols are secondary metabolites from various plants and are widely found in foods and beverages of plant origin, and more than 8000 polyphenols have been identified. Due to their antioxidant, anti-inflammatory and immunomodulatory activities, they play an essential role in the management of human health. In recent years, they have attracted much attention from nutritionists and food scientists due to their nutritional and therapeutic value (83). According to chemical structure, polyphenols can be divided into four major groups: Flavonoids, Stilbenes, Phenolic acids, and Lignans. Flavonoids comprise 15 carbon atoms, including two benzene rings (A Ring and B Ring) and a heterocycle (C Ring), abbreviated as C6-C3-C6, with the presence of flavan nucleus. Depending on the oxidation level of the C ring and hydroxylation patterns, flavonoids can be classified as flavonols, flavones, flavanones, anthocyanin, flavan-3-ols, and isoflavones (84). Non-flavonoids, including Stilbenes, Phenolic acids, and Lignans. Phenolic acids are the simplest compounds in the family of polyphenols because they have only one phenolic ring, such as ferulic acid, caffeic acid, and gallic acid (85). Stilbenes is a plant

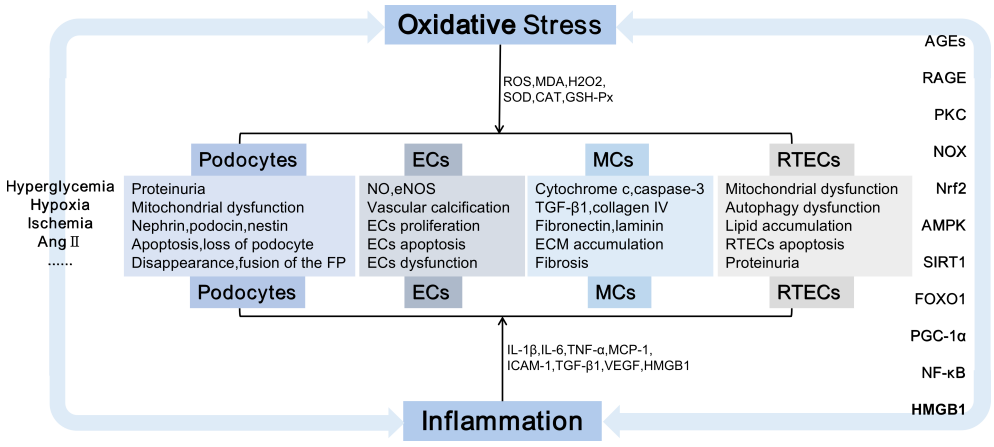

FIGURE 1
Kidney damage is caused by oxidative stress and inflammation. Damaged cells including podocytes, ECs, MCs, and RTECs, causing podocyte injury, the accumulation of extracellular matrix,epithelial-mesenchymal transition, cell apoptosis, etc., which eventually lead to irreversible glomerular fibrosis and tubular damage, exacerbate the progression of DN.Ang II, Angiotensin II; FP, Foot processes; ECs, endothelial cells; MCs, mesangial cells; RTECs, renal tubular epithelial cells; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; TGF-β, transforming growth factor-β; ECM, extracellular matrix;IL-1, interleukin-1; TNF-α, tumor necrosis factor-α; MCP-1, monocyte chemoattractant protein-1; ICAM-1, intercellular adhesion molecule-1; VEGF,vascular endothelial growth factor; AGEs, advanced glycation end products; RAGE, receptor for advanced glycation end products; NOX, nicotinamide adenine dinucleotide phosphate oxidase; PKC, protein kinase C; Nrf2, nuclear factor E2-related factor 2;AMPK, AMP-activated protein kinase; SIRT1, Sirtuin-1; FOXO, forkhead transcription factor O; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1α;NF-κB, nuclear factor-kappaB; DN, diabetic nephropathy;HMGB1,high mobility group box 1.

polyphenol with a diphenylethylene backbone. Resveratrol is the most representative compound among the stilbenes (86). Lignans are compounds produced by the oxidative dimerization of two phenyl propane units. Differences in the number of phenol units and how they are combined lead to different physical, chemical, and biological properties (87–89). In particular, the stability of polyphenols is also affected by many structural features, such as The hydrogenation of the C2=C3, hydroxylation, and methoxylation (90) (Figure 2).

3.2 The action of polyphenols with oxidative stress induced inflammation

Oxidative stress induced by ROS accumulation plays a pro-inflammatory role in various diseases, and NOX is considered a significant source of ROS. Inflammation inducers activate membrane recognition receptors of the innate immune system, such as TLR, which activate NOX and produce superoxide anion, which is rapidly converted to H_2O_2 by SOD, and H2O2 activates the Nrf2 signaling pathway to promote the release of antioxidant enzymes such as HO-1. More importantly, H2O2 activates inflammatory signaling pathways such as NF-κB, intensifying the release of pro-inflammatory mediators and stimulating inflammatory responses (91) (Figure 3).

Polyphenols have long been known for their antioxidant power to scavenge ROS. However, the impact of polyphenols goes far beyond this. Polyphenols can be indirectly anti-inflammatory through antioxidant effects or directly alleviate inflammation by modulating signaling pathways. The antioxidant capacity of polyphenols is mainly attributed to the direct scavenging of reactive oxygen species and the inhibition of reactive oxygen species production. Polyphenols inhibit the activity of NOX and significantly inhibit the production of superoxide anion (92). The phenolic ring structure of polyphenols can directly neutralize the free radicals generated by lipid peroxidation (93). Polyphenols can chelate metal ions, such as Fe3+, and prevent the conversion of H2O2 to highly toxic HO• (94). More importantly, polyphenols can promote the expression of antioxidant enzymes by enhancing endogenous antioxidant capacity induced by the Nrf2 pathway (95). Pomegranate polyphenols activate Nrf2 synapse, inhibit NF-κB suppression, reduce cell ROS production, and protect against drug-induced apoptosis (96). Resveratrol may activate the SIRT1/Nrf2 pathway to reduce inflammation and endoplasmic reticulum stress, effectively delaying the structural and functional decline of various tissues and organs due to aging (97). Polyphenols also enhance cellular antioxidant activity, inhibit pyroptosis and alleviate metabolic disorders and inflammatory responses through the Nrf2-NLRP3 axis (98).

While most studies suggest that polyphenols alleviate inflammation thanks to their antioxidant and free radical scavenging abilities, polyphenols can also directly modulate inflammatory signaling pathways. Many polyphenols, such as curcumin and resveratrol, have been reported to be potent inhibitors of NF-κB, inhibiting NF-κB translocation to the nucleus, inhibiting its binding from targeting DNA and subsequent transcription of pro-inflammatory cytokines, inhibiting phosphorylation or ubiquitination of signaling molecules, and inhibiting degradation of IκB (99). Polyphenols also target TLR4, the upstream of NF-κB, mitigating the inflammatory response through the TLR4/NF-κB signaling pathway (100). Resveratrol can suppress excessive inflammatory responses by interfering with HMGB1-mediated activation of the TLR4/NF-κB signaling pathway (101). Resveratrol suppresses HMGB1 expression through upregulation of miR-149, inhibits the ferroptosis formation pathway, and ameliorates injury (102).

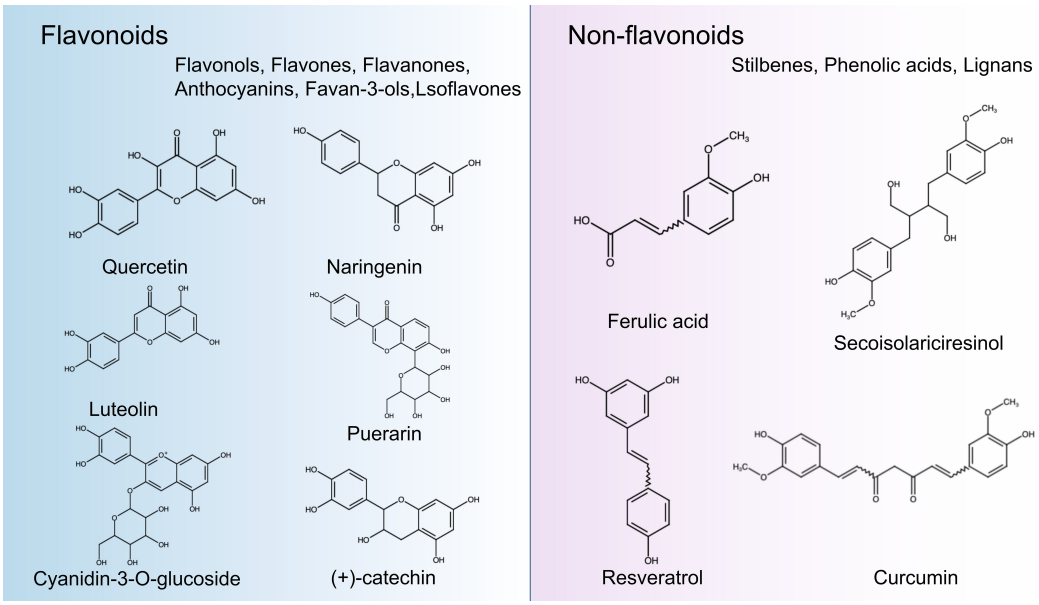

FIGURE 2
Classification and chemical structure of polyphenols. The structures of common polyphenol representatives (Created with BioRender.com.).

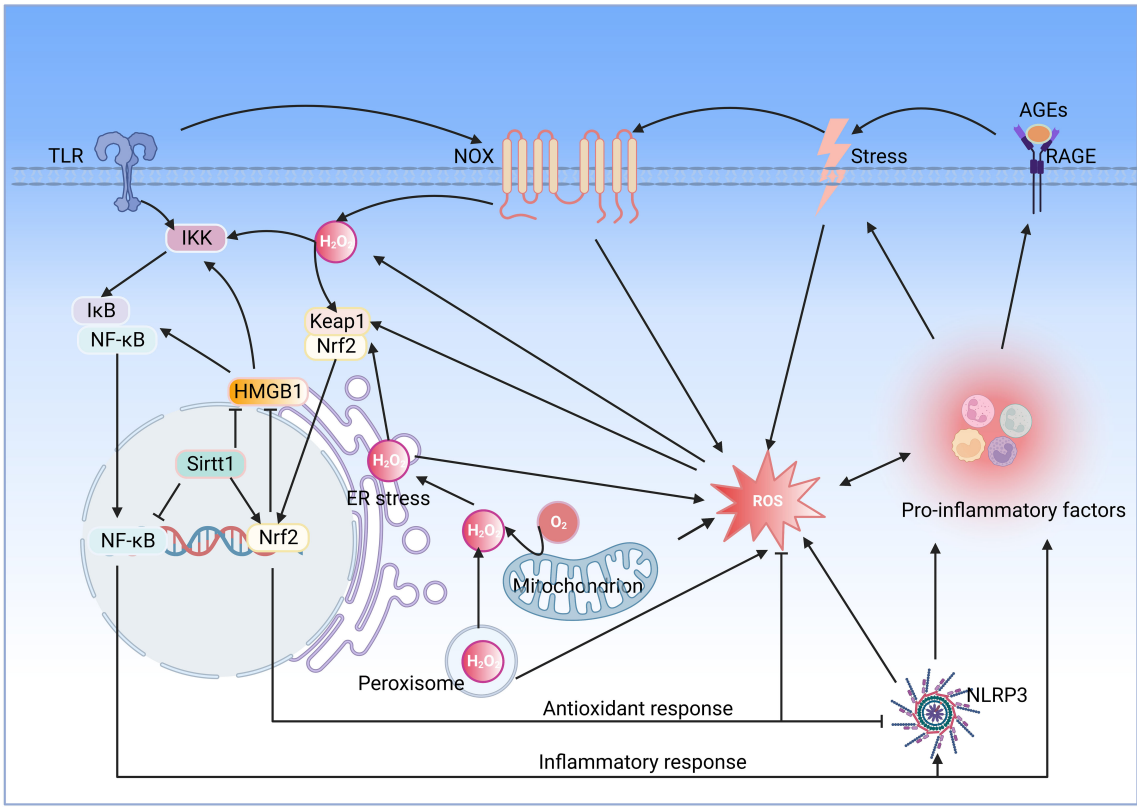

FIGURE 3
Interaction between oxidative stress and inflammation. Oxidative stress and inflammation interact with each other, and both of which constitute a vicious oxidative stress-inflammation cycle.TLR, toll-like receptor; IKK, IkB kinase; NF-κB, nuclear factor-kappaB; HMGB1, high mobility group box 1; Sirt1, sirtuin 1; Nrf2, nuclear factor E2-related factor 2; Keap, Kelch-like ECH-associated protein; NOX, NADPH oxidase; RAGE, receptor for advanced glycation end products; ER, endoplasmic reticulum; ROS, reactive oxygen species; NLRP3, NOD-like receptor family pyrin domain containing 3 (Created with BioRender.com.).

Also, resveratrol can mitigate downstream inflammatory responses by activating sirt1 and inhibiting the nucleoplasmic translocation and extracellular release of HMGB1 (103). Interestingly, a metabolomic study demonstrated the different roles of polyphenols in lipopolysaccharide-induced mouse embryonic fibroblast cells, with Theobroma cacao exerting mainly antioxidant properties and Lippia citriodora exerting anti-inflammatory effects mainly based on the reduction of pro-inflammatory cytokine and MCP-1 production, it was confirmed that the anti-inflammatory function of polyphenols could not be attributed to one mechanism of action but rather the coordination between different pathways and mechanisms (104) (Figure 4).

3.3 Polyphenols in DN

Some polyphenols have been widely used to repair molecules after free radical damage and modulate various dysregulated mediators and pathways, such as directly or indirectly blocking the production of ROS and alleviating the process of oxidative stress and inflammatory response, suggesting that polyphenols are an alternative approach to mitigate DN progression. In this review, “polyphenols”, “flavonoids”, “stilbenes”, “phenolic acids”, “lignans”, “flavonols”, “flavones”, “flavanones”, “anthocyanin”, “flavan-3-ols”, “isoflavones”, “diabetic nephropathy”, “diabetic kidney disease” as search topic words in Web of Science, and combining, de-duplicating to screen polyphenols as the primary intervention preclinical studies. (Table 1) (Figure 5).

3.3.1 Flavonoids

3.3.1.1 Quercetin

Quercetin, a polyphenol flavonoid, is ubiquitous in plants. Studies have shown that quercetin has a variety of biological activities, including scavenging free radicals and preventing lipid peroxidation in the body. Furthermore, it has anti-inflammatory properties and prevents diabetes complications (151, 152). Quercetin in streptozotocin (STZ)-induced DN rats can effectively reduce BUN and Scr levels, increase the formulation of nephrin and podocin while decreasing desmin in DN rats, relieve podocyte effacement (125) and improve renal pathological changes, including glomerular volume atrophy, high ECM and glycogen deposition, basement membrane thickening and tubulointerstitial fibrosis (123, 126). Further research illustrated that quercetin upregulated the formulation of SIRT1, activated the Nrf2/HO-1 pathway (128), inhibited the AGE-RAGE pathway (153), enhanced the expression of SOD, GSH-Px, and CAT (127), decreased the

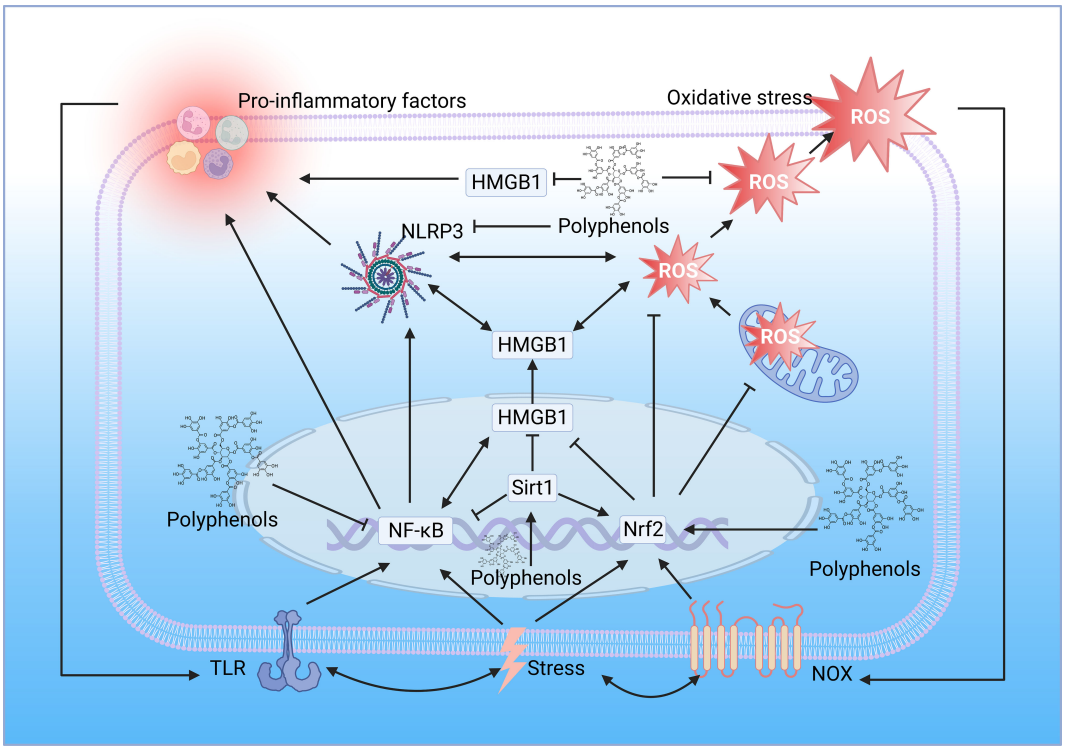

FIGURE 4
Polyphenols regulate oxidative stress and inflammation. The location of the polyphenol structure diagram in the figure is the critical node of polyphenol regulation. TLR, toll-like receptor; NF-κB, nuclear factor-kappaB; HMGB1, high mobility group box 1; Sirt1, sirtuin 1; Nrf2, nuclear factor E2-related factor 2; NOX, NADPH oxidase; ROS, reactive oxygen species; NLRP3, NOD-like receptor family pyrin domain containing 3 (Created with BioRender.com.).

MDA, elevated the HDL (high density lipoprotein) content, decreased the expression of triglyceride (TG) and low-density lipoprotein (LDL), and regulated renal lipid accumulation. Additionally, quercetin decreased the expression of NF-κB, TNF-α, IL-1β, ICAM, and ICAM-1 (124). Vitro studies indicated that quercetin promoted the expression of SOD, GSH-Px, CAT, and SIRT1, and reduced the formulation of MDA, TNF-α, IL-1β, IL-6, TGF-β1, Smad 2/3, type IV collagen and laminin (154–156). There is consistent with a recent systematic study that reported that quercetin significantly decreased renal index, Scr, BUN, urine albumin, MDA, TNF-α, and IL-1β and increased the activities of SOD. Furthermore, CAT alleviated the degree of oxidative stress in animal models of DN (157). It is also proposed that the optimal dose for preclinical experiments is 90-150 mg/kg/day, and the administration time is 2-4 months (158).

3.3.1.2 Puerarin

Puerarin is a natural isoflavone of Pueraria lobata (gegen), extensively found in food and Chinese herb medicine in East Asian countries. Puerarin has cardioprotective, antioxidant, and anti-inflammatory properties. Moreover, it exerts anti-diabetic activity by insulin secretion and maintaining metabolic homeostasis in STZ-induced diabetic mice (159). Furthermore, it can improve diabetes and other complications, including DN, by reducing the formation of AGEs and delaying oxidative stress (160). The SIRT1/FOXO1 signaling pathway is firmly related to the progression of DN. Studies have noted that activating the SIRT1/FOXO1 signaling pathway can increase SOD, CAT, and GSH-Px concentration and ameliorate renal injury in DN (161). Additionally, the SIRT1/FOXO1 signaling pathway can induce the expression of mitochondrial PGC-1α and improve mitochondrial function and ROS production (133). SIRT1 inhibited the NF-κB activity through deacetylation, and the expression of IL-6, TNF-α, and NOX4 was decreased, blood glucose, BUN, Scr, albuminuria, and urinary albumin to creatinine ratio (UACR) was ameliorated (134). Moreover, Puerarin directly diminished the formulation of ROS and FP effacement, restored the regular expression of nephrin and podocin of podocytes, decreased the formulation of matrix metalloproteinase-9 (MMP-9) and type IV collagen, and restored podocyte injury (135).

3.3.1.3 Other flavonoids

In DN models, baicalin (162), apigenin, and silybin have been found to promote Nrf2 translocation into the nucleus. Genes induced by ARE are activated, such as HO-1 and NQO-1, accompanied by various antioxidant enzymes, such as GSH-Px, SOD, and CAT. However, the activity of MDA has been inhibited (39). Moreover, the concentration of IL-1β, IL-6, MCP-1, and TNF-α has been decreased, and the levels of FN and TGF-β1 that induced renal fibrosis have been reduced (129). Luteolin (163) promotes the expression of HO-1; reduces the expression of MDA, FN, α-SMA, collagen I, and collagen IV; and decreases the proliferation of MCs

TABLE 1 Effects of polyphenols on DN.

Name	Model	Dosage	Effect↓	Effect↑	Ref
Resveratrol	db/db	10mg/kg/d,12 w	FBG,SCr,BUN,TC,TG,MDA,desmin,	SOD,podocin,nephrin	(105)
	STZ	5mg/kg/d,8 w	FBG,SCr,TGF-β1,MDA,FN,NF-kB,	CAT,SOD, GPx,GSH,Nrf2,Sirt1, FoxO1	(106)
	db/db	20mg/kg/d,12w	TGF-β1, ECM, Bax	AMPK,PPARα,FoxO1,FoxO3a, PGC-1α,eNOS,Bcl-2	(107)
	STZ	5 mg/kg/d,30d	FBG,TNF-α, IL-1β, IL-6,NF-κB,superoxide anion,hydroxyl radical, NO,Keap1	CCR, SOD, CAT, GPx, Nrf2, GR, HO-1	(108)
	STZ	0.1,1mg/kg/d,7d	FBG,BUN,SCr,TG,the superoxide anion, carbonyl, IL-1β,	AMPK	(109)
	STZ	30mg/kg/d,16 w	FBG, MDA,UP-24H	CCR,SIRT1,SOD,CAT,FOXO3	(110)
	STZ	30mg/kg/day,12w	FBG,BUN,TC,proteinuria,MDA,	Mn-SOD,SIRT1,PGC-1α	(111)
	STZ	5,10 mg/kg/d,2w	FBG, SCr, BUN,	MDA, CCR, GSH, SOD, CAT,	(112)
	STZ	20 mg/kg/d,8w	FBG, urea,SCr, MDA,	SOD, GPX	(113)
	db/db	20 mg/kg/d,12w	FBG, SCr, albuminuria, type IV collagen, TGF-β1, Bax,caspase-3	CCR,AMPK, SIRT1,PGC-1α, FOXO3a,Bcl-2,SOD	(114)
	db/db	40 mg/kg/d,12w	FBG,SCr,BUN,MDA,NOX4,EMT,α-SMA,E-cadherin,TGF-β,IGF-1R	SOD, HRD1	(115)
Curcumin	STZ	80,130 mg/kg/ d,60 d	FBG, SCr, urea, TC, TG, LDL-C, MDA, ROS,	HDL-C, TAC, NO	(116)
	OLETF	100 mg/kg/d,20w	FBG, UACR, MDA,	SOD,Nrf2/Keap1,HO-1,AMPK	(117)
	STZ	100 mg/kg/d,8w	FBG,Cr,BUN,UACR,PKC,NOX4,MDA,TGF-β1,CTGF, type IV collagen,FN,VEGF,p300	CCr, GPx	(118)
	STZ	15,30 mg/kg/d,2w	FBG,SCr,BUN,proteinuria,BUN,MDA	CCR, GSH, SOD, CAT	(119)
	STZ	10mg/kg/d,56d	24hUP,FN,TGF-β1,8-hydroxy-2'-deoxyguanosine		(120)
	STZ	50,100,200mg/kg/ d, 8w	24hUP,UACR,MDA,ROS,caspase-3,Bax	CCR, SOD, Bcl-2	(121)
	STZ	100mg/kg/d,12w	FBG,urea,BUN,SCr,collagen II/III,TGF-β1,cytochrome-c,caspase-3, NF-κB,PKC,.NADPH oxidase	CCR, MnSOD, GSH, Bcl-2, Nrf2, FOXO-3a	(122)
Quercetin	STZ	50 mg/kg/d,8w	FBG,24hUP,BUN,Scr,TNF-α,IL-1β,AGEs,MDA	SOD, GSH-Px	(123)
	STZ	10mg/kg/d,8w	FBG,BUN,Scr,MDA,TG,LDL,ACR,ICAM-1	SOD, HDL	(124)
	STZ	50,100mg/kg/ d,12w	BUN,TG,MDA,desmin,TGF-β1,Smad2, Smad3	Ccr, SOD, GSH, nephrin, podocin	(125)
	STZ	10 mg/kg/d,4w	FBG, TC, TG, proteinuria, urea,Cr, superoxide anions		(126)
	STZ	50mg/kg/d,5w	FBG,urea,BUN,SCr,TGF-β,TNF-α, IL-6	TAC, GSH and CAT	(127)
	STZ	100mg/kg/d,15d	FBG,urea,BUN,MDA,NF-kB,	SOD,CAT,SIRT1	(128)
Baicalin	STZ	40mg/kg/d,7d	TNF-α,NLRP3,MDA,P65,α-SMA,FN,TGF-β1	SOD,CAT,GPX	(129)
	db/db	400mg/kg/d,8w	ACR,AER,MDA, IL-1β, IL-6, MCP-1,TNFα	GSH-PX,SOD,CAT,Nrf2,HO-1, NQO-1	(39)
Naringenin	STZ	25,50mg/kg/d,4w	MDA	GSH,CAT,SOD	(130)
	STZ	5,10mg/kg/d,10w	TC,TG,LDL, VLDL, SCr,UA,TGF-β1,IL-1β	HDL,SOD,CAT,GSH	(131)
Silibinin	db/db	15,30 mg/kg/ d,10w	BUN, SCr,UA, MDA	SOD, GSH-Px	(132)
Puerarin	STZ	20,40,80mg/kg/ d,8w	FBG,BUN, Scr,24hUP,IL-6,TNF-α, ROS,NF-κB	MnSOD,CAT,SIRT1,FOXO1, PGC-1α	(133)
	STZ	20 mg/kg/d,8w	UACR,NOX4,NF-κB	SIRT1	(134)

(Continued)

TABLE 1 Continued

Name	Model	Dosage	Effect↓	Effect↑	Ref
	STZ	100mg/kg/d,7d	FP effacement,ROS,MMP-9,collagen IV	nephrin,podocin	(135)
Cyanidin-3-O-glucoside	STZ	10,20mg kg/d,8w	Scr,BUN,UA,ROS,MDA,TNF-α, MCP-1, IL-1β,IL-6,NF-κB, TGF-β1, Smad2,Smad3	SOD,GPX,CAT,Smad7	(136)
	db/db	10,20mg kg/d,12w	BUN,SCr,UA,ACR,collagen IV, FN,TGF-β1, MMP9,TG,TC,TNF-α, IL-1α, MCP-1	GCLC,GCLM,GSH	(137)
Apigenin	STZ	25,50mg/kg/d,30d	MDA,IL-1β,IL-6,TNF-α	Nrf2, HO-1,SOD,CAT	(138)
Luteolin	db/db	10mg/kg/d,12w	Fn, α-SMA, collagenI,collagen IV,IL-1β,IL-6,TNF-α,IL-17A,MDA, STAT3	SOD	(139)
	STZ	200 mg/kg/d,8w	BUN, SCr,24h UP,TC,TG,LDL,MDA	HDL,SOD,HO-1	(140)
Mangiferin	STZ	40mg/kg/d,30d	BUN,SCr,UA,ROS,Collagen,PKCs,MAPKs, NF-κB,TGF-β1,TNF-α, caspase 8,MMP	GSH,CAT, SOD, GPX,GR,Bcl-2, Bcl-xl	(141)
	STZ	15,30,60mg/kg/d,4w	FBG,TG,TC,BUN,SCr,UA,FN,collagen I,α-SMA,TNF-α,IL-6,IL-1β, MDA,ROS,TGF-β1,PI3K, Akt	SOD,CAT,GSH-Px	(142)
	STZ	15,30,60mg/kg,9w	AER,BUN,AGEs,RAGE,MDA	GSH	(143)
Ferulic Acid	STZ	50mg kg/d,8w	BUN,SCr,UACR,AGEs,ROS,NO,MDA,MAPK,TNF-α, IL-1β, and IL-6, MCP-1,ICAM-1,VCAM-1,NF-κB,	SOD2,CAT,beclin-1,LC3-II	(144)
	STZ	100mg kg/d,8w	BUN,Cr,FBG,TC,TG,MDA,NF-κB p65,TNF-α,TGF-β1,collagen IV	SOD, CAT, GPx, nephrin, podocin	(145)
	OLETF	10mg/kg/d,20w	24h UP, ACR, MDA, MCP-1, TGF-β1, collagen IV, ROS		(146)
Caffeic Acid	alloxan	50mg kg/d,7d	TC, TG, LDL,VLDL, MDA	HDL	(147)
Ellagic acid	STZ	50,100,150 mg/kg/d,4w	MDA,TNF-α,TLR4,IRAK4,TRAF6,IKKβ,NF- Kb	T-SOD	(148)
Chlorogenic acid	STZ	10, 20 mg/kg/d for 6 weeks	FBG, BUN, Cr, MDA	SOD, GSH-Px, CAT, Bcl-2	(149)
	STZ	10 mg/kg daily for 8 weeks,	BUN,proteinuria,MDA,IL-6,TNF-α,IL-1β,NF-κB	CCR, SOD, GSH-Px, Nrf2, HO-1	(150)

STZ, streptozotocin; FBG, fasting blood glucose; Scr, serum creatinine; BUN, blood urea nitrogen; TG, triglyceride; TC, total cholesterol; SOD, superoxide dismutase; MDA, malondialdehyde; TGF-β, transforming growth factor-β; GSH-Px, glutathione peroxidase; CAT, catalase; FN, fibronectin; NF-κB, nuclear factor-kappaB; Nrf2, nuclear factor E2-related factor 2; HO-1, hemoxygenase 1; Keap1, Kelch-like ECH-associated protein; SIRT1, Sirtuin-1; FOXO, forkhead transcription factor O; PPARα, peroxisome proliferator-activated receptor α; ECM, extracellular matrix; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1α; NO, nitric oxide; eNOS, endothelial NO synthase; Bcl-2, B-cell lymphoma-2; Bax, BCL-2 associated X; CCr, creatinine clearance; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; GR, glutathione reductase; UP24H, 24h urine protein; EMT, epithelial-mesenchymal transition; α-SMA, α-smooth muscle actin; IGF-1R, IGF-1 receptor; HRD1, 3-hydroxy-3-methylglutaryl reductase degradation; HDL, high density lipoprotein; LDL, low density lipoprotein; TC, total cholesterol; VLDL, very-low-density lipoprotein; CCr, creatinine clearance; TOS, total oxidant status; TAC, total antioxidant capacity; UACR, urinary albumin to creatinine ratio; GGT, γ-glutamyltranspeptidase; CTGF, connective growth factor; ROS, eactive oxygen species; H2O2, hydrogen perxide; NADPH, nicotinamide adenine dinucleotide phosphate; NOX, NADPH oxidase; AGEs, advanced glycation end products; PKC, protein kinase C; AMPK, AMP-activated protein kinase; VEGF, vascular endothelial growth factor; 24hUP, 24-hour urinary protein; MnSOD, manganese superoxide dismutase; ACR, albumin/creatinine ratio; ICAM-1, intercellular adhesion molecule 1; OLETF, Otsuka-Long-Evans-Tokushima Fatty; AER, albumin excretion rate; NQO-1, quinone oxidoreductase-1; MCP-1, monocyte chemotactic protein-1; GCLC, glutamate-cysteine ligase catalytic subunit; GCLM,glutamate-cysteine ligase modifier subunit; MMP, mitochondrial membrane potential; TLR4, Toll-like receptor 4. Effect↓: decrease; Effect↑: increase.

and excessive accumulation of ECM (140). The expression of STAT3 is highly correlated with oxidative stress and inflammatory response. Luteolin effectively inhibits the activation of STAT3 (139). Mangiferin inhibits the activation of the AGEs/RAGE axis, and PKCs reduce the phosphorylation of PI3K/Akt and improve the inflammation and oxidative stress in DN (141). Furthermore, similar to naringenin (131), the levels of TC, TG, LDL, and VLDL(very-low-density lipoprotein) were decreased, and the HDL level is raised, which improves lipid peroxidation by targeting oxidative stress and inflammation, cyanidin-3-O-glucoside (C3G) decreases the formulation of Smad2 and Smad3 while enhancing Smad7. It also improves renal pathological and functional changes, including the degree of renal fibrosis, SCr, BUN, urinary albumin, and ACR (137, 164). Eriodictyol can obstruct the production of NOX2, NOX4, directly shorten the production of ROS and MDA and protect MCs from HG stimulation by inhibiting the production of FN, collagen IV, and ECM (165). Notably, phloretin can directly restore nephrin and podocin levels and improve the disappearance of FP of podocytes (166). Furthermore, the inhibition of oxidative stress by apigenin *in vitro* has demonstrated the activation of Nrf2, HO-1, SOD, and CAT activities; downregulated the generation of TNF-α, IL-1β, and IL-6; inhibited the Nrf2, leading to the disruption of apigenin contrary to HG-induced oxidative damage was disrupted (167).

3.3.2 Stilbenes

3.3.2.1 Resveratrol

Resveratrol is a natural polyphenolic antioxidant. It has various biochemical and physiological properties, including antioxidant, anti-inflammatory, anti-diabetic, anti-obesity, cardiovascular

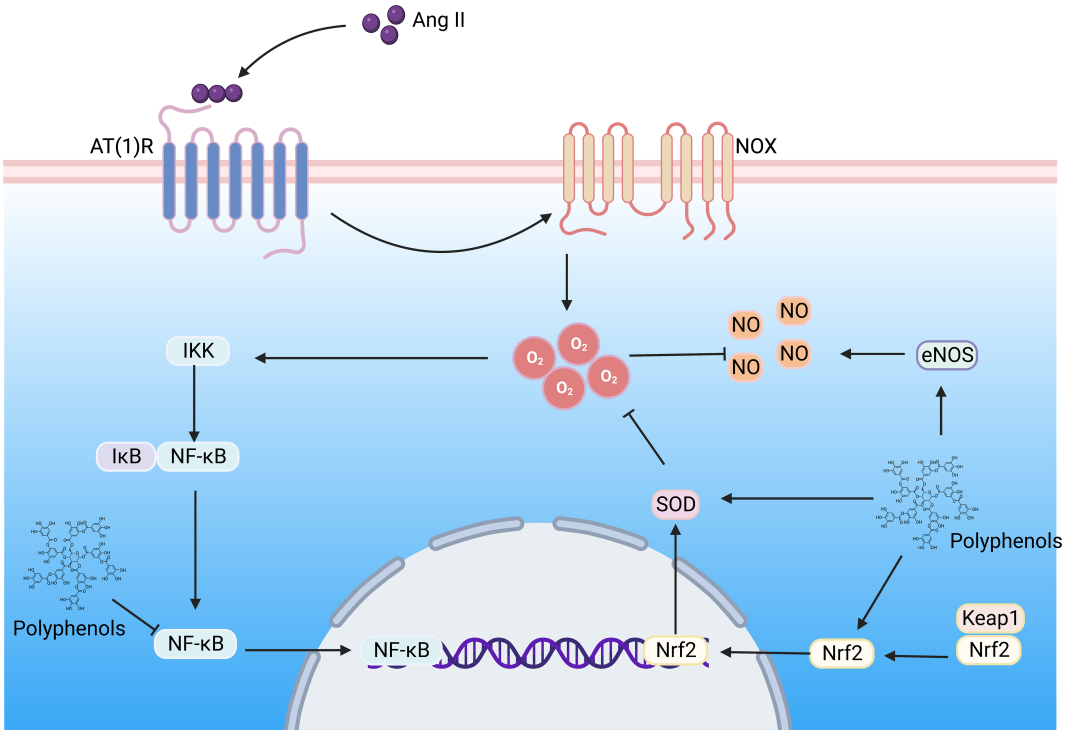

FIGURE 5
The polyphenols improve DN by regulating the Ang II signaling pathway. Hyperglycemia induces the formation of large amounts of Ang II, which binds to the AT(1)R) in the kidney, activating NOX, contributing to superoxide formation, triggering oxidative stress and inflammation, and resulting in renal vascular remodeling. Ang II, Angiotensin II; AT(1)R, Ang II type 1 receptor; NOX, NADPH oxidase; NF-κB, nuclear factor-kappaB; Nrf2, nuclear factor E2-related factor 2; NLRP3, NOD-like receptor family pyrin domain containing 3; IKK, IkB kinase; Keap, Kelch-like ECH-associated protein; SOD, superoxide dismutase; NO, nitric oxide;eNOS, endothelial nitric oxide synthase. (Created with BioRender.com.).

protection, and anti-tumor features (168). Resveratrol exerts its renoprotective effects through multiple mechanisms, including reducing oxidative stress and AGE production, inhibiting endoplasmic reticulum stress and inflammation; improving lipotoxicity; stimulating autophagy (169); and activating a variety of pathways, including Nrf2, AMPK, SIRT1and FOXO3a. A systematic review showed that resveratrol could apply its antioxidant activity by reducing the production of MDA and restoring the action of SOD, CAT, and GSH-Px (170); decreasing 4-hydroxynonenal, an indicator of lipid peroxidation (171); and inhibiting the formation of ROS and lip peroxidation (172). Resveratrol activates antioxidant enzymes and decreases the secretion of superoxide anion, hydroxyl radical, TNF-α, IL-1β, IL-6, and NF-κBp65 by targeting the Nrf2-Keap1 signaling pathway; moreover, it improves the thickened basement membrane, leading to loss of FP (108). AMPK activation also has ameliorating effects on oxidative stress (169).AMPK inhibits HG-induced expression of NOX; decreases the content of FN and the proliferation of MCs; prevents EMT of RTECs (173, 174); restores podocyte-related proteins to normal, including nephrin, podocin, and desmin; and ameliorates mesangial matrix expansion, tubular basement membrane thickening, and glomerular hypertrophy in db/db mice of DN (105). Additionally, AMPK mediates the upregulation of SIRT1; activates the SIRT1/peroxisome proliferator-activated receptor α(PPARα)/FoxO pathway; heightens the generation of CAT, SOD, and GSH-Px; and decreases the expression of MDA, TGF-β1, FN, NF-kBp65, SCr, and urinary protein. In Silencing SIRT1, the protective effects are suppressed (106, 107, 110). Resveratrol elevates the concentration of Mn-SOD. Furthermore, it repairs the expression of SIRT1 and peroxisome proliferator-activated receptor-γ coactivator-1α(PGC-1α) and reestablishes the activity of respiratory chain complexes I and III and mitochondrial membrane potential. Furthermore, it inhibits the transport of Cyto C from the mitochondria to the cytoplasm and delays the progression of apoptosis in glomerular podocyte and tubular epithelial cells (111).

3.3.2.2 Curcumin

Curcumin is an acidic polyphenol with unsaturated aliphatic and aromatic groups as the main chain, which is more common in turmeric (Curcuma longa L.) and Curcumae Rhizoma. The Pharmacological activities of Curcumin include antioxidant, anti-inflammatory, immunomodulatory, and anti-renal fibrosis. A previous study reported that Curcumin mediates the upregulation of GSH-Px, SOD, and other antioxidant factors by activating the antioxidant Nrf2/HO-1 pathway. Furthermore, it downregulates the expression of MDA and ROS effectively promotes improvement of the urinary protein excretion rate (175), elevates the expression of nephrin, and repairs podocyte injury (176). Additionally, Curcumin protects RTECs from HG-induced EMT *via* Nrf2-mediated upregulation of HO-1, which consequently knocks down Nrf2 and inhibits the upregulation of HO-1 (177). Curcumin activates

the phosphorylation of AMPK, significantly increases the protein rate of Nrf2/Keap1, increases the activity of the antioxidant enzyme, and attenuates the expression of MDA, kidney injury molecule-1, and neutrophil gelatinase-associated lipocalin (116, 117). furthermore, it reduces the incidence of lipid accumulation, tubular dilatation, and glomerular sclerosis (178). Curcumin prohibited the activity of PKC and the expression of NF-κB, NADPH oxidase, collagen I/III, and TGF-β1. Also, it increases the protein levels of FOXO-3a, Nrf2, manganese SOD, GSH-Px, and B-cell lymphoma-2(Bcl-2); inhibits the generation of cytochrome-c and the production of caspase-3; and prevents damage to the mitochondria, renal tubules and mesangial cell (179). A systematic review of randomized displayed that Curcumin had some beneficial effects on various parameters, including inflammation or oxidative stress, in patients with renal disease (180). A randomized, double-anonymized trial assessing the redox status of dietary Curcumin supplementation (320 mg/d) in patients with non-diabetic or diabetic proteinuria confirmed that Curcumin enhanced the antioxidant capacity of diabetic proteinuric patients while attenuating lipid peroxidation in non-diabetic patients, suggesting a therapeutic effect of dietary Curcumin supplementation (181). *In vitro* studies have also found that Curcumin effectively reduces oxidized LDL isolated from human plasma-related markers such as conjugated diene, lipid peroxides, and lysolecithin, preventing oxidation and lipid modification of LDL (182).

3.3.3 Phenolic acid

Multiple phenolic acids have been used, including ferulic, chlorogenic, caffeic, ellagic, etc. Phenolic acids have various functions, such as regulating blood sugar and lipids, anti-oxidation, anti-inflammatory, and anti-fibrosis. Hyperglycemia triggers oxidative stress, leading to the formulation of mitogen-activated protein kinases (MAPK). Ferulic acid inhibits the activation of MAPKs and decreases the expression of ROS, NO, carbonyl, MDA, and inflammatory factors and adhesion molecules, including NF-κB, TNF-α, IL-1β, and IL-6, MCP-1, ICAM-1 and vascular cell adhesion molecule-1. Moreover, it increases the levels of Beclin-1 and LC3-II (144). Long-term treatment with ferulic acid significantly downregulated the expression of p-NF-κB p65, TNF-α, TGF-β1 and type IV collagen protein, and MDA in renal tissue; increased the activity of SOD, CAT, GPx; and upregulated the levels of nephrin and podocin, which improved podocyte injury and reduced the serum levels of BUN, Cr, fasting blood glucose (FBG), TC (total cholesterol), TG by alleviating oxidative stress, inflammation and fibrosis in STZ-induced DN rats (145). Chlorogenic acid and caffeic acid increased renal formulation of Nrf2 and HO-1 and increased the activity of SOD and GSH-Px. Furthermore, they decreased the formulation of NF-κB, IL-6, TNF-α, and IL-1β (150, 183). Chlorogenic acid can also alleviate endoplasmic reticulum stress, promote the formulation of anti-apoptosis factors, such as Bcl-2, attenuate the proliferation of MCs and mesangial expansion, and reduce the levels of FBG, BUN, and Cr (149). Caffeic acid reduces the activation of NOX; decreases the generation of ROS; and heightens the action of antioxidant enzymes, including T-SOD, GPx, and GSH (184), thereby reducing the levels of MDA (148). Mainly, ellagic acid significantly inhibits the activation of renal NF-κB, IL-1β, IL-6, and TNF-α. Additionally, it decreases the generation of TGF-β and fibronectin in renal tissue (185).

4 Conclusion and prospect

This review summarizes oxidative stress and oxidative stress-associated inflammation that lead to changes in renal cell structure and function in DN. Oxidative stress is recognized as a powerful mechanism for the occurrence and progression of DN.DN, a common complication of DM, has become a significant cause of end-stage renal disease. Even though the pathogenesis of DKD is complex, oxidative stress has been proposed to be central to the pathogenesis of DKD. Hyperglycemia activates signaling pathways such as AGE/RAGE and Ang II, which induce large production of ROS. Large amounts of ROS activate NF-κB, TGF-β1, AMPK, and other pathways, which induce renal inflammation, autophagy, and fibrosis, triggering abnormal kidney structure and function. In addition, oxidative stress also interacts with other factors in disease progression. ROS are not only messengers of various signaling pathways but also regulators of various cellular metabolism, proliferation, differentiation, and apoptosis. Oxidative stress is accompanied by tissue inflammation, apoptosis, and tissue fibrosis, and in particular, oxidative stress and inflammation are considered to be in a causal relationship, with many factors exacerbating the progression of DKD (186). Oxidative stress induces excessive production of ROS, activates signaling pathways and transcription factors, mediates the infiltration and recruitment of inflammatory cells, such as macrophages and monocytes, and promotes the production of inflammatory factors. Conversely, the formulation of inflammatory mediators can further produce ROS, which aggravates oxidative stress, and oxidative stress and inflammation collaborate to affect the progression of DN. As a natural compound with antioxidant and anti-inflammatory properties, polyphenols can ameliorate or reverse cell damage and pathological changes of DN and are considered to be attractive drugs against DN by targeting signaling pathways, including Nrf2, NF-κB, AMPK, and SIRT1. However, the complex regulatory mechanism of polyphenols in DN has not been thoroughly demonstrated, and inhibition of renal damage and restoration of structure and function should be the core of alternative polyphenol therapy. Therefore, more studies are needed to elucidate polyphenols and how to repair kidney injury and improve kidney function.

Polyphenols exhibit various biological activities, such as antioxidant and anti-inflammatory. Multiple clear experimental evidence suggests that long-term intake of polyphenols can effectively improve the progression of chronic diseases, including DN. However, there are still some limitations, polyphenols are famous for their potent antioxidant properties, but in some cases, such as in reaction systems rich in redox-active metals (e.g., iron, copper), flavonoids have a pro-oxidant effect, reducing the clearance of ROS and inducing mitochondrial toxicity (187). In addition, the lack of pharmacokinetic measurements of ingested polyphenols and

the correct dose remains a challenge, so the application should be tailored to the purpose and the selection of the appropriate dose and mode of action. Moreover, the evaluation of the efficacy of polyphenols in combination with other conventional treatments for DN and the specific mechanism of action remains to be investigated. Many natural products did not enter clinical trials or were terminated due to reduced oral utilization or activity, which may limit their widespread clinical application, and further studies are needed to overcome these limitations and to bridge the gap between preclinical and clinical studies of natural products. It is worth noting that the clinical translation of DN therapies from natural products is still a long process.

Author contributions

QJ: Conceptualization, Writing-Original draft, Investigation, Visualization. TL: Conceptualization, Investigation. YQ: Investigation. DL: Investigation. LY: Investigation. HM: Investigation. FM: Investigation. YW: Investigation. LP: Writing-review & editing. YZ: Writing-review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Glossary

DM	Diabetes mellitus
T1D	type 1 diabetes
T2D	type 2 diabetes
DN	Diabetic nephropathy
DKD	diabetic kidney disease
CKD	chronic kidney disease
ESRD	end-stage renal disease
Ang II	Angiotensin II
ROS	reactive oxygen species
HG	high glucose
SGLT2	sodium-glucose cotransporter 2
Nrf2	nuclear factor E2-related factor 2
ARE	antioxidant response element
HO-1	heme oxygenase 1
SOD	superoxide dismutase
CAT	catalase
H_2O_2	hydrogen perxide
NO	nitric oxide
NADPH	nicotinamide adenine dinucleotide phosphate
NOX	NADPH oxidase
AGEs	advanced glycation end products
PKC	protein kinase C
GSH-Px	glutathione peroxidase
GR	glutathione reductase
MDA	malondialdehyde
RAGE	receptor for advanced glycation end products
NF-κB	nuclear factor-kappaB
NLRP3	NOD-like receptor family pyrin domain containing 3
IL-1β	interleukin-1β
TNF-α	tumor necrosis factor-α
MCP-1	monocyte chemoattractant protein-1
ICAM-1	intercellular adhesion molecule-1
TGF-β	transforming growth factor-β
ECs	endothelial cells
MCs	mesangial cells
RTECs	renal tubular epithelial cells
NQO-1	NADPH quinone oxidoreductase-1
HMGB1	high mobility group box 1
TLR4,	Toll-like receptor 4
FP	foot processes
FN	fibronectin
ECM	extracellular matrix
EMT	epithelial-mesenchymal transition
BUN	blood urea nitrogen
Keap1	Kelch-like ECH-associated protein
AMPK	AMP-activated protein kinase
Sirt1	Sirtuin-1
FOXO	forkhead transcription factor O
STZ	streptozotocin
HDL	high density lipoprotein
TG	triglyceride
LDL	low density lipoprotein
UACR	urinary albumin to creatinine ratio
TC	total cholesterol
VLDL	very-low-density lipoprotein
UACR	urinary albumin to creatinine ratio
MMP-9	matrix metalloproteinase-9
C3G	Cyanidin-3-O-glucoside
ACR	albumin/creatinine ratio
PPARα	peroxisome proliferator-activated receptor α
PGC-1α	peroxisome proliferator-activated receptor-γ
Bcl-2	B-cell lymphoma-2
MAPKs	mitogen-activated protein kinases
FBG	fasting blood glucose.

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Allogeneic cord blood regulatory T cells decrease dsDNA antibody and improve albuminuria in systemic lupus erythematosus

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Background: Lupus nephritis (LN) constitutes the most severe organ manifestations of systemic lupus erythematosus (SLE), where pathogenic T cells have been identified to play an essential role in 'helping' B cells to make autoantibodies and produce inflammatory cytokines that drive kidney injury in SLE. Regulatory T cells (Tregs), responsible for decreasing inflammation, are defective and decreased in SLE and have been associated with disease progression. We hypothesize that treatment with allogeneic, healthy Tregs derived from umbilical cord blood (UCB) may arrest such an inflammatory process and protect against kidney damage.

Methods: UCB-Tregs function was examined by their ability to suppress CellTrace Violet-labeled SLE peripheral blood mononuclear cells (PBMCs) or healthy donor (HD) conventional T cells (Tcons); and by inhibiting secretion of inflammatory cytokines by SLE PBMCs. Humanized SLE model was established where female Rag2$^{-/-}\gamma c^{-/-}$ mice were transplanted with 3 × 10^6 human SLE-PBMCs by intravenous injection on day 0, followed by single or multiple injection of UCB-Tregs to understand their impact on disease development. Mice PB was assessed weekly by flow cytometry. Phenotypic analysis of isolated cells from mouse PB, lung, spleen, liver and kidney was performed by flow cytometry. Kidney damage was assessed by quantifying urinary albumin and creatinine secretion. Systemic disease was evaluated by anti-dsDNA IgG Ab analysis as well as immunohistochemistry analysis of organs. Systemic inflammation was determined by measuring cytokine levels.

Results: *In vitro*, UCB-Tregs are able to suppress HD Tcons and pathogenic SLE-PBMCs to a similar extent. UCB-Tregs decrease secretion of several inflammatory cytokines including IFN-γ, IP-10, TNF-α, IL-6, IL-17A, and sCD40L by SLE PBMCs in a time-dependent manner, with a corresponding increase in secretion of suppressor cytokine, IL-10. *In vivo*, single or multiple doses of UCB-Tregs led to a decrease in CD8$^+$ T effector cells in different organs

and a decrease in circulating inflammatory cytokines. Improvement in skin inflammation and loss of hair; and resolution of $CD3^+$, $CD8^+$, $CD20^+$ and $Ki67^+$ SLE-PBMC infiltration was observed in UCB-Treg recipients with a corresponding decrease in plasma anti-double stranded DNA IgG antibody levels and improved albuminuria.

Conclusions: UCB-Tregs can decrease inflammatory burden in SLE, reduce auto-antibody production and resolve end organ damage especially, improve kidney function. Adoptive therapy with UCB-Tregs should be explored for treatment of lupus nephritis in the clinical setting.

KEYWORDS

umbilical cord blood (UCB), allogeneic, regulatory T cells (Tregs), systemic lupus erythematosus (SLE), lupus nephritis (LN), dsDNA antibodies, albuminuria

Highlights

- UCB-Tregs suppress pathogenic SLE cells, decrease $CD19^+$B cells and monocytes, increase IL-10, and decrease IFN-γ, IP-10, TNF-α, IL-6, IL-17A, and sCD40L.
- UCB-Tregs decrease $CD8^+$T and $CD20^+$B cell tissue infiltration, inflammatory cytokines, anti-dsDNA IgG Ab, and albuminuria in lupus xenografts.

Introduction

Systemic lupus erythematosus (SLE) is known to be a B-cell-mediated autoimmune disorder with multi-organ involvement, including skin rash, pulmonary fibrosis, joint pain, neurological dysfunction, vasculitis, and renal failure (1, 2). Recent data support the contribution of autoreactive, pathogenic T cells in the perpetuation of the autoimmune process including autoantibody production and tissue inflammation in SLE (3–5). In fact, adoptive transfer of T cells overexpressing LFA-1 can induce lupus-like disease including glomerulonephritis in naïve recipient mice (6).

Lupus nephritis (LN), a fatal complication of SLE, is thought to be caused by an inflammatory response to immunogenic, endogenous chromatin and driven by the autoreactive leukocytes, immune complexes (ICs), and IL-17-producing T helper 17 cells (TH17 cells) in collaboration with various cytokines, chemokines, and growth factors, where the treatment usually consists of immunosuppressive therapy including steroids (7).

$CD4^+CD25^+CD127^{low}$ regulatory T cells (Tregs) expressing the lineage-specific transcription factor forkhead box P3 (FoxP3) regulate the activation and expansion of auto-reactive T cells and other harmful immune cells in the peripheral lymphatic organs and prevent and control inflammation and autoimmunity (8). The FoxP3 protein is the master regulator for development and function of $CD4^+CD25^+CD127^{low}$ Treg cells, where mutations of FoxP3 gene impair their development and function that may result in severe autoimmune disease (9–11). Previously, it has been reported that SLE patients have a lower percentage of $CD4^+CD25^+CD127^{low}$ Tregs when compared to a healthy population, and $CD4^+CD25^+CD127^{low}$ Tregs derived from SLE patients show defect in their suppressor function (12–15) and are functionally exhausted (16, 17). Additionally, the SLE pathogenic T effector (Teff) cells develop resistance to $CD4^+CD25^+CD127^{low}$ Treg-induced suppression (18). Such a "double whammy" leads to a heavily inflammatory microenvironment and a continuous loop of tissue destruction resulting in end organ damage, especially kidney damage (19).

Findings from these studies suggest that adoptive therapy with $CD4^+CD25^+CD127^{low}$ Tregs may represent a potential therapeutic strategy for treating inflammatory processes in SLE. We have previously shown that adoptive therapy with allogeneic, umbilical cord blood (UCB)-derived $CD4^+CD25^+CD127^{low}$ Tregs can prevent graft vs. host disease (20, 21), resolve lung inflammation (22), treat COVID-19-associated acute respiratory distress syndrome and multi-organ failure (23), and show early survival benefit (24).

We now hypothesize that $CD4^+CD25^+CD127^{low}$ UCB-Tregs can treat LN, leveraging their unique properties including (i) lack of plasticity when exposed to inflammatory micro-environments; (ii) no requirement for HLA matching with the recipients; (iii) long shelf life of the cryopreserved cells; and (iv) immediate product availability for on-demand treatment (22).

Materials and methods

SLE and healthy donor PBMCs

Human SLE-peripheral blood mononuclear cells (SLE-PBMCs) (ASTARTE Biologics, Bothell, WA, USA) or healthy donor PBMCs (HD-PBMCs) (Gulf Coast Blood Bank, Houston, TX, USA) were purified using Lymphoprep (StemCell Technologies, Vancouver, BC, Canada) and then cultured in X-VIVO 15 medium (Lonza

Biowhittaker, Morristown, NJ, USA) with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, USA), 2 mM L-glutamine (Sigma-Aldrich, St Louis, MO, USA), 1% penicillin–streptomycin (Thermo Fisher Scientific), and 1,000 IU/ml IL-2 (Clinigen Inc., Yardley, PA, USA) in the presence of CD3/CD28 beads (Thermo Fisher Scientific) for 3–7 days.

UCB-Treg cell isolation and *ex vivo* expansion

$CD4^+CD25^+CD127^{low}$ Treg cells were isolated from UCB units and cultured and cryopreserved as described previously (22). Additionally, frozen $CD4^+CD25^+CD127^{low}$ UCB-Tregs were provided by Cellenkos Inc (Houston, TX, USA). Cryopreserved $CD4^+CD25^+CD127^{low}$ UCB-Tregs were thawed and cultured in X-VIVO 15 medium supplemented with 10% FBS, 2 mM L-glutamine, 1% penicillin–streptomycin, and 1,000 IU/ml IL-2 for 3–7 days.

Flow cytometry analysis

APC-eFluor 780-conjugated mouse anti-human CD45 antibody (Ab) (HI30), Alexa Fluor-532-conjugated mouse anti-human CD3 Ab (UCHT1), FITC-conjugated mouse anti-human CD3 Ab (UCHT1), PerCP-Cyanine5.5-conjugated mouse anti-human CD8a Ab (RTA-T8), Super Bright 600-conjugated mouse anti-human CD19 Ab (SJ25C1), PE-conjugated mouse anti-human CD25 Ab (BC96), PE-Cy5-conjugated mouse anti-human CD127 Ab (eBioRDR5), APC-conjugated mouse anti-human CD56 Ab (CMSSB), FITC-conjugated mouse anti-human CD16 Ab (eBioCB16(CB16)), PerCP-eFluor 710-conjugated mouse anti-human CD14 Ab (61D3), PE-Cy7-conjugated mouse anti-human HLA-DR Ab (LN3), and LIVE/DEAD™ fixable Blue dye were purchased from Thermo Fisher Scientific. BV650-conjugated mouse anti-human CD4 Ab (L200), BV510-conjugated mouse anti-human CD8 Ab (RPA-T8), PE-CF594-conjugated mouse anti-human CD27 Ab (M-T271), Alexa Fluor 700-conjugated mouse anti-human IgD Ab (IA6-2), BV421-conjugated mouse anti-human CD62L Ab (SK11), Alexa Fluor 647-conjugated Armenian hamster anti-Helios Ab (22F6), Alexa Fluor 647-conjugated mouse anti-human FoxP3 Ab (259D/C7), and PerCP-Cy5.5-conjugated mouse anti-human FoxP3 Ab (236A/E7) were purchased from BD Biosciences. Pacific Blue-conjugated mouse anti-mouse CD45.1 Ab (A20) was purchased from SouthernBiotech (Birmingham, AL, USA). The antibodies per test were used according to the vendor's instruction.

Events in the t-distributed stochastic neighbor embedding (t-SNE) were overlaid with manually gated lymphocytes, $CD3^+CD19^-$, $CD4^+$ T, $CD4^+CD25^+$ T, $CD4^+CD25^+CD127^{low}$ Treg, $CD4^+CD8^+$ T, $CD8^+$ T, $CD3^-CD19^+$ B, $CD27^-IgD^-$ double negative (DN) B, $CD27^+IgD^-$ Memory B, $CD27^-IgD^+$ Naïve B, $CD27^+IgD^+$ Plasma, $CD56^+$ NK cells, and $CD14^+$ monocytes and displayed for all treatments in the concatenated file. Stained cells were acquired on Cytek Aurora flow cytometer (Cytek Biosciences, Fremont, CA, USA) or a BD LSRFortessa flow cytometer (BD Biosciences) and analyzed using FlowJo software (FlowJo, LLC, Ashland, OR, USA).

Suppression assay

$CD4^+CD25^-$ conventional T cells (Tcons) or SLE-PBMCs were stained with CellTrace Violet (CTV) (Thermo Fisher Scientific) following the manufacturer's instruction. CTV-labeled Tcons or SLE-PBMCs were co-cultured with different ratios of unlabeled $CD4^+CD25^+CD127^{low}$ UCB-Tregs in the presence of CD3/CD28 beads. Proliferation of CTV-labeled Tcons or SLE-PBMCs was assessed by LSRFortessa Cell Analyzer as described previously (22).

Detection of human cytokines in cell culture supernatants

HD-PBMCs, SLE-PBMCs, or $CD4^+CD25^+CD127^{low}$ UCB-Tregs, or a combination were cultured for 3 or 7 days; culture supernatants were collected for cytokine analysis; and human IL-10, IFN-γ, IP-10, TNF-α, IL-6, and IL-17A were assessed using Human Cytokine ELISA kits (Thermo Fisher Scientific) according to the manufacturer's instructions. Levels of sCD40L, IL-1α, and IL-21 were measured using Human Cytokine/Chemokine 71-plex Discovery Assay Array kit (Eve Technologies, Calgary, AB, Canada).

SLE xenograft model

Animal procedures were performed according to an approved protocol by MD Anderson Cancer Center IACUC. *Rag2/IL2rg* compound mutant mice ($Rag2^{-/-}$ $\gamma c^{-/-}$ mice) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) at 4 weeks of age and were transplanted with 3×10^6 SLE-PBMCs by intravenous tail vein (t.v.) injection (25). After mice displayed human immune cells, 10×10^6 $CD4^+CD25^+CD127^{low}$ UCB-Treg cells were injected intravenously on day 7 for single treatment ($n = 3$) or on days 30, 35, 45, and 51 for multiple treatment ($n = 7$). PB SLE cells as well as production of anti-double-stranded DNA IgG antibody were detected in SLE xenografts. Mice were assessed weekly for the reconstitution level of human immune cells by flow cytometry of the peripheral blood. Mice were monitored twice per week for weight loss and survival. At the time of euthanasia, organs of SLE xenografts were aseptically harvested, homogenized, and filtered using a nylon mesh to obtain single-cell suspensions. After RBC lysis, tissue cells were collected by centrifugation and phenotypic analysis of cells was performed by analysis of surface or intracellular markers. Data acquired by LSRFortessa Cell Analyzer (BD Biosciences) were analyzed with BD FACSDiva 8.0.1 software and FlowJo software (FlowJo LLC, Ashland, Oregon, USA).

Detection of urinary albumin in SLE xenografts

Urine samples were collected every other week for the assessment of kidney function in SLE xenografts and the levels of albumin and creatinine were assessed using the Exocell Albumin M

assay kit (Ethos Biosciences, Logan Township, NJ, USA) according to the manufacturer's instructions.

Detection of anti-human double-stranded DNA IgG antibody in SLE xenografts

Plasma samples were collected weekly from the EDTA-treated peripheral blood of untreated and *ex vivo* expanded $CD4^+CD25^+CD127^{low}$ UCB-Tregs-infused SLE mice. The level of anti-human double-stranded DNA IgG Ab in the plasma was assessed using the Abnova assay kit (Abnova, Neihu District, Taipei City, Taiwan) according to the manufacturer's instruction.

Detection of human cytokine/chemokine in the plasma of SLE xenografts

Plasma samples were collected weekly from the EDTA-treated PB of SLE xenografts and levels of inflammatory cytokines were measured using Human cytokine 42-plex Discovery assay kit (Eve Technologies).

Histopathology and immunohistochemistry

Harvested organs were fixed with 10% buffered formalin and embedded in paraffin for processing into 5-µm tissue sections. De-paraffinized and rehydrated tissue sections were stained with Hematoxylin & Eosin, and evaluated by an institution pathologist, who was blinded to the treatment arms. For immunohistochemistry (IHC), de-paraffinized and rehydrated tissue sections were subjected to heat-mediated antigen retrieval with sodium citrate buffer (pH 6), permeabilization, and blocking prior to staining with primary antibodies including human CD3 (Cat #A0452, Clone F7.2.38) (DAKO, Santa Clara, CA, USA), CD4 (Cat #CD4-368-L-CE, Clone 4B12) (Leika Biosystems Inc., Buffalo, Grove, IL, USA), CD8 (Cat #MS-457s, Clone C8/144B) (Thermo Fisher Scientific), CD20 (Cat #MO755, Clone L26) (DAKO), and Ki-67 (Cat #M7240, Clone MIB-1) (DAKO). Appropriate horseradish peroxidase-conjugated secondary antibodies were used and the stained tissue slides were analyzed using Aperio ImageScope (Leica Biosystems Inc., Buffalo Grove, IL, USA). IHC images were analyzed at ×40 magnification using HALO 3.3 software (India Labs, Albuquerque, NM, USA), and H-score was defined by the percentage of strongly positive stain × 3 + moderately positive stain × 2 + weakly positive stain × 1. A final value of 0–300 was also calculated using HALO software.

Statistical analysis

All statistical analyses were done with GraphPad Prism 9 software (San Diego, CA, USA). Data are presented as mean ± SEM. *p*-values were obtained using one or two-way analysis of variance (ANOVA) with Tukey multiple comparison test, *F*-test, or two-tailed unpaired *t*-test with 95% confidence interval for evaluation of statistical significance compared with the untreated controls. $p < 0.05$ was considered statistically significant.

Results

$CD4^+CD25^+CD127^{low}$ UCB-Tregs suppress SLE-PBMCs proliferation and decrease $CD19^+$ B cells

UCB-Tregs phenotype was consistent with $CD4^+CD25^{high}CD127^{low}FoxP3^+$ (22). Since pathogenic SLE PBMCs may be resistant to Treg-mediated suppression (18), we compared the ability of $CD4^+CD25^+CD127^{low}$ UCB-Tregs to suppress proliferation of target cells. As shown in Figure 1A, no differences were observed in the ability of the $CD4^+CD25^+CD127^{low}$ UCB-Treg cells to suppress HD-Tcons vs. SLE-PBMCs at a 1:1 ratio. Initial analysis of the expression levels of surface and intracellular markers, including CD45, CD14, CD3, CD4, CD25, CD127, CD8, CD19, IgD, CD27, CD62L, Helios, FoxP3, CD56, and HLA-DR marker on the tSNE map, allowed for grouping of the cell populations into monocytes and lymphocytes including $CD4^+$T, $CD8^+$T, and $CD4^+CD8^+$T cells; $CD4^+CD25^+CD127^{low}$Treg cells; $CD19^+$B cells; and $CD56^+$NK cells (Figure 1B). As shown in Figures 1C, D, $CD4^+CD25^+CD127^{low}$ UCB-Tregs decrease the inflammatory cell population of SLE-PBMCs. After stimulation with CD3/CD28 beads and IL-2 for 3 days, an increase in the percentage of the $CD4^+CD25^+CD127^{low}$-expressing Treg cells was observed in SLE-PBMCs, whereas their distribution rearrangement was observed in $CD4^+CD25^+CD127^{low}$ UCB-Tregs where an overall decrease in the $CD8^+$ only expressing T cells with an actual increase in the $CD4^+$ and $CD8^+$ co-expressing cells was observed in UCB-Tregs : SLE-PBMCs co-culture. Additionally, a decrease in $CD19^+$ B cells, $CD56^+$ NK cells, and $CD14^+$ monocytes was also observed.

$CD4^+CD25^+CD127^{low}$ UCB-Tregs increase IL-10 and decrease inflammation

$CD4^+CD25^+CD127^{low}$ Tregs can suppress Teff cell proliferation by secreting inhibitory cytokine IL-10 (26). As shown Figure 2A, high levels of soluble IL-10 were detected in $CD4^+CD25^+CD127^{low}$ UCB-Tregs supernatant at days 3 and 7 (11,169 ± 118 pg/ml and 8,019 ± 221 pg/ml, respectively), whereas minimal IL-10 secretion was detected in HD-PBMC- or SLE-PBMC-alone supernatant at day 3 (223 ± 130 pg/ml and 1,010 ± 38 pg/ml, respectively) and day 7 (494 ± 59 pg/ml and 1,391 ± 111 pg/ml, respectively). Co-culture of HD-PBMCs or SLE-PBMCs with $CD4^+CD25^+CD127^{low}$ UCB-Tregs increased IL-10 production on day 3 (7,995 ± 182 pg/ml and 7,802 ± 372 pg/ml, respectively) and day 7 (8,086 ± 86 pg/ml and 6,466 ± 152 pg/ml) (two-way ANOVA $p < 0.0001$).

As shown in Figures 2B–I, $CD4^+CD25^+CD127^{low}$ UCB-Tregs were able to suppress several inflammatory cytokines secreted by SLE-PBMCs in their co-culture supernatants on days 3 and 7, including the following: (i) IFN-γ: Significantly higher IFN-γ levels of 29,264 ± 1,867 pg/ml and 72,080 ± 2,379 pg/ml were secreted by SLE-PBMCs alone at days 3 and 7,

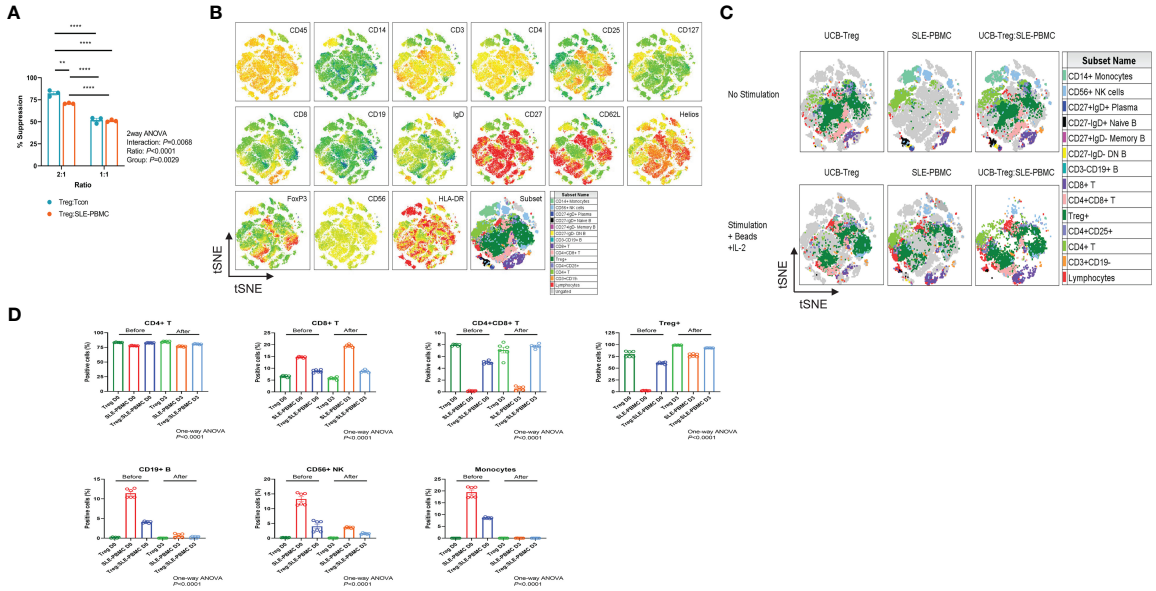

FIGURE 1
CD4$^+$CD25$^+$CD127^{low} UCB-Tregs suppress SLE-PBMC and shift their cell population distribution. **(A)** Functional analysis of *ex vivo*-expanded day 14 CD4$^+$CD25$^+$CD127^{low} UCB-Tregs on suppression of Tcon cells from healthy donor and SLE-PBMCs. Two-way ANOVA demonstrated that ratio ($p < 0.0001$), group ($p = 0.0029$), and interaction ($p = 0.0068$) between UCB-Treg : Tcon and UCB-Treg : SLE-PBMC were statistically significant. Data are presented as mean ± SEM ($n = 3$). $p < 0.05$ was considered statistically significant. **$p < 0.01$; ****$p < 0.0001$ by two-way ANOVA with Tukey multiple comparison tests. **(B)** Expression levels of surface and intracellular markers on tSNE map. Unstimulated or stimulated CD4$^+$CD25$^+$CD127^{low} UCB-Tregs, SLE-PBMCs, or CD4$^+$CD25$^+$CD127^{low} UCB-Treg plus SLE-PBMC co-cultures (1:1) with CD3/CD28 plus IL-2 were stained with Live/Dead dye, CD45, CD14, CD3, CD4, CD25, CD127, CD8, CD19, IgD, CD27, CD62L, Helios, FoxP3, CD56, and HLA-DR antibodies. Stained live CD45$^+$ cells from all six treatments were gated, down-sampled to 10,000 cells per sample which were concatenated. tSNE was run on six samples and the resulting tSNE plots were displayed expression intensities of surface and intracellular markers for all treatments in the concatenated file. **(C)** Subset analysis on tSNE map. Events in the tSNE embeddings were overlaid with manually gated lymphocytes, CD3$^+$CD19$^-$ T, CD4$^+$ T, CD4$^+$CD25$^+$ T, CD4$^+$CD25$^+$CD127^{low} Treg, CD4$^+$CD8$^+$ T, CD8$^+$ T, CD3$^-$CD19$^+$ B, CD27$^-$IgD$^-$ DN B, CD27$^+$IgD$^-$ Memory B, CD27$^-$IgD$^+$ Naïve B, CD27$^+$IgD$^+$ Plasma, CD56$^+$ NK cells, and CD14$^+$ monocytes and displayed for all treatments in the concatenated file. Stained cells were acquired on a Cytek Aurora flow cytometer and analyzed using FlowJo software. **(D)** Quantification analysis of subsets. Unstimulated or stimulated CD4$^+$CD25$^+$CD127^{low} UCB-Tregs, SLE-PBMCs, or CD4$^+$CD25$^+$CD127^{low} UCB-Treg plus SLE-PBMC co-cultures (1:1) with CD3/CD28 plus IL-2 were stained with Live/Dead dye, CD45, CD14, CD3, CD4, CD25, CD127, CD8, CD19, IgD, CD27, CD62L, Helios, FoxP3, CD56, and HLA-DR antibodies. CD4$^+$ T, CD8$^+$ T, CD4$^+$CD8$^+$ T, CD4$^+$CD25$^+$CD127^{low} Treg, CD3$^-$CD19$^+$ B, CD56$^+$ NK cells, and CD14$^+$ monocytes were quantified. Data are presented as mean ± SEM ($n = 6$). $p < 0.05$ was considered statistically significant. $p < 0.0001$ by one-way ANOVA test.

respectively, when compared to CD4$^+$CD25$^+$CD127^{low} UCB-Tregs alone levels of 32 ± 3 pg/ml and 149 ± 16 pg/ml at the same time points ($p < 0.0001$). Significantly decreased IFN-γ levels of 3,155 ± 310 pg/ml at 3 days ($p = 0.0002$) and 7,767 ± 271 pg/ml at 7 days ($p < 0.0001$, Figure 2B) were detected in their co-culture supernatants. (ii) IP-10: Significantly higher IP-10 levels of 4,941 ± 140 pg/ml and 7,505 ± 33 pg/ml were secreted by SLE-PBMCs alone at days 3 and 7, respectively, when compared to CD4$^+$CD25$^+$CD127^{low} UCB-Tregs alone levels of 22 ± 2 pg/ml and 33 ± 2 pg/ml at the same time points ($p < 0.0001$). Addition of CD4$^+$CD25$^+$CD127^{low} UCB-Tregs to the proliferating SLE-PBMCs significantly decreased the secreted IP-10 levels to 1,068 ± 77 pg/ml at 3 days ($p < 0.0001$) and 1,340 ± 35 pg/ml IP-10 at 7 days ($p < 0.0001$, Figure 2C). (iii) TNF-α: Significantly higher TNF-α levels of 9,342 ± 202 pg/ml at 3 days and 13,426 ± 333 pg/ml at 7 days were secreted by SLE-PBMCs when compared to CD4$^+$CD25$^+$CD127^{low} UCB-Tregs alone levels of 358 ± 45 pg/ml and 355 ± 24 pg/ml, at the corresponding time points ($p < 0.0001$). Addition of CD4$^+$CD25$^+$CD127^{low} UCB-Tregs to the proliferating SLE-PBMCs significantly decreased the secreted TNF-α levels to 5,071 ± 97 pg/ml at 3 days ($p < 0.0001$) and 5,564 ± 50 pg/ml at 7 days ($p < 0.0001$, Figure 2D). (iv) IL-6: Significantly higher IL-6 levels of 1,150 ± 30 pg/ml at 3 days and 4,747 ± 67 pg/ml IL-6 at 7 days were secreted by SLE-PBMCs when compared to CD4$^+$CD25$^+$CD127^{low} UCB-Tregs alone levels of 0 pg/ml for both the corresponding time points ($p < 0.0001$). The addition of CD4$^+$CD25$^+$CD127^{low} UCB-Tregs to the proliferating SLE-PBMCs significantly decreased the secreted IL-6 levels to 213 ± 3 pg/ml at 3 days ($p < 0.0001$) and 273 ± 5 pg/ml at 7 days ($p < 0.0001$, Figure 2E). (v) IL-17A: Significantly higher IL-17A levels of 9,770 ± 616 pg/ml at 3 days and 39,402 ± 374 pg/ml at 7 days were secreted by SLE-PBMCs when compared to CD4$^+$CD25$^+$CD127^{low} UCB-Tregs alone levels of 14 ± 1 pg/ml and 13 ± 2 pg/ml at the corresponding time points ($p < 0.0001$). The addition of CD4$^+$CD25$^+$CD127^{low} UCB-Tregs to the proliferating SLE-PBMCs significantly decreased the secreted IL-17A levels to 4,705 ± 43 pg/ml at 3 days ($p = 0.0012$) and 10,438 ± 668 pg/ml at 7 days ($p < 0.0001$, Figure 2F). (vi) sCD40L: Significantly higher sCD40L levels of 241 ± 1 pg/ml at 3 days and 327 ± 24 pg/ml sCD40L at 7 days were secreted by SLE-PBMCs when compared to CD4$^+$CD25$^+$CD127^{low} UCB-Tregs alone levels of 29 ± 8 pg/ml and 13 ± 3 at the corresponding time points ($p < 0.0001$). The addition of CD4$^+$CD25$^+$CD127^{low} UCB-Tregs to the proliferating SLE-PBMCs significantly decreased the secreted sCD40L level to 124 ± 3 pg/ml at 3 days ($p = 0.0006$) and 177 ± 13 pg/ml at 7 days ($p = 0.0336$; Figure 2G). (vii) IL-1α: CD4$^+$CD25$^+$CD127^{low} UCB-Tregs

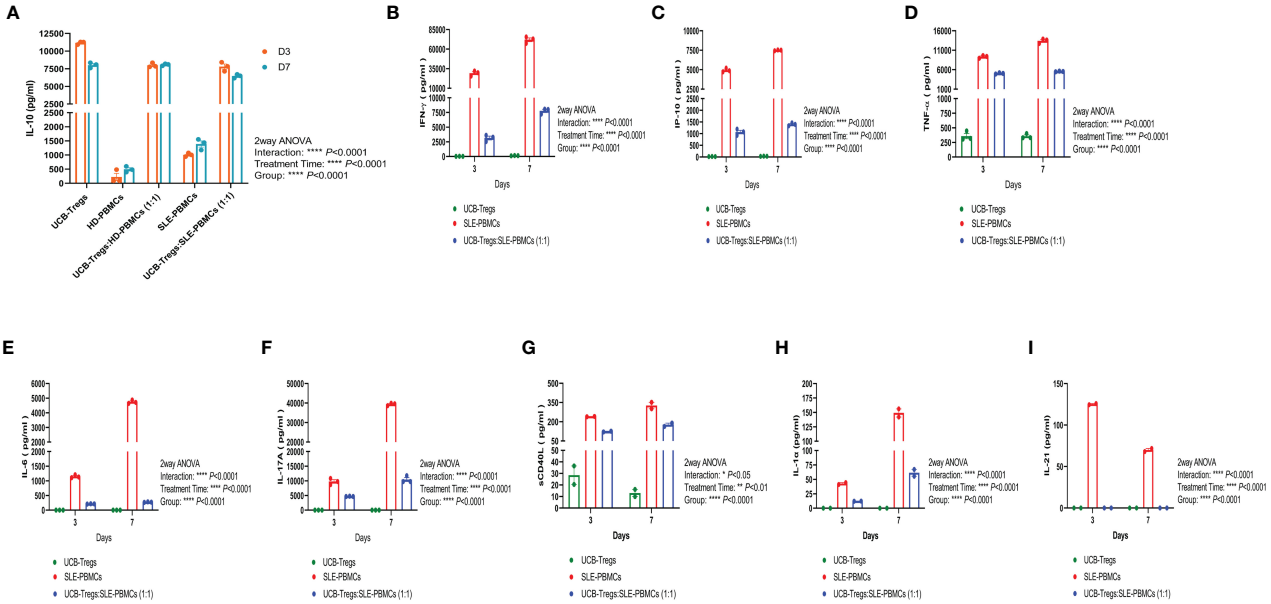

FIGURE 2
$CD4^+CD25^+CD127^{low}$ UCB-Tregs increase IL-10 secretion and decrease inflammatory cytokines in co-culture with SLE-PBMCs. $CD4^+CD25^+CD127^{low}$ UCB-Tregs, HD-PBMCs, SLE-PBMCs, or UCB-Tregs plus SLE-PBMC co-cultures (1:1) were seeded at 1×10^6 cells per well in six-well plates and were stimulated with Human T-Activator CD3/CD28 in a 1 cell:1 bead ratio in X-VIVO 15 medium supplemented with 10% FBS, 2 mM L-glutamine, 1% penicillin–streptomycin, and 1,000 IU/ml IL-2. After 3 days or 7 days, cell culture supernatants were collected for cytokine analysis. Production levels of human IL-10 **(A)**, IFN-γ **(B)**, IP-10 **(C)**, TNF-α **(D)**, IL-6 **(E)**, and IL-17A **(F)** in the cell culture supernatants were assessed using Human Cytokine ELISA kits according to manufacturer's instructions and production levels of sCD40L **(G)**, IL-1α **(H)**, and IL-21 **(I)** were measured using human cytokine/chemokine 71-plex discovery assay array. Data are presented as mean ± SEM (n = 2–3). $p < 0.05$ was considered statistically significant. *$p < 0.05$, **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ by two-way ANOVA with Tukey multiple comparison tests.

did not produce IL-1α. SLE-PBMCs alone produced 43 ± 2 pg/ml at 3 days and 149 ± 7 pg/ml IL-1α at 7 days ($p = 0.0046$, when compared to $CD4^+CD25^+CD127^{low}$ UCB-Tregs). The addition of $CD4^+CD25^+CD127^{low}$ UCB-Tregs to the proliferating SLE-PBMCs significantly decreased the secreted IL-1α levels to 12 ± 0 pg/ml at 3 days ($p = 0.0029$) and 61 ± 6 pg/ml at 7 days ($p = 0.0106$; Figure 2H). (viii) IL-21: $CD4^+CD25^+CD127^{low}$ UCB-Tregs did not produce IL-21. SLE-PBMCs alone produced 126 ± 1 pg/ml at 3 days and 70 ± 2 pg/ml IL-21 at 7 days. The addition of $CD4^+CD25^+CD127^{low}$ UCB-Tregs to the proliferating SLE-PBMCs led to undetectable IL-21 levels at day 3 ($p < 0.0001$) and day 7 ($p = 0.0007$; Figure 2I).

Single injection of $CD4^+CD25^+CD127^{low}$ UCB-Tregs slows SLE development *in vivo*

SLE xenograft was established as described previously (25), where single t.v. injection of $CD4^+CD25^+CD127^{low}$ UCB-Tregs was administered at 7 days after SLE-PBMC injection (Figure 3A). When compared to SLE-PBMC only recipients (Control-single), a single injection of $CD4^+CD25^+CD127^{low}$ UCB-Tregs (Treatment-single) led to an improvement in weight gain (Figure 3B) and survival (63 median survival days for the Control-single group and 90 median survival days for the Treatment-single group, Figure 3C). At 11 weeks, the Treatment-single group demonstrated a significant reduction in the circulating human $CD45^+$ cells ($p = 0.0025$; Figure 3D) and human $CD8^+$ T cells ($p < 0.0001$; Figure 3E). At 8 weeks, Treatment-single demonstrated an increase in circulating human $CD4^+$ cells (93.4% vs. 61.7%) and $CD4^+CD25^{high}CD127^{low}FoxP3^+$ cells (16.7% vs. 0.8%) (Figure 3F). Such an increase in the $CD4^+$ cell populations was not evident at the 11-week time point (data not shown).

Widespread skin erythema seen in the Control-single group (Figure 3G, left upper panel) that correlated with histologic findings of widespread parakeratosis (Figure 3G, middle upper panel) resolved upon the addition of $CD4^+CD25^+CD127^{low}$ UCB-Tregs (Figure 3G, left lower panel), which correlated with preservation of the epidermal and subdermal layer, and resolution of the $CD8^+$ cell infiltrate and intact adipose tissue layer (Figure 3G, middle and right lower panel) in the Treatment-single group. Bulky spleen with histologic evidence of lymphoid (white pulp) hyperplasia in the Control-single mice (Figure 3H, upper panel) was resolved with the addition of $CD4^+CD25^+CD127^{low}$ UCB-Tregs (Figure 3H, lower panel) that occurred in conjunction with a reduction of $CD3^+$, $CD8^+$, $CD20^+$, and $Ki67^+$ cells as detected by the immunohistochemical (IHC) staining of spleen tissue of the Treatment-single group. Quantification of the IHC staining also showed a significant reduction in both human marker and the H-score for the respective stains ($p < 0.0001$) (Figure 3I). As shown in Figure 3J, extensive lymphoid infiltrate involving parenchyma and hilar stroma in the kidney tissue of the Control-single group (left upper panel) was notably decreased by the single injection of $CD4^+CD25^+CD127^{low}$ UCB-Tregs (Treatment-single group) (left lower panel), with preservation of the renal glomeruli. Additionally, a decrease in the total IHC-positive cells and H-score for human CD8, CD20, and Ki67 marker was also

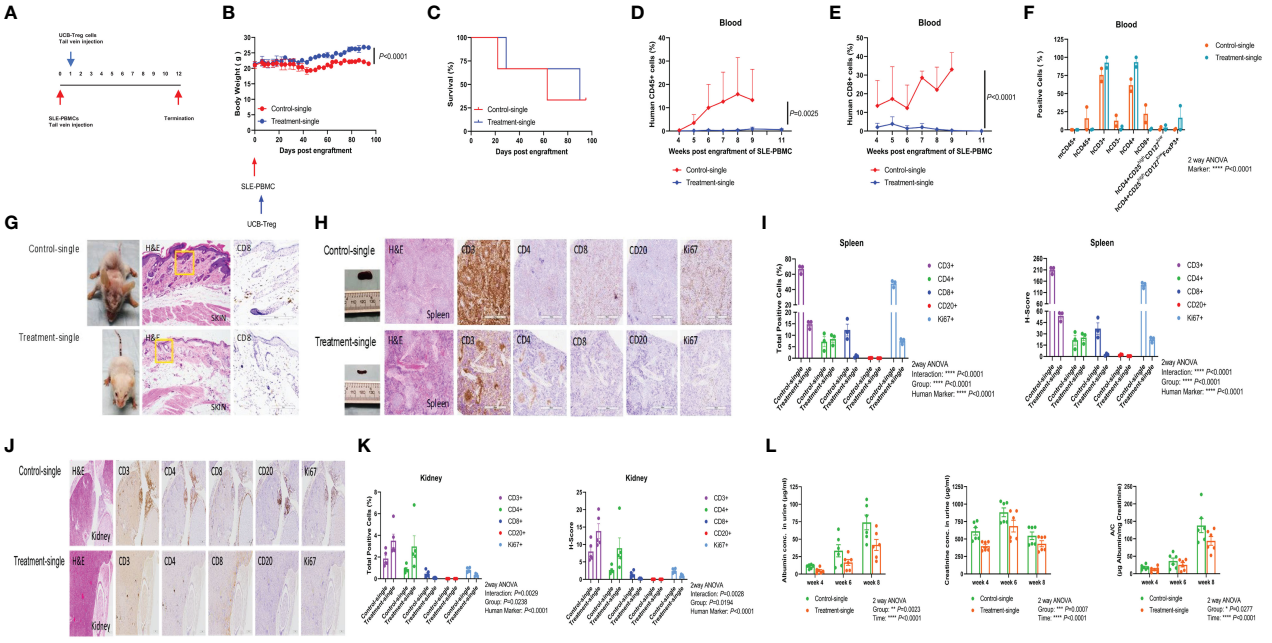

FIGURE 3
Single injection of $CD4^+CD25^+CD127^{low}$ UCB-Tregs halts disease progression in SLE xenografts. **(A)** Schematic summary of single injection of $CD4^+CD25^+CD127^{low}$ UCB-Treg cell therapy for SLE in a SLE xenograft model. Female $Rag2^{-/-}$ $\gamma c^{-/-}$ mice were transplanted with 3 × 10^6 human SLE-PBMCs by intravenous injection on day 0 and divided into two groups (Control-single and Treatment-single, n = 3 per group). Single injection of 10 × 10^6 *ex vivo* expanded $CD4^+CD25^+CD127^{low}$ UCB-Tregs was administered through t.v. on day 7 after SLE-PBMCs injection. **(B)** $CD4^+CD25^+CD127^{low}$ UCB-Tregs improve body weight in SLE xenografts. Change of body weight was monitored twice per week until termination of experiments. Data are presented as mean ± SEM (n = 3). $p < 0.05$ by Student t-test was considered statistically significant. **(C)** $CD4^+CD25^+CD127^{low}$ UCB-Tregs improves median survival days. Kaplan–Meier analysis was performed to estimate median survival times. **(D)** $CD4^+CD25^+CD127^{low}$ UCB-Tregs decrease human $CD45^+$ cells. Mouse PBMC was procured from Control-single and Treatment-single recipients and analyzed for $CD45^+$ cells as measured by flow cytometry at the indicated time points. $p < 0.05$ by two-tailed unpaired Student t-test was considered statistically significant. **(E)** $CD4^+CD25^+CD127^{low}$ UCB-Tregs decrease human $CD8^+$ cells. Mouse PBMC was procured from Control-single and Treatment-single recipients and analyzed for $CD8^+$ cells as measured by flow cytometry at the indicated time points. Data are presented as mean ± SEM. p-values were obtained using two-tailed unpaired t-test with 95% confidence interval for evaluation of statistical significance compared with the untreated controls. $p < 0.05$ was considered statistically significant. **(F)** Comparison of phenotypes between Control-single and Treatment-single recipients at 8 weeks post engraftment of SLE-PBMCs. $p < 0.05$ was considered statistically significant. $p < 0.0001$ by one-way ANOVA test. **(G)** Photograph of representative mouse Control-single and Treatment-single arm exhibiting skin changes. Representative H&E and CD8 staining of mouse-affected skin tissue sections from Control-single and Treatment single arm. **(H)** Photograph of spleen and representative H&E, CD3, CD4, CD8, CD20, and Ki67 staining of spleen tissue sections from representative mouse Control-single and Treatment-single arm. **(I)** Quantification analysis of positive cells and H-score of spleen tissue sections. Immunochemistry images of human $CD3^+$, $CD4^+$, $CD8^+$, $CD20^+$, and $Ki67^+$ cells were analyzed at ×40 magnification using HALO 3.3 software. Quantification analysis of human $CD3^+$, $CD4^+$, $CD8^+$, $CD20^+$, and $Ki67^+$ cells and H-scores for human CD3, CD4, CD8, CD20, and Ki67 positivity were calculated using HALO 3.3 software. Data are presented as mean ± SEM (n = 3). $p < 0.05$ was considered statistically significant. $^{****}p < 0.0001$ by two-way ANOVA with Tukey multiple comparison tests. **(J)** Single injection of $CD4^+CD25^+CD127^{low}$ UCB-Tregs decreased renal inflammation *in vivo*. Representative H&E, CD3, CD4, CD8, CD20, and Ki67 staining of kidney tissue sections. **(K)** Quantification analysis of positive cells and H-score of kidney tissue sections. Immunochemistry images of human $CD3^+$, $CD4^+$, $CD8^+$, $CD20^+$, and $Ki67^+$ cells were analyzed at ×40 magnification using HALO 3.3 software. Quantification analysis of human $CD3^+$, $CD4^+$, $CD8^+$, $CD20^+$, and $Ki67^+$ cells and H-scores for human CD3, CD4, CD8, CD20, and Ki67 positivity were calculated using HALO 3.3 software. Data are presented as mean ± SEM (n=5). $p < 0.05$ was considered statistically significant. **(L)** Single injection of $CD4^+CD25^+CD127^{low}$ UCB-Tregs decreased albuminuria in SLE xenografts. Expression levels of urinary albumin, creatinine, and albumin/creatinine. Data are presented as mean ± SEM (n = 6). $p < 0.05$ was considered statistically significant. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ by two-way ANOVA with Tukey multiple comparison tests.

observed in the Treatment-single group whereas both total $CD3^+$ and $CD4^+$ cells were increased in the Treatment-single group. Group for total positive cells (p = 0.0238) and H-score (p = 0.0194) was significant. Furthermore, human marker for both total positive cells and H-score was significant ($p < 0.0001$) (Figure 3K). Concurrently, a significant improvement in albuminuria (Group: p = 0.0023; Time: $p <$ 0.0001), creatinuria (Group: p = 0.0007; Time: $p < 0.0001$), and urine albumin/creatinine (A/C) ratio (Group: p = 0.0277; Time: $p < 0.001$) was also demonstrated (Figure 3L).

Multiple injections of $CD4^+CD25^+CD127^{low}$ UCB-Tregs can resolve SLE pathology

Using the same SLE xenograft model, we allowed for 4 weeks to establish human disease in immune-deficient mice followed by multiple t.v. injections of $CD4^+CD25^+CD127^{low}$ UCB-Tregs administered on days 30, 35, 45, and 51 (Figure 4A). The Treatment-multiple group demonstrated a significant reduction in the circulating human $CD45^+$ cells (Figure 4B, $p < 0.01$). The percentage of circulating

$CD4^+CD25^{high}CD127^{low}$ Treg cells was significantly increased following multiple $CD4^+CD25^+CD127^{low}$ UCB-Tregs injections and lasted up to day 62 (Figure 4C, $p < 0.0001$). Mice were euthanized at 12 weeks where the phenotype analysis of PB and aseptically harvested single cell organ suspensions showed a significant decrease in human $CD8^+$ T cells in PB ($p = 0.0096$), spleen ($p = 0.0187$), and liver ($p = 0.002$); human $CD45^+$ cells in PB ($p = 0.0251$) and spleen ($p = 0.0187$); and human $CD3^+$ cells in PB ($p = 0.0434$) and liver ($p = 0.0003$) in the treatment group (Treatment-multiple), when compared to the control arm (Control-multiple) (Figure 4D). Mice in the control group developed malar, discoid and erythematous skin rash, and/or hair loss (Figure 4E, upper panel), similar to that observed in human disease (27). Such aggressive cutaneous manifestations were not observed in the treatment group (Treatment-multiple) (Figure 4E, lower panel). Histopathological examination of affected skin biopsy of control mice (Control-multiple) showed lymphocytic infiltrate, subdermal edema, loss of hair follicles, and widespread parakeratosis and hair follicle loss when compared to intact hair follicles and preservation of the subdermal layer in the tissue obtained from the affected site of the Treatment-multiple group mouse (Figure 4F).

As shown in Figure 5A, histopathological examination of the spleen showed diffuse lymphocytic infiltration with loss of red- and white-pulp demarcation in the Control-multiple group, whereas spleen tissue architecture was well preserved in the Treatment-multiple group. The total number of human $CD3^+$, $CD4^+$, $CD8^+$, $CD20^+$, and $Ki67^+$ cells and the H-score for spleen IHC stains were significantly decreased in the Treatment-multiple group compared with the Control-multiple group ($p < 0.0001$) (Figure 5B). A significant reduction in the total number of human $CD3^+$, $CD4^+$, $CD8^+$, $CD20^+$, and $Ki67^+$ cells and the H-score for all liver IHC stains in the Treatment-multiple group was also observed ($p < 0.0001$) (Figures 5C, D).

$CD4^+CD25^+CD127^{low}$ UCB-Tregs improve renal function and decrease disease activity

Since anti-double-stranded DNA (dsDNA) antibodies are known to contribute to pathogenesis of LN (28), we measured

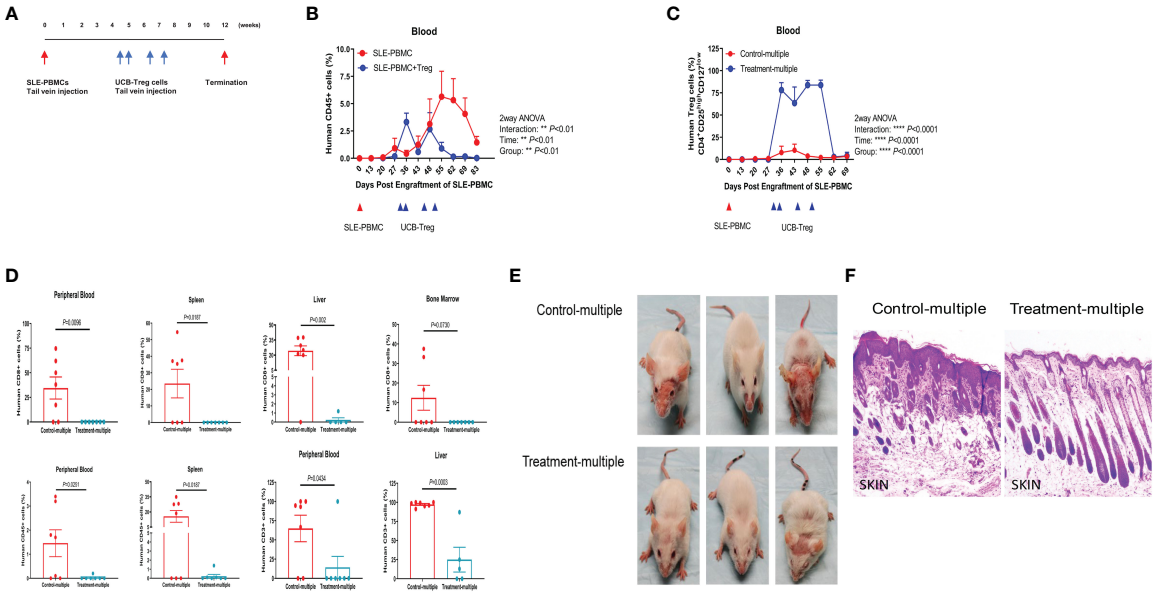

FIGURE 4
Multiple injections of $CD4^+CD25^+CD127^{low}$ UCB-Tregs increase Tregs, decrease $CD8^+$ T cells, and improve skin inflammation in the SLE xenogeneic model. **(A)** Schematic summary of multiple $CD4^+CD25^+CD127^{low}$ UCB-Treg cell therapy for SLE in a SLE xenograft model. Female $Rag2^{-/-}$ $\gamma c^{-/-}$ mice were transplanted with 3×10^6 human SLE-PBMCs by intravenous injection. After mice displayed human immune cells, they were divided into two groups (control and treatment, $n = 7$ mice/group), and 10×10^6 *ex vivo* expanded $CD4^+CD25^+CD127^{low}$ UCB-Tregs were infused into SLE xenografts intravenously on day 30, day 35, day 45, and day 51 for treatment. **(B)** Sustained decrease in PB human $CD45^+$ cells in $CD4^+CD25^+CD127^{low}$ UCB-Treg recipients. Mouse PBMC was procured from Control-multiple and Treatment-multiple recipients and analyzed for $CD45^+$ cells by flow cytometry at the indicated time points. Data are presented as mean ± SEM ($n = 7$). $p < 0.05$ was considered statistically significant. $^{**}p < 0.01$ by two-way ANOVA with Tukey multiple comparison tests. **(C)** Sustained increase in PB human $CD4^+CD25^{high}CD127^{low}$ Treg cells in $CD4^+CD25^+CD127^{low}$ UCB-Treg recipients. Mouse PBMC was procured from Control-multiple and Treatment-multiple recipients and analyzed for $CD4^+CD25^{high}CD127^{low}$ Treg cells by flow cytometry at the indicated time points. Data are presented as mean ± SEM ($n = 7$). *p*-values were obtained using two-way analysis of variance (ANOVA) with Tukey multiple comparison test. $p < 0.05$ was considered statistically significant. **** $p < 0.0001$ by two-way ANOVA with Tukey multiple comparison tests. **(D)** $CD4^+CD25^+CD127^{low}$ UCB-Tregs decrease $CD8^+$ T, $CD45^+$, and $CD3^+$ T cells in multiple organs. At the time of euthanasia, PB and organs from Control-multiple and Treatment-multiple recipients were harvested and liquified and analyzed for human $CD8^+$ T cells, $CD45^+$ cells, and $CD3^+$ T cells by flow cytometry at the indicated time points. Data are presented as mean ± SEM ($n = 5$-7). $p < 0.05$ by Student *t*-test was considered statistically significant. **(E)** $CD4^+CD25^+CD127^{low}$ UCB-Tregs decrease skin disease burden in SLE xenografts. Photographs of Control-multiple (upper panel) and Treatment-multiple (lower panel) were compared at 12 weeks. **(F)** Representative H&E staining of mouse skin tissue sections of Control-multiple (epidermal ulceration, subdermal lymphocyte infiltrate, disruption of hair follicles, and loss of subdermal adipose tissue) and Treatment-multiple (epidermal layer intact and clear visualization of the subdermal layers including hair follicles and adipose tissue).

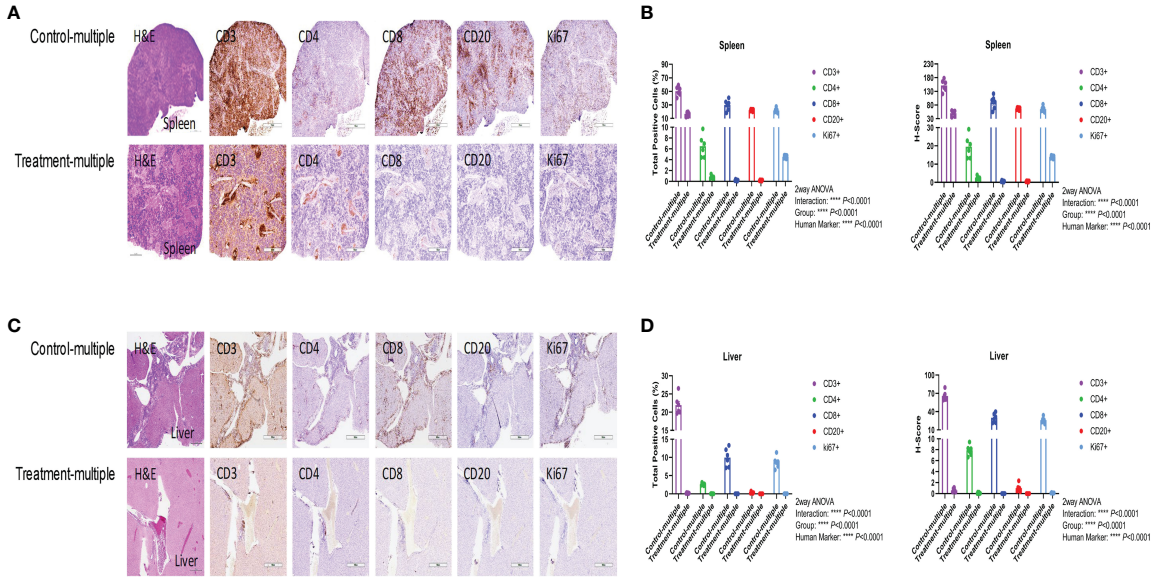

FIGURE 5
$CD4^+CD25^+CD127^{low}$ UCB-Tregs decrease tissue inflammation in SLE xenografts. To assess pathology and cellular infiltrates in the tissue, human $CD3^+$ cells, $CD4^+$ T cells, $CD8^+$ T cells, $CD20^+$ B cells, or $Ki67^+$ cells in the spleen **(A, B)** and liver **(C, D)** of untreated (Control-multiple) and treated mice (Treatment-multiple) were detected by immunohistochemistry. Spleen **(A)** and liver **(C)** tissues were harvested, fixed with 10% buffered formalin, and embedded in paraffin for processing into 5-µm tissue sections. To assess pathology and cellular infiltrates in the tissues, de-paraffinized and rehydrated tissue sections were stained with Hematoxylin & Eosin. For immunohistochemistry, de-paraffinized and rehydrated tissue sections were subjected to heat-mediated antigen retrieval with sodium citrate buffer (pH 6), permeabilization, and blocking prior to staining with primary antibodies against human CD3, CD4, CD8, CD20, and Ki-67, and appropriate horseradish peroxidase-conjugated secondary antibodies were used to determine the subset of human cells in the mouse tissues. Immunochemistry images of human $CD3^+$, $CD4^+$, $CD8^+$, $CD20^+$, and $Ki67^+$ cells were analyzed at ×40 magnification using HALO 3.3 software in spleen **(B)** and liver **(D)**. Quantification analysis of human $CD3^+$, $CD4^+$, $CD8^+$, $CD20^+$, and $Ki67^+$ cells and H-scores for human CD3, CD4, CD8, CD20, and Ki67 positivity were calculated using HALO 3.3 software. Data are presented as mean ± SEM (n = 6). $p < 0.05$ was considered statistically significant. $p < 0.0001$ by two-way ANOVA with Tukey multiple comparison tests.

their levels in the Control-multiple and Treatment-multiple arm. As shown in Figure 6A, multiple injections of $CD4^+CD25^+CD127^{low}$ UCB-Tregs significantly reduced circulating levels of human anti-dsDNA IgG antibody at 12 weeks (p = 0.0241). Histopathological evaluation of kidney tissue at the time of euthanasia revealed intense lymphocytic infiltrate in the Control-multiple arm compared to preservation of tissue architecture in the Treatment-multiple arm (Figure 6B, left panel). IHC staining of renal tissue showed a significant decrease in the total number of $CD3^+$, $CD4^+$, $CD8^+$, $CD20^+$, and $Ki67^+$ cells and the H-score for all kidney IHC stains in the Treatment-multiple arm when compared to the Control-multiple arm ($p < 0.0001$) (Figures 6B, C). Since SLE-induced renal inflammation leads to organ dysfunction as captured by albuminuria (29), we measured secretion of albumin in mouse urine. As shown in Figure 6D, a significant reduction in albuminuria was observed in the Treatment-multiple arm when compared to the Control-multiple arm (p = 0.02).

$CD4^+CD25^+CD127^{low}$ UCB-Tregs resolve SLE inflammation *in vivo*

As shown in Figure 7, at the time of euthanasia, mouse plasma showed a reduction in the levels of circulating inflammatory cytokines including IFN-γ, IP-10, TNF-α, IL-17A, sCD40L, and IL-1α, which overlapped with those impacted in *in vitro* studies (Figures 2B–I).

Discussion

Here, we provide proof of concept of using adoptive therapy with $CD4^+CD25^+CD127^{low}$ UCB-Tregs for the treatment of LN by decreasing systemic and renal inflammation, and overall disease burden as shown by a decrease in anti-dsDNA Ab and improvement in albuminuria. The superiority of $CD4^+CD25^+CD127^{low}$ UCB-Tregs is highlighted by their ability to suppress both pathogenic SLE cells and healthy donor Tcon cells, to a similar degree, and thus provides proof that $CD4^+CD25^+CD127^{low}$ UCB-Tregs are not impacted by the potential Teff resistance, well described as a possible point of failure in using $CD4^+CD25^+CD127^{low}$ Treg adoptive therapy in SLE (14). At a fundamental level, the co-culture of $CD4^+CD25^+CD127^{low}$ UCB-Tregs with SLE-PBMC shifts the dominance of $CD8^+$ Teffs and $CD19^+$ lupus B cells towards the $CD4^+CD25^+CD127^{low}$ Treg phenotype and $CD4^+CD8^+$ dual expressing T cells, which have been shown to have a suppressive effect on the production of autoantibodies including anti-dsDNA Ab in SLE (30). $CD4^+CD25^+CD127^{low}$ UCB-Tregs decrease the percentage of pathogenic monocytes, which have been shown to be associated with deterioration of kidney function in lupus patients (31). In fact, our $CD4^+CD25^+CD127^{low}$ UCB-Tregs were able to continue

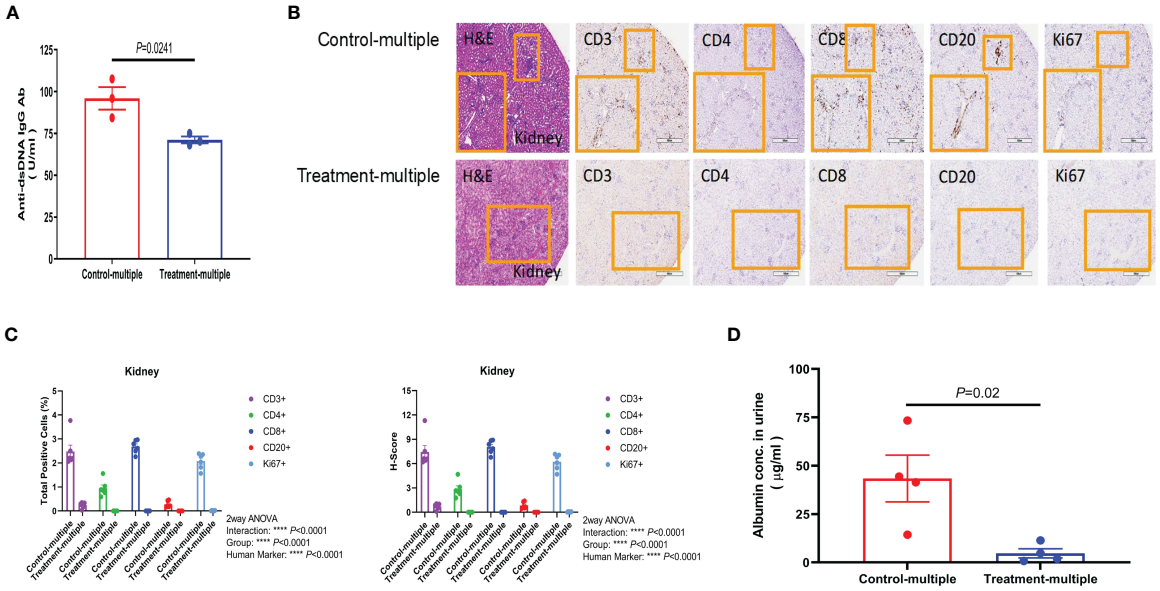

FIGURE 6
$CD4^+CD25^+CD127^{low}$ UCB-Tregs decrease anti-dsDNA IgG Ab and improve renal function in SLE xenografts. **(A)** Multiple injections of $CD4^+CD25^+CD127^{low}$ UCB-Tregs decrease anti-human dsDNA Ab. Plasma samples were collected from the EDTA-treated PB of untreated (Control-multiple) and $CD4^+CD25^+CD127^{low}$ UCB-Tregs-injected mice (Treatment-multiple). Levels of anti-human dsDNA IgG Ab were measured using the Abnova assay kit. Data are presented as mean ± SEM (n = 3). P-values were obtained using two-tailed unpaired t-test with 95% confidence interval for evaluation of statistical significance compared with the untreated controls. $P < 0.05$ was considered statistically significant. **(B)** Representative H&E, CD3, CD4, CD8, CD20, and Ki67 staining of kidney tissue sections of Control-multiple (upper panel) and Treatment-multiple (lower panel). **(C)** Quantification analysis of human $CD3^+$, $CD4^+$, $CD8^+$, $CD20^+$, and $Ki67^+$ cells and H-scores for human CD3, CD4, CD8, CD20, and Ki67 positivity. Total positive cells and H-score were calculated using HALO 3.3 software. Data are presented as mean ± SEM (n = 6). $p < 0.05$ was considered statistically significant. ****$p < 0.0001$ by two-way ANOVA with Tukey multiple comparison tests. **(D)** $CD4^+CD25^+CD127^{low}$ UCB-Tregs decrease albuminuria in SLE xenografts. At the time of euthanasia, at 12 weeks, urine samples were collected from Control-multiple and Treatment-multiple and levels of urinary albumin were assessed using the Exocell Albumin M assay kit. Data are presented as mean ± SEM (n = 4). p-values were obtained using two-tailed unpaired t-test with 95% confidence interval for evaluation of statistical significance compared with the untreated controls. $p < 0.05$ was considered statistically significant.

their IL-10 secretion, a well-described suppressive cytokine, to inhibit the proliferation of pathogenic SLE-PBMCs *in vitro*. IL-2-dependent IL-10 expression in human $CD4^+CD25^+CD127^{low}$ Tregs has been shown to be mediated by Stat5 recruitment to a Stat-binding motif in the fourth intron of IL-10 (I-SRE) (32), which can, in turn, induce suppression of Teffs (33). Recently, efforts have been made to use external IL-2 injection to increase $CD4^+CD25^+CD127^{low}$ Treg cells *in vivo* in lupus patients (34–36). However, our data do not support such an approach since the stimulation of SLE-PBMCs with IL-2 and CD3/28 beads did increase in $CD4^+$ $CD25^+CD127^{low}$ Treg cell population, but it did not translate into suppressor function as there was no increase in the secretion of IL-10. Therefore, such an acquired phenotype may not correlate with functional $CD4^+CD25^+CD127^{low}$ Treg cells and caution must be exercised when evaluating systemic IL-2 drug treatment as an attempt to stimulate and/or increase $CD4^+CD25^+CD127^{low}$ Tregs in patients suffering from SLE and other autoimmune diseases, since the correlation of *in vivo* expansion of $CD4^+CD25^+CD127^{low}$ Treg cells in response to low-dose IL-2 in SLE patients with a clinical response in open-label studies did not translate into clinical efficacy in randomized controlled trials (37). In fact, stimulation of human $CD4^+CD25^+CD127^{low}$ Treg cells under inflammatory conditions in the presence of IL-2 carries the risk of converting them into TH17 cells (38).

Co-culture with $CD4^+CD25^+CD127^{low}$ UCB-Tregs also decreased the percentage of $CD56^+$ NK cells in the SLE-PBMC population, implicated in excessive IFN-γ production in patients with active SLE (39), where elevated levels of IFN-γ have been shown to be associated with nephrotic syndrome (40). Independently, co-culture with our $CD4^+CD25^+CD127^{low}$ UCB-Tregs significantly decreased IFN-γ secretion by the pathogenic lupus cells *in vitro* and multiple injections of $CD4^+CD25^+CD127^{low}$ UCB-Tregs in SLE xenografts decreased circulating IFN-γ levels with a corresponding improvement in kidney function. Recently, the role of IFN-γ and IFN-γ-inducible GBP1 gene has been shown to mediate the development of SLE in a gene expression study (41). IFN-γ is a major proinflammatory cytokine that regulates the functions of several important immune system cells, including B cells and T cells (42), directly inhibits $CD4^+CD25^+CD127^{low}$ Treg cell function (43), and contributes significantly to the development of SLE (44). Our $CD4^+CD25^+CD127^{low}$ UCB-Treg cells were able to overcome the inhibitory effect of IFN-γ, which may additionally contribute to its multi-dimensional mechanism of action as a therapeutic agent for SLE patients.

$CD4^+CD25^+CD127^{low}$ UCB-Tregs decreased $CD19^+$ B-cell population in the SLE-PBMCs. B cells have been identified to drive lupus pathogenesis and are the target of currently approved biologics treatment for SLE (45, 46). Recently, CD19-targeted CAR T cells

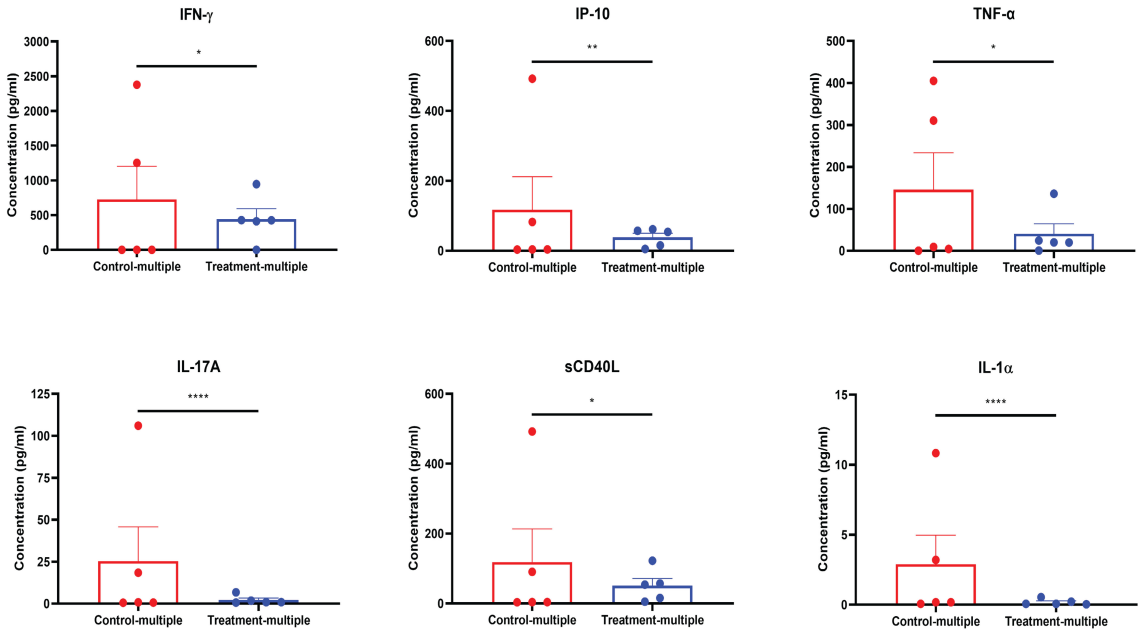

FIGURE 7
$CD4^+CD25^+CD127^{low}$ UCB-Tregs decrease systemic inflammation in SLE xenografts. Plasma samples were collected from the EDTA-treated PB of untreated (Control-multiple) and $CD4^+CD25^+CD127^{low}$ UCB-Tregs-infused mice (Treatment-multiple). Levels of human IFN-γ, IP-10, TNF-α, IL-17A, sCD40L, and IL-1α in the plasma of SLE xenografts were measured using the Human cytokine 42-plex Discovery assay kit. Data are presented as mean ± SEM (n = 5). $p < 0.05$ by F-test was considered statistically significant. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ by two-way ANOVA with Tukey multiple comparison tests.

treatment was shown to induce clinical remission with a decrease in proteinuria in a patient with refractory SLE, where the expansion of CAR T cells preceded the complete and sustained depletion of circulating B cells, resulting in a decrease in anti-dsDNA autoantibodies level (47). A similar coupled decrease of anti-dsDNA IgG Ab and albuminuria was also observed *in vivo* in response to treatment with multiple injections of our $CD4^+CD25^+CD127^{low}$ UCB-Tregs.

The active role of monocytes in accelerating inflammation and injury in kidney glomerular lesions has been identified in SLE (48, 49). Monocytes in SLE have been shown to engage the CD40/CD40L signaling pathways to contribute to lupus pathogenesis (50). Our $CD4^+CD25^+CD127^{low}$ UCB-Tregs decreased monocytes in the SLE-PBMC population as well as reduced soluble CD40L *in vitro* and *in vivo*. Additionally, $CD4^+CD25^+CD127^{low}$ UCB-Tregs also decreased several inflammatory cytokines implicated in lupus pathogenesis both *in vitro* and *in vivo*, including IP-10 (51), TNF-α (52), IL-6 (53), IL-17A (54), and IL-1 (55). The crosstalk between the circulating and tissue-resident cytotoxic $CD8^+$ T cells and widespread tissue destruction including skin, spleen, and kidney, as well as systemic and tissue inflammation, clearly evident in our xenogeneic lupus model, is supported by the published data where local cytokine, chemokine, and adhesion molecule production has been shown to encourage the further influx of inflammatory cells and the production of proinflammatory cytokines, ultimately resulting in tissue inflammation, tissue injury, and, eventually, fibrosis (56).

The remarkable ability of our $CD4^+CD25^+CD127^{low}$ UCB-Treg cells to interrupt the vicious inflammation–injury loop provides proof of concept of their activity in SLE. Previous reports have shown the role of anti-dsDNA isotypes and anti-C1q antibody in the diagnosis of SLE and their association with disease activity and LN (57, 58). Our results suggest that the correlation of $CD4^+CD25^+CD127^{low}$ UCB-Tregs administration with decreases in known disease measures such as anti-dsDNA IgG Ab as well as the quantification of the end organ damage including albuminuria and its multimodal mechanism of action makes them ideal for the treatment of LN.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by The University of Texas M.D. Anderson Cancer Center Institutional Review Board. The studies were conducted in accordance with the local legislation and institutional requirements. The animal study was approved by The University of Texas M.D. Anderson Cancer Center Institutional Animal Care & Use Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

Conception and design of the study: M-AL and SP. Acquisition, analysis, and interpretation of data: M-AL, XT, JDK, MGR, MH, MN, KZ, HM, CRF, and SP. Drafting and revising the manuscript: M-AL and SP. All authors contributed to the article and approved the submitted version.

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Conflict of interest

JDK received research funding from Stemline Therapeutics, Angle, and Kiromic BioPharm. SP has an equity interest in, holds patents for, receives royalties and research funding from, and is a member of the board of directors/advisory committee for Cellenkos Inc. CRF received research funding from 4D, AbbVie, Acerta, Adaptimmune, Allogene, Amgen, Bayer, Celgene, Cellectis, EMD, Gilead, Genentech/Roche, Guardant, Iovance, Janssen Pharmaceutical, Kite, Morphosys, Nektar, Novartis, Pfizer, Pharmacyclics, Sanofi, Takeda, TG Therapeutics, Xencor, Ziopharm, Burroughs Wellcome Fund, and Eastern Cooperative Oncology Group, National Cancer Institute, and Cancer Prevention and Research Institute of Texas: CPRIT Scholar in Cancer Research and and consulting fees from AbbVie, Bayer, BeiGene, Celgene, Denovo, Biopharma, Epizyme, Genentech/Roche, Genmab, Gilead, Karyopharm, Pharmacyclics/Janssen, SeaGen, and Spectrum. TS was employed by the company Cellenkos Inc.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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NETosis: an emerging therapeutic target in renal diseases

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Introduction: Neutrophil extracellular traps (NETs) are web-like structures composed of nuclear and granular components. The primary role of NETS is to prevent the dissemination of microbes and facilitate their elimination. However, this process is accompanied by collateral proinflammatory adverse effects when the NET release becomes uncontrollable, or clearance is impaired. Although NET-induced organ damage is conducted primarily and indirectly via immune complexes and the subsequent release of cytokines, their direct effects on cells are also remarkable. NETosis plays a critical pathogenic role in several renal disorders, such as the early phase of acute tubular necrosis, anti-neutrophil cytoplasmic antibody-mediated renal vasculitis, lupus nephritis, thrombotic microangiopathies, anti-glomerular basement membrane disease, and diabetic nephropathy. Their substantial contribution in the course of these disorders makes them a desirable target in the therapeutic armamentarium. This article gives an in-depth review of the heterogeneous pathogenesis and physiological regulations of NETosis and its pivotal role in renal diseases. Based on the pathogenesis, the article also outlines the current therapeutic options and possible molecular targets in the treatment of NET-related renal disorders.

Methods: We carried out thorough literature research published in PubMed and Google Scholar, including a comprehensive review and analysis of the classification, pathomechanisms, and a broad spectrum of NET-related kidney disorders.

Conclusions: NETosis plays a pivotal role in certain renal diseases. It initiates and maintains inflammatory and autoimmune disorders, thus making it a desirable target for improving patient and renal outcomes. Better understanding and clinical translation of the pathogenesis are crucial aspects to treatment, for improving patient, and renal outcomes.

KEYWORDS

NETosis, neutrophil extracellular traps, renal diseases, lupus nephritis, ANCA associated vasculitis

1 Introduction

Neutrophil granulocytes are crucial members of innate immunity. Their antimicrobial arsenal includes 1) the release of granular proteins such as neutrophil elastase (NE) and myeloperoxidase (MPO), 2) the phagocytosis via the production of reactive oxygen species (ROS) inside the phagosome, and 3) the formation of neutrophil extracellular traps (NETs) (1–3).

NETs are web-like structures of nuclear and granular components released from the membrane of activated neutrophils. The granular components are the contents of the primary (azurophilic) granules, such as NE, cathepsin G, MPO, and LL-37 (cathelicidin) as well as the secondary and tertiary granules (lactoferrin, gelatinase) (4). Highly decondensed chromatin fibers and citrullinated histone proteins make up the majority of the nuclear components. Four main forms of NETosis (the mechanism of releasing NETs) are known today: lytic (suicidal), non-lytic (vital), caspase 11/4-mediated, and mitochondrial NETosis.

Traditionally, cell death is categorized either as accidental or programmed cell death. Accidental cell death – *necrosis* – is an uncontrolled process that occurs as a result of an overwhelming stimulus that is accompanied by inflammatory responses caused by releasing components of the dying cell such as heat shock proteins, uric acid, and nuclear proteins. Programmed cell deaths include apoptosis, pyroptosis, necroptosis, and autophagy. *Apoptosis* occurs in non-inflammatory conditions and is precisely conducted by a cascade of molecular events (membrane blebbing, size reduction, chromatin condensation, and DNA fragmentation) ended with the engulfment of the granule-packed cellular components by phagocytes, without breaking the cell membranes (5). *Pyroptosis* is a process, driven by the inflammasome and Gasdermin-D, during which the cell swells until its membrane eventually breaks down (6). *Necroptosis* is a lytic form of cell death and mimics both the characteristics of apoptosis and necrosis and it is mediated by the receptor-interacting protein kinase (RIPK3) and the mixed lineage kinase domain-like (MLKL) pseudokinase. During *autophagy*, the primary role is to meet metabolic needs and to recycle certain cytoplasmic proteins and organelles by engulfing and covering them in vesicles that fuse with lysosomes to digest their contents. In contrast to the other canonical forms of programmed cell death, NETosis can proceed differently depending on the stimuli and the environment (7, 8).

The first evidence of NETosis was described in 1996 by Takei et al., who found that phorbol-12-myristate-13-acetate (PMA)-induced neutrophils died by a previously unknown mechanism other than apoptosis or necrosis (9). Later, in 2004, Brinkmann et al. revealed that activated neutrophils release their nuclear and granular contents to the extracellular space, where they form a web-like network to immobilize and kill bacteria (4). It is well-known that NETs can bind microbes to prevent them from spreading and eliminate them, but they may also convey unfavorable effects in tissue damage, atherosclerosis, thrombosis, or malignancies (10–12).

Furthermore, the role of NETosis was already described in autoimmune conditions. Enhanced production or decreased clearance of the NETs causes dysregulated immunity, autoantigen modification and externalization, and tissue damage (13). The importance of NETosis has been demonstrated in the pathogenesis of a variety of disorders, such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV), psoriasis and gout (14).

In this review article, we summarize the heterogeneous pathogenesis and physiological regulations of NETosis and its pivotal role in renal diseases. Based on the pathogenesis, the article also discusses current therapeutic options and possible molecular targets in the treatment of NET-related renal disorders.

2 Pathomechanisms

2.1 Lytic (suicidal) NET formation

The most extensively studied type of NET formation is called "suicidal" or "lytic", also referred as *NETosis*. This is indeed a form of cell death because once the neutrophils release their contents, they eventually perish (Figure 1A).

Based on the involvement of nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase, lytic NETosis was used to be divided into *NADPH-dependent* and *NADPH-independent* forms. Later it became clear that the NADPH-independent pathway is conducted by mitochondrial ROS (mtROS) production, hence, the term, "NADPH-independent" was modified to "mitochondria-dependent NETosis" (15). On the other hand, since NADPH-oxidase may be induced by mtROS - NADPH-oxidase can engage in both processes.

Furthermore, triggering stimuli have a pivotal role and the signalization of NETosis depends on them. Proteomic analysis showed diversity in terms of NET-constitution and post-translational modifications, which suggests that the stimulus determines the biological effects (16).

Conventional lytic NETosis is usually initiated by complement proteins (such as C3b, C5a), cytokines, and ligands binding to Toll-like receptors (TLR) or IgG-Fc receptors (17). Upon receptor activation, calcium storage is released from the endoplasmic reticulum. Increased cytoplasmic calcium levels activate protein kinase C (PKC) via the RAF-MEK-MAPK/ERK signaling pathways and induce the phosphorylation of gp91phox. This stimulates the membrane-bound subunits of NADPH-oxidase to assemble into the functional enzyme in the cytoplasmic or phagosome membranes. Thus, the reactive oxygen species (ROS) generation is initiated (18, 19).

Simultaneously, the disintegrated azurophilic granules, MPO, NE, and other lytic enzymes are released into the cytosol. By oxidizing it, MPO facilitates the release of NE into the cytoplasm, however, the exact role of the lytic enzymes in this process still needs to be elucidated (20, 21).

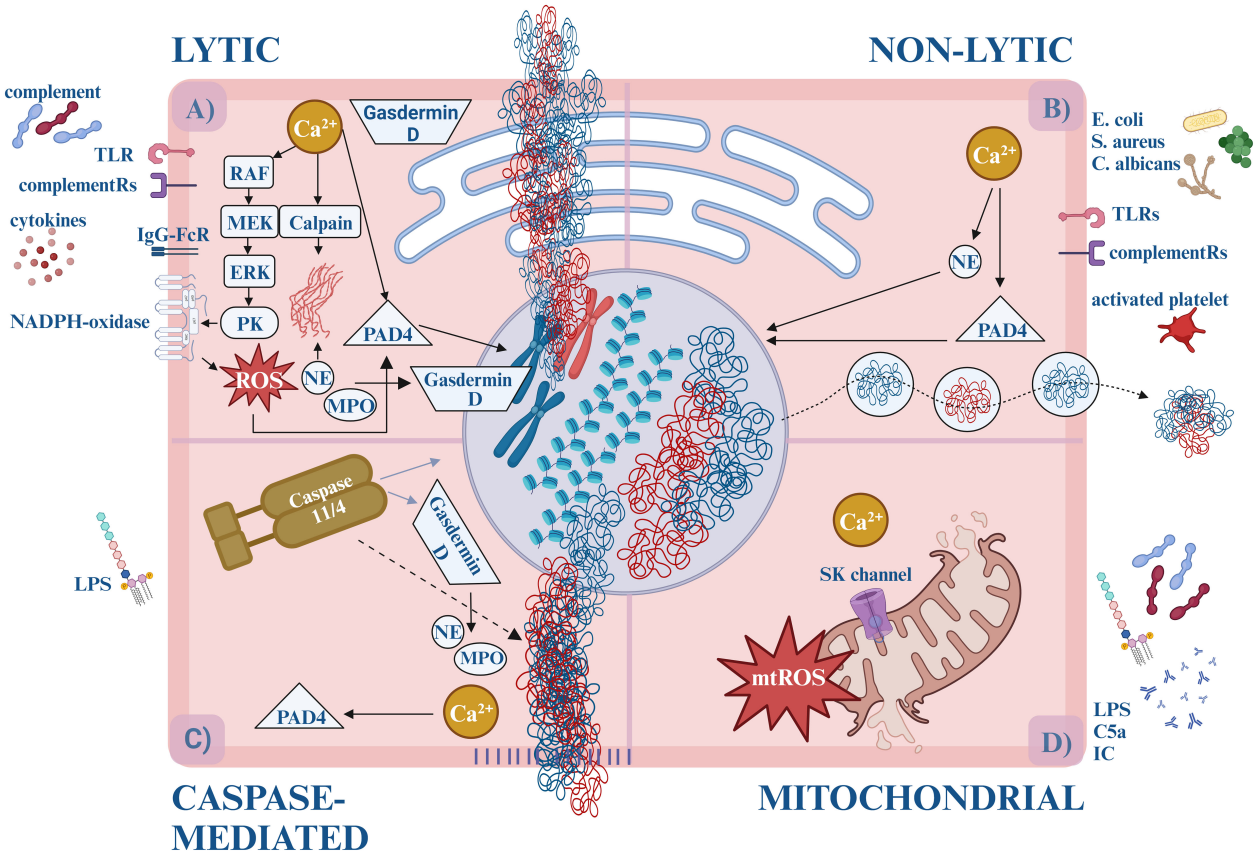

FIGURE 1
The forms of NETosis. **(A)** Lytic (suicidal) NET formation. By the activation of TLR (e.g., TLR2, TLR4, TLR7, TLR9) or IgG-Fc receptors (FcγRIIa and/or FcγRIIIb) calcium is released from the ER, activating PKC via the RAF-MEK-MAPK/ERK pathway. PKC triggers the assembly of the membrane-bound subunits of NADPH-oxidase, which starts generating ROS. ROS and/or calcium activates PAD4, which translocates to the nucleus and induces the citrullination of arginine amino acids, loosening the histone-DNA interaction, and inhibits the H1 histone-mediated compaction causing chromatin decondensation. Simultaneously, enzymes (MPO, NE) are released from the granules. NE and the calcium-activated calpain degrade the cytosolic filamentous network and the nuclear lamina. With the participation of Gasdermin-D, NE destructs the nuclear envelope and enables water influx. NE cleaves histones causing further chromatin decondensation. Eventually, Gasdermin D-mediated pore opening of the plasma membrane leads to the externalization of the DNA, cytosolic and granular proteins. **(B)** Non-lytic (vital) NET formation. Different stimuli induce non-lytic NET formation, like bacteria, and fungi via TLRs (e.g., TLR2, TLR4, TLR7, TLR9) or complement receptors (e.g., CR1, CR3, CR4, C5aR1). Calcium influx starts through the SK-channels leading to PAD4 and NE activation. They translocate to the nucleus and decondense the chromatin. Unlike lytic NETosis, NADPH oxidase is not involved in the process, and the nuclear content is released from the cell in vesicles. It takes approximately 5-60 minutes, compared to the lytic form, which takes about 3-4 hours. **(C)** Caspase-mediated NETosis. LPS activates caspase-11/4 via an uncanonical inflammasome pathway. Then caspase-11/4 activates Gasdermin D, which starts forming pores on the nuclear-, plasma- and granular membranes, enabling calcium influx and NE, MPO release. Caspase-11/4 enters the nucleus and cleaves histones, while the calcium-activated PAD4 citrullinates the arginine amino acids. Although NE, MPO, and PAD4 facilitate the process, their activities are not prerequisites for NETosis. **(D)** Mitochondrial NETosis. LPS, GM-CSF, C5a or IC induction elevates cytosolic calcium level, which opens SK channels and leads to mtROS production, and mitochondrial DNA release. ER, endoplasmatic reticule; DNA, deoxyribonucleic acid; GM-CSF, granulocyte-macrophage colony-stimulating factor; IC, immune complexes; IgG-FcR, immune globulin G – fragment crystallizable receptor; LPS, lipopolysaccharides; MPO, myeloperoxidase; mtROS, mitochondrial reactive oxygen species; NAPDH oxidase, nicotinamide adenine dinucleotide phosphate oxidase; NE, neutrophil elastase; PAD4, protein arginine deiminase 4; PKC, protein kinase-C; ROS, reactive oxygen species; SK-channels, small conductance calcium-activated potassium channels; TLR, Toll-like receptor.

The cytosolic fibrous network also falls victim to degradation. The disassembly of actin and vimentin molecules is promoted by calcium influx. Activated by calcium level increase and citrullination, calpain - a serine protease – facilitates the decondensation of micro- and intermedier filaments and nuclear lamin as well (22). The intactness of the microtubular network, however, does not affect the NETosis (23). NE binds and degrades the actin fibers (preventing concurrent phagocytosis), thus making its way to the nucleus. Cooperating with Gasdermin-D, NE destructs the nucleus envelope and enables water influx (21). Here NE proceeds to cleave histones which eventually leads to chromatin decondensation (24–26).

By binding calcium ions or activated by ROS, protein-arginine deiminase 4 (PAD4) adopts its catalytically active form. PAD4 translocates to the nucleus which induces citrullination of the arginine amino acids, thus reducing the positive charge and loosening the histone-DNA interaction. PAD4 also has direct effects on chromatin decondensation via inhibition of linker

histone-mediated compaction (23). Ultimately, these processes lead to the decondensation of the chromatin structure (27, 28). Gasdermin D-mediated pore openings induce the progressive permeabilization of the plasma membrane which leads to the externalization of the DNA, cytosolic and granular proteins (29). Of note, pore formation changes the intracellular calcium gradient, which further promotes PAD4 activation (30).

2.2 Non-lytic (vital) NET formation

During "vital" or "non-lytic" NET formation neutrophils maintain their viability and antimicrobial functions (e.g., engaging in recruitment, chemotaxis, and phagocytosis) after they release the nuclear or mitochondrial DNA (Figure 1B).

Non-lytic NETosis is initiated by the detection of stimuli of complement receptors and activated platelets. Staphylococcus aureus, Escherichia coli, and Candida albicans can also cause non-lytic NET formation by activation of complement receptor (CR) -1, -3, -4 and TLR - 2, -4 ligands NADPH independently. Calcium influx starts through the small conductance potassium channel member three (SK) and activates PAD4 and NE. They translocate to the nucleus where they decondense the chromatin. Eventually, chromatin is expelled to the extracellular space by vesicular transport, in which the blebs fuse with the plasma membrane again. This mechanism maintains the integrity of the plasma membrane and the anucleated neutrophils (cytoplasts) stay alive, keeping their ability to do their antimicrobial functions: migrate and phagocytose. This process takes approximately 5-60 minutes, compared to the lytic form of NETosis, which requires 3-4 hours (20).

2.3 Other types of NETosis

The third form of NET formation is conducted by caspases. Cytosolic lipopolysaccharide (LPS) induction of neutrophils activates murine caspase-11 or the human ortholog caspase-4 via an uncanonical inflammasome pathway to enable Gasdermin D cleavage into the pore-forming fragments. Upon entering the nucleus, caspase-11/4 degrades the histones and chromatin. Gasdermin D forms pores on granules as well, liberating NE/MPO. Calcium influx through the pores activates PAD4 and citrullinates histones. Although NE, MPO, and PAD4 facilitate the process, their activities are not prerequisites for NETosis – caspase-induced chromatin cleavage converges in a similar molecular pathway as lytic NETosis (31) (Figure 1C).

Another pathway that leads to NETosis is the Ca^{2+}-ionophore-induced mitochondrial ROS (mtROS) production. Elevated cytosolic calcium level opens SK channels, mediates mtROS production, and induces apoptosis and NET formation (15, 32). Granulocyte-macrophage colony-stimulating factor (GM-CSF), LPS, complement component 5a (C5a), or ribonucleoprotein-containing immune complexes also activate neutrophils to release mitochondrial DNA and ROS (33). Oxidized mtDNA has potent proinflammatory and type I interferon stimulatory properties that seem to play a pivotal role in the pathogenesis of lupus nephritis (LN) (34). Although mtROS-induced NETosis is known as NADPH-oxidase-independent NETosis, the crosstalk with NADPH-oxidase to produce additional ROS has already been demonstrated (32) (Figure 1D).

In conclusion, the contribution of PAD4 and ROS are the most relevant factors to group the processes. Lytic NETosis is ROS-dependent, and the participation of PAD4 is not obligatory, while non-lytic NET formation occurs in the absence of ROS with the important role of PAD4. Table 1 summarizes the main characteristics of NET formation.

2.4 Clearance of NETs

The control of NET formation and elimination is inevitable to maintain tissue homeostasis. Lytic neutrophil remnants are mainly taken up by macrophages. NET-contents function as danger-associated molecules and they are recognized by membrane-bound receptors (intracellular adhesion molecule 1, 3, liver X receptor, Mer tyrosine kinase) or soluble pattern recognition proteins (galectin-3, C3, C1q, annexin A1, factor-H related protein, C-reactive protein, clusterin, milk fat globule EGF factor 8). Scavenging macrophages identify NETotic neutrophils by "eat-me" signals or by the loss of "don't eat-me" signals and phagocytosis takes place. Interestingly, neutrophil LL-37 interacts with extracellular anionic molecules such as dsDNA or dsRNA and aids their uptake by macrophages. Typically, the degradation of the apoptotic (and the NETotic) neutrophils creates a net anti-inflammatory microenvironment, hence this process differs from the traditional phagocytosis, so the term, "efferocytosis" was coined (35). After NETosis, interferons and other proinflammatory cytokines prime macrophages to M1 macrophages. M1 macrophages are characterized by the ability to secrete inflammatory cytokines and costimulatory molecules. Furthermore, upon interaction with NETs, M1 macrophages release DNA in a PAD4-dependent manner (36). Hence, they not only phagocyte the intercellular debris but also exacerbate inflammation. However, over time, M1 macrophages also activate their own caspase-activated DNase to degrade the surrounding extracellular DNA, and, the macrophage phenotype shifts toward an anti-inflammatory effector function, to the M2 macrophages (36). Although the molecular background of phenotype shifting is not fully unraveled, a crosstalk between the M1 and M2 polarizing pathways is proved. The increase of cell death by M1 macrophages is recognized by M2 phenotypes and they establish a predominantly anti-inflammatory milieu, therefore, the microenvironment commits to tissue remodeling and immune tolerance (37–39). Interestingly, M2 macrophages also contribute to the initial M1 polarization by releasing inflammatory cytokines upon encountering NETs (36). This highlights that macrophages could be further subdivided based on their functionally distinct roles (40).

The complement system also facilitates the clearance of cellular debris. All three complement pathways are involved in the removal of NETs; however, the classical pathway components, C3, C4, C5,

TABLE 1 Comparison of NETosis forms.

	Lytic (suicidal)	Non-lytic (vital)	Caspase-mediated	Mitochondrial
Duration	3-4 hours	50-60 minutes	depends on stimuli	depends on stimuli (C. albicans: 30 min; mito-antigens: 3-4h)
Stimuli	crystals (cholesterol, monosodium urate, calcium carbonate) auto-Abs, ICs viruses, bacteria, fungi, parasites DAMPS (histone, LDL) protein fibers tumor cells cytokines (TNFa, CXCL2, IL-1, IL-8, IL-18) LPS, activated platelets, PAF NO, H2O2 PMA	Bacteria: E. coli, S. aureus Fungi: C. albicans LPS IL-8 PAF ICs complements (e.g., C3b, C5a)	cytosolic LPS cytosolic G(-) bacteria	Ca^{2+}-ionophores GM-CSF LPS C5a ribonucleoprotein-containing ICs UV
Receptors	TLRs (e.g., TLR2, TLR4, TLR7, TLR9), NOD-like Rs C-type lectin Rs, complement Rs, FcRs (e.g., FcγRIIa and/or FcγRIIIb), chemokine Rs (e.g., CXCR1, CXCR2 and CXCR4) Siglec, RAGE, PSGL1	CRs (e.g., CR1, CR3, CR4, C5aR1) TLRs (e.g., TLR2, TLR4, TLR7, TLR9) activated platelets	to be identified	to be identified TLRs (e.g., TLR7, TLR9) CRs (e.g., C5aR1)
ROS	dependent	independent	independent (see details in text)	mtROS-dependent
PAD4	independent	dependent	not obligatory	
NE	dependent		not obligatory	
MPO	dependent		not obligatory	
In disease	AAV		sepsis, RA, cancer	SLE, LN

Empty cells indicate that no matching data was found in the literature.
AAV, anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis; C5a, complement 5a; CR, complement receptor; CXCL2, chemokine (C-X-C motif) ligand 2; CXCR, chemokine receptor; DAMP, damage-associated molecular patterns; GM-CSF, granulocyte-macrophage colony-stimulating factor; H2O2, hydrogen peroxide; ICs, immune complexes; IL, interleukin; mtROS, mithocondrial reactive oxygen species; LN, lupus nephritis; LPS, lipopolysaccharides; NE, neutrophil elastase; NO, nitrogen oxide; NOD, nucleotide oligomerization domain; PAD4, protein arginine deiminase 4; PAF, platelet-activating factor; PMA, phorbol-12-myristate-13-acetate; PSGL-1, P-selectin glycoprotein ligand-1; R, receptor; RA, rheumatoid arthritis; RAGE, receptor for advanced glycation end products; ROS, reactive oxygen species; SLE, systemic lupus erythematosus; TLR, Toll-like receptor; TNF-α, tumor necrosis factor alfa; UV, ultraviolet.

and C1q have a higher affinity toward secondary necrotic cells like NETs. They either interact directly with DNA and mitochondrial DAMPs (damage-associated molecular patterns) or via immune complexes. Complement members do not only take part in opsonization but also aid the removal of NET remnants by activating serine proteases C1r and C1s (35).

Since DNA forms the backbone of NETs, DNases are central participants in degradation and digestion. The DNase complex contains three different enzymes (DNase I, DNase II, and DNase1-like 3 protein) with different functions (41). DNase I is responsible for the removal of protein-free DNA; while DNase1-like 3 protein degrades protein-associated DNA, including DNA packed in microvesicles, and DNase II digests the DNA from apoptotic cells (41, 42). Plasminogen, on the other hand, penetrates necrotic cells, accumulates in the cytoplasm and nucleus, and activates plasmin by tissue-type or urokinase-type plasminogen activator. Plasmin degrades histone H1 and facilitates internucleosomal DNA cleavage by DNase1 (43).

During NETosis, a substantial amount of serine proteases (e.g., PR3, MPO, NE, cathepsin G) are released into the extracellular space increasing the detrimental effects. To oppose this, ceruloplasmin disrupts MPO and limits MPO-dependent ROS generation, and alpha1-antitrypsin inhibits NE, PR3, and cathepsin G by forming complexes with them (44, 45).

3 The pathological roles of NETosis

3.1 Autoimmunity

While the beneficial effects of NETs have long been recognized, more recent studies have revealed that NETs also play a role in various pathological conditions, some of which may not be beneficial. The possibility that NETs may be involved in autoimmune diseases was initially suggested in 2004 by Brinkmann et al., leading to an increase in research on the subject (4). The role of NETosis in systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and ANCA-associated vasculitis (AAV) is well known today, but authors suggest that NETosis may also play a crucial role in other autoimmune and autoinflammatory conditions, such as type 1 diabetes mellitus, inflammatory bowel disease, gout, antiphospholipid syndrome, and in other rheumatic diseases (46–50).

Autoimmune diseases arise from the combination of genetic and environmental factors. NETs can be involved in breaking immune tolerance and triggering autoimmunity in a variety of pathways. After the initiation of an environmental factor, the propagation phase is characterized by inflammation and tissue damage. NETs contribute to propagation by epitope spreading

and NET-forming cellular components (such as histones, DNA, and granular proteins) can serve as autoantigens for later antibody production.

Furthermore, NETs interplay with the adaptive immune system. LL-37-DNA complexes enhance autoantibody production on B-cells via TLR9 (51). Activated neutrophils release B cell-activating factor (BAFF) which upregulates the CD21 and CD19 co-receptor expression and prolongs the B-cell survival by decreasing proapoptotic proteins (52). The NET-derived immunoglobulins bind to the FcγR of the neutrophils and initiate further NETosis, creating a vicious circle (53). NETs prime CD4+ T-cells directly on T-cell receptors with a lower threshold, thus T-cell response is increased upon suboptimal NET component stimulation. On the other hand, T-cell response is also elicited in a dendritic cell-mediated manner. NETs induce costimulatory CD80 and CD86 on dendritic cells which interacts with T-cell CD28 for activation and survival, as well as interleukin (IL) production.

Interferon overproduction is a hallmark in the pathogenesis of several autoimmune disorders. Plasmacytoid dendritic cells are the primary interferon-producing cells, and they can be activated by NET-induced LL-37, HMGB1 (high mobility group box 1 protein), and DNA via TLR9 (54). NETs also mediate the activation of caspase-1, leading to inflammasome activation via the NLRP3 (NOD-like receptor family, pyrin domain containing) in macrophages. Inflammasome activation results in IL-18 and -1β secretion which further promotes NET formation (55).

Autoimmunity also occurs in case of failure to remove apoptotic and NETotic cells. As an example, NADPH-oxidase or PAD4 knock-out mice develop lupus-like disorders which contradict the studies that suggested improvement in the case of administration of NADPH-oxidase or PAD4 inhibitors in NET-related autoimmune diseases (56). Both enzymes are required for cellular debris removal by macrophages and the total absence of them exacerbates autoimmunity instead of ameliorating it (57). Furthermore, DNA accumulation as a result of the lack of DNase activity leads to prolonged inflammation and the presence of NET autoantigens. In susceptible individuals self-tolerance breaks, and an autoimmune response develops (41).

3.2 Autoinflammation

Based on the current classification, autoimmune and autoinflammatory diseases are positioned at opposite ends of a spectrum. In autoinflammatory diseases, immune tolerance remains intact and the pathomechanism is not driven by autoantigen-autoantibody interactions. Local and external factors, such as infections, mechanical damage, or temperature effects have a significant impact in triggering the disease, leading to innate immune responses and tissue damage (58, 59). These pathologies can be classified based on their genetic origin, distinguishing between monogenic and polygenic diseases, where the NF-κB, the NLRP3 inflammasome, and the IL-1β pathway play an important role in the signalization (60, 61).

Based on Matzinger's "danger-theory", in autoinflammatory diseases damage-associated molecular patterns (DAMPs) and pathogene-associated molecular patterns (PAMPs) serve as trigger factors for the innate immune system (62). Neutrophils engage in the process, however, the exact role of NETosis is still elusive. The various immunogenic proinflammatory factors released during NETosis can maintain sterile inflammation as an amplification loop, partly by stimulating the innate immune system to further recruitment and cytokine production by netting DNA, histone, and antimicrobial peptides; and partly via the activation of NLRP3-caspase 1 inflammasome system and IL-1β production (63, 64). matrix metalloproteinase (MMP)-mediated endothelial injury is a direct tissue-damaging consequence of histones and LL-37 (65). Prolonged IL-1β and IL-18 production can trigger T-cell differentiation and IL-18 can induce T-helper 1 and B cells. Accordingly, in some immunological cases, we can speak of autoinflammatory-autoimmune diseases of mixed etiology (like juvenile idiopathic arthritis, rheumatoid arthritis, Behcet's disease, or adult-onset Still syndrome) (59, 66).

3.3 Thrombosis and atherosclerosis

Arterial and venous thrombotic and thromboembolic events can also occur via NETosis. NETs can serve as a scaffold for platelets to aggregate (10). Local hypoxia, NET compounds stimulate the endothelium further to release procoagulant factors and promote thrombus and NET formation. Additionally, tissue factor (TF) is released along with DNA, activating the extrinsic coagulation cascade pathway and increasing the risk of blood clots in the arterial and venous systems (67, 68). Along with the immunogenic effects, NETs also significantly contribute to vascular injury. Cytokines and extracellular histones damage endothelial cells directly as well as promote endothelial-mesenchymal transition (69). The NET-compound MMP-9 activates endothelial MMP-2 leading to endothelial cell death and augmenting the activity of collagenolysis, while interferons inhibit the endothelial progenitor's differentiation (65, 70). NET proteins also modify the high-density lipoprotein (HDL) via oxidation, stirring it into a proatherogenic direction (71). Endothelial cells have limited capacity to take up the remnants of NETs, and persistent exposure to them provokes vascular leakage by demolishing intercellular junction proteins (69). Ultimately, these changes result in compromised endothelial function and vascular damage. Macrophages are recruited to remove NETs and promote plaque formation (11). Taken together, all these alterations lead to atherosclerotic diseases and an augmented potential to rupture and thrombus formation.

3.4 Tumorigenesis

The cancerogenic and tumorigenic roles of NETosis were observed as well (72). NETs can be found in large quantities around the tumorous microenvironment. NETs abolish tumor cells through their lytic enzymes and disrupt the extracellular matrix and intercellular connections, which can facilitate the migration of tumor cells (12, 73). NETs also assist the spread of cancer by capturing and

helping metastatic cells to adhere to the tissue. They can also serve as a physical barrier, protecting tumor cells from cytotoxicity (73).

3.5 Kidney injury

The effect of NETosis on renal cells is primarily conducted by the immune complexes and the consequently induced cytokines. However, the direct effect of NETosis on renal cells is not fully elucidated and only a limited number of studies are available. The releasing endogenous antigens from NETs are aggravating the inflammation since they act as DAMPs and further prime neutrophils and trigger NET production (37). Most importantly, extracellular histones convey significant cytotoxic effects with cytokines such as interferon and induce epithelial-mesenchymal and PEC (parietal epithelial cell) progenitor proliferation, thus crescent formation (74). Histones activate TLR-2,4 and NLRP3. NLRP3 mediates caspase-1 molecular complex (inflammasome) activation and promotes IL-1 and IL-18 cleavage to mature forms. Caspase-1 elicits pyroptosis in endothelial cells and it also contributes to platelet activation, aggregation, and microthrombus formation thus hemodynamic disturbances (75). NETs cause changes in the slit diaphragm-associated proteins, such as podocin and nephrin, provoking podocyte effacement and

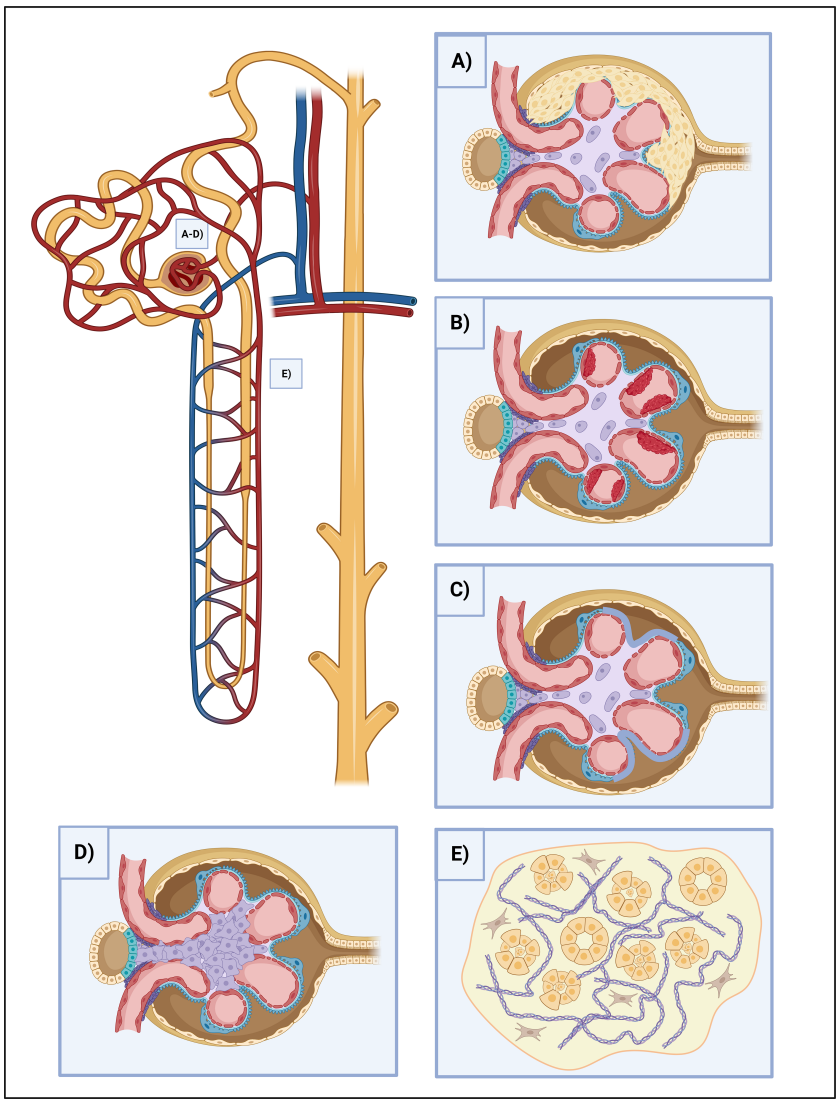

FIGURE 2
The effects of NETosis in kidney injury. **(A)** Crescent formation. Some NETosis products, such as histones and cytokines cause crescent formation by the induction of epithelial-mesenchymal and PEC progenitor proliferation. **(B)** Thrombosis. NETosis is heavily involved in clot formation. Besides NETs can serve as a scaffold for platelets, their contents damage the endothelium directly, oxidize HDL and stir it towards a proatherogenic direction, demolish the intracellular junctions and provoke vascular leakage, as well as stimulate the endothelium for further procoagulant factor (TF) release. **(C)** Podocyte foot process effacement. NETs disrupt the integrity of the slit-diaphragm by damaging the junctional proteins (podocin and nephrin), leading to the dysfunction of the glomerular filtration barrier, and causing proteinuria. **(D)** Mesangial proliferation. NETs cause ECM proliferation and the increment of mesangial cell count, leading to fibrosis. **(E)** Tubular injury. NETs play multiple roles in maintaining the destructive loop of tubular injury. NETosis-associated cytokine release causes tubuloepithelial cell necrosis, while histones have direct cytotoxic effects and help to prime neutrophils for further NETosis. ECM, extracellular matrix; HDL, high-density lipoprotein; NET, neutrophil extracellular traps; PEC, parietal epithelial cells; TF, tissue factor.

inducing consequential proteinuria. They induce podocyte cell hypertrophy, mitotic catastrophe, and eventually podocyte cell death as well (76). NETs hasten tubular epithelial cell apoptosis and further NET formation (74, 77) (Figures 2A-E).

4 Renal disorders with NET formation

One of the major objectives of this review was to summarize renal diseases in which NET formation is involved in the pathogenesis of the diseases. In the following paragraphs, we elaborate on these renal disorders.

4.1 SLE and lupus nephritis

SLE is a multiorgan autoimmune disease characterized by the dysregulation of both the innate and adaptive immune systems, leading to inflammation and severe tissue damage across the body. About 30-50% of SLE patients develop lupus nephritis, which is a severe complication with proteinuria, kidney function loss, and increasing risk of mortality. 10-30% of the patients will progress to end-stage renal disease in 5 years. Impaired tolerance, aberrant response to self-antigens, and type I interferons are considered crucial in SLE development and pathogenesis, in which NETosis plays a critical role (4) (Figure 3A).

Low-density granulocytes (LDGs) are highly granular pathogenic granulocytes displayed in a high concentration in SLE patients. These neutrophils can be characterized by their ability to produce high amounts of proinflammatory cytokines, including type I interferons, and their increased propensity to undergo spontaneous NETosis (70, 78). Moreover, LDGs have a hyperability to produce mitochondrial ROS, which - as mentioned above - is adequate to generate NETs even in the lack of NADPH-oxidase and further stimulates interferon gene transcription (79).

This process is perpetuated by nucleic-acid-containing immune complexes. They activate neutrophils via FcγR pathways and induce mitochondria-dependent NETosis (79, 80). It is widely known that ultraviolet (UV) radiation exacerbates the pre-existing lupus disease. UV radiation induces NETosis wavelength- and dose-dependently. Penetrating the epidermis, UV-A and blue light induce ROS-production and subsequent MPO- and NE-dependent NETosis (81). UV-C radiation triggers mtROS generation, mtDNA decondensation, and caspase 3 cleavage, hence, features of both apoptosis and NETosis. Nevertheless, it is important to bear in mind that UV-C does not have biological importance since it is completely absorbed by the ozone layer (82)

On the other hand, oxidized mtDNA, exposed LL-37, and HMGB1-DNA complexes activate plasmacytoid dendritic cells via TLR9/TLR7, as well as endocytosed nucleic acid-autoantibody complexes can initiate type I interferon production, contributing to the prominent interferon signature in SLE (69, 83, 84). LL-37 and other NET proteins activate the NLRP3 inflammasome in macrophages, increasing the IL-1 and IL-18 secretion. As positive feedback, IL-18 and interferon α further stimulate the process of NET formation (2).

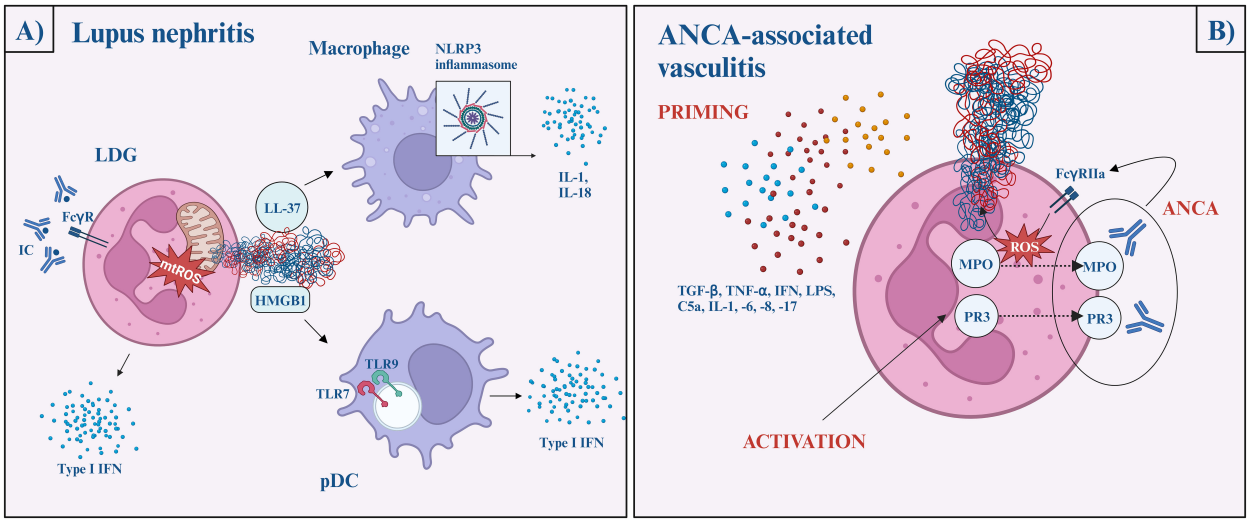

FIGURE 3
The role of NETs in lupus nephritis **(A)** and ANCA-associated vasculitis **(B)**. Lupus nephritis **(A)** Immune complexes activate neutrophils via the FcγR pathway and induce mitochondria-dependent NETosis. The NET-content (oxidized mtDNA, LL-37, and HMGB1-DNA complexes) later activates plasmacytoid dendritic cells via TLR7 or TLR9 and initiate type I interferon production, while macrophages are activated via the NLRP3 inflammasome pathway and facilitate IL-1 and IL-18 secretion. ANCA-associated vasculitis **(B)** Neutrophil priming occurs in response to various cytokines (TGFβ, TNFα, IFN, LPS, C5a, IL-1, -6, -8, -17), which reduces the neutrophil activation threshold. Then, MPO and PR3 are released onto the cell surface, where ANCA crossbinds them to neutrophil FcγRIIa and induces uncontrolled ROS and lytic enzyme bursts. After the burst, MPO and NE migrate to the nucleus, where NETosis begins. ANCA, anti-neutrophil cytoplasmic antibodies; AAV, ANCA-associated vasculitis; C5, complement factor 5; FcγR, fragment crystalline gamma receptor; HMGB1, high mobility group box 1; IFN, interferon; IL, interleukin; LDG, low-density granulocytes; LPS, lipopolysaccharide; MPO, myeloperoxidase; mtDNA, mitochondrial DNA; NE, neutrophil elastase; NLRP3, NLR family pyrin domain containing 3; pDC, plasmacytoid dendritic cell; PR3, proteinase 3; ROS, reactive oxygen species; TGF-β, transforming growth factor beta; TLR, Toll-like receptor; TNF-α, tumor necrosis factor alpha.

To conclude, NET formation in SLE is mostly NADPH-oxidase independent and induced primarily by immune complexes through FcγR signaling (85).

The accumulation of NETs is a common phenomenon in SLE and increases the exposure of nucleic acids and proteins to B-cells and plasmacytoid dendritic cells to generate high levels of interferons. There are three known ways in which the clearance of NETs may be impaired: 1) mutations and polymorphisms of DNase I, which lead to inadequate enzyme function, 2) inhibition of DNase by autoantibodies, and 3) the presence of anti-NET antibodies, that hide the binding sites from DNase I (86–89). In the latter form, autoantibodies recruit complement; C1q binds to DNA and prevents DNase I from degrading NETs (90). Furthermore, oxidized DNA is more resilient to DNase degradation (91). In summary, the altered clearance results in a longer exposure time to NETs and autoantigens, therefore it correlates with the severity of the disease. In parallel with this, the amount of DNase I in the kidney and urine decreases as lupus nephritis progresses (92).

4.2 Anti-neutrophil cytoplasmic antibody-associated vasculitis

Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is a necrotizing small vessel vasculitis characterized by the presence of circulating ANCAs. Based on the circulating antibodies and clinical manifestations, three main forms of AAV are distinguished: microscopic polyangiitis (MPA), granulomatosis with polyangiitis (GPA), and eosinophil granulomatosis with polyangiitis (EGPA). Renal involvement with nephritis syndrome (kidney function loss, hematuria, high blood pressure) is a common manifestation of AAVs occurring in most cases of MPA and frequently in GPA, however, it is rare in EGPA (93). The diagnostic hallmark of AAV is antibodies targeted against granular proteins of neutrophils: MPO and PR3.

Neutrophil priming is presumed to be the key step in NET formation in AAV. Priming is a process in which the neutrophil response is enhanced by an activating stimulus. Priming undergoes with the help of proinflammatory cytokines (such as transforming growth factor-beta, tumor necrosis factor α, interferon-α, -γ, IL-1, -6, -8, -17) complements (C5a), and LPS. After neutrophil activation, MPO and PR3 exteriorize on the cell surface. ANCA crossbinds these antigens with neutrophil FcγRIIa and induces uncontrolled ROS and lytic enzyme bursts. After the burst, MPO and NE migrate to the nucleus and NETosis begins and run its course in an NADPH-oxidase-dependent pathway, via the lytic NETosis. This is a pivotal difference compared to SLE (94, 95).

Although ANCA stimulation is suggested to be a key to NET formation, recent studies highlight a possibility of an ANCA-independent manner of NETosis. NETosis still occurs when ANCA IgG and IgA are depleted, or when the C5a receptor is inhibited. It does not correlate with serum tumor necrosis factor α (TNF-α), IL-6, -8 (priming cytokines), or conventional inflammatory markers like CRP or erythrocyte sedimentation rate. In fact, the level of NETosis is higher in ANCA-negative AAV patients. Additionally, even though relapse in AAV is commonly triggered by a concurrent infection, NET formation in AAV patients was lower during severe infection than in patients with relapsing AAV. To sum up, the exact mechanism of NET formation in AAV patients remains unknown and the increased level of NETs is linked to autoimmunity and clinical disease activity rather than concomitant infection (96) (Figure 3B).

4.3 Diabetic nephropathy

Diabetes can affect kidney function through several mechanisms. Classic diabetic nephropathy is considered a microvascular complication of diabetes, which will lead to proteinuria and glomerulosclerosis. Although diabetes is closely associated with inflammation and oxidative stress, the role of neutrophils in this process is almost neglected. Increased glucose level upregulates PKC activity and induces NADPH-oxidase overstimulation and oxidative burst regardless of the type of diabetes. As described above, oxidative burst is a pivotal step in NET formation. Inflammatory cytokines and free fatty acids inhibit insulin signaling by phosphorylation of inhibitors of nuclear factor kappa-B kinase (IKKβ) and c-Jun N-terminal kinase 1 (JNK1), which are also inflammatory pathway mediators, and induce NFκβ (nuclear factor kappa-light-chain-enhancer of activated B cells) translocation to the nucleus, resulting in various proinflammatory gene activation which is necessary for the priming process (97). Moreover, high extracellular glucose polarizes macrophages to a proinflammatory M1 phenotype. Interacting with NETs, M1-macrophages not only exacerbate the proinflammatory response but also go under apoptosis and release extracellular DNA. This mechanism is initiated specifically by NETs and contributes to an augmented load of free DNA and the progression of the disease (36). Although the underlying pathomechanisms in the development of diabetic kidney disease are complex and go beyond the scope of this review, it is clear that NETosis is noticeably involved in the process.

4.4 Acute tubular necrosis

Acute tubular necrosis (ATN) is the most common form of acute kidney injury (AKI), and it is characterized by the destruction of the tubular epithelial cells leading to a rapid kidney function loss with oliguria or anuria. It may occur as a result of ischemic or toxic impacts. Acute tubular necrosis is accompanied by a massive inflammatory response including recruitment, activation of immune cells, and increased proinflammatory cytokine production. Renal cell necrosis releases necrotic cell debris which is introduced as DAMPs. The innate immune system and especially neutrophils are the major responder and effector cells in AKI and play a role in the crescendo-type inflammation (98). DAMPs, such as histones generate a secondary autoimmune amplification loop by further priming neutrophils, activating them to form NETs, further deteriorating the kidney injury and exposing even more endogenous epitopes (77). Uromodulin is a constitutively secreted protein by epithelial cells in the thick ascending limb and the distal tubule into the tubular lumen.

Uromodulin binds and aggregates cytokines in the lumen and stays inert inside the luminal compartment. However, in case of tubular damage, uromodulin compiles in the interstitium as crystal-like structures and they can activate antigen-presenting cells and elicit the NLRP3-inflammasome-caspase-1 pathway (99). In the late phase of the injury, macrophages infiltrate the injured tissue and in the proinflammatory environment, they undergo an M1-phenotype shift contributing to necroinflammation. Contrary to necrosis, apoptosis is a balanced and regulated physiological mechanism that halts the exaggerated inflammatory process. Apoptosis of the recruited immune cells polarizes phagocytes to M2 phenotype and favors an anti-inflammatory milieu by secreting Il-10, TGF-β, and growth factors. This environment promotes not only suspending the inflammatory vicious circle but also contributes to the regeneration of epithelial and vascular healing and fibrosis (37).

4.5 Anti-glomerular basement membrane disease

Anti-glomerular basement membrane (GBM) disease is a rare autoimmune disorder in which antibodies are produced against type IV collagen and presents with rapidly progressive glomerulonephritis and alveolar hemorrhage (100). Anti-GBM IgG binds FcγRIIa in a shear-force-dependent manner. FcγRIIa binding causes F-actin polymerization via the Abl/Src mediated pathway. Their interaction and actin polymerization leads to an endothelial CD18 integrin (Mac-1) activation, and integration-mediated adhesion takes over selectin-mediated rolling. Thus, neutrophil attachment to the endothelial cells in the capillaries is sustained. Eventually, FcγR engagement triggers intravascular ROS, protease, and NET generation (101, 102). This mechanism with the extensive NET formation and the consequent exaggerating necroinflammation explains the substantial renal damage in anti-GBM glomerulonephritis which not only confines to the glomeruli but also affects the interstitium and the tubules.

4.6 Hemolytic uremic syndrome

Hemolytic uremic syndrome (HUS) is a thrombotic microangiopathy caused by enterohemorrhagic Shiga toxin (Stx) - producing bacteria (Shigella dysenteriae or enterohemorrhagic Escherichia coli). The syndrome is characterized by thrombocytopenia, hemolytic anemia, and acute renal failure. It is one of the main causes of acute kidney injury in children and has no specific treatment.

In hemolytic uremic syndrome, neutrophils are prone to undergo NETosis. There are several mechanisms behind it: 1) Shiga toxin is believed to be a potent neutrophil activator and NET inducer. 2) Uric acid is released from the injured cells in a great quantity, crystallizes, and triggers NET formation. 3) Higher dose of Stx induces NETosis in a ROS-dependent manner. 4) Stx enhances P-selectin expression on LPS-treated platelets and promotes neutrophil activation and aggregation (102).

Furthermore, exaggerated endothelial damage and thrombosis formation can be observed in HUS. Besides the excessive release of endothelial toxic NET contents, LPS, and Stx-treated platelets also contribute to endothelial damage (102). The role of neutrophil extracellular traps in atypical hemolytic uremic syndrome and thrombotic microangiopathies, however, is unclear.

4.7 Autoinflammatory diseases with renal involvement

Gout is an autoinflammatory condition characterized by monosodium urate (MSU) crystal deposition in the joints and the kidneys. High uric acid concentration is also a risk for nephrolithiasis.

MSUs are taken up by phagocytes and either directly damage the cell membrane causing necrosis and necrotic debris release leading to inflammatory cell recruitment, cytokine and chemokine production, or activating the NLRP3-caspase-1 inflammasome system resulting IL-1β release (50, 103). Recruited neutrophils undergo NADPH-dependent *lytic* NETosis, partly via direct activation by MSUs and partly by the inflammatory mediators (104). Uniquely, NETosis has a dual role in gout. Besides perpetuating inflammation, NETosis also limits it by engulfing MSUs preventing them from further phagocyte activation. MSU-stimulated neutrophils form aggregated NETs, which can proteolytically degrade and inactivate cytokines and chemokines stopping gout attack and resolving inflammation (105, 106).

Familial Mediterranean Fever (FMF) is a monogenic autoinflammatory disease, with clinical characteristics of fever attacks, joint pain, and skin rashes. During fever attacks, neutrophils produce large amounts of NETs which perpetuate inflammation via directly derived IL-1β and by inducing polymorphonuclear cells for further IL-1β production. However, NETs also have a self-limiting, therefore anti-inflammatory effect, as they can prevent further NETosis and stop the fever attack (107, 108). The most common kidney damage caused by FMF is amyloidosis, but IgA nephropathy and mesangioproliferative glomerulonephritis were also described (109).

5 Potential therapeutic targets in NETosis

Since the signalization of NETosis is sprawling, the number of potential therapeutic targets is extensive. Nevertheless, NET inhibition in renal disorders is not exhausted yet, most agents are yet to be trialed in kidney diseases. Here we list the most examined and most promising drug candidates in clinical practice.

5.1 Disease-modifying antirheumatic drugs

5.1.1 Conventional synthetic DMARDs

5.1.1.1 Recombinant DNase

DNase is responsible for the disassembly of the NET-related nucleoproteins and facilitates the clearance by macrophages. Recombinant DNase eliminates the NETosis products and delays the development of antibodies against them. Nevertheless, recombinant DNase I treatment is controversial. It does have a good efficacy to reduce NETs in COVID-triggered acute respiratory

distress syndrome via inhaled route or when administered intravenously in lupus nephritis patients and anti-MPO ANCA mouse model, however, intravenous or subcutaneous administration failed to achieve sufficient bioactive serum concentration in humans (94, 110, 111). Recombinant DNase I is inactivated rapidly by G actin by forming a complex with it. To overcome this limitation, adeno-associated virus vectors were used in a lupus-prone mice (NZBWF1) model. This way, DNaseI activity was maintained for more than 6 months, and renal neutrophils, NETs, IgG, and C3 were significantly reduced. However, this way of rhDNase administration still did not extend lifespan and preserved renal function (112).

5.1.1.2 Toll-like receptor inhibitors

Hydroxychloroquine (HCQ) and chloroquine (CQ) are anti-malarial drugs frequently used in autoimmune diseases, due to their immunomodulatory effects. They inhibit NET formation by antagonizing TLR-3, - 7, -8, -9 (113). Furthermore, hydroxychloroquine also has an inhibitory effect on cyclic GMP-AMP synthase (cGAS), a cytosolic DNA sensor responsible for type I interferon response. Taken together, the downstream proinflammatory cytokine production will be suppressed (114). Chloroquine has been shown to reduce autophagy and increase the pH in lysosomes, impeding antigen presentation on MHC class II molecules for CD4+ T-cells. Additionally, hydroxychloroquine is suggested to inhibit PAD4 as well (115). Utilizing these pharmacological effects, hydroxychloroquine and chloroquine are demonstrated to reduce SLE and especially lupus nephritis flares, help to maintain remission and delay the onset of complications (116, 117). Hydroxychloroquine attenuated anti-GBM nephritis in WKY rats as well which was attributed to the suppression of JNK/p38 MAPK phosphorylation (118).

The dual TLR 7/8 inhibitor enpatoran was well-tolerated in a phase 1 study in healthy participants and a current WILLOW study is evaluating its efficacy in SLE patients, however, patients with active lupus nephritis have been excluded (NCT05162586) (119). TLR inhibition with GIT27 (VGX-1027) mitigated kidney injury in a diabetic experimental mouse model. GIT27 targets primarily TLR4 but also interferes with TLR2/6 signaling pathways in macrophages which results in a lower profibrotic and proinflammatory profile, lower albuminuria, and mesangial expansion (120). TAK-242 and eritoran are selective TLR4 inhibitors while NI-0101 is a humanized monoclonal antibody against TLR4 (121). TAK-242 ameliorated rhabdomyolysis, acetaminophen, and contrast-induced acute kidney injury (122–124). TAK-242 also reduced serum creatinine and blood urea nitrogen concentration in sepsis-mediated kidney injury (125). Nevertheless, it is important to bear in mind that TLR4 activation and the subsequent proinflammatory response are crucial for bacterial clearance in sepsis.

Taken together, Toll-like receptor inhibitors are promising therapeutic agents in NETosis-related diseases, however, in previous clinical trials none has yet been proven clinically useful (126, 127).

5.1.1.3 Calcineurin inhibitors

Cyclosporin, tacrolimus, and voclosporin are calcineurin pathway inhibitors widely used in organ transplants and autoimmune disorders. They bind cytophilin and downregulate the transcription factor nuclear factor of activated T cells (NFAT) and inhibit the calcineurin pathway. Studies proved a significant influence on neutrophils suppressing chemokinesis, adhesion, angiotensin II response, phagocytic activity reduction, and reducing the extent of IL-8 induced NETosis (128). Calcineurin inhibitors also block T-cell overactivation and podocyte alteration. This results in proinflammatory mediator release from T-cells and cytoskeleton stabilization and apoptosis inhibition in podocytes emphasizing their powerful roles in autoimmune renal disorders (129). Although calcineurin inhibitors are not recommended as first-line therapy in lupus nephritis, cyclosporin, and tacrolimus can be used in combination with mycophenolate-mofetil in nephrotic range proteinuria. The combination of voclosporin and mycophenolate-mofetil was associated with a higher rate of complete remission at 6 months as compared with mycophenolate alone (130).

Tacrolimus improved albuminuria and tubulointerstitial damage, and ameliorated macrophage infiltration and proinflammatory cytokine expression in diabetic db/db mice (131). Tacrolimus was proved to recover the nephrin expression, thus maintaining the structural and functional integrity of podocytes in diabetic Sprague-Dawley rats (132).

5.1.1.4 Colchicine

Colchicine is an alkaloid derivative that has pleiotropic anti-inflammatory effects. It attaches to soluble tubulin in an almost irreversible manner and inhibits the elongation of the microtubules. In higher concentrations, it also promotes microtubule depolymerization, alters endothelial E-selectin distribution, promotes L-selectin shedding on neutrophils, inhibits superoxide production and NLRP3. Colchicine inhibits immune response by dampening neutrophil chemotaxis, recruitment, adhesion, inflammasome activation, and NETosis as well. Colchicine blocks inflammatory response via regulating NF-kB and caspase-1 (133). The advantageous effects of colchicine in NETosis were proved in a small subset of patients, however, its potential renal toxicity in comprised kidney function limits its use in possible utilization in NET-related kidney disorders (134–136). Colchicine was studied in a randomized controlled double-blind clinical trial in diabetic nephropathy where colchicine decreased neutrophil-related chronic inflammation; however, it did not lower creatinine, urinary albumin/creatinine ratio and did not prevent overt nephropathy either (137).

5.1.1.5 PAD4-inhibitors

Inhibition of PAD4 via Chlor-amidine (Cl-amidine) irreversibly blocks the active sites of the enzyme via covalent modification (138). GSK484 and GSK199 are selective and reversible inhibitors that bind to PAD4 with a high affinity (139).

Cl-amidine significantly inhibits NET formation in NZM model of murine lupus, reduces complement consumption, glomerular IgG deposition, and although not significantly but reduces albuminuria. Cl-amidine administration to NZM mice improved endothelium-dependent vasorelaxation and delayed arterial thrombosis which was partly attributed to the reduction of NET formation (140). Following PAD inhibition, immune complex

deposition, interstitial inflammation, and urine albumin/creatinine ratio decreased in MRL/lpr lupus nephritis model as well (141, 142).

Cl-amidine attenuated kidney injury in a rabbit model of LPS-induced acute kidney injury. Cl-amidine decreased histopathologic signs of acute kidney injury, as well as elevated creatinine and blood urea nitrogen (143). GSK484 mitigated renal ischemia-reperfusion injury in C57BL/6 mice by reducing NETosis (144).

Although PAD4 inhibition proved to be effective in the suppression of NETosis in several *in vitro* and *in vivo* animal models, clinical trials have not been conducted yet. On the other hand, inhibition strategy with PAD4 is a double-edged sword since it inhibits essential NET formation as well.

5.1.2 Targeted synthetic DMARDs

5.1.2.1 Tyrosine kinase and JAK-inhibitors

Bosutinib is a selective dual Abl/Src inhibitor used in chronic myeloid leukemia (CML) to inhibit the Bcr-Abl fusion protein. However, based on this function, the utilization of bosutinib might be reevaluated and repurposed. It proved to be beneficial in anti-GBM glomerulonephritis since it averted an early step of inflammation. Bosutinib reduced the FcγRIIa-mediated adhesion, neutrophil recruitment, and NET formation in an *in vitro* experiment (101). Another selective Abl/Src inhibitor dasatinib significantly reduced neutrophil and macrophage influx in skin wounds, thus accelerating healing and reducing scarring (145). On the other hand, it is important to note that the multi-tyrosine kinase inhibitor ponatinib increases NET production which contributes to vascular toxicity in CML patients (146).

Neutrophil Bruton's tyrosine kinase (Btk) mediates reactive oxygen species generation via TLR4-signals, and integrin-mediated recruitment and activation (147–149). Btk inhibitors reduced proteinuria and improved glomerular histopathology in lupus-prone animal models (150–153). Elsubrutinib is currently under the scope for efficacy in SLE patients including lupus nephritis alone or in combination with upadacitinib compared to placebo (NCT03978520). Nevertheless, previous studies with Btk inhibitors (fenebrutinib, evobrutinib) did not bring the expected results in SLE patients, though patients with active LN were excluded (154, 155).

Spleen tyrosine kinase (Syk) is an adapter protein that is responsible for phosphorylating several other adapter proteins to propagate signalization cascades resulting in cytokine/chemokine release via NF-κB, actin cytoskeleton remodeling, proliferation, and differentiation via the MAPK pathway and also for survival via the NFAT (nuclear factor of activated T cells) pathway (156). Syk is activated by phosphorylation upon ANCA binding to FcγRIIa in TNF-α–primed neutrophils and induces respiratory burst. Syk is also an essential regulator in B-cell receptor-mediated signalization and enhanced function is related to the pathogenesis of autoimmune disease and B-cell leukemias and lymphomas as well (157). Fostamatinib is a highly selective Syk inhibitor, and it was proven to improve MPO-ANCA glomerulonephritis in a pre-clinical ANCA vasculitis model in Wistar Kyoto rats (158). Fostamatinib delayed renal progression in NZB/NZW mice in lupus nephritis model and anti-GBM glomerulonephritis in Sprague-Dawley and WKY rats (159–161). Fostamatinib has not undergone a clinical investigation yet. A trial had commenced years ago that targeted SLE patients, but it was withdrawn before it could have been started (NCT00752999).

Downstream inhibition of the JAK/STAT pathway with tofacitinib is proven to lower NET formation (162). Tofacitinib decreased proteinuria, reduced mesangial cell proliferation, and glomerular IgG deposition. Furthermore, after tofacitinib administration in MRL/lpr mice, TGF-β receptor 1 was reduced in renal tissues, thus tofacitinib alleviated renal fibrosis in MRL/lpr mice model (163). Other JAK inhibitors, like baricitinib, peficitinib, upadacitinib and filgotinib are approved as disease-modifying antirheumatic drugs (164).

Baricitinib was a promising agent in SLE, however, it failed to decrease the SLEDAI (Systemic Lupus Erythematosus Disease Activity Index) or the BILAG class in SLE-BRAVE II study (165). Although lupus nephritis patients were excluded from this trial, other ongoing studies are taking chances on baricitinib and LN patients (NCT05686746). Brepocitinib and upadacitinib are other JAK inhibitors that are currently subjects to SLE studies, however, renal involvement with brepocitinib is among the exclusion criteria (NCT03845517, NCT03978520). The phase II result of the upadacitinib study is expected to be published soon (NCT03978520).

An abundance of evidence showed the progression of diabetic kidney disease is boosted by inflammation that is mediated by the JAK-STAT pathway. Baricitinib decreased the urinary albumin/creatinine ratio from baseline at 6 months compared to placebo (166). This finding is supported by two previous trials in which patients with diabetic nephropathy received monocyte chemoattractant protein 1 (MCP1) inhibitors (167, 168). Ruxolitinib contributed to lower proinflammatory cytokine levels and regulated podocyte autophagy in diabetic kidney disease (169, 170). Although it has been proven that ruxolitinib blunts NET formation in chronic myeloproliferative neoplasms and its NET inhibiting role has not been investigated in renal disorders, it is suspected that NET suppression also contributes to the amelioration of diabetic nephropathy (171, 172).

Tofacitinib attenuated LPS-induced acute kidney injury by inducing intrarenal proinflammatory cytokine production and reducing oxidative stress (173). The same effect was reached when C-X-C Motif Chemokine Receptor 1 and 2 (CXCR1/2) was inhibited by the chemokine C-X-C motif ligand 8 (CXCL8) antagonist, G31P (174).

5.1.3 Biological DMARDs

5.1.3.1 C5-inhibitors

Priming via C5a-C5a receptor interaction is crucial in NET formation. The C5a receptor antagonist, avacopan, and the anti-C5a monoclonal antibody, eculizumab have been both proven to reduce NET formation (2). The orally administered avacopan was approved as an adjunct therapy for microscopic polyangiitis and granulomatosis with polyangiitis which permits a lower glucocorticoid exposure and reduces the glucocorticoid-related toxicity, thus improving patient quality of life (175). Eculizumab is an anti-C5 monoclonal antibody that inhibits complement-mediated thrombotic microangiopathy and

significantly improves renal function in patients with atypical hemolytic-uremic syndrome (176). Eculizumab is a therapeutic option in individuals with lupus nephritis who developed subsequent thrombotic microangiopathy and patients with refractory lupus nephritis might benefit from supplementary eculizumab therapy as well (177). Moreover, in a case report eculizumab was used as rescue therapy and improved renal outcome in two patients with anti-GBM glomerulonephritis (178).

5.1.3.2 Interferon pathway inhibitors

As previously described, type I interferons elicit numerous immunological effects, including the assistance of NET formation. Therefore, interferon blockade is an ideal pharmacological approach to decrease NET production. Sifalimumab and rontalizumab are monoclonal antibodies against interferon α and aid their neutralization. Both of them underwent trials in SLE patients and reached superiority above placebo, however, in these trials, patients with renal involvement were excluded (179, 180). Anifrolumab is a human immunoglobulin targeted against subunit 1 of the interferon α receptor, conveying a general inhibition signal (181). Anifrolumab decreased the BILAG (British Isles Lupus Assessment Group)-based Composite Lupus Assessment (BICLA) index in SLE patients in the TULIP study, however, this study also excluded lupus nephritis patients (182). Nevertheless, the TULIP-LN study evaluated the efficacy of anifrolumab in renal involvement. Unfortunately, the primary endpoint of the study was not met, there was no significant improvement in the 24-hour protein/creatinine ratio from baseline to 52 weeks in anifrolumab-treated patients versus placebo. Authors explain the results by the suboptimal dosing and the increased clearance of anifrolumab associated with proteinuria (183).

5.1.3.3 Depletion of B-lymphocytes

B-cells contribute to NET formation mainly via antibody production and immune complexes; however, they are also potent sources of cytokines for priming. The primary B-lymphocyte depleting agents are rituximab (anti-CD20 monoclonal antibody) and belimumab (B-lymphocyte stimulator (BLyS)/BAFF inhibitor human monoclonal antibody). They are used mostly individually but sequential therapy also has its rationale: belimumab targets CD20+ and CD20- plasmablasts but spares the CD27+ memory cells, while rituximab reduces CD20+ cells effectively - including CD27+ memory cells- but does not affect CD20- plasmablast cells (184). Obinutuzumab is a new-generation anti-CD20 monoclonal antibody designed to overcome rituximab resistance. Obinutuzumab has a greater affinity for FcγRIII and employs different mechanisms of action. It evokes a greater direct B-cell death by a more potent antibody-dependent cellular toxicity and phagocytosis (185).

Rituximab used to be a promising agent in proliferative lupus nephritis, but the renal response rate was not superior over mycophenolate-mofetil and glucocorticoids at 1 year (LUNAR study) (186). Nevertheless, it may still be considered in persistent disease activity or in repeated flares (187). On the contrary, belimumab helped to reach renal response beside standard therapy (BLISS-LN study) so it was included in Kidney International Improving Global Outcomes (KDIGO) guidelines as the first on-label monoclonal antibody in SLE treatment (187, 188). The combination of belimumab and rituximab effectively reduced NET formation in human SLE by reducing the circulating immune complexes (189). An ongoing randomized controlled study (SynBioSe-2) is investigating the combination treatment protocol of belimumab followed by rituximab in lupus nephritis patients compared to prednisolone and mycophenolate-mofetil induction (NCT03747159). Nevertheless, an *in vitro* study contradicted this result and showed enhanced NETosis after Rituximab treatment (190). The NOBILITY trial was designed to evaluate the hypothesis that adding obinutuzumab to the standard glucocorticoid and mycophenolate-mofetil treatment improves the rate of complete renal remission compared to the treatment without obinutuzumab. Obinutuzumab add-on therapy was superior to placebo in proliferative lupus nephritis to reach complete renal response at 2 years (191).

The induction and maintenance protocol of ANCA-associated glomerulonephritis also involves rituximab. Beside glucocorticoids, the standard induction therapy is either cyclophosphamide and/or rituximab (187). Rituximab is also highly efficient in relapsing disease and anti-PR3 ANCA-positive patients resulted in a higher remission rate post-rituximab treatment (192, 193). Belimumab was studied as a maintenance agent, but it did not reduce the risk of relapse. However, utilizing the two agents' additive synergistic effects, an ongoing study (COMBIVAS - NCT03967925) uses rituximab and belimumab combination therapy as induction in PR3 vasculitis. Obinutuzumab was reported to reach remission successfully in three cases of ANCA-associated vasculitis. An ongoing randomized, double-blind study (ObiVas) is currently recruiting participants for a phase II study to compare the efficacy of rituximab and obinutuzumab in AAV patients [ISRCTN13069630].

Rituximab use has been reported for the treatment of anti-GBM with various outcomes. Although B-cell depletion inhibition is not part of the KDIGO treatment guidelines and standard-of-care approaches in anti-GBM glomerulonephritis, rituximab may be initiated in cases of refractory to standard therapy or in relapsed disease (187, 194). Although anti-GBM antibody vanishes after rituximab therapy in the majority of cases, it cannot halt kidney injury or reverse dialysis dependency (194). However, the negative renal outcome is an inherent consequence of the rapid pathomechanism of anti-GBM glomerulonephritis not the inefficacy of immunosuppressive therapies.

5.1.3.4 Interleukin antagonists

Tocilizumab is a humanized monoclonal antibody against the interleukin-6 receptor. It decreases endothelial dysfunction and oxidative stress and downregulates low-density granulocytes in rheumatoid arthritis.

Tocilizumab has already been proven effective in large-vessel vasculitis and a few reports also reported remission in microscopic polyangiitis with tocilizumab. SATELITE study is going to evaluate the efficacy in patients with granulomatosis with polyangiitis (NCT04871191).

Tocilizumab showed a promising clinical and serological response in SLE patients; however, patients with fulminant renal

involvement were excluded. PF-04236921 is a fully-humanized monoclonal antibody against IL-6 that was not significantly different from placebo for reducing the disease activity (195).

Tocilizumab ameliorated proteinuria in streptozotocin-induced diabetic nephropathy rat model and attenuated histological changes and was also beneficial for podocytes in mice (196, 197). Tocilizumab mitigated kidney function deterioration in a case report, however, clinical trials have been not conducted yet (198).

Anakinra (human IL-1 receptor antagonist), canakinumab (human monoclonal antibody against IL-1β) and rilonacept (soluble decoy receptor) are used mostly in the treatment of autoinflammatory diseases (e.g., juvenile idiopathic arthritis, gout). *In vitro*, pre-treatment with anakinra successfully reduced NET formation in PMA-induced NETosis in a time- and dose-dependent manner (199). Anakinra was successfully used to partially inhibit NET production in experimental gout models. Both anakinra and canakinumab attenuated MSU-induced NETosis via altering NLRP3-inflammasome activation and IL-1β release (50, 200). Rilonacept is an IL-1α and IL-1β trap, which is approved in the treatment of cryopyrin-associated periodic syndromes and recurrent pericarditis (201), however, exact effect on NETs is not well described.

The anti-IL-18 monoclonal antibody GSK1070806 have been tested in renal transplant delayed graft function (202), obesity and type 2 diabetes (203), but was not proven effective. Recombinant human IL-18 binding protein, which neutralize IL-18 along with IL-37 had more success in experiments and clinical trials in psoriasis, rheumatoid arthritis, hemophagocytic lymphohistiocytosis, and adult onset Still disease (204). To date, there is no approved treatment targeting IL-18.

5.1.4 Miscellaneous

5.1.4.1 Antioxidants

Amino salicylates scavenge ROS superoxide and recover SOD activity, NF-κB, MPO, and proinflammatory cytokine production. The inhibitory effect of 5-aminosalicylic acid and acetylsalicylic acid on NETosis was proved, making them a potentially ideal supplementary therapy in NET-related kidney disorders (205, 206).

N-acetylcysteine reduces NETosis by modulating ROS production (207).

N-acetylcysteine (NAC) slightly reduces albumin/creatinine ratio and suppresses renal fibrosis in diabetic nephropathy in diabetic rat models (208, 209). High-dose NAC decreased SLEDAI scores and BILAG classes significantly in a randomized double-blind clinical trial, though patients with acute flare threatening vital organs were excluded (210). Pre- and post-treatment with NAC in septic rats were protective against kidney injury while long-term administration of NAC worsened organ failure and did not improve albuminuria. As a matter of fact, high-dose NAC administration in patients with over 24-hour duration sepsis resulted in cardiac depression, hypotension, and worsened acute kidney injury (211–215). Interestingly, NAC employed protective effects when administered in short-term ischemia in Wistar rats. NAC treatment decreased renal vascular resistance, thus increased renal blood flow and prevented histopathological changes when ischemia was applied for 30 minutes compared to 45 minutes. This suggests that NAC treatment might have a therapeutic window to utilize the nephroprotective effect (216). NAC also failed to offer renal protection in patients with stage 3 chronic kidney disease at risk of contrast-induced nephropathy (217).

Besides being a potent antidiabetic drug, metformin has mTOR and consequential mtROS production inhibitory effects. During PMA- and/or Ca-induced NETosis, metformin effectively reduced NETosis via inhibition of NADPH-oxidase and the membrane translocation of PKC-βII (218).

Metformin improves renal function in MRL/lpr lupus-prone mice. It decreased the proinflammatory cytokines and the levels of anti-nuclear antibody and anti-double stranded DNA antibodies. Metformin administration also attenuated histopathological damages (219). A *post hoc* analysis demonstrated that metformin decreased the incidence of flares in SLE patients, though patients with renal impairment were excluded (220). A study aimed to evaluate the preventive effects of metformin in lupus nephritis (NCT04145687), however, the current status is unknown.

Metformin inhibits mitochondrial respiratory complex I, and reduces ATP synthesis, hence increases AMP/ATP and ADP/ATP ratios. It leads to the activation of adenosine-monophosphate-activated kinase (AMPK). AMPK inhibits hepatic gluconeogenesis, lowering glucose toxicity. Metformin alleviates the glucose- and advanced glycation product (AGE)-induced NF-κB activation, ROS production, and the subsequent proinflammatory cytokine production (221). By this, metformin does not only repress intrarenal inflammation but also proliferation and fibrosis (222).

Idebenone is a ubiquinone analog antioxidant agent with ROS scavenger properties, hence a mitochondrial ROS inhibitor. It defends membranes from lipid peroxidation and restores ATP production in the mitochondria (223). In MitoTEMPO study it was proved to inhibit spontaneous NET formation of low-density granulocytes and decrease disease activity in SLE (224).

5.1.4.2 Vitamin D

Vitamin D is a fat-soluble secosteroid with pleiotropic function. The role in NETosis was shown by Handono et al. who studied SLE patients with hypovitaminosis D. Results showed that vitamin D3 substitution inhibited NETosis activation and decreased consequential endothelial damage, however, the pathomechanism remains unknown (225). Vitamin D was significantly lower in patients with lupus nephritis compared to patients with either active SLE without nephritis or inactive SLE (226). Another study demonstrated an inverse correlation between SLEDAI and serum 25-hydroxy-cholecalciferol and proved that higher levels of vitamin D were associated with a decrease in urine protein/creatinine ratio (227).

Although a negative correlation was observed between 25-hydroxy-cholecalciferol and Birmingham vasculitis activity score (BVAS) in AAV, no difference was revealed in 25-hydroxy-cholecalciferol levels among AAV patients with and without renal involvement (228).

A double-blind, randomized-controlled study demonstrated that the vitamin D analog paricalcitol decreased urinary albumin

excretion significantly compared to placebo (229). Another randomized-controlled trial with type 2 diabetic patients showed a faster decline in estimated glomerular filtration rate in patients with 25-hydroxy-cholecalciferol deficiency (230).

Macrophage and lymphocyte infiltration in ischemia-induced kidney injury was proven to be significantly higher and the risk of acute kidney injury was 1.2- and 1.5-fold higher in patients with 25-hydroxy-cholecalciferol insufficiency and deficiency (231, 232). Although vitamin D has a pleiotropic effect in acute kidney injury, the participation of NETosis cannot be omitted either (231).

5.1.4.3 Antibiotics

Azithromycin exerts a dose-dependent ROS inhibition, hence preventing the NET release. Gentamicin and chloramphenicol also decrease NETosis, the latter probably via decreasing MPO activity. Nevertheless, their efficacy was tested merely preclinically (233, 234).

TABLE 2 Potential therapeutic targets of NETosis.

Disease-modifying antirheumatic drugs (DMARDs)		
Conventional synthetic DMARDs (csDMARDs)		
Recombinant DNase		dismantles NETs and accelerates clearance
Toll-like Receptor inhibitors	Hydroxychloroquine	inhibits NET formation by antagonizing Toll-like receptors (TLR-3, 7, 8, 9); has inhibitory effects on cyclic GMP-AMP synthase (cGAS), and has PAD4 inhibitory functions
	Chloroquine	inhibits NET formation by antagonizing Toll-like receptors (TLR-3, 7, 8, 9); reduces autophagy, and increases the pH in lysosomes, impeding antigen presentation on MHC class II molecules for CD4+ T-cells
	Enpatoran	dual TLR7/8 inhibitor
	GIT27 (VGX-1027)	targets primarily TLR4 but also interferes with TLR2, TLR6 signaling pathways in macrophages
	TAK-242	selective TLR4 inhibitor
	Eritoran	selective TLR4 inhibitor
Calcineurin-inhibitors	Cyclosporin	suppresses chemokinesis, adhesion, and angiotensin II response, hence reduces phagocytic activity, blocks T-cell overactivation and podocyte alteration
	Tacrolimus	suppresses chemokinesis, adhesion, and angiotensin II response, hence reduces phagocytic activity
	Voclosporin	suppresses chemokinesis, adhesion, and angiotensin II response, hence reduces phagocytic activity
Colchicine		inhibits immune response by dampening neutrophil chemotaxis, recruitment, adhesion, inflammasome activation, and NETosis.
PAD-4 inhibitors	Chlor-amidine	irreversibly blocks PAD4 active site via covalent modification
	GSK484	selectively and reversibly binds to PAD4
	GSK199	selectively and reversibly binds to PAD4
Targeted synthetic DMARDs (tsDMARDs)		
JAK/STAT inhibitors	Tofacitinib	decreases proteinuria, reduces mesangial cell proliferation and glomerular IgG deposition; in MRL/lpr mice reduces TGF-β receptor 1 in renal tissues, thus alleviates renal fibrosis
	Ruxolitinib	alleviates renal fibrosis in MRL/lpr mice model; lowers proinflammatory cytokine levels and regulates podocyte autophagy in diabetic kidney disease
	Baricitinib	decreases the urinary albumin/creatinine ratio from baseline at 6 months compared to placebo
	Peficitinib	
	Upadacitinib	
	Filgotinib	
	Brepocitinib	
Tyrosine kinase inhibitors	Bosutinib	Abl/Src inhibitor: reduces the FcγRIIa-mediated adhesion, neutrophil recruitment, and NET formation
	Dasatinib	reduces neutrophil and macrophage influx in skin wounds
Bruton tyrosine kinase inhibitor	Elsubrutinib	reduces proteinuria and improves glomerular histopathology in lupus-prone animal models
	Upadacitinib	reduces proteinuria and improves glomerular histopathology in lupus-prone animal models

(Continued)

TABLE 2 Continued

Disease-modifying antirheumatic drugs (DMARDs)		
Spleen tyrosine kinase inhibitors	Fostamatinib	highly selective Syk inhibitor, improves MPO-ANCA glomerulonephritis in a pre-clinical ANCA vasculitis model in Wistar Kyoto rats; delays renal progression in NZB/NZW mice in lupus nephritis model and anti-GBM glomerulonephritis in Sprague-Dawley and WKY rats
Biological DMARDs (bDMARDs)		
Complement 5 inhibitors	Eculizumab	inhibits complement-mediated thrombotic microangiopathy and significantly improves renal function in atypical HUS
	Avacopan	anti-C5a receptor antagonist
Interferon pathway inhibitors	Sifalimumab	monoclonal antibody: binds to interferon alpha and aids their neutralization
	Rontalizumab	monoclonal antibody: binds to interferon alpha and aids their neutralization
	Anifrolumab	human immunoglobulin: binds to the subunit 1 of interferon alpha receptor
Depletion of B-lymphocytes	Belimumab	anti-BlyS/BAFF, reduces circulating immunocomplexes
	Rituximab	anti-CD20, reduces circulating immunocomplexes
	Obinutuzumab	anti-CD20, reduces circulating immunocomplexes
Interleukin antagonists	Tocilizumab	anti-IL-6R antibody; improves endothelial function and decreases oxidative stress in RA, reduces the low-density granulocytes and NETosis
	Canakinumab	monoclonal antibody against IL-1β
	Anakinra	IL-1R blocker; reduces NET formation in a time- and dose-dependent manner, has a calcium-dependent role in the MPO and NE activation
	Rilonacept	soluble decoy receptor, which acts as an IL-1α and IL-1β trap
	GSK1070806	anti-IL-18 monoclonal antibody
	Rh-IL-18 BP	neutralizes IL-18 along with IL-37
Other	NI-0101	humanized monoclonal antibody against TLR4
Miscellaneous		
Antioxidants	N-acetylcysteine	modulates ROS production
	Amino salicylates	scavenges ROS superoxide, recovers SOD activity, NF-κB, MPO and proinflammatory cytokine production
	Metformin	mTOR and mtROS inhibitor; inhibits the NADPH oxidase and the membrane translocation of PKC-βII
	Idebenone	ubiquinone analog antioxidant; scavenges ROS, defends membranes from lipid peroxidation and restores ATP production in the mitochondria
	DPI	NADPH oxidase inhibitor; binds to the subunits and prevents electron flow and ROS production
	MitoTEMPO	Mitochondrial ROS scavenger; decreases spontaneous NETosis serum and anti-dsDNA levels, lowers proteinuria and renal IC deposition
Vitamin D		unknown pathomechanism
Antibiotics	Azithromycin	inhibits ROS
	Chloramphenicol	decreases MPO activity
	Gentamicin	unknown pathomechanism
PKC inhibitors	Ro-31-8220	pan-PKC inhibitor; blocks PMA-induced NET formation
	Go 6976	PKC-α and β inhibitor, reduces ROS production in PMA-induced NETosis
	LY333531	PKC inhibitor with a high selectivity for PKC-β, reduces ROS production by blocking the p47phox phosphorylation of NADPH-oxidase in PMA-induced NETosis
PA-dPEG24		peptide inhibitor of C1; dose-dependently blocks the MPO pathway of NET formation
Prostaglandins	PGE2	cAMP–PKA pathway modulators; limits NETosis in an exchange protein activated by cAMP- and protein kinase A-dependent manner (EP2 and EP4 Gαs-coupled receptors)
Dibutyryl cAMP		cell-permeable cAMP analog

(Continued)

TABLE 2 Continued

Disease-modifying antirheumatic drugs (DMARDs)		
Rolipram		PDE4 inhibitor
Butaprost		EP2 receptor agonist
LDC7599		Gasdermin-D inhibitor; inhibits pore-formation in the nuclear and plasma membrane and prevents NETosis
Disulfiram		Gasdermin-D inhibitor; inhibits pore-formation in the nuclear and plasma membrane and prevents NETosis

AMP, adenosine monophosphate; ANCA, anti-neutrophil cytoplasmic antibodies; ATP, adenosine triphosphate; BAFF, B-cell activating factor; BLyS, B lymphocyte stimulator; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; dsDNA, double-stranded deoxyribonucleic acid; dsRNA, double-stranded ribonucleic acid; IC, immune complex; IL, interleukin; MHC, major histocompatibility complex; MPO, myeloperoxidase; mTOR, mammalian target of rapamycin; mtROS, mitochondrial reactive oxygen species; NADPH oxidase, nicotinamide adenine dinucleotide phosphate oxidase; NE, neutrophil elastase; NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells; PAD4, protein arginine deiminase 4; PDE4, phosphodiesterase-4 inhibitor; PKC-βII, protein kinase C beta II; PMA, phorbol myristate acetate; RA, rheumatoid arthritis; Rh-IL-18 BP, recombinant human IL-18 binding protein; ROS, reactive oxygen species; Syk, spleen tyrosine kinase; SOD, superoxide dismutase; TGF-β, transforming growth factor beta.

5.1.4.4 Gasdermin D inhibition

Inhibition of Gasdermin D effectively halts the cascade of inflammatory molecule release in NETosis. Disulfiram suppresses pyroptosis by covalently modifying and inhibiting Gasdermin D (235). LDC7559 is a small molecule that was also proven to inhibit Gasdermin D and delay NETosis (236).

5.1.4.5 Protein-kinase C inhibition

The protein-kinase C isoenzyme family plays a role in the downstream pathways of NETosis. The pan-PKC inhibition with Ro-31, PKC-α, and β-inhibitor Go 6976, and PKC-β inhibition with LY333531 reduce NET formation (237). PKC-δ inhibitor rottlerin prevented glucose-induces ERK activity and decreased TGF-β1-induced collagen synthesis in mesangial cells in an *in vitro* diabetic nephropathy model but it did not affect NET formation according to another study (237, 238).

Table 2 summarizes the potential therapeutical targets of NET formation.

6 Conclusions

The pivotal role of neutrophil extracellular traps in the pathomechanism of renal disorders is an evolving hot topic (239, 240). NETs are not only generated by infectious stimuli, but they are also essential in several autoimmune and acute kidney disorders. A more in-depth understanding of the formation, regulation, and dysregulation of NETs offers potential therapeutic targets in kidney diseases leading to better patient and renal outcomes.

Author contributions

MJ: Investigation, Visualization, Writing – original draft. AM: Investigation, Visualization, Writing – original draft. ZJ: Software, Writing – review & editing. NL: Conceptualization, Funding acquisition, Resources, Supervision, Writing – review & editing.

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Conflict of interest

All authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Unleashing the power of complement activation: unraveling renal damage in human anti-glomerular basement membrane disease

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Anti-glomerular basement membrane (GBM) disease is a rare but life-threatening autoimmune disorder characterized by rapidly progressive glomerulonephritis with or without pulmonary hemorrhage. Renal biopsies of anti-GBM patients predominantly show linear deposition of IgG and complement component 3 (C3), indicating a close association between antigen-antibody reactions and subsequent complement activation in the pathogenesis of the disease. All three major pathways of complement activation, including the classical, lectin, and alternative pathways, are involved in human anti-GBM disease. Several complement factors, such as C3, C5b-9, and factor B, show a positive correlation with the severity of the renal injury and act as risk factors for renal outcomes. Furthermore, compared to patients with single positivity for anti-GBM antibodies, individuals who are double-seropositive for anti-neutrophil cytoplasmic antibody (ANCA) and anti-GBM antibodies exhibit a unique clinical phenotype that lies between ANCA-associated vasculitis (AAV) and anti-GBM disease. Complement activation may serve as a potential "bridge" for triggering both AAV and anti-GBM conditions. The aim of this article is to provide a comprehensive review of the latest clinical evidence regarding the role of complement activation in anti-GBM disease. Furthermore, potential therapeutic strategies targeting complement components and associated precautions are discussed, to establish a theoretical basis for complement-targeted therapies.

KEYWORDS

anti-glomerular basement membrane disease, complement, autoimmune nephropathy, complement activation, complement therapeutics, emerging therapies

1 Introduction

Over 100 years ago, it was discovered that complement serves as a mediator to activate the innate immune pathway, playing a crucial role in defending against microbial invasion and immune surveillance. However, upon exposure to pathogens or contact with foreign surfaces, improper activation, and immune dysregulation can cause the complement system to shift from a defense mechanism to an attacking system, thereby becoming a primary pathogenic mechanism for numerous diseases (1). Specifically, autoimmune system dysregulation leads to the accumulation of immune complexes that cannot be cleared from the body. Subsequently, the complement system remains continuously activated due to stimulation by immune complexes, recruiting immune cells to launch attacks against self-tissues, resulting in tissue and organ damage and the occurrence of autoimmune diseases (2).

Anti-glomerular basement membrane (anti-GBM) disease is a rare autoimmune disease characterized by the deposition of anti-GBM antibodies in the kidneys and/or lungs, primarily affecting the glomerular capillaries or pulmonary capillaries. The annual incidence of the disease is 1.64 per million, with a 5-year renal survival rate of 34% (3, 4). It is worth noting that low levels of anti-GBM antibodies can also be detected in the general population, even when they fall below the "clinically positive" range (5).

In addition to anti-GBM disease, autoimmune disorders such as rheumatoid arthritis and systemic lupus erythematosus have also exhibited immune responses targeting self-antigens several years before the clinical onset (6, 7). Undeniably, antibodies have the potential to induce immune system attacks and directly damage target organs. However, in cases where antibodies persist for a prolonged period before clinical symptoms appear, complement system dysregulation may serve as a major contributing factor in the amplification of pathogenic factors, ultimately disrupting immune tolerance and leading to disease progression (5, 8). It is worth noting that the presence of antineutrophil cytoplasmic antibody (ANCA) positivity in patients with anti-GBM disease may be associated with more severe clinical manifestations and a poorer prognosis (9, 10). The coexistence of these two conditions could be attributed to intermolecular epitope spreading or the emergence of new antigenic epitopes (11, 12). However, the exact relationship between this phenomenon and complement system activation is currently unclear. Given that the kidney is highly sensitive to complement-mediated damage, it becomes particularly important to clarify the role of complement in anti-GBM disease. Furthermore, targeting the complement system to protect cells or tissues from immune system attack may provide benefits that outweigh the potential harms associated with suppressing complement and weakening the body's defenses (13). Therefore, targeted therapies aimed at the complement system may hold tremendous potential in the treatment of autoimmune kidney diseases.

2 Overview of anti-GBM disease

The main characteristic of anti-GBM disease is the production of pathogenic autoantibodies called anti-GBM antibodies, which primarily target the type IV collagen antigen in the glomerular and alveolar basement membranes (14). Research has revealed that type IV collagen is a triple-helical structure composed of α3, α4, and α5 chains, and the interactions between non-collagenous domains, such as hydrogen bonds, ionic bonds, and van der Waals forces, lead to the formation of hexameric structures in the GBM (15). The hexameric structure of the GBM serves as a barrier to prevent the interaction between antigens and antibodies. However, under the influence of specific factors, the hexameric structure becomes disrupted, resulting in the exposure of the cryptic antigenic α3 chain non-collagenous domain (α3NC1). This exposed domain is recognized by anti-GBM antibodies, leading to the formation of immune complexes and serving as the initial trigger for the autoimmune response (16). Subsequently, the complement system is activated, leading to a cascade reaction, generating complement cleavage products and inflammatory mediators, recruiting neutrophils to the damaged glomerular basement membrane area, and releasing cytokines and chemokines to attract and activate other immune cells, amplifying the inflammatory response (17, 18). Eventually, the excessive inflammatory reaction causes damage to the glomerular basement membrane and alveolar basement membrane, resulting in acute glomerulonephritis and pulmonary hemorrhage syndrome (19, 20) (Figure 1).

It is commonly postulated that a complex interplay of genetic factors, environmental influences, infections, and medications may contribute to the onset of anti-GBM disease (21). It has been observed that α3(IV)NC1 is predominantly localized to the basement membranes of the glomeruli, alveoli, brain, eyes, and inner ear, exhibiting a relatively restricted distribution (22). According to available data, around 90% of patients exhibit renal involvement, primarily presenting as acute glomerulonephritis, and in severe cases, it can progress to renal failure (23). About 40%-60% of patients experience pulmonary inflammation and injury, with pulmonary hemorrhage being the initial symptom in severe cases. Failure to intervene promptly can result in respiratory distress and pose a life-threatening risk (23–25). It is reassuring to note that this percentage has been trending downward in recent studies (15%-35%) due to early detection and early intervention of the disease (3, 4, 26). The treatment of anti-GBM disease, along with other autoimmune disorders in this category, remains a significant challenge. Although treatment modalities such as high-dose corticosteroids, plasma exchange, and immunosuppressive agents can mitigate the progression of anti-GBM disease, they do not offer a definitive cure (14). Of greater concern, our understanding of the disease's recurrence patterns and triggers remains limited. Anti-GBM disease may relapse at various time points or be influenced by different triggering factors, ultimately leading to the occurrence of end-stage renal failure.

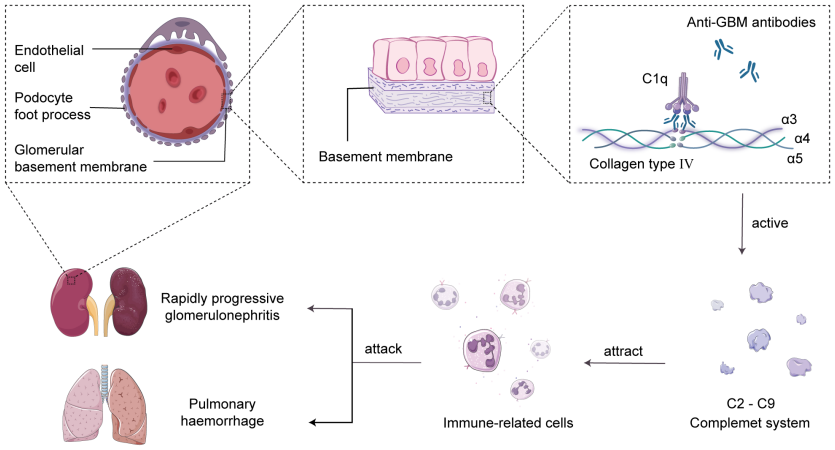

FIGURE 1
Complement-mediated acute glomerulonephritis and pulmonary hemorrhage. In the human glomerular basement membrane (GBM), the non-collagenous domain of the α3 chain of type IV collagen binds to self-anti-GBM antibodies, forming immune complexes and activating the complement system in situ. This triggers the recruitment of immune cells to attack the GBM and alveolar basement membrane, leading to the development of acute glomerulonephritis and pulmonary hemorrhage syndrome.

3 Complement activation found in anti-GBM disease

Complement, a group of endogenously produced proteins, constitutes an essential component of the immune system, closely intertwined with both innate and adaptive immune responses. The primary functions of the complement system encompass pathogen elimination, inflammatory reactions, antibody-mediated cytotoxicity, and immune modulation (27). Particularly in autoimmune diseases, including anti-GBM disease, the excessive activation of the complement system plays a pivotal role in the onset and progression of the disease. Research suggests that in a mouse model of anti-GBM-mediated glomerulonephritis, mice lacking the Fc-γ receptors chain, which is essential for complement receptor binding, in either inflammatory cells or resident kidney cells, exhibit near-complete complement system paralysis (28). Consequently, no urinary albumin was observed following kidney injury. As anticipated, the absence of albuminuria was also observed in FcR-γ chain and component 3 (C3) double-knockout mice, providing robust evidence for the involvement of the complement system in the pathogenesis of anti-GBM disease (28). Complement primarily induces localized rather than systemic immune responses leading to renal damage (29). Additionally, the levels of serum C3 deposition in glomeruli are positively correlated with the risk of renal failure (30). Complement appears to be linked not only to the extent of renal injury but also to the formation of crescents. For example, glomeruli with crescents demonstrate more intense complement deposition and greater severity of renal damage compared to glomeruli without crescents (31). Specifically, complement activation triggers the formation of the membrane attack complex (MAC), which subsequently mediates endothelial cell apoptosis and paves the way for crucial damage caused by neutrophils and other immune cells to the GBM (32, 33). Ultimately, crescents consisting of endothelial cells, immune cells, and cellular debris are formed within the glomeruli, exacerbating glomerular injury and deterioration of renal function (34–36). Conversely, in mice deficient in C6, the inability to assemble the C6-C9 complex and form the MAC helps them resist renal damage induced by anti-GBM antibodies. This study's findings offer further confirmation, from an alternative standpoint, of the complement system's role in renal injury in anti-GBM disease (37). Next, we will discuss the role of complement in anti-GBM disease by examining each of the three complement pathways individually. And evidence for complement activation in anti-GBM patients is presented in Table 1.

4 Complement activation pathways in anti-GBM disease

4.1 The classical pathway

The complement system is primarily activated through three main pathways: the classical pathway, the lectin pathway, and the alternative pathway. In patients with anti-GBM disease, IgG antibodies are produced, which subsequently target self-antigens, forming immune complexes that bind with C1q. This sequential activation involves complement components such as C4 and C2, leading to the formation of C3 convertase and C5 convertase. Ultimately, the terminal pathway of the three pathways is activated, resulting in the cleavage of C5 into C5b, which then associates with C6, C7, C8, and C9 to form the MAC (46). The MAC formation creates pores on the cell surface, inducing cell lysis and subsequent death of cells or pathogens. This process is regarded as the activation of the classical complement pathway (Figure 2).

Research has demonstrated that in the presence of C1q, administration of anti-C1q antibodies results in the binding and deposition of C1q and anti-C1q antibodies in the glomeruli, leading to increased renal damage in anti-GBM antibody-pre-treated mice

TABLE 1 Evidence for complement activation in anti-GBM disease.

Evidence	References	Key findings
Complement involved in the pathogenesis of anti-GBM	(28)	In a mouse model of anti-GBM-mediated glomerulonephritis, FcR-gamma-chain (-/-) mice were unable to bind to complement receptors, which in turn failed to activate the complement system and therefore failed to produce albuminuria.
	(29)	Patients with anti-GBM diseases have elevated plasma and urine levels of the terminal complement complex C5b-9.
	(30)	Levels of C3 deposition in glomeruli positively correlate with risk of renal failure.
	(31)	Higher incidence of complement component deposition in crescent-shaped glomeruli.
	(37)	C6-deficient mice are unable to form membrane attack complex, which attenuates anti-GBM antibody-induced kidney injury.
Activation of the Classical pathway	(28)	C1q knockout mice have significantly reduced kidney injury.
	(38)	C1q and anti-C1q antibodies bind to form immune complexes and are deposited in the glomeruli, leading to increased renal injury in mice pretreated with anti-GBM antibodies.
	(39)	C1q was detected in the glomeruli of almost all anti-GBM patients examined, where it was deposited linearly along the glomerular capillary wall and Bowman's capsule and co-localized with the terminal complement complex C5b-9.
Activation of the lectin pathway	(29)	MBL levels are elevated in plasma and urine in anti-GBM patients.
	(39, 40)	Renal biopsy shows diffuse deposition of MBL in glomeruli.
	(41)	Renal tissue injury is attenuated in both MASP-2-deficient and MASP-2 inhibitor-treated mice
	(42, 43)	Circulating anti-GBM antibodies (mainly IgG4 subclass) activate the lectin complement pathway
Activation of the alternative pathway	(28, 44)	When the classical and lectin pathways are blocked, complement can be activated by alternative pathways.
	(45)	Factor B is deposited linearly and granularly along the glomerular capillary wall and is highly co-localized with C5b-9.
		In crescentic glomeruli, factor B is deposited at a higher rate.

anti-GBM, Anti-glomerular basement membrane; MBL, Mannose-binding lectin; MASP-2, MBL-associated serine proteases 2.

(38). Moreover, in patients with the anti-GBM disease, C1q deposition along the glomerular capillary wall (GCW) and Bowman's capsule is detectable in nearly all examined glomeruli, and it co-localizes well with the terminal complement complex C5b-9 (39). In contrast, in a model of renal injury induced by exogenous anti-GBM antibodies, mice deficient in C1q exhibit significantly reduced renal damage (28). This outcome can be partially attributed to the blockade of classical pathway initiation following C1q knockout, thereby inhibiting complement activation and preventing renal injury. However, conventional renal biopsy examinations often fail to demonstrate C1q deposition in immunofluorescence tests (47). Additionally, other research has not found a correlation between the intensity of C1q deposition and the clinical characteristics of patients (39). One potential explanation is that, compared to other autoimmune diseases such as systemic lupus erythematosus, anti-C1q antibodies in anti-GBM patients are generally present at lower levels, making it difficult for them to bind to C1q and deposit in the kidneys (38).

4.2 The lectin pathway

Mannose-binding lectin (MBL) is an important soluble pattern recognition molecule that activates the lectin pathway. In contrast to the classical pathway, which primarily relies on antigen-antibody interactions, the lectin pathway primarily depends on the binding of lectins on the pathogen surface to activated lectin receptors on the cell surface, and this binding typically occurs on immune cell surfaces (48). Specifically, when the MBL complex binds to the surface of pathogens, MBL-associated serine proteases (MASP-1 and MASP-2) are activated, initiating the complement cascade, leading to the cleavage and activation of C4 and C2, and subsequently activating the complement system (49) (Figure 2). The lectin pathway primarily contributes to the inflammatory response by activating immune cells and releasing inflammatory mediators to combat pathogens (50). In contrast, the classical pathway not only triggers an inflammatory response but also directly destroys pathogens and marks them for clearance by immune cells. Overall, these two pathways play complementary roles in the immune system.

In anti-GBM patients, the elevation of plasma and urinary MBL levels indicates the involvement of the lectin pathway (29). It has been reported that MBL diffusely deposits in the glomerular capillary walls, mesangial areas, GBM, and even crescents (39, 40). MBL co-localizes with partial C4d but not with C5b-9. Based on this, a study suggests that the lectin pathway may not be involved in complement activation in human anti-GBM disease (45). However, considering that MBL primarily participates in the early stages of complement activation, its triggering role in complement activation cannot be ruled out, even though it does not co-localize with C5b-9. Indeed, both MASP-2 deficient mice and mice treated with MASP-2 inhibitors exhibit reduced tubulointerstitial damage and decreased proteinuria, demonstrating the protective effect of inhibiting the lectin pathway on the kidneys (41). Previously, anti-

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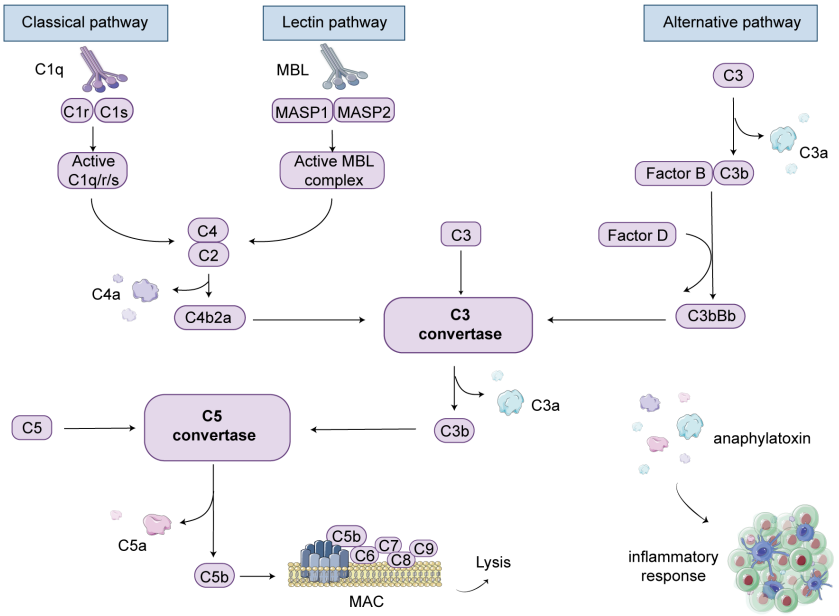

FIGURE 2
Complement cascade schematic illustration. There are three pathways involved in complement-mediated renal involvement, namely the classical, lectin, and alternative pathways. The classical pathway can be activated by the complex formation of antigens and antibodies. When mannose-binding lectin (MBL) binds to serine proteases, known as mannose-associated serine proteases (MASP1 and MASP2), the lectin pathway is activated. The alternative pathway is initiated when complement C3 covalently binds to microbial surfaces and undergoes cleavage, resulting in the formation of C3b. Each pathway ultimately generates active C3 convertases, leading to the cleavage of C3 into C3a and C3b fragments. C3b can then interact with C4b2a or C3bBb, generating C5 convertases. Under the action of C5 convertases, C5 is cleaved into C5a and C5b. C5b binds to cell membranes and, along with C6, C7, C8, and C9, forms the membrane attack complex (MAC), leading to cell lysis. Additionally, complement products such as C3a, C4a, and C5a act as anaphylatoxins and chemotaxis, attracting and activating immune cells, thereby inducing an inflammatory response.

GBM antibodies were thought to mainly belong to the immunoglobulin G subclass 1 (IgG1). Interestingly, there have been reports suggesting the presence of circulating anti-GBM antibodies predominantly of the IgG4 subclass, which may lead to false-negative results in immune detection of anti-GBM disease (51, 52). Despite IgG4 subclass antibodies being considered to have lower complement activation capacity due to their inability to bind to C1q, they can still activate the lectin complement pathway by binding to MBL, leading to complement system activation and substantial deposition in the kidneys (42, 43).

4.3 The alternative pathway

Compared to the other two pathways, the alternative pathway does not require the involvement of antibodies or lectins. Instead, it primarily relies on the activation or cleavage of endogenous molecules within the body, such as complement C3, factor B, factor D, and others (53). This also means that the activation of the alternative pathway is largely a spontaneous and non-specific process. Specifically, C3 in the plasma is cleaved into C3a and C3b. C3b, along with blood proteins like factor B and factor D, binds to specific surface structures of microbes and self-molecules in the bodily fluid, forming the C3 convertase and activating the terminal complement pathway (Figure 2). It is important to note that the alternative pathway and the classical pathway are not isolated entities; they can mutually influence and cross-activate each other under certain circumstances (54). For example, specific surface structures of pathogens may simultaneously activate these two pathways, thereby triggering a more robust immune response (55).

In the anti-GBM model, the occurrence of inflammatory cell infiltration and renal damage has been observed in mice with a specific knockout of the shared complement component C4 in the classical and lectin pathways. Similarly, in mice with a double knockout of C1q and C4, deposition of C3 complexes can also be detected (28, 44). These facts demonstrate that despite the blockade of the classical and lectin pathways, complement activation can still occur solely through the alternative pathway, ultimately leading to kidney injury. Factor B, which is exclusive to the alternative complement pathway, plays a distinct role in differentiating it from the other two pathways. It has been reported that Factor B can deposit along the GCW in a linear and granular pattern and exhibits good co-localization with C5b-9 (45). Compared to non-crescentic glomeruli, crescentic glomeruli exhibit significantly stronger deposition of Factor B (45). These findings provide clearer indications of alternative complement pathway activation. Notably, one major function of the alternative pathway is to amplify the activation of the classical pathway. Once the classical pathway is activated, components of the alternative pathway, such as Factor B, Factor D, and C3b, are recruited and participate in the complement activation process, interacting with the activation products of the classical pathway to further enhance complement activation (53, 54).

Therefore, through this amplification loop, the alternative pathway not only engages in the complement activation process but also reinforces the activation effect of the classical pathway (56).

5 Comparing complement pathways in anti-GBM disease and other autoimmune disorders

Autoimmune diseases refer to a group of conditions in which the immune system abnormally turns against the body's own healthy tissues and cells. In addition to anti-GBM disease, this category encompasses disorders such as systemic lupus erythematosus (SLE), rheumatoid arthritis, ANCA-associated vasculitis (AAV), and more (57). In comparison to anti-GBM disease, other autoimmune disorders also involve the activation of multiple complement pathways, but the antibodies forming immune complexes differ significantly. These include antinuclear antibodies, double-stranded DNA antibodies, ANCA, and rheumatoid factors, marking a fundamental distinction from anti-GBM disease (58, 59). In addition, in the context of anti-GBM disease, the activation of complement and the deposition of its components occur specifically on the glomerular basement membrane of the kidneys. This triggers a cascade of inflammatory reactions and cellular damage, ultimately resulting in the development of acute glomerulonephritis. However, in other autoimmune diseases, this pathological process occurs across multiple organs and tissues, such as the kidneys, skin, and joints, leading to a wide range of inflammatory responses and tissue injuries (60, 61). Notably, in SLE, complement levels reflect disease activity and severity to some extent, whereas measuring complement components in anti-GBM diseases has limited application in diagnostic and therapeutic monitoring (62, 63). In the management of these diseases, interventions targeting the complement pathway exhibit variations. In patients with anti-GBM disease, the primary approach involves the utilization of immunosuppressants and plasma exchange to suppress the production of self-antibodies and reduce complement activation (64, 65). Conversely, for other autoimmune diseases, treatment strategies may encompass the administration of immunosuppressants, nonsteroidal anti-inflammatory drugs (NSAIDs), and biologic agents to alleviate inflammatory responses and modulate the functionality of the immune system (66, 67). Excitingly, there is a growing anticipation that complement modulators hold promising potential as a potential therapeutic target for the majority of autoimmune diseases, including anti-GBM disease (57, 67, 68).

6 Double-seropositive for ANCA and anti-GBM antibodies

Among all autoimmune diseases, anti-GBM patients are most prone to develop anti-neutrophil cytoplasmic antibody (ANCA) positivity, typically in the form of anti-myeloperoxidase (MPO) antibodies. These individuals, referred to as antibody double-seropositive patients (DPPs), comprise approximately 20-35% of the total anti-GBM population (22). Conversely, up to 10% of ANCA-positive patients exhibit circulating anti-GBM antibodies as well (9, 69). In this patient population, a confluence of features is observed, combining ANCA-associated vasculitis and anti-GBM disease traits. These include severe renal illness, pulmonary hemorrhage, and diverse symptoms across various organs and tissues due to vascular damage (such as fever, fatigue, sinusitis, joint pain, etc.). Unfortunately, the majority of these patients demonstrate a suboptimal response to treatment and experience a poorer renal prognosis (70, 71). However, studies have also reported that DPPs have a higher average age compared to single-antibody-positive patients, and it is believed that the prognosis of DPPs depends on the titers of the antibodies (26, 72). Currently, known factors predicting survival rates include severe renal failure, advanced age, and the presence of pulmonary hemorrhage at the onset of the disease (73). Interestingly, ANCA positivity can also occur in circulating antibody-negative anti-GBM disease patients (74). Although the mechanisms and underlying connections between the simultaneous occurrence of these two diseases are not fully elucidated, research suggests that there are no significant specific differences in the antigens present in the serum between double-seropositive and single-antibody-positive patients (anti-GBM antibody or ANCA) (75). However, whether these two diseases involve interconnected complement activation pathways or mutually influence each other's complement activation mechanisms remains uncertain.

Based on available data, anti-GBM antibodies can appear years to decades after ANCA antibodies. Both antibodies can coexist and persist for several years before experiencing a sharp increase in levels weeks to months before clinical disease onset (5). Traditionally, it has been believed that the self-degradation and remodeling of basement membranes lead to the exposure of hidden epitopes (76). However, recent research in AAV has uncovered that proteases released by ANCA-activated neutrophils digest Col (IV), leading to the exposure of α3(IV)NC1. CD11c+ macrophages then present GBM epitopes, triggering the host's immune system to generate anti-GBM antibodies (12). In simple terms, AAV disrupts the hexameric structure of the GBM, exposing self-antigens stored within the GBM and triggering an anti-GBM response. Furthermore, due to varying affinities of antibodies for different tissues, both clinically and histologically, double-seropositive patients (DPPs) exhibit extrarenal manifestations similar to those observed in AAV patients, while their renal manifestations resemble those seen in anti-GBM disease patients (70). However, the precise role of the complement system in this process remains inadequately understood. Reports indicate a decrease in serum complement levels and an increase in renal complement deposition in patients with ANCA-associated glomerulonephritis (77, 78). Furthermore, in comparison to single antibody-positive patients, DPPs exhibit a lower trend in serum C3 and C4 levels, indicating a higher degree of complement activation in DPPs (79). And DPPs demonstrate a higher rate of primary disease recurrence, suggesting that the cascading complement activation might be one of the reasons for the more severe pathological changes observed in DPPs (73). It is worth noting that the total complement capacity appears to be

limited within a short period, which may explain the less pronounced decrease in complement levels in DPPs compared to single antibody-positive diseases.

7 The impact of therapeutic plasma exchange on the complement system

Standard treatment for anti-GBM disease includes cyclophosphamide, corticosteroids, and therapeutic plasma exchange (TPE), a combined therapy that was first proposed in 1976 and continues to be used today (80). Currently, guidelines still recommend adopting the mentioned approach for treatment, even in the absence of pulmonary hemorrhage, except in cases of limited renal function (those on dialysis at presentation, with 100% crescents or > 50% global glomerulosclerosis in an adequate biopsy sample, and no pulmonary hemorrhage) (81, 82). Additionally, as long as renal function is viable, TPE should be continued until anti-GBM titers are no longer detectable (81). Some researchers have even proposed that high-dose intravenous glucocorticoids may not be necessary for anti-GBM disease following timely TPE and other necessary treatments (23). TPE involves the removal of plasma along with large molecular substances such as self-antibodies, immune complexes, and toxins through centrifugation or filtration. Studies have shown that the addition of TPE to corticosteroids and cyclophosphamide treatment improves overall survival, particularly in anti-GBM patients with initial treatment presenting a serum creatinine level higher than 6.8 mg/dL and concurrent pulmonary hemorrhage when conventional therapy fails to improve renal outcomes (83). DPPs exhibit multi-organ damage, including renal involvement, and TPE has been demonstrated to rapidly and effectively remove antibodies and inflammatory mediators, including complement (84). Immunoadsorption (IAS) is an alternative therapy to TPE, offering increased specificity. After blood separation in separator device, plasma passes through highly selective adsorption columns, targeting specific antibodies or complement molecules for precise and rapid removal of pathogenic factors. The impact of immunoadsorption on anti-GBM antibody titers is currently being investigated (NCT02765789). Existing research has demonstrated that IAS can effectively remove cellular cytokines, such as complement C3a and tumor necrosis factor-α. Moreover, IAS has shown comparable efficacy to TPE in clearing anti-GBM antibodies and promoting renal repair (85, 86).

In fact, most studies primarily focus on the clearance rate of antibodies during TPE, while the clearance effect on complement remains understudied. Furthermore, despite improvements, non-self materials can still activate complement during the process of blood passing through dialyzers and extracorporeal circuits, leading to dialysis-related inflammation (87, 88). Currently, complement C3 inhibitors (such as peptide-based matrix metalloproteinase inhibitor 101 or complement c5 convertase inhibitor 40) may hold promise in intervening in dialysis-related inflammation for patients undergoing chronic dialysis (89, 90). Additionally, the risks associated with dialysis have also spurred the development of complement inhibitors for non-dialysis patients.

8 The application of complement regulators in anti-GBM disease

There is no doubt that significant progress has been made in the development of drugs targeting the complement system. Serine proteases, including Cinryze, Cetor, Berinert, Ruconest, and C5 inhibitors such as eculizumab, have been approved for the treatment of complement-related disorders (2, 91). Eculizumab, an anti-C5 humanized monoclonal antibody, can block the cleavage of C5 and inhibit the formation of C5b-9 (92). Currently, it is primarily approved for paroxysmal nocturnal hemoglobinuria and atypical hemolytic uremic syndrome, or used for its non-specific anti-inflammatory effects in the intervention of AAV (93). Considering the crucial role of C5 in complement activation, targeting C5 may represent an effective therapeutic approach for anti-GBM disease, although C5 blockade does not reverse the initial driving factors of the disease. High circulating levels of C5b-9, along with positive staining in renal biopsies of anti-GBM patients, confirm the activation of the terminal complement cascade, providing a rationale for the use of Eculizumab. It is important to note that the C5-blocking effect of Eculizumab can alleviate glomerular inflammation and reduce proteinuria, but it does not decrease complement deposition in the kidneys (94). In life-threatening organ damage seen in Goodpasture syndrome, Eculizumab even has the potential to block complement-driven lung injury (95). Therefore, despite the high cost of treatment, C5 and C5a inhibitors may serve as targeted anti-inflammatory agents to mitigate renal injury.

In addition to treating primary anti-GBM disease, further research is warranted on targeting the complement system to improve outcomes related to post-transplant recurrence. Among 224 patients who underwent renal allograft transplantation for anti-GBM disease between 1963 and 2010, six patients experienced a recurrence of anti-GBM disease, with graft failure occurring in two cases (96). As early as 20 years ago, when immunosuppressive agents were limited, studies reported a high recurrence rate of 14% in anti-GBM disease (97). Therefore, considering the higher incidence of disease recurrence following allograft kidney transplantation in the presence of anti-GBM antibodies, it is crucial to ensure low titers of anti-GBM antibodies before transplantation. Indeed, despite delaying transplantation and implementing standardized immunosuppressive regimens, sporadic cases of recurrence still occur (98, 99). Furthermore, disease recurrence has been observed even in the absence of serum anti-GBM antibodies (100, 101). The occurrence of such cases may be attributed to the presence of specific subtypes of anti-GBM antibodies that are not detectable by the tests currently employed (102, 103). Additionally, it cannot be ruled out that the levels of anti-GBM antibodies may fall below the detection range, while the amplifying effect of the complement cascade could

potentially contribute to disease recurrence. Currently, research has shown that blocking complement activation through the use of serine protease inhibitors, such as C1 esterase inhibitors, or terminal complement pathway inhibitors can prevent delayed graft function and reduce rejection reactions, ultimately improving graft survival (104, 105). Currently, the use of eculizumab is being investigated in kidney transplant recipients to counteract antibody-mediated rejection and complement-mediated injury (NCT02113891, NCT01327573).

9 Considerations for complement-targeted therapy

The deficiency of complement components can also lead to the accumulation of immune complexes and cellular debris, reflecting the intricate role of complement in renal diseases (106). For instance, in an experimental animal model of anti-GBM disease, the knockout of C3 worsened glomerular injury (28). This may be attributed to the loss of C3-mediated clearance of antigen-antibody complexes, resulting in increased deposition of immune complexes and exacerbation of glomerular injury. Furthermore, studies have reported that defects in factor H of the alternative pathway lead to uncontrolled complement activation and deposition of complement on glomeruli, while supplementation of factor H can inhibit C3 deposition on the GBM (106, 107). Therefore, not all detected complement components are pathogenic, and the activation of the complement system is not always harmful to the body. When using complement-targeted therapies, it is essential to rigorously evaluate the effects on complement to avoid the inappropriate use of complement modulators. Furthermore, inhibiting or modulating the function of the complement system may reduce the body's ability to resist infections, making patients more susceptible to pathogen invasion. Studies have reported that patients treated with eculizumab may face a significant risk of severe infections caused by microorganisms such as gonococcus and meningococcus (108, 109). Therefore, during complement-targeted treatment, it is crucial to closely monitor the risk of treatment-related infections and assess the potential benefits and risks. The continuation of this treatment approach is only meaningful when the benefits outweigh the risks.

Notably, complement inhibition therapy is an emerging treatment strategy, and currently, there are no available biomarkers to predict the response of renal diseases to complement inhibition (110). Thus, the identification of biomarkers that can predict the response of kidney disease patients to complement blockade therapy is one of the current research priorities. Due to the complexity of renal diseases and individual variations, the response of patients to complement inhibition therapy can vary significantly. The use of complement inhibitors requires individualized strategies based on the severity of the condition and complement levels. For instance, during the acute phase of complement-mediated renal diseases, the choice of complement modulators tends to focus on directly targeting key steps in complement activation, such as C3/C5 antibodies or C3a/C5a receptor antagonists. These drugs can effectively inhibit the effects of C3a/C5a and the formation of MAC, thereby reducing the release of inflammatory mediators and cell damage (110). Conversely, in the chronic phase, the treatment goal is to alleviate complement-mediated inflammation and fibrosis while preserving renal function stability. For example, the therapeutic effects of small interfering RNA may be delayed but could prove cost-effective in long-term strategies (111). Overall, in different scenarios, the selection of appropriate complement modulators may require a more comprehensive consideration of the underlying pathological mechanisms of the disease and individual characteristics.

10 Conclusion

In conclusion, the role of complement in anti-GBM disease is both complex and crucial. It plays a dual role in regulating the antigen-antibody immune response and activating the inflammatory process through complement cascade reactions, thereby directly or indirectly causing damage to the glomeruli. It is evident that all three complement activation pathways are involved in the pathogenesis of anti-GBM disease. Moreover, complement activation is present not only in patients with classic anti-GBM disease, but also in patients who are double positive for ANCA and anti-GBM antibodies. The degree of complement activation is positively correlated with the severity of the disease. Research on the complement system contributes to a deeper understanding of the pathogenesis of anti-GBM disease and provides a foundation for the development of complement-targeted therapeutics.

Author contributions

This study was a collaborative effort among all authors. AT and XZ were involved in writing the manuscript. TT, DX, and BX contributed to manuscript revisions. YH, and ML provided final approval. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Genetic link between primary sclerosing cholangitis and thyroid dysfunction: a bidirectional two-sample Mendelian randomization study

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Background: Observational studies have demonstrated an association between primary sclerosing cholangitis (PSC) and thyroid dysfunction (TD). However, the causal relationship between PSC and TD remains uncertain. The purpose of this study is to investigate the causal associations and specific direction between these two conditions. Gaining insight into the potential causal relationship between PSC and TD is valuable for elucidating the pathogenesis of PSC and for devising innovative approaches for the prevention and treatment of PSC and its associated complications.

Methods: We conducted a bidirectional two-sample Mendelian randomization (MR) analysis to investigate the causal association between PSC and TD, such as autoimmune thyroid disease (AITD), thyroid cancer (TC), thyroid stimulating hormone (TSH), thyrotropin-releasing hormone (TRH), among others. PSC was the exposure variable, while TD was the outcome variable. To identify suitable instrumental variables (IVs), we utilized genome-wide association study (GWAS) datasets to select potential candidate single-nucleotide polymorphisms (SNPs). The primary statistical approach employed was the inverse-variance weighted (IVW) method, which was complemented by a series of sensitivity analyses to assess the robustness of the results by estimating heterogeneity and pleiotropy.

Results: We found that the causal associations between genetically predicted PSC and Graves' disease (GD), hyperthyroidism (IVW OR=1.230, 95%CI: 1.089–1.389, P=0.001; IVW OR=1.001, 95%CI: 1.000-1.002, P=0.000) were statistically significant. The reverse MR analysis indicated that genetic susceptibility to hyperthyroidism (P=0.000) and hypothyroidism (p=0.028) might be the risk of PSC. There was no statistically significant causal association observed between PSC and other TD (IVW P>0.05), with the exception of GD, hyperthyroidism, and hypothyroidism as determined through bidirectional two-sample analysis. To ensure the reliability of our findings, additional sensitivity analyses were conducted, including the leave-one-out (LOO) test, heterogeneity test, and pleiotropic test.

Conclusion: In this study, we conducted an investigation into the causal association between PSC and TD. Our findings indicate that PSC significantly elevates the susceptibility to GD and hyperthyroidism from a statistical perspective. These results shed light on the etiology of PSC and have implications for the management of patients with PSC.

KEYWORDS

primary sclerosing cholangitis, thyroid dysfunction, hyperthyroidism, hypothyroidism, Mendelian randomization, causal relationship

Introduction

Primary sclerosing cholangitis (PSC) is characterized by autoimmune cholestatic liver disease, resulting in the development of multifocal biliary strictures through inflammation and fibrosis (1, 2). The rarity of PSC is evident, as it affects fewer than 200,000 individuals in the United States (US) and less than 5 per 10,000 inhabitants in the European Union (EU). The prevalence and annual incidence rate of PSC are estimated to be approximately 1 per 10,000 and 0.4 to 20 cases per 100,000 per year in the US and Northern Europe, respectively (3, 4). Furthermore, population-based studies have indicated a yearly rise in the incidence and prevalence of PSC, potentially attributed in part to the utilization of magnetic resonance cholangiography (MRC) (5–8). PSC is distinguished by the progressive deterioration of liver function, often accompanied by a heightened susceptibility to cholangiocarcinoma and colorectal cancer. In cases of advanced-stage PSC, liver transplantation (LT) becomes necessary. However, there is a possibility of disease recurrence following LT or surgical intervention (9). Despite the proposal of various mechanistic theories, the pathogenesis of PSC remains unclear and intricate. Consequently, comprehending the potential pathogenesis of PSC is of utmost importance in order to facilitate the development of efficacious therapeutic approaches and enhance prognosis.

It is well-established that patients with PSC exhibit distinct characteristics that are linked to a range of extrahepatic autoimmune diseases, such as rheumatoid arthritis (RA), scleroderma, inflammatory bowel disease (IBD), and autoimmune thyroid disease (AITD) (10, 11). A comparative study has examined the prevalence of TD in PSC, revealing a rate of 11%, and an incidence of 2.1 patients per 100 person-years (12). The findings of this study revealed that TD was unexpectedly prevalent among patients with PSC, surpassing the expected prevalence in the general population (12). Therefore, additional research on the association between thyroid disease and PSC is justified. Furthermore, a separate investigation involving PSC patients reported a prevalence of thyroid diseases in 8.4% of the 119 participants (13). Indeed, the relationship between PSC and AITD has been examined through descriptive and observational studies (14). In addition, there exists a significant correlation between thyroid hormones and liver function, and it is crucial to promptly diagnose and manage TD in patients with PSC to enhance the quality of their healthcare. A case report has documented the occurrence of hyperthyroidism, specifically Graves' disease (GD), in a 19-year-old male patient with PSC and a history of ulcerative colitis. This observation suggests a shared immunogenetic predisposition between PSC and GD, potentially influenced by the presence of a human leukocyte antigen (HLA) phenotype (15). This finding demonstrates that patients with PSC are at an elevated risk for AITD, encompassing both GD and Hashimoto's thyroiditis, also referred to as autoimmune thyroiditis (AT). Research indicates that PSC patients exhibit higher levels of serum T3, T4, and thyroid-stimulating hormone (TSH) in comparison to control groups (16). However, the establishment of a causal relationship between PSC and TD is derived from observational studies, wherein the presence of reverse causality, selection bias, and particularly unobserved confounding factors may obscure genuine causal connections. Consequently, it is crucial to investigate the correlation between PSC and TD in order to gain insights into the fundamental mechanisms of these diseases and improve their therapeutic approaches and quality of life.

Priority of the double-blind randomized controlled trial (RCT) may be compromised due to inherent disadvantages including challenging ethical approval, substantial time, manpower, and financial investments (17). In our study, Mendelian randomization (MR) was employed as a quasi- RCT design for analyzing causal effects.

MR can be conducted by utilizing single-nucleotide polymorphisms (SNPs) as instrumental variables (IVs) to establish a causal association between exposure and outcome (18, 19). This approach is widely acknowledged in observational studies, relying on the assumptions of IVs (20, 21). MR leverages genotypes that remain unaltered by confounding factors, thus serving as a corrective measure for the bias introduced by the presence of confounding factors in observational studies (22). The natural advantage of MR is determined by the principles of Mendel's law of inheritance. The process of meiosis entails the random distribution of homologous chromosomes and the subsequent combination of gametes, leading to the random assortment of genetic variations in the absence of external influences (23, 24). In contrast to alternative research methodologies, MR demonstrates a reduced level of measurement error concerning genetic variation and its subsequent effects. Furthermore, the utilization of genome-wide association studies (GWAS) in the context of MR is relatively uncomplicated, particularly in

bidirectional two-sample MR (25). In this scenario, MR is based on the premise that if a causal connection exists between PSC and TD, SNPs linked to the exposures will also demonstrate an association with the outcomes, thereby establishing a causal link between PSC and TD through the mediation of exposures. The IVs must adhere to the following criteria (1): demonstrate a robust correlation with the exposure factors (2), should not be linked to any other potential confounding factors, and (3) should not possess a direct association with the outcome (24). Therefore, we employed MR analyses utilizing valid SNPs as IVs to ascertain and evaluate the causal relationship between PSC and TD. This investigation was based on twelve extensive GWAS summary statistics, predominantly focusing on the European population's data pertaining to PSC and TD, including AITD (GD and AT), hyperthyroidism, hypothyroidism, thyroid cancer (TC), TSH, thyrotropin-releasing hormone (TRH), thyroxine-binding globulin (TBG), thyroid hormone receptor alpha (THRα), thyroid peroxidase (TP) and thyroglobulin (TG). In addition, in order to ensure the reliability of our findings, we conducted various sensitivity analyses, encompassing the heterogeneity test, pleiotropy test, leave-one-out (LOO) test, and reverse-direction MR analyses.

Methods

Source of data and study design

The analysis was conducted using publicly accessible summary-level data from GWAS that specifically examined traits of interest, primarily in individuals of European ancestry, encompassing both males and females. A total of twelve datasets on PSC and TD traits were collected. The PSC dataset (n= 14890) was obtained from the International PSC Study Group (IPSCSG) as reported by Sun-Gou Ji et al., which investigated the relationship between the quantity of PSC and SNPs (26). AITD dataset includes both AT (n=212453) and GD (n=NA). Hyperthyroidism dataset (n=337159) was obtained from Neale Lab Consortium. Hypothyroidism dataset contained 463010 individuals from MRC-IEU in European population. TC dataset (n=1080) was obtained from the data reported by Aleksandra Köhler et al (27). TSH, TRH, THRα, TP and TG dataset all contained 3301 individuals in European population (28). TBG dataset was obtained from GWAS through the human blood plasma proteome (29).

The study design is visually presented in Figure 1, illustrating the schematic representation and the three core assumptions of MR. These assumptions are as follows: (A) a robust association exists between SNPs and the exposure variable (TD/PSC); (B) SNPs are not influenced by any known confounding factors; and (C) the impact of SNPs on the outcome variable (PSC/TD) is solely mediated through the exposure variable (TD/PSC).

Selection and validation of IVs

A series of quality control procedures were implemented to identify eligible SNPs that exhibit improved statistical power in the analysis of genetic variants. Initially, IVs were utilized to examine

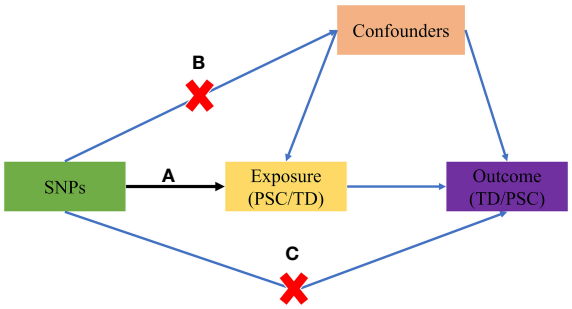

FIGURE 1
The basic principles of the MR study. **(A)** SNPs are strongly associated with exposure; **(B)** SNPs are independent of confounders; **(C)** SNPs must only affect outcome via exposure. MR, Mendelian randomization; PSC, primary sclerosing cholangitis; TD, thyroid dysfunction; SNP, single-nucleotide polymorphism.

preprocessed exposure data and determine genetic variants that met the genome-wide association threshold ($P<5*10^{-8}$) (30, 31). In the event that the number of filtered SNPs was insufficient, it was feasible to modify the threshold to $P<1*10^{-5}$. Following this, a manual search was conducted using PhenoScanner V2 to mitigate the potential impact of confounding factors and identify SNPs associated with the outcome (32). Thirdly, variants within a physical proximity of less than 10000 kb and with an $R^2<0.001$ were excluded to reduce the presence of linkage disequilibrium (LD). We also excluded SNPs for the existence of palindromic sequences with intermediate allele frequencies. Moreover, the F-statistic was calculated to evaluate the effectiveness of individual SNPs (33). SNPs with F-statistics exceeding 10 were deemed sufficiently robust to counteract potential biases (30). SNPs with a minor allele frequency below 5% were ultimately excluded from the analysis. The present analysis did not require ethics approval as all GWAS data included in the study were publicly available and had already obtained approval from the respective ethical review boards.

MR design and statistical analyses

The exposure and outcome datasets were harmonized in order to ensure consistent reference to the same allele (34). By selecting the SNPs as IVs, it was necessary for them to be valid. As the primary analysis, the inverse-variance weighted (IVW) method was employed, along with four supplementary methods (MR-Egger, weighted median, weighted mode, and simple mode), to investigate the potential causal effect between PSC and TD. Additionally, reverse-direction MR was conducted to evaluate the potential reverse causal association of TD on PSC.

Sensitivity analysis

A sensitivity analysis was conducted to assess potential deviations from the model assumptions in the MR analysis. This analysis involved three tests (1): the LOO test (2), the heterogeneity test, and (3) the pleiotropic test (31, 32). The LOO test was

employed to examine if individual SNPs disproportionately influenced the overall estimates. This was achieved by sequentially removing each SNP and applying IVW method to the remaining SNPs (32). Heterogeneity among the IVs was assessed using Cochrane's Q-value (35). The results of the heterogeneity test revealed the presence of heterogeneity when the p-value was below 0.05. In order to address the potential influence of heterogeneity on the outcomes, the multiplicative random-effects model was utilized. Alternatively, the fixed effects model was employed to evaluate the causal relationship between PSC and TD. Furthermore, MR-Egger regression test and MR-PRESSO global test were utilized to assess the impact of horizontal pleiotropy and ensure that the selected IVs met the fundamental assumptions of the MR analysis (36). When the p-value is less than the predetermined significance level of 0.05, the obtained results demonstrate instability (36). Furthermore, the MR-PRESSO outlier test was used to correct SNPs by removing outliers. MR-PRESSO distortion test was employed to ascertain the presence of a statistically significant disparity in casual estimation before and after the correction of outliers. All statistical analyses were conducted using the "TwoSampleMR" (version 0.5.4) and "MRPRESSO" (Version 1.0) packages in R version 4.0.3 (R Foundation for Statistical Computing, Vienna, Austria). Statistical significance was defined as a p-value less than 0.05. The flowchart about the screening of IVs and the steps of MR analysis was shown in Figure 2.

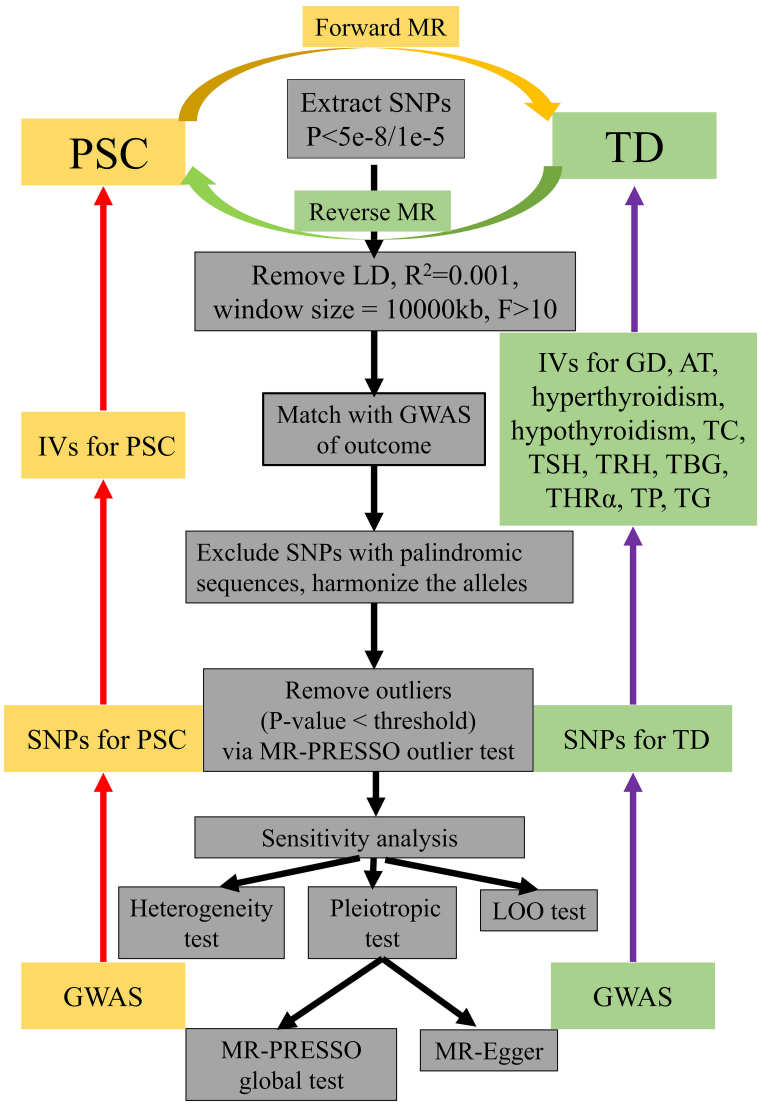

FIGURE 2
A flowchart about the screening of IVs and how the MR analysis was performed. IVs, instrumental variables; MR, Mendelian randomization; PSC, primary sclerosing cholangitis; TD, thyroid dysfunction; LD, linkage disequilibrium; GWAS, genome-wide association study; LOO test, leave-one-out test; SNP, single-nucleotide polymorphism; GD, Graves' disease; AT, autoimmune thyroiditis; TC, thyroid cancer; TSH, thyroid stimulating hormone; TRH, thyrotropin-releasing hormone, TBG, thyroxine-binding globulin; THRα, thyroid hormone receptor alpha; TP, thyroid peroxidase; TG, thyroglobulin.

Results

In summary, the studies incorporated in this analysis were published within the timeframe of 2013 to 2021 and primarily focused on the European population, as per the specified selection criteria (Supplementary Table S1).

The causal effect of PSC on TD via forward MR

In order to conduct a thorough assessment of the connections between PSC and the likelihood of TD development, MR analyses were employed to validate associations with a statistically significant P-value of less than 0.05. The presence of genetic variations in PSC was found to be causally linked to the risk of TD. Considering the criteria for selection, specific SNPs associated with TD were retrieved for the purpose of investigating the causal impact of PSC on TD (Supplementary Table S2). We observed a significant causal association using the IVW method between PSC and TD (PSC and GD, IVW OR = 1.230, 95% CI 1.089-1.389, P=0.001; PSC and hyperthyroidism, IVW OR = 1.001, 95% CI 1.000-1.002, P=0.000) (Figures 3, 4; Supplementary Table S2). Significant and consistent results between PSC and GD (P=0.017), PSC and hyperthyroidism (P=0.004) were also observed in the weighted median method (Supplementary Table S2). The robustness of the MR analysis findings can be further substantiated by employing the LOO method of sensitivity analyses to ascertain the impact of individual genetic variants on the overall outcomes (Figures 3B, 4B). The absence of significant horizontal pleiotropy was indicated by the MR-Egger regression test and MR-PRESSO global test (PSC for GD, P=0.562; P=0.334) (Figure 3C; Supplementary Table S4). These results may suggest causal relationship between PSC and GD was stable and unbiased. On the contrary, there had a significant horizontal pleiotropy in PSC on hyperthyroidism via MR-PRESSO global test (P<0.001) while after distorting outliers using the MR-PRESSO distortion test, there was still significant difference between results before and after outlier's correction in PSC on hyperthyroidism (P<0.001) (Figure 4C; Supplementary Table S4). This may indicate causal link between PSC and hyperthyroidism was unstable and biased. Cochran's Q statistics test identified statistical heterogeneity using IVW (IVW PSC on GD and hyperthyroidism; Cochran's Q=11.121, P=0.267; Cochran's Q=62.468, P=0.023), as presented in Figures 3D, 4D; Supplementary Table S4. Hence, IVW

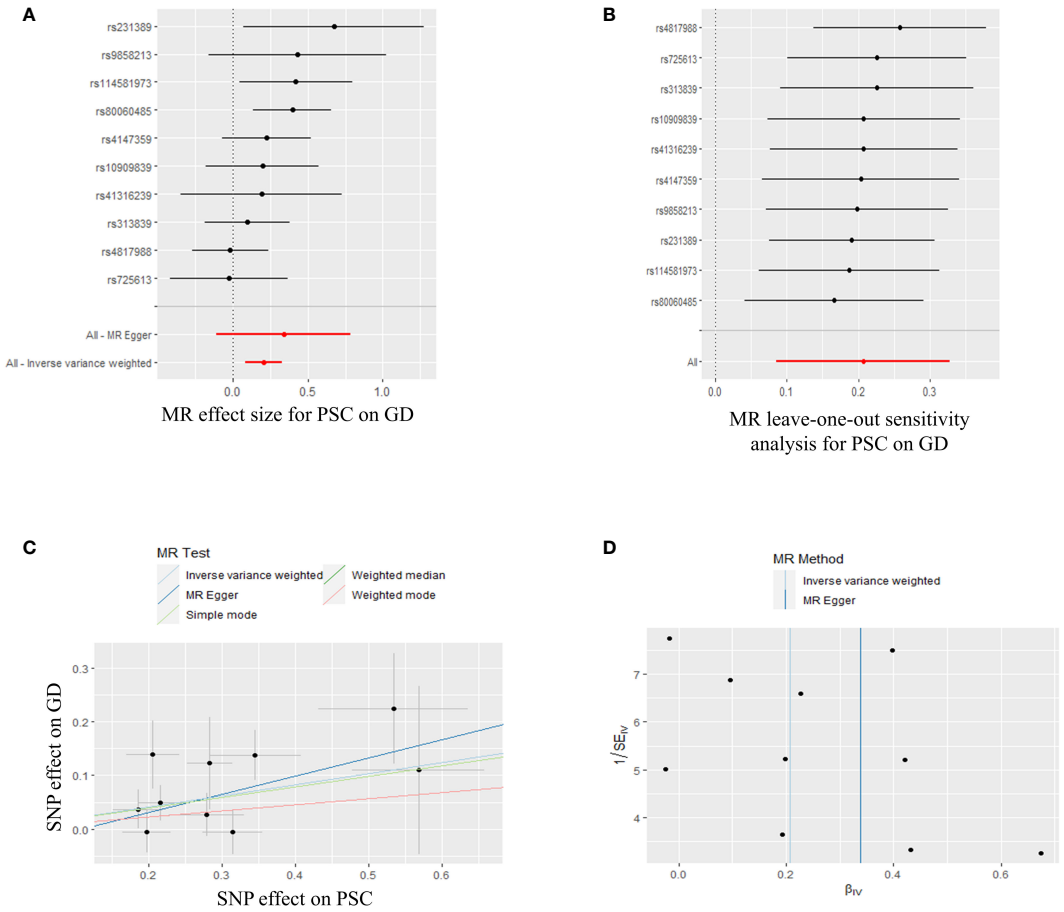

FIGURE 3
Plots of MR estimates of the causal relationship between PSC and GD. **(A)** The forest plot of SNPs associated with PSC and their risk on GD. **(B)** LOO sensitivity analysis of the association of PSC on GD. **(C)** Scatter plot of the association of PSC on GD. **(D)** Funnel plot of the association of PSC on GD. MR, Mendelian randomization; PSC, primary sclerosing cholangitis; GD, Graves' disease; SNP, single-nucleotide polymorphism; LOO, leave-one-out.

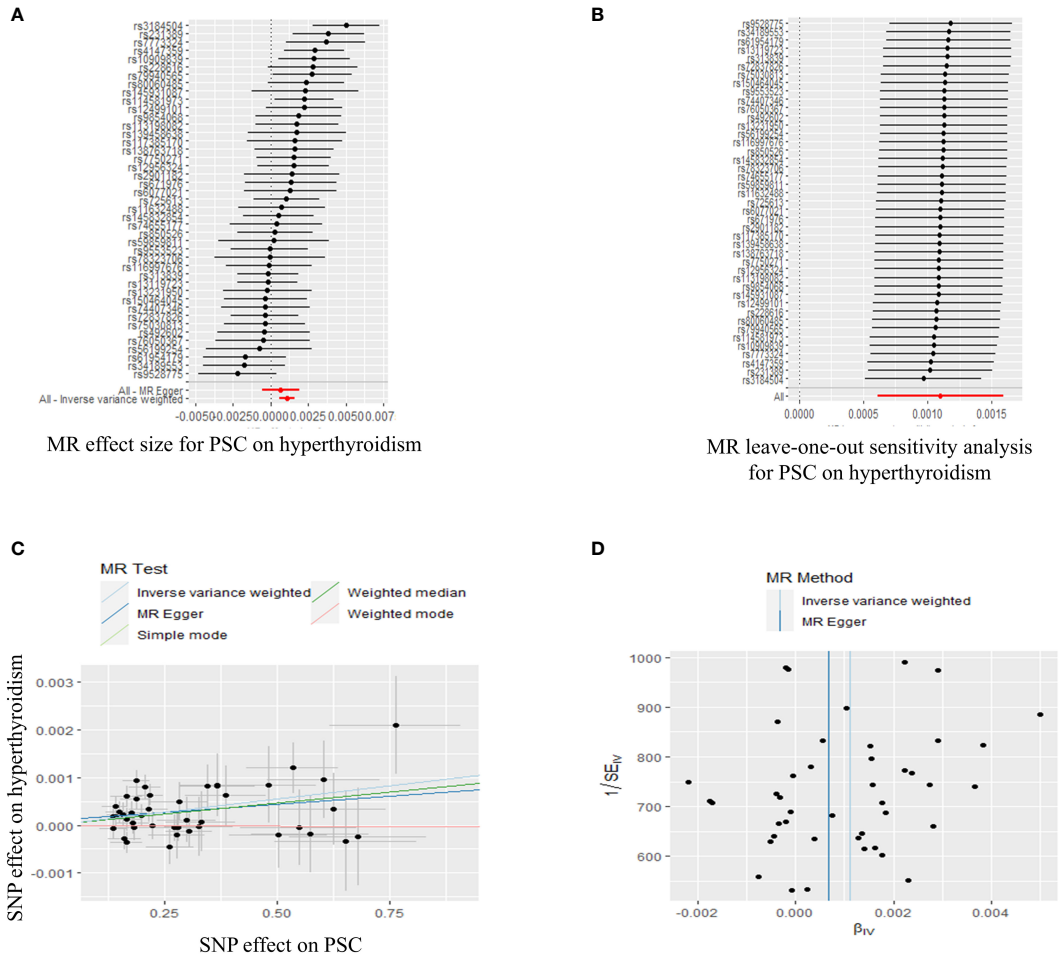

FIGURE 4
Plots of MR estimates of the causal relationship between PSC and hyperthyroidism. **(A)** The forest plot of SNPs associated with PSC and their risk on hyperthyroidism. **(B)** LOO sensitivity analysis of the association of PSC on hyperthyroidism. **(C)** Scatter plot of the association of PSC on hyperthyroidism. **(D)** Funnel plot of the association of PSC on hyperthyroidism. MR, Mendelian randomization; PSC, primary sclerosing cholangitis; SNP, single-nucleotide polymorphism; LOO, leave-one-out.

method used a fixed-effect model between PSC and GD, while they used a multiplicative random effect model to infer causal relationship between PSC and hyperthyroidism. MR analysis showed that there had no causal association between PSC and hypothyroidism, AT, TC, TSH, TRH, TBG, THRα, TP and TG (Supplementary Figures S1, S4-S7, Supplementary Tables S2, S4).

In summary, the findings from the PSC and GD, hyperthyroidism causally linked to MR estimation outcomes, along with the inclusion of all sensitivity analyses as qualitative control measures, collectively indicate a weak bias in the causal association.

The causal effect of TD on PSC via reverse MR

In the context of reverse MR analysis, we conducted a study using TD as the exposure and PSC as the outcome in order to investigate the causal effects of TD on PSC. Supplementary Table S3 presents the selected SNPs utilized as IVs to detect causality. The IVW analysis revealed a significant association between genetically predicted occurrence of two out of the eleven TD-related diseases or biomarkers and PSC, including hyperthyroidism (IVW P=0.000) and hypothyroidism (IVW P=0.028) (Supplementary Figures S2, S3, Supplementary Table S3). The LOO method was used to assess robustness of these results (Supplementary Figures S2B, S3B). MR-Egger regression test was executed to assess horizontal pleiotropy, and the results revealed that the presence of horizontal pleiotropy was unlikely to bias the causality of hyperthyroidism (intercept=0.056, P=0.329) (Supplementary Figure S2C, Supplementary Table S4) and hypothyroidism with PSC (intercept=0.045, P=0.155) (Supplementary Figure S3C, Supplementary Table S4). It is worth noting that there had significant difference between results before and after outlier's correction in hyperthyroidism and hypothyroidism for PSC after distorting outliers using the MR- PRESSO distortion test (P<0.001). Heterogeneity test using IVW revealed that there had heterogeneity in the estimations of the SNPs included. Hence, we used a multiplicative random effect model to test causal association (hyperthyroidism on PSC, Cochran's Q=80.234, P=0.000)

(Supplementary Figure S2D, Supplementary Table S4). There had no obvious heterogeneity in hypothyroidism on PSC. Therefore, we used a fixed effects model to infer causal link between hypothyroidism on PSC (Cochran's Q=21.034, P=0.072) (Supplementary Figure S3D, Supplementary Table S4).

Moreover, the summary data accessed from IVW analysis implied no evidence to measure causality between GD (P=0.945), AT (P=0.757), TC (P=0.396), TSH (P=0.841), TRH (P=0.770), THRα (P=0.901), TP (P=0.111), TG (P=0.469) and PSC (Supplementary Figures S4-S7, Supplementary Tables S3, S4). It is worth mentioning that the number of SNPs for IVs was too small based on our screening criteria, so we did not determine the causal relationship between TBG and PSC.

Forest plot, the results of the LOO sensitivity analysis, scatter plot, and the funnel plot of the link between TD and PSC are shown in Supplementary Figures S4-S7, respectively, where similar results can be observed.

Discussion

As is known to all, this is the first study to use two-sample MR analysis and large-scale GWAS datasets to conclude a causal relationship between PSC and TD. Our study found statistical evidence that genetically predicted PSC had a positive causal effect on GD and hyperthyroidism risk, suggesting that PSC per se may play a causal role in the pathogenesis of TD. However, there was no causal effect between PSC and AT, hypothyroidism, TC, TSH, TRH, TBG, THRα, TP, TG. Moreover, the reverse MR analysis suggested that genetic susceptibility to hyperthyroidism and hypothyroidism might affect the risk of PSC. A series of sensitive analyses supported the findings mentioned above.

In terms of the causal relationship between PSC and GD, our analysis revealed that PSC may have a potential cause-and-effect influence on GD, as indicated by MR analyses. However, the reverse-direction MR analyses did not yield any significant findings. From this review, we can infer that the genome-wide significant risk loci (chromosome: 2q33, candidate risk genes: CD28 and CTLA4, lead SNP: rs7426056) are shared between PSC and GD, excluding the HLA complex on chromosome 6 (9). Consistent with our findings, an engaging case have shown that there was combined pathogenesis rather than a coincidence in a PSC patient with GD (15). As expected, our results are consistent with previous retrospective studies (12). Prevalence of TD in patients with PSC was 11% (95% CI 0.060-0.200). Of these patients, five (6%) had hypothyroidism and four (5%) had hyperthyroidism (12).

The bidirectional two-sample MR design was employed to establish a causal relationship between PSC and both hyperthyroidism and hypothyroidism. Our results are analogous to a case report (37). M Zeniya also showed that hypothyroidism was observed in both autoimmune hepatitis and primary biliary cirrhosis (PSC), at 12% and 5.8%, respectively in Japan. The occurrence of PSC and hyperthyroidism or hypothyroidism might be caused by genetic factors (38, 39). Similarly, PBC, an autoimmune liver disease (AILD) similar to PSC, was also found to be causally related to AITD, TSH, hypothyroidism and TC by MR analysis (40). It is worth noting that the causal effect is weak between PSC and hyperthyroidism, as the OR is close to 1 (IVW OR=1.001), even though it is statistically significant. This is why it is rare in the publications and has only been reported in case reports so far.

Multiple hypotheses have been proposed regarding the causal relationship between hypothyroidism and PSC. One such hypothesis suggests that the shared occurrence of the HLA phenotype is frequently observed in individuals with both PSC and hypothyroidism (chromosome:12q24, candidate risk genes: SH2B3 and ATXN2, lead SNP: rs3184504) and the presence of several autoantibodies suggest a common immunogenetic predisposition. On the other hand, we investigated the presence of missense mutations or loss of immunostaining for 7H6, a tight junction (TJ) protein specific to bile canaliculi in hepatocytes, in patients with PSC. This protein is closely linked to paracellular permeability and bile duct injury. The clinical features of patients with TJ mutations included hypothyroxinemia (41, 42). In fact, there are few studies on PSC and hyperthyroidism/hypothyroidism in view of weak causal association. In conclusion, given the established causal relationship between PSC and hyperthyroidism/hypothyroidism, our findings make a valuable contribution to the ongoing investigation of the pathogenesis of PSC.

In our analysis using MR, we observed a significant and consistent causal relationship between PSC and TD (GD and hypothyroidism) through the use of the IVW method as the primary approach. However, the results obtained from alternative methods such as MR Egger, weighted median, simple mode, and weighted mode showed little significance. Moreover, OR values are very close to 1 with weak causal effect, although the relationship between PSC and hyperthyroidism indicates the statistical significance. This lack of significance may be attributed to the low statistical power of these methods or the potential underestimation of the causal effect due to inflated type I error when all genetic variants are assumed to be valid (43–45). With the exception of IVW and weighted median, all methods indicate no significant association between PSC and hyperthyroidism. Moreover, the use of methods to examine the causal direction of PSC on other TD did not yield statistically significant results (P>0.05).

Our results have the potential to enhance the understanding of the diagnosis and management of patients with PSC and TD. Specifically, our findings provide insights into the occurrence of TD in PSC patients, offer guidance for the treatment of TD in PSC patients, and contribute to the improvement of the quality of life for individuals with PSC. The data from our study demonstrated a causal link between PSC and TD (GD and hyperthyroidism). Consistent monitoring of thyroid-related hormones and prompt diagnosis and treatment of TD can provide valuable insights for the management of PSC and lifestyle interventions. It would be meaningful for clinicians to monitor the diagnostic markers of TD in patients diagnosed with PSC, particularly those who have risk factors associated with TD. Additionally, it is crucial to exercise caution when administering thyroid hormone-related drugs to TD patients in order to safeguard liver function and AILD. Furthermore, future research should focus on establishing the relationship between metabolites and PSC, with the aim of identifying novel and effective biomarkers that can aid in the

exploration of the potential pathogenesis of PSC in TD (46). However, the results need further validation *in vivo*.

Our study is subject to several limitations. First, the use of MR analysis does not allow for the complete elimination of potential unmeasured confounding factors. Consequently, the presence of unknown confounders may compromise the validity of our IVs assumptions, thereby introducing bias into our results. Next, the participants in our study predominantly consisted of individuals from the European population, as evidenced by the GWAS data. Our conclusions in MR analysis may not apply to other populations. Moreover, there may be an overlap in the population included in both the exposure and outcome of our analysis, which could potentially affect the reliability of the results. Therefore, further investigations should be conducted on a more diverse population to ensure the generalizability of our findings. Furthermore, the IVW OR obtained from MR analysis of PSC in causal relation to hyperthyroidism was found to be 1.001. This value, being in close proximity to 1, suggest a weak causal effect. This implies that PSC is less likely to be associated with hyperthyroidism potentially due to smaller populations and lack of uniformity in diagnostic criteria, etc. Finally, it is widely acknowledged that the incidence of PSC may vary based on sex. Therefore, future studies should carefully consider the influence of sex on causality when examining this relationship.

In summary, the results of our study demonstrate that PSC can lead to TD, particularly in the forms of GD and hyperthyroidism. The identification of a potential causal association between PSC and TD offers a new avenue for investigating the origins and progression of PSC.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Since this MR study is based on publicly available GWAS summary statistics, written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

WZ: Writing – review & editing, Conceptualization, Data curation, Formal Analysis, Investigation, Software, Supervision, Validation, Visualization, Writing – original draft. RL: Funding acquisition, Methodology, Resources, Supervision, Validation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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SUPPLEMENTARY FIGURE 1
Plots of MR estimates of the causal relationship between PSC and hypothyroidism. **(A)** The forest plot of SNPs associated with PSC and their risk on hypothyroidism. **(B)** LOO sensitivity analysis of the association of PSC on hypothyroidism. **(C)** Scatter plot of the association of PSC on hypothyroidism. **(D)** Funnel plot of the association of PSC on hypothyroidism. MR, Mendelian randomization; PSC, primary sclerosing cholangitis; SNP, single-nucleotide polymorphism; LOO, leave-one-out.

SUPPLEMENTARY FIGURE 2
Plots of MR estimates of the causal relationship between hyperthyroidism and PSC. **(A)** The forest plot of SNPs associated with hyperthyroidism and their risk on PSC. **(B)** LOO sensitivity analysis of the association of hyperthyroidism on PSC. **(C)** Scatter plot of the association of hyperthyroidism on PSC. **(D)** Funnel plot of the association of hyperthyroidism on PSC. MR, Mendelian randomization; PSC, primary sclerosing cholangitis; SNP, single-nucleotide polymorphism; LOO, leave-one-out.

SUPPLEMENTARY FIGURE 3
Plots of MR estimates of the causal relationship between hypothyroidism and PSC. **(A)** The forest plot of SNPs associated with hypothyroidism and their risk on PSC. **(B)** LOO sensitivity analysis of the association of hypothyroidism on PSC. **(C)** Scatter plot of the association of hypothyroidism on PSC. **(D)** Funnel plot of the association of hypothyroidism on PSC. MR, Mendelian randomization; PSC, primary sclerosing cholangitis; SNP, single-nucleotide polymorphism; LOO, leave-one-out.

SUPPLEMENTARY FIGURE 4
Forest plot of the association of PSC on AT **(A)**, TC **(B)**, TSH **(C)**, TRH **(D)**, TBG **(E)**, THRα **(F)**, TP**(G)**, TG (H). Forest plot of the association of GD **(I)**, AT **(J)**, TC **(K)**, TSH **(L)**, TRH **(M)**, THRα **(N)**, TP **(O)**, TG **(P)** on PSC. PSC, primary sclerosing cholangitis; AT, autoimmune thyroiditis; TC, thyroid cancer; TSH, thyroid stimulating hormone; TRH, thyrotropin-releasing hormone, TBG, thyroxine-binding globulin; THRα, thyroid hormone receptor alpha; TP, thyroid peroxidase; TG, thyroglobulin.

SUPPLEMENTARY FIGURE 5
LOO sensitivity analysis of the association of PSC on AT **(A)**, TC **(B)**, TSH **(C)**, TRH **(D)**, TBG **(E)**, THRα **(F)**, TP**(G)**, TG **(H)**. LOO sensitivity analysis of the association of GD **(I)**, AT **(J)**, TC **(K)**, TSH **(L)**, TRH **(M)**, THRα **(N)**, TP **(O)**, TG **(P)** on PSC. PSC, primary sclerosing cholangitis; AT, autoimmune thyroiditis; TC, thyroid cancer; TSH, thyroid stimulating hormone; TRH, thyrotropin-releasing hormone, TBG, thyroxine-binding globulin; THRα, thyroid hormone receptor alpha; TP, thyroid peroxidase; TG, thyroglobulin.

SUPPLEMENTARY FIGURE 6
Scatter plot of the association of PSC on AT **(A)**, TC **(B)**, TSH **(C)**, TRH **(D)**, TBG **(E)**, THRα **(F)**, TP**(G)**, TG **(H)**. Scatter plot of the association of GD **(I)**, AT **(J)**, TC **(K)**, TSH **(L)**, TRH **(M)**, THRα **(N)**, TP **(O)**, TG **(P)** on PSC. PSC, primary sclerosing cholangitis; AT, autoimmune thyroiditis; TC, thyroid cancer; TSH, thyroid stimulating hormone; TRH, thyrotropin-releasing hormone, TBG, thyroxine-binding globulin; THRα, thyroid hormone receptor alpha; TP, thyroid peroxidase; TG, thyroglobulin.

SUPPLEMENTARY FIGURE 7
Funnel plot of the association of PSC on AT **(A)**, TC **(B)**, TSH **(C)**, TRH **(D)**, TBG **(E)**, THRα **(F)**, TP**(G)**, TG **(H)**. Funnel plot of the association of GD **(I)**, AT **(J)**, TC **(K)**, TSH **(L)**, TRH **(M)**, THRα **(N)**, TP **(O)**, TG **(P)** on PSC. PSC, primary sclerosing cholangitis; AT, autoimmune thyroiditis; TC, thyroid cancer; TSH, thyroid stimulating hormone; TRH, thyrotropin-releasing hormone, TBG, thyroxine-binding globulin; THRα, thyroid hormone receptor alpha; TP, thyroid peroxidase; TG, thyroglobulin.

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Immune-mediated cholangiopathies in children: the need to better understand the pathophysiology for finding the future possible treatment targets

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Cholangiopathies are defined as focal or extensive damage of the bile ducts. According to the pathogenetic mechanism, it may be immune-mediated or due to genetic, infectious, toxic, vascular, and obstructive causes. Their chronic evolution is characterized by inflammation, obstruction of bile flow, cholangiocyte proliferation, and progression toward fibrosis and cirrhosis. Immune-mediated cholangiopathies comprise primary sclerosing cholangitis (PSC), autoimmune cholangitis and IgG4-associated cholangitis in adults and biliary atresia (BA), neonatal sclerosing cholangitis (NSC) in children. The main purpose of this narrative review was to highlight the similarities and differences among immune-mediated cholangiopathies, especially those frequent in children in which cholangiocyte senescence plays a key role (BA, NSC, and PSC). These three entities have many similarities in terms of clinical and histopathological manifestations, and the distinction between them can be hard to achieve. In BA, bile duct destruction occurs due to aggression of the biliary cells due to viral infections or toxins during the intrauterine period or immediately after birth. The consequence is the activation of the immune system leading to severe inflammation and fibrosis of the extrahepatic biliary tract, lumen stenosis, and impairment of the biliary flow. PSC is characterized by inflammation and fibrosis of intra- and extrahepatic bile ducts, leading to secondary biliary cirrhosis. It is a multifactorial disease that occurs because of genetic predisposition [human leukocyte antigen (HLA) and non-HLA haplotypes], autoimmunity (cellular immune response, autoantibodies, association with inflammatory bowel disease), environmental factors (infections or toxic bile), and host factors (intestinal microbiota). NSC seems to be a distinct subgroup of childhood PSC that appears due to the interaction between genetic predisposition (HLA B8 and DR3) and the disruption of the immune system, validated by elevated IgG levels or specific antibodies [antinuclear antibody (ANA), anti-smooth muscle antibody (ASMA)]. Currently, the exact mechanism of immune cholangiopathy is not fully understood, and further data are required to identify individuals at high risk of developing these conditions. A better understanding of the immune mechanisms and

pathophysiology of BA, NSC, and PSC will open new perspectives for future treatments and better methods of preventing severe evolution.

KEYWORDS

immune-mediated cholangiopathies, biliary atresia, sclerosing cholangitis, cellular senescence, autophagy

1 Introduction

Cholangiopathies are focal or extensive damage of the bile ducts due to genetic, infectious, immune, environmental, or unknown causes. All of these disorders have a chronic evolution characterized by inflammation, biliary fibrosis with the obstruction of bile flow, cholangiocyte proliferation, and progression toward fibrosis and cirrhosis (1–3). According to the pathogenetic mechanism, cholangiopathies are divided into genetic disorders, immune-mediated, infectious, toxic, vascular, and obstructive cholangiopathies. In adults, the most common immune-mediated cholangiopathies are primary biliary cholangitis (PBC), primary sclerosing cholangitis (PSC), autoimmune cholangitis (AC), and IgG4-associated cholangitis (IAC), while, in children, is biliary atresia (BA) (Figure 1) (3–12). PBC was named after the first patients presenting with cirrhosis described in 1949 by Dauphinee and Sinclair (13). However, the terminology of the disease changed in 2016 from cirrhosis to cholangitis due to early diagnosis in asymptomatic patients after 2000 (14). PBC is a chronic, autoimmune, progressive liver disorder that is characterized by the presence of antimitochondrial antibodies (AMAs) in serum, cholestasis and specific liver histology (15), while secondary biliary cirrhosis develops after different pathogenic injuries and can result in chronic biliary obstruction (16).

Bile canaliculi surround hepatocytes except for the side next to a sinusoid. The bile canaliculi are composed of hepatocytes' walls. Bile secreted by hepatocytes circulates through canaliculi toward the center of the liver lamina and flows into hepatic ductules. The ductules fuse into gradually larger ducts. Liver segments are divided by its biliary drainage. The right lobe is branched into anterior (segments V and VIII) and posterior sections (segments VI and VII) (17), and each is divided into superior (VIII and VII) and inferior segments (V and VI). The left lobe is branched into medial (segment IV) and lateral (segments II and III) sections. Biliary segmental ducts are named third-order ducts, sectoral bile ducts are second-order ducts, and the main right and left ducts are considered first-order ducts. The hepatic ducts are located near the portal vein and hepatic artery, which form the portal triad (18).

Immune pathogenesis is operative in all types of immune cholangiopathies leading to chronic inflammation of the bile duct and progressing to cirrhosis (10–12, 19–21). BA, PBC, and PSC are secondary to the increased cholangiocyte senescence process, while AC and IAC are associated with the overproduction of antibodies (19, 20). Cellular senescence, autophagy, and apoptosis are distinct cellular responses to stress. Autophagy plays a significant role in preventing cell damage by maintaining a lysosomal turnover of cellular components. It favors the senescence process and delays cell apoptosis, determining the recovery and repair of normal cell function (19–21). Typically, cellular senescence is produced by combining several factors: DNA deterioration, telomere shortening, and oxidative stress. Senescence is a process in which a cell can no longer replicate or proliferate and is irreversibly arrested in the G1 phase. Senescent cells remain metabolically active and adopt an immunogenic phenotype of pro-inflammatory status (20). PSC, BA, and NSC evolve with severe inflammation, bile duct obstruction, and fibrosis, predominantly extrahepatic biliary ducts in BA or including the entire biliary tree in PSC (2, 21). This narrative review aims to analyze the similarities and differences in the pathological mechanisms of PSC, BA, and NSC, the most frequently encountered immune-mediated cholangiopathies in children.

2 Biliary atresia

BA represents the most common cause of cholestasis in infants and the first indication for liver transplantation in children due to its severe and irreversible evolution (21–23). The incidence of BA varies according to geographic region, with 1 to 10–19,000 live births in Europe and North America, while in Asian countries is much higher (1–3,000 live births) (21–24).

2.1 The etiopathogenesis of BA

Currently, it is not known precisely what triggers this severe process of obstruction of bile flow. Two major hypotheses underlie

Abbreviations: PSC, primary sclerosing cholangitis; BA, biliary atresia, NSC, neonatal sclerosing cholangitis; IAC, IgG4-associated cholangitis; PBC, primary biliary cholangitis; AC, autoimmune cholangitis; AMA, antimitochondrial antibody; NK cells, natural killer cells; Th, T helper lymphocytes; MHC, major histocompatibility complex; TCR, T-cell receptor; IL, interleukin; M, macrophage; TGF-β, transforming growth factor-beta; HSCs, hepatic stellate cells; PAMPs, pathogen-associated molecular patterns; IFN-γ, interferon-gamma; TNF-β, tumor necrosis factor-beta; HLA; human leukocyte antigen; CMV, cytomegalovirus; EBV, Epstein–Barr virus; NIMA, non-inherited maternal antigen; ECM, extracellular matrix; ANA, antinuclear antibody; ASMA, anti-smooth muscle antibody; IBD, inflammatory bowel disease; AIH, autoimmune hepatitis.

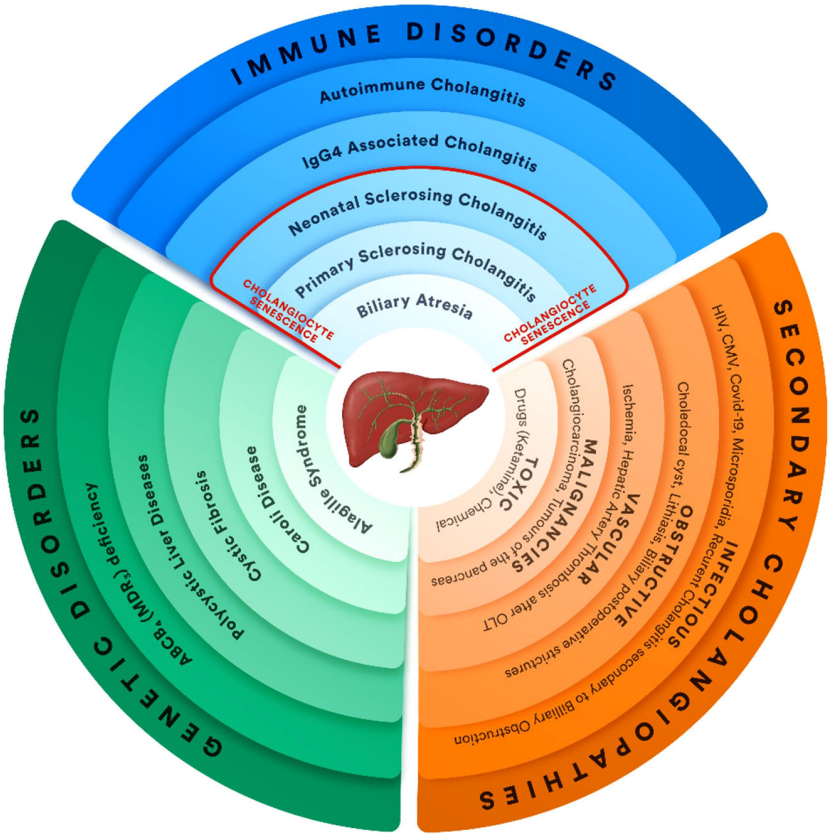

FIGURE 1
Cholangiopathy classification: immune, genetic disorders, secondary cholangiopathies (OLT, orthotopic liver transplantation) (3–12).

the etiology of BA. The first one is that BA appears during organogenesis and the development of the liver, while the second theory supports the hypothesis of an immune disorder (25, 26). Starting from these, BA is classified into embryonal/fetal or syndromic BA (10%–20%) and non-syndromic or acquired/perinatal BA (80%–90%) (24). Embryonal BA is due to defective development of the extrahepatic biliary tract and is often associated with other congenital anomalies such as midline or left-sided liver, asplenia or polysplenia, cardiac malformation (tetralogy of Fallot, dextrocardia, anomalies of the pulmonary or cardiac vessels, atrial and ventricular septal defects), and interrupted inferior vena cava (27, 28). The heterozygous transition of CFC1:c433G>A located in exon 5 was founded in 5 of 10 patients with BA splenic malformation (polysplenia) syndrome, suggesting that genetic predisposition contributes to the disease (29). In those forms of BA, a lack of ductal plate remodeling during bile duct morphogenesis was demonstrated with the persistence of periportal epithelial sleeves (ductal plaque malformation) (30). The existence of cases of BA in the same family and the higher incidence in some regions of the globe (Asian countries) have raised the idea of genetic inheritance (29, 30).

In non-syndromic BA, a combination of factors triggers the inflammatory process (immune system, local and environmental factors). This process appears due to aggression of the biliary cells, mainly due to infections or toxins during the intrauterine period or immediately after birth. The consequence is a severe process of inflammation and fibrosis of the extrahepatic biliary tract, determining lumen stenosis and impairment of the biliary flow (25, 31).

2.2 The immune system in BA

The immune system represents perhaps the most important factor in the etiopathogenesis of BA. The liver is an important immunological organ with many properties, such as innate immunity and hematopoiesis in the fetus, immune tolerance, and poor adaptive immune response versus overreactive autoimmunity (32). These immune functions are provided by T lymphocytes, natural killer (NK) cells, macrophages (Kupffer cells), or dendritic cells (DCs) (33). Involvement of the immune system in BA etiopathogenesis was proven by evidence of bile duct damages and inflammatory cells found near them and in cholangiocytes. Also, at the level of the portal spaces, numerous inflammatory cells were revealed: NK cells (CD8), T helper cells (CD4), and histiocytes (CD68). CD4 has more functional subsets, including helper T (Th1, Th2, Th17, and follicular helper), which promotes innate and adaptive immune responses, and the Regulatory T cell (Treg), which suppresses the inflammation resulting from innate and adaptive immunity (34). CD4 marker is typically found on the

surface of immune cells such as T helper cells, monocytes, macrophages, and DCs and serves as a co-receptor for the T-cell receptor (TCR). The complex TCR-CD4 binds to distinct regions of the antigen-presenting major histocompatibility complex (MHC) class II molecule. The major role of CD4 is to send signals to other types of immune cells, including cluster of differentiation 8 (CD8) (35). CD8 is a transmembrane glycoprotein co-receptor for the TCR (35). CD8 includes NK cells, the leading cell killer in adaptive immunity, and CD8 Treg cells, which inhibit the activity of Th cells' immune responses to infection (36, 37) (Table 1).

2.2.1 The innate immune system in BA

The innate immune represents the first line of defense against different infections (nonself) or tissue injury (damaged self) (68). In the liver and biliary ducts, this system is represented by the epithelial cells that prevent physical and chemical aggressions. After viral infections, cholangiocyte inflammation represents the immune system's first response. The process of acute inflammation is initiated by specialized cells (macrophages, DCs, histiocytes, Kupffer cells) that will release inflammatory mediators (cytokines and chemokines) responsible for the clinical signs of inflammation and tissue damage (69). Neutrophils have a major role in the initial inflammatory response to tissue injury. They accumulate near intrahepatic bile ducts in the early phases of viral hepatic injury and produce reactive oxygen species and leukotriene, amplifying cellular destruction and inflammation. The initial immune phase is followed by a second adaptive immune phase mediated by lymphocytes, a process controlled by interleukin 12 (IL-12) (69, 70). Macrophages also play an important role in the pathogenesis of BA. These special cells involved in the detection, phagocytosis, and destruction of different antigens are divided into pro-inflammatory macrophages (M1) and restorative macrophages (M2), with a significant role in tissue regeneration (34). In BA, the balance tilts especially toward pro-inflammatory M1, which, once activated, will produce new chemokines. They will recruit other inflammatory cells producing mediators [IL-1β, IL-6, IL-8, tumor necrosis factor (TNF)-α], thus creating a vicious circle responsible for perpetuating inflammation and liver fibrosis (70, 71). IL-6 and IL-8 (CXCL8) are increased in the serum of patients with BA, indicating ongoing inflammation (71). Macrophages and cholangiocytes mainly produce IL-8. Its role is to recruit inflammatory cells (neutrophils, basophils, T cells), mediate innate immune activation, regulate granulocyte recruitment along the vascular wall and degranulation, and activate them. IL-8 mediates liver injury in BA by promoting bile duct reaction and liver fibrogenesis and induces alpha-smooth muscle actin (α-SMA), a marker responsible for collagen synthesis (72). IL-6 is involved in acute and chronic

TABLE 1 Pathogenesis and treatment of the most frequent cholangiopathies in children–BA, PSC, NSC.

Characteristics	Biliary atresia	Neonatal sclerosing cholangitis	Primary sclerosing cholangitis
Genetic predisposition	- heterozygous transition of CFC1:c433G>A (29)	- autosomal recessive disorder (38) - CLDN1 variant (38) - KMT2D or KDM6A gene variants (39) - HLA B8 and DR3 (40)	- HLA class II (HLA-DR, -DQ, or -DP) (41, 42) - NKG2D gene polymorphisms (43)
Viruses, bacteria, fungi	- Cytomegalovirus (44, 45), *Rotavirus*, herpesvirus, adenovirus, reovirus, Epstein–Barr virus (46–49)	- Cytomegalovirus (39)	- intestinal microbiota (50, 51)
Toxic	- biliatresone (52) - plants from *Dysphania* (25, 52)	- bile acids (53)	- bile acids (53)
Anatomy and local factors	- fetal anatomy (54)	–	- enterohepatic circulation (50)
Innate and adaptive immunity	- cholangiocytes (55), macrophages (35), dendritic cells, histiocytes, Kupffer cells (33)	- cholangiocytes (56)	- cholangiocytes, endothelial cells macrophages, dendritic cells and natural killer cells (41, 57, 58)
Cellular immunity (mediated by T lymphocytes)	- natural killer cells (CD8+T) (59) - helper T cells (CD4+) (35)	- helper T cells (CD4+) (40)	- natural killer cells (CD8+T) (57) - helper T cells (CD4+) (60)
Humoral immunity (antibody-mediated immunity)	- local antibodies against the basal membrane of the biliary epithelium (34, 61) - serum autoantibodies against α-enolase (34, 62)	- antinuclear antibodies (43) -anti-smooth muscle antibody (43)	- Anti-neutrophil cytoplasmic antibodies (63) - antimitochondrial antibodies (41, 63) - anti-thyroperoxidase antibodies (41, 63)
Maternal microchimerism	Yes	No	No
Current therapy	- Kasai intervention (64) - postoperative steroid administration - ursodeoxycholic acid administration - liver transplantation (65)	- liver transplantation (66)	- ursodeoxycholic acid administration - liver transplantation (67)

inflammation and is important in the pathophysiology of graft-versus-host disease (GVHD) and BA (35). The macrophages also release transforming growth factor-beta (TGF-β), which will stimulate hepatic stellate cells (HSCs) to synthesize collagen, thus determining fibrosis and progression to cirrhosis (73). Innate immunity expresses Toll-like receptors (TLRs), a class of proteins responsible for inflammatory response, which recognizes pathogen-associated molecular patterns (PAMPs) and realizes an essential connection between innate and adaptive immunity (73, 74).

2.2.2 The adaptive immune system in BA

Adaptive immunity (acquired immunity) supposes an immune response triggered by repeated exposure to an antigen with two main mechanisms: humoral immunity (antibody-mediated immunity) and cellular immunity (mediated by T cells) (75). Adaptive immunity is the body's response against non-self-processed peptide antigens or self-antigens when this antigen is presented *via* MHC class I and II molecules (76). Normally, antigens are expressed on the cell surface of the antigen-presenting cell after a viral infection. Helper T cells (CD4) activated through the MHC–antigen complex produce cytokines that promote immune defense against virally infected cells. Most Th0 become Th1, which promotes cell-mediated inflammatory responses by inducing the activation of antigen-presenting cells (CD4), cytotoxic cells (CD8), macrophages, or NK cells (77–79). Some T cells will differentiate into cytotoxic T cells (CD8) that express TCRs involved in recognizing specific antigens. This process occurs in the thymus during the development of immature T cells (80). Once activated, CD8 cells will perpetuate inflammation by producing cytokines (IL-2), chemokines [Macrophage inflammatory protein (MIP)-1α, MIP-1β, Regulated upon Activation, Normal T cell Expressed, and Secreted (RANTES), interferon-gamma (IFN-γ), and TNF-α] and by stimulating the macrophage activation that determines infected cells' destruction (81, 82). The virus also leads to portal tract inflammation mediated by Th1-cell and periductular inflammation secondary to accumulation of the immune cells with progressive obliteration of the bile ducts and fibrosis (83, 84). With the progression of the inflammatory process, macrophages and DCs will determine activation and proliferation of other Th0 cells, which will turn into Th1 cells after encountering IL-12, IL-18, and interferon type 1 (IFN-α, β, or γ) or Th2 cells after encountering IL-4 (84). Th1 cells produce IL-2, IL-12p70, IL-12p40, IFN-γ, and tumor necrosis factor-beta (TNF-β), while Th2 cells produce IL-4, IL-5, IL-6, and IL-10 (81). Therefore, one of the most important roles of these inflammatory processes in the bile ducts returns to Th1 cells and CD8 T cells, found in over 90% of children with BA (77, 81, 84). Due to this reason, BA has many similarities with GVHD seen after allogeneic hematopoietic-like lymphocytic infiltrate in portal space (Kupffer cells, CD4 T cells, and CD8 T cells), with predominance of CD24+ T helper (Th1) cells and a high number of cell adhesion molecules or human leukocyte antigen (HLA class II) markers (26). All of these processes were described in BA and are not encountered in other cholangiopathies (82). Many other pro-inflammatory cytokines were significantly higher in BA, such as TNF-α, IFN-γ, IL-12, IL-23, IL-32, and IL-33. These will perpetuate the inflammatory cascade and extend bile duct injuries (80). IL-12p40 is a subunit of IL-12 and IL-23, and its role is to promote the migration of macrophages or DCs and is a well-known inducer of the Th1 response (77, 83, 84). The level of IL-12p40 is significantly increased in BA and can predict the success of the Kasai intervention: serum level was higher in children with successful Kasai compared to those in which the intervention failed (64, 78, 79). IL-32 stimulates the synthesis of other pro-inflammatory cytokines, thus perpetuating periductal inflammation (61). In parallel with the high levels of these pro- or anti-inflammatory cytokines, lower levels of growth factors have been found in patients with BA, which reflect inflammation (64). Serum levels of those cytokines may serve as noninvasive biomarkers for the disease progression after surgery and could help to identify patients at high risk for poor outcomes. Regarding humoral immunity in BA, quite a few are known. The presence of antibodies (IgM, IgG) was detected in the basal membrane of the biliary epithelium. Also, the serum of patients with BA seems to contain autoantibodies against α-enolase, a cytoplasmic glycolytic enzyme expressed in various cells, including biliary epithelial cells and hepatocytes (61, 62).

2.3 The microchimerism in BA

During the last few years, more studies described the role of maternal microchimerism in BA etiopathogenesis. Microchimerism supposes maternal transplacental bidirectional cell trafficking between the mother and the fetus. This process normally occurs in approximately 40% of pregnancies and continues after birth (62). During normal pregnancy, three types of microchimerism occur: fetal microchimerism (transfer of fetal cells from the fetal circulation into the maternal circulation), maternal microchimerism (transfer of maternal cells into the fetal circulation during pregnancy or parturition), or microchimerism in twins (exchange of cells between the fetuses in the uterus) (55, 85). Maternal microchimerism is supposed to trigger biliary inflammation (27, 28), as maternal alloantigens induce the development of fetal T cells with pro-inflammatory potential and protective immune responses. Fetal T cells with pro-inflammatory potential are born in a tolerogenic environment and form the fetus's immune system (55, 86). In BA, maternal cells passed to the fetus in the liver become semiallogeneic and persist after birth for a variable period causing insults. The antigen-presenting cells located in the bile duct epithelium, or the vascular endothelium of the portal space, will become the target of maternal effector lymphocytes, thus triggering hepatic and biliary tissue destruction (27). This process was described by Suskind et al. (26), who compared liver tissue derived from children with BA to those with neonatal hepatitis, detecting a high number of maternal cells in the liver of children with BA. Also, Leveque proposed that ductal injuries start in the fetal period with the transfer of maternal chimeric cells and their adherence to bile duct epithelial cells or endothelial cells in the fetus's liver, causing acute inflammation of bile ducts (87). As a response to maternal antigen, native T fetal cells will differentiate

predominantly in Th1, which accumulates in the liver and lymphoid organs and determine chronic inflammation by suppressing Th17 cells, with an important role in maintaining mucosal barriers. The loss of Th17 cell populations at mucosal surfaces determines chronic inflammation and is associated with multiple inflammatory and immune disorders, including BA (88). In neonates, there is a deficit in circulating Tregs in peripheral blood and a dysfunction of them (34). Also, the reduction of lithocholic acid in the gut of patients with BA will decrease Th17 and Th1 (88). As Th1 decreases, Th2 will proliferate, releasing cytokines (IL-4, IL-5, IL-9, IL-13) that stimulate collagen synthesis and fibrosis and are responsible for intrahepatic and extrahepatic bile duct proliferation (44, 88). In 2014, Toshihiro Muraji (27) also demonstrated the presence of a higher number of chimeric maternal cells in the portal area and sinusoids. He elaborated on a few hypotheses explaining the role of chimeric cells in triggering BA (27, 44, 88). The first refers to a primary insult triggered by the migration of chimeric cells in the first semester of pregnancy, along with intrahepatic and extrahepatic duct development (27, 44, 88). Chimeric cells will attach to the portal biliary and endothelial cells from different liver segments. The fetus will develop tolerance against them over time by the production of TGF-β at the level of the lymph nodes. The TGF-β will stimulate Treg fetal synthesis, a subpopulation of T cells, working in time as memory T cells for maternal antigens and causing immunotolerance later in life. Treg cells suppress cytokine production of B, CD4, CD8, and DCs and protect tissues against autoimmunities (27, 45). Various aggressions like cytomegalovirus (CMV) infection can reduce the number and activity of Treg cells determining the loss of immune tolerance and triggering the inflammatory process. Moreover, repeated exposure of the mother's immune system to these antigens will trigger the immune attack and distort bile duct architecture (44, 45). Exposure to non-inherited maternal antigens (NIMAs) in fetal life and the development of tolerance to the maternal cells (NIMA effect) can be another trigger for bile duct destruction in BA. The last theory is about the injury of the fetal liver determined by maternal lymphocytes during the Kasai intervention (27, 44, 45). Along with this intervention, chimeric cells in the liver become semiallogenes causing GVHD interactions and new injuries of hepatic structures. Complement C4d deposits are present at the level of the endothelial cells in the portal space like renal complexes encountered in renal peritubular capillaries of patients with kidney transplants. This is an important indicator for acute antibody-mediated rejection (AMR) (89, 90). These findings suggest that complement activation led to progressive portal vein damage and stimulated BA fibrogenesis (89, 90).

2.4 Environmental factors in BA

Studies in animal models described the important role of environmental factors in BA etiopathogenesis. Viral infections with CMV, *Rotavirus*, reovirus, herpesvirus, adenovirus, and Epstein–Barr virus (EBV) are the leading cause of BA. In 1974, Benjamin Landing elaborated the first theory according to which BA and a few other infantile obstructive cholangiopathies were caused by a viral infection of the liver or biliary ducts (46–49). In non-syndromic BA, the disease often occurs sporadic, with genetic factors having a minor influence, but there is an involvement of the environmental factors and the immune system. Perinatal BA occurs after a viral infection with biliary tropism, which causes the activation of the immune system and determines inflammation and damage to the bile duct, leading to cholangitis associated with ductal and periductal fibrosis (24, 32). The virus must infect neonates, cause viremia, replicate in cholangiocytes, and determine the inflammatory immune response from the host (49). CMV infection is considered an important cause of BA, with a prevalence of 25% (91). Both intrauterine and perinatally CMV infections have been proven to be etiopathogenetic factors in BA (91). CMV injury of cholangiocytes leads to altered immune response, chronic inflammation, and fibrosis of the biliary ducts (92). CMV infection determines the increase of T helper cells (Th1 and Th17) and decrease of regulatory T cells (Th2 and Tregs), causing an exaggerated autoimmune response and bile duct obstruction. CMV also causes the increase of cytokines (IFN-γ, Th1 cytokines, and TNF-α) with inflammation and tissue damage (93). In patients with BA and CMV infection, liver fibrosis is more severe, episodes of cholangitis are more frequent, and progression to cirrhosis is more accelerated compared with patients with BA but without CMV. Moreover, CMV infection seems to be an important prognostic factor in the postoperative evolution of children with BA and Kasai procedure, the evidence of infection predicting an unfavorable prognosis (94). Other authors support that BA is only a genetic abnormality and CMV would not have any role in BA (91).

Reovirus is a member of the family Reoviridae, which determines cholangiocyte infection, followed by necrosis and inflammation in newborn mice used for experimental studies. Reovirus infection also reduces the number of Tregs in the liver, making the bile ducts susceptible to the virus and the immune system (91, 95–97). Reovirus antibodies IgM and IgG have been detected in 55% of cases with BA, but a direct causal relationship has not yet been proven (47, 96).

Another important virus in BA etiopathogenesis is *Rotavirus*, another genus in the family Reoviridae (98). Recent findings on mice indicate that *Rotavirus* induces BA only if the infectious dose is high enough for the virus to escape into the circulation, infecting many cholangiocytes and triggering the immune response (96). An important finding is related to the time of infection. Studies on pregnant female mice infected with *Rotavirus* proved that the infection did not cause BA in the pups, although the viremia was very high. Instead, the postnatal infection can determine Treg cell paucity, disturbing the balance between immune activation and immune tolerance and making the bile ducts susceptible to infection. Another theory postulates that *Rotavirus* infection in the first days of life determines BA due to an immature neonatal murine immune system (47).

Exogenous toxins are possible environmental factors implicated in BA; their role being proven only in animals. Environmental toxins, such as plants from *Dysphania* species, cause biliary damage in Australian newborn lambs or mice. Thus, in 2007, Park et al. noticed that more lambs developed BA after they were fed with *Dysphania* plants found in some arid areas. Also, Michael Pack and Rebecca Wells's team isolated another plant toxin (isoflavone or

biliatresone) that causes extrahepatic bile duct destruction in mammalian cells, supposing the hypothesis that the same process could happen in humans (25, 52).

2.5 The local factors in BA

The local factors, cholangiocytes, HSC, or fetal anatomy, are important in BA etiopathogenesis. The main functions of cholangiocytes consist of forming and secreting the primary bile into canaliculi, transport of bile, various ions, solutes, and water across the biliary tree (54, 99, 100). Besides these, cholangiocytes represent the first line of defense of the biliary tract. After viral or bacterial infection, cholangiocytes express a variety of surface innate immune receptors (TLRs), which activate intracellular signaling cascades stimulating the expression of adhesion molecules and the release of cytokines, chemokines, IFN, or other inflammatory mediators (99). IL-8 and IL-15 are the most produced ILs, essential in the chemotaxis and activation of NK cells (59). Cholangiocytes express many cytokines, chemokines, MHC class I and II, CD 44 or TLR-4, and TLR-9 (44, 101). TLRs are transmembrane proteins, expressed on the cell surface, that recognize PAMPs expressed on infectious agents and stimulate the production of cytokines necessary for innate and adaptive immunity (44, 101). TLR recognizes bacterial DNA and distinguishes it from self-DNA (44, 101). Furthermore, in infected cholangiocytes, the release of type 1 IFN-induced apoptosis processes determined cell death and the release of late mediators (34). Hepatic stellate cells (HSCs) determine the progression of liver fibrosis and play an important role in BA evolution (102). HSCs act as antigen-presenting cells and promote NK cell proliferation (102). Once activated, HSCs, together with bone marrow-derived cells, portal fibroblasts, and hepatocytes, become myofibroblasts, which in turn produce extracellular matrix (ECM) composed of glycosaminoglycans and proteoglycans, structural proteins (collagen and elastose), and adhesion proteins, which facilitate binding of the cells to the matrix. In a healthy liver ECM, these components are well balanced. But in BA, excessive liver healing leads to disproportionate deposition of ECM and promotes liver fibrosis (103). The non-syndromic isolated type of BA may result from fetal anatomy (104). There is a defective remodeling of the transition zone between the extrahepatic and intrahepatic bile ducts, called the porta hepatis. Fetal anatomy of the liver could explain the segmental distribution of inflammation and fibrosis of bile ducts. BA starts before the 15th week of gestation when the umbilical vein drains blood flow from the placenta, predominantly in the left branch of the portal vein. That is why, in some cases, the inflammation and fibrosis were more severe on the left lateral segment of the liver (22, 27).

3 Neonatal sclerosing cholangitis

Neonatal sclerosing cholangitis (NSC) is a rare autosomal recessive disorder characterized by inflammation and obliterative fibrosis of intrahepatic and often extrahepatic bile ducts, with dilation of preserved segments. Characteristically, the extrahepatic biliary tree is not atretic (38). NSC and BA have many similarities in clinical and histopathological manifestations, and the distinction between the two can be hard to achieve (38). Both have similar features in the first days of life, the particularities becoming more evident during the disease (38). The image scan for the gallbladder is normal in NSC, and histopathology reveals cholangitis at the level of intrahepatic and extrahepatic ducts and sometimes on the pancreatic duct (38, 43, 105). But, similar to BA, the mechanism of tissue damage remains unknown (43).

3.1 The etiopathogenesis of NSC

A characteristic of NSC is represented by autoimmune features like antinuclear antibody (ANA) or anti-smooth muscle antibody (ASMA) seropositivity. NSC can be associated with immunodeficiency, inflammatory bowel disease, Langerhans cell histiocytosis, psoriasis, cystic fibrosis, and sickle cell anemia. Also, NSC can be part of two complex syndromes: neonatal ichthyosis-sclerosing cholangitis (NISCH) syndrome and Kabuki syndrome (38). NISCH syndrome is a rare genetic disorder secondary to the CLDN1 variant that determines claudin-1 deficiency and affects hepatic tight junctions (TJs) (38). TJ proteins are localized on the surface of hepatocytes, cholangiocytes, and epidermis and are important for bile secretion, creating a barrier between the blood and bile flow, but also for skin integrity (56). In NISCH syndrome, hepatic variants of TJ molecules increased paracellular permeability for bile acids leading to inflammation and fibrosis of the intra- and extrahepatic bile ducts and biliary cirrhosis (53). Kabuki syndrome is a genetic disorder secondary to KMT2D or KDM6A gene variants (39) characterized by facial dysmorphism, developmental delay, growth hormone deficiency, skeletal anomalies, hypotonia, and congenital heart defect (38).

3.2 The immune system in NSC

Even though the name of NSC and PSC is almost similar, the pathogenetic mechanism is different. NSC seems to be a distinct subgroup of childhood PSC, especially the forms that associate specific autoimmune phenomena like elevated IgG levels and a high titer of ANA and ASMA (43). NSC is associated with increased incidence of HLA B8 and DR3, molecules that present antigens to CD4 T helper lymphocytes, initiating the immune response, which denotes the importance of genetic and immunologic factors in the disease etiopathogenesis (40).

4 Primary sclerosing cholangitis

PSC is a chronic liver disease characterized by inflammation and fibrosis of intra- and extrahepatic bile ducts, leading to secondary bile cirrhosis. The etiopathogenesis of PSC is not yet fully known, but like BA or NSC, genetic predisposition, infections, and host immunity seem to have significant roles. In addition, an important role in PSC belongs to the intestinal microbiota, with a high association with

inflammatory bowel disease (IBD) (40). Up to 75% of people with PSC also have IBD, especially ulcerative colitis (UC) (106). Unlike BA, PSC is relatively rare in children, with an incidence lower than 20%. The disease predominantly affects men aged 30–40 and has a particular geographic distribution (more frequent in Northern Europe than Southern Europe and Asia) (40, 106).

4.1 The etiopathogenesis of PSC

In adults, sclerosing cholangitis is divided into PSC with unknown etiology and secondary sclerosing cholangitis (SSC) with a direct causative agent (41, 107). PSC is a multifactorial disease that occurs because of a cycle of immune-mediated cholangiocyte injury leading to fibrosis. Genetic predisposition (associations with HLA and non-HLA haplotypes involved in bile homeostasis and associated with inflammatory regulatory pathways), autoimmunity (involvement of cellular immune response, the presence of various autoantibodies, association of IBD), and environmental factors (infections, selenium or vitamin D deficiency, toxic bile) are the main mechanisms that trigger and maintain inflammation in PSC (41, 107). SSC arises because of the action of a well-known factor that triggers a chronic inflammatory process of bile ducts: infections (CMV, EBV, bacteria, or cryptosporidiosis), drugs (floxuridine), lithiasis, congenital disorders (choledochal cyst, cystic fibrosis), surgical trauma of the bile ducts, ischemia (hepatic artery occlusion after liver transplantation), or malignancies (41, 107).

Unlike BA, genetic predisposition plays a significant role in PSC onset (107). Evidence for genetic susceptibility in PSC is obvious, supported by the different prevalence in certain regions such as Northern and Southern Europe, by a 100 times higher incidence risk for first-degree relatives of PSC patients compared to the general population, of the strong connection with IBD and the presence of autoantibodies (108). PSC occurs because of genetic polymorphisms that determine susceptibility to the disease. The MHC is an important genetic susceptibility locus for the development of PBC. Several HLA molecules are central in the disease coordinating immune responses through the T-cell response (107). Commonly, hepatocytes and cholangiocytes express only HLA class I molecules. Still, in PSC, some HLA class II are aberrantly expressed: HLA-DR on the bile duct epithelium and vascular endothelium, HLA-DP on the bile duct epithelium, but not on the vascular endothelium and HLA-DQ on the bile ducts (109). A more critical role of genetics is linked to cholangiocarcinoma progression: NKG2D gene polymorphisms detected in patients with PSC were associated with a higher risk of cholangiocarcinoma and could identify patients at risk (110). Also, genetic variants of the steroid and xenobiotic receptor (SXR), which protects against bile acid-induced liver injury in mice, are associated with more severe forms of PSC (111).

4.2 The immune system in PSC

It is well known that the immune system plays a central role in the etiopathogenesis of PSC, considered an autoimmune disease, even if it does not respect the classic picture of autoimmunity. Thus, it is twice as frequent in men and does not respond to immunosuppressive therapy. However, it presents many features like autoimmunity, such as specific autoantibodies, HLA haplotypes, the association of IBD (70%) or AIH (50%–96%), and the histopathological aspect that describes the presence of a lymphocytic infiltrate in portal areas (42, 112). Mixed inflammatory cells (lymphocytes, plasma cells, and neutrophils) accumulate around the bile ducts. But as the disease progresses, the inflammatory infiltrate is reduced simultaneously with the progressive reduction of bile ducts and the appearance of periductal fibrosis. These changes give the "onion skin" appearance of medium-sized or larger bile ducts, a morphological feature considered characteristic of the disease (113–115).

4.3 The innate immune system in PSC

The exposure to toxic bile and the dysregulation of the intestinal microbiota found in PSC patients suggest that innate immunity plays a significant role in PSC etiopathogenesis. The cellular immune response is a primary event in the pathogenesis of PSC, one of the specific features being the abundance of T lymphocytes in the portal spaces, especially CD4, because of the action of different antigens (60). Bacteria, or bacterial constituents (lipopolysaccharide, lipoteichoic acid, peptidoglycans), cross the inflamed intestinal wall and reach the portal circulation (42, 113, 114). Once entered in the portal circulation, these antigens will attract inflammatory cells, such as macrophages, DCs, and NK cells, which will be activated through pattern recognition receptors. All of these activated cells will secrete pro-inflammatory and chemotactic cytokines to maintain the inflammation (57, 58, 116, 117). Also, they will determine MAdCAM-1 expression on portal endothelial cells that plays a central role in recruiting intestinal mucosal lymphocytes to the liver during intestinal inflammation (58). This time, the peripheral blood T-cell level is normal (60). Other authors described a predominance of CD8 T cells or an equal CD4/CD8 ratio. The explanation is related to the areas of the liver where inflammation predominates: CD4 in the portal spaces and CD8 in the lobular areas (57). Cholangiocytes and endothelial cells are important in PSC, as they create a vicious circle leading to inflammation and fibrosis progression (57, 58, 116). Cholangiocytes are the main targets of immune attacks in PSC. After an infection or injury of the biliary ducts, they can secrete pro-inflammatory and pro-fibrotic cytokines and chemokines (IL-1β, IL-6, TNF-α, and IFN-γ) (63, 117–119). Itself, cholangiocytes will be exposed to the action of inflammatory mediators produced by infiltrating inflammatory cells that migrate to the site of the bile duct injury, thus creating a vicious circle that will lead to ductular cholestasis and chronic inflammation (63, 117–119). Bo and his team reported an increased level of TNF-α and IL-1β, both pro-inflammatory cytokines required for activating the innate immune response, mediating the recruitment, activation, and adherence of circulating phagocytic cells (macrophages and neutrophils) and terminating the innate immune response (116, 117). They also

described a decreased level of Th-2 mediators (IL-2) in the serum of a patient with PSC (63, 117, 118). Activated cholangiocytes will determine chemotaxis of other immune, mesenchymal, and endothelial cells, all implicated in tissue injury, persistent inflammation, apoptosis, angiogenesis, tissue remodeling processes, and fibrosis (63, 116–120). According to some authors, cholangiocyte activation can also induce the upregulation of HLA molecules and stimulate T and B cells, but more studies are necessary to confirm this hypothesis (121–123). Similar to BA, some T cells will differentiate into CD8 cells that express TCRs with a role in antigen recognition. In PSC, TLRs are found in a large number, in both the biliary and intestinal mucosa. They could be used for predicting the prognosis of the disease due to the correlation with Ludwig fibrosis scores in PSC patients (123). Another similarity with BA is related to cholangiocyte senescence. Normally, biliary epithelial cells have a limited replication capacity in the adult liver. After bile duct injuries, their replication rate increases considerably, thus preventing ductopenia. This property is also valid in liver damage when cholangiocytes become facultative liver stem cells and favor hepatic tissue regeneration (124). In the end stages of PSC, these cells have an increased secretion capacity of numerous cytokines, chemokines, growth factors (IL-6, IL-8, monocyte chemoattractant protein-1, plasminogen activator inhibitor-1, growth factors, and ECM), all this maintaining the inflammation and favoring the progression of fibrosis. Aberrant proliferation and senescence of reactive cholangiocytes in response to a chronic injury underlie new hypotheses regarding the pathogenesis of PSC (125). They also have a high potential for malignant transformation, contributing to the development of cholangiocarcinoma (125). In PSC, blood vascular endothelial cells (BECs) are important in the immune reaction. BECs control the immune response by regulating blood flow and immune cell recruitment by synthesizing cytokines, enzymes, and HLA molecules. Pro-inflammatory cytokines released by BEC stimulate cholangiocytes to secrete chemokines, cytokines, and growth factors that will cause inflammation and fibrosis (107, 113). Like cholangiocytes, BEC does not express HLA class II molecules under normal conditions, but in PSC, these molecules are excessively expressed, especially HLA-DR (109, 115). Some authors support the hypothesis that BECs are antigen-presenting cells, with antibodies attaching to them and stimulating IL-6 synthesis and CD44 expression, a transmembrane glycoprotein involved in cellular aggregation and migration, lymphocyte activation, lymphopoiesis, angiogenesis, and release of cytokines (120). HSCs and portal fibroblasts (PFs) play a major role in hepatic fibrosis by promoting collagen synthesis that begins in the peri-ductular region extending over time throughout the parenchyma (126).

4.4 The adaptive immune system in PSC

In PSC, adaptive immunity involves specialized immune cells and antibodies that attack and destroy cholangiocytes. It is a complex process, also found in BA but less in NSC, consisting of responses to specific antigens or self-antigens presented by MHC molecules to antigen-presenting cells to T cells with specialized receptors (TCRs). In PSC, these T cells, especially CD4 cells, infiltrate the portal spaces, while CD8 cells are found in interface hepatitis (110, 129). This finding coincides with a reduced level of peripheral blood CD4+ T cells or defective apoptosis of activated T cells, which may be part of the immune dysregulation observed in patients with PSC (127–129). After the initial response to an antigen, some T cells persist in the body and become long-living memory T that does not require antigen stimulation to proliferate, so they do not need a signal *via* MHC (127–129). Similarities between bacterial antigens from the bowel and self-peptides can trigger the activation of specific T or B cells that can cross-react with self-epitopes responsible for initiating the PSC (130). Activated T cells and macrophages will trigger cholangiocytes' apoptosis and senescence. The result is immune-mediated damage of the bile ducts, fibrosis, and an increased risk for cholangiocarcinoma (130). Some of the intestinal T lymphocytes will persist as memory cells. They will cross in the portal circulation and, at some point, in the presence of various antigens, can trigger inflammation of the liver and bile ducts. Memory B cells are plasma cells that can produce antibodies for a long time (131). The role of B cells in PSC pathogenesis is not entirely understood. Still, the presence of so many antibodies in the serum of patients with PSC suggests that these cells certainly have a role in the etiopathogenesis of the disease (130). Also, B cells can be detected in the inflammatory infiltrate surrounding bile ducts in PBC, leading to the destruction of intrahepatic bile ducts (132).

The humoral immune response is often encountered in PSC, but in many situations, the meaning of each antibody is not known precisely. Anti-neutrophil cytoplasmic antibodies (ANCAs) are present in the serum of 80% of patients with PSC (117). Other antibodies that can be found in PSC are antimitochondrial (<10%) and anti-thyroperoxidase (7%–16%), and less often, ANA, ASMA, anti-endothelial cell antibody (AECA), or anti-cardiolipin antibody (41, 116). A particular category is represented by antibodies against the pancreatic zymogen granule glycoprotein 2 (GP2) identified in both disorders, PSC and IBD (63). According to some authors, their presence in PSC confirms the involvement of the gut–liver axis in the etiopathogenesis because glycoprotein is commonly expressed in human enterocytes and is not found in hepatobiliary tissue (118).

4.5 Environmental factors in PSC

Regarding environmental factors, in PSC, viral infections are not as important a risk factor as in BA. Several viruses have been analyzed, including EBV, CMV, mumps, measles, coxsackie 1-6, and hepatitis B and C viruses, but no association with the disease was detected (133–136). The involvement of toxins in PSC etiopathogenesis was also described. Bile acids (BA) are incriminated in the pathogenesis and progression of chronic fibrosing cholangiopathies, and more importantly, they accelerate carcinogenesis, increasing the risk of cholangiocarcinoma (50, 129, 136–138). Considering that 10%–20% of patients with PSC will develop cholangiocarcinoma, this may be the basis of new therapeutic methods to prevent it (51). Other theories refer to a defect in the hepatobiliary transport system or to arteriosclerosis of

the bile duct found especially in those at risk of vascular disease. Altered biliary lipid oxidation and secretion or exposure of endothelial cells to toxic luminal lipid content initiate and favor both the atherosclerosis process and the inflammation in PSC (139).

A significant role in the etiopathogenesis of PSC belongs to the intestinal microbiota. The association between PSC and IBD supports the implications of immune-mediated mechanisms in the initiation and progression of PSC, considered an immune-mediated disease like UC (136). The increased intestinal wall permeability of patients with IBD causes the easy translocation of intestinal bacteria into the portal vein, leading to portal bacteremia and activation of cholangiocytes (136). Kupffer cells in the liver will release chemokines and cytokines attracting other inflammatory cells (macrophages, monocytes, lymphocytes, neutrophils, and fibroblasts) into the portal tracts and peribiliary space, leading to chronic inflammation, periductal fibrosis, obstructive strictures, and finally to secondary biliary cirrhosis (136). At the level of the inflamed intestinal wall but also in the liver, numerous adhesion molecules that attract T cells will be aberrantly expressed (137). These memory T cells accumulated in the inflamed intestine can persist as long-lived memory cells, enter the circulation to the liver, and will trigger the inflammation of portal spaces (50). Clinical studies support the role of intestinal microbiota in PSC: the gut microbiome in patients with PSC and PSC and IBD is different from that in healthy controls and IBD patients only. Also, the frequent association of PSC with IBD demonstrates the role of the gut–liver axis in the disease (50, 51). Experimental studies confirm the implication of the microbiome in PSC pathogeny, proving the involvement of some specific molecules produced by the microbiota in triggering epithelial injury (51, 139).

Comparing BA and PSC as entities secondary to the increased cholangiocyte senescence process, in Figure 2, we summarized the main characteristics of the pathogenesis.

5 Conclusions

Despite recent advances in identifying essential aspects regarding the natural history of the most frequent cholangiopathies in children, even with the increasing availability of animal models, a clear and

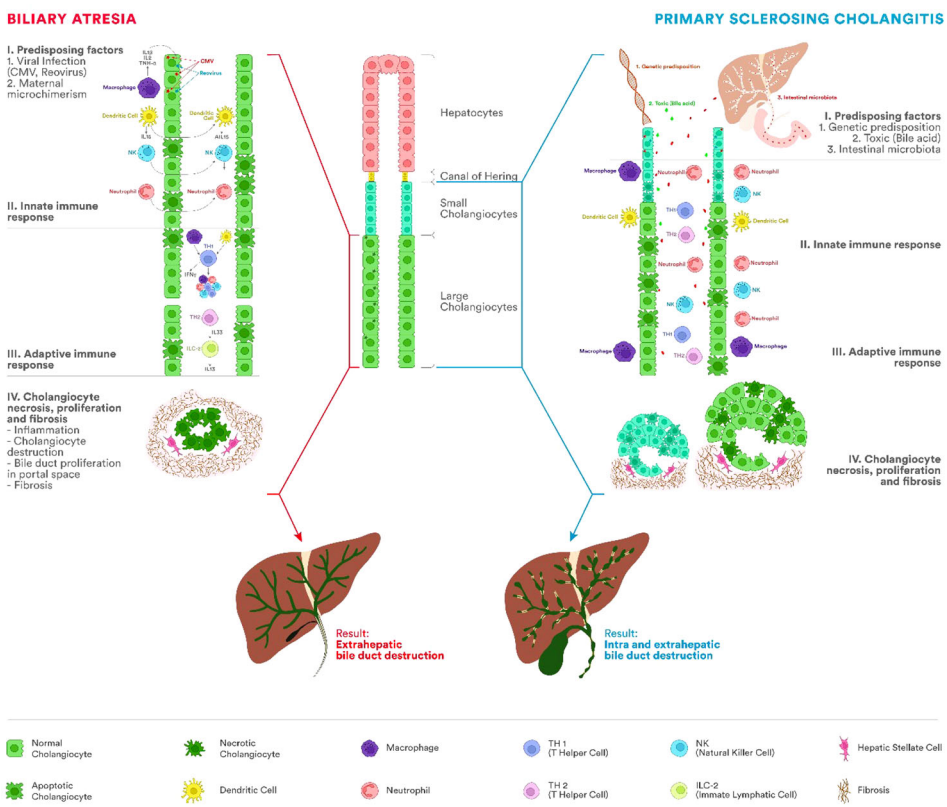

FIGURE 2
Etiopathogenesis of BA and PSC. The immune system represents one of the most important factors in the etiopathogenesis of BA and PSC, leading to chronic inflammation of the bile duct and progressing to cirrhosis. In BA, the process appears most frequently after viral infections with cytomegalovirus (CMV), *Rotavirus*, or reovirus. PSC occurs in people with a genetic predisposition and different antigens acting as triggers (bile acids, bacteria from the intestinal microbiota). Involvement of innate and adaptive immunity in both diseases has been proven by the presence of innate immune cells, including T lymphocytes, natural killer (NK) cells, macrophages (Kupffer cells), or dendritic cells. In BA, fetal cholangiocyte infection leads to a severe process of inflammation and fibrosis of the extrahepatic biliary tract with stenosis and impairment of the biliary flow. There will be a proliferation of the bile ducts in the portal space, fibrosis, and cirrhosis. Unlike BA, in PSC, the extrahepatic biliary tree is not atretic. In PSC, mixed inflammatory cells (lymphocytes, plasma cells, and neutrophils) are present around the intra- and extrahepatic bile ducts. As the disease progresses, the inflammatory infiltrate decreases simultaneously with the progressive reduction of bile ducts and the appearance of periductal fibrosis and progression to cirrhosis.

conclusive report concerning disease initiation is still lacking. The most frequently encountered cholangiopathies in children (BA, NSC, and PSC) probably involve genetic predisposition, environmental factors, and the immune system's dysregulation. Understanding the pathophysiology of different cholangiopathies better is essential to improve the outcome. At present, there is no effective therapy in these entities. Based on the novel findings, future research should aim to find therapies (pharmaceutical or cellular) targeting the disease mechanism at the molecular level. Careful clinical observational studies of clearly defined pediatric patients, together with novel animal models, should further improve our knowledge on the pathogenesis of the most common cholangiopathies in children.

Author contributions

AG, AM, CS, GB and TP contributed to the conception and design of the study. AG wrote the first draft of the manuscript. AG, AM, CS, GB and TP wrote sections of the manuscript. AG, CS and TP revised the final draft. All authors contributed to the manuscript revision, read, and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Regulation of CD47 expression on CD14$^+$ monocytes by interferon-α in PBC patients

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Background: Primary biliary cholangitis (PBC) is a chronic intrahepatic cholestatic autoimmune liver disease characterized by inflammatory injury of small and medium-sized bile ducts in the liver. The pathogenesis of PBC has yet to be entirely understood. CD47/signal-regulatory protein alpha (SIRPα) is closely related to developing autoimmune diseases by promoting inflammatory response. However, the effect of CD47/SIRPα on inflammatory response in PBC patients is still unclear.

Objective: We investigated the expression of CD47/SIRPα and the effect of inflammatory cytokines on the CD47 expression, analyzed potential autoantibodies against CD47 and the effect of anti-CD47 antibody on the inflammatory response in PBC, provided laboratory basis for the study of the pathogenesis and targets for non-invasive diagnosis and treatment on PBC.

Methods: The expression levels of CD47 and SIRPα on peripheral blood mononuclear cells (PBMC) were measured in 14 patients with PBC (the PBC group) and 13 healthy subjects (the Control group) by flow cytometry (FCM). The PBMC derived from healthy subjects were stimulated with healthy subjects' serum, PBC patients' serum, IFN-α or TNF-α, and the CD47 expression level on CD14$^+$ monocytes was detected by FCM. The level of serum anti-CD47 antibody or IFN-α in PBC patients and healthy subjects was analyzed by ELISA. FCM was used to examine the TNF-α expression level in CD14$^+$ monocytes of healthy subjects stimulated with isotype control antibody, anti-CD47 antibody, LPS or LPS combined with CD47 antibody.

Results: The CD47 expression level on the CD14$^+$ monocytes in PBC patients was statistically higher than that in the Control group (P<0.01). Compared with the Control group (PBMC+healthy serum), the CD47 expression on CD14$^+$ monocyte stimulated with the PBC patients' serum (PBMC+PBC patients' serum) was increased (P<0.001); the CD47 expression on CD14$^+$ monocyte stimulated with IFN-α (PBMC + IFN-α) increased gradually with the increased concentration of IFN-α (P<0.05). However, there was no similar trend on CD14$^+$ monocyte stimulated with the TNF-α (PBMC+TNF-α) (P>0.05). The levels of serum anti-CD47 antibody and IFN-α in the PBC patients were higher than those in healthy subjects (P<0.05). The TNF-α expression level in CD14$^+$ monocyte

stimulated with the LPS (PBMC+LPS) or anti-CD47 antibody+LPS group (PBMC +LPS+anti-CD47 antibody) was significantly increased than that in the Control group (PBMC+isotype control antibody) ($P<0.01$ and $P<0.001$, respectively). The TNF-α expression level in $CD14^+$ monocyte stimulated with the anti-CD47 antibody + LPS was higher than that with the LPS ($P< 0.05$).

Conclusion: The CD47 may be related to the pathogenesis of PBC by inflammatory response. The CD47/SIRPα signal were imbalanced in PBC patients. The presence of serum anti-CD47 antibodies in PBC patients provides a laboratory basis for clinical diagnosis and treatment.

KEYWORDS

PBC, cd47, SIRPα, immune checkpoint, inflammatory response

1 Introduction

Primary biliary cholangitis (PBC) is a chronic intrahepatic cholestatic autoimmune liver disease characterized by inflammatory injury of small and medium-sized bile ducts in the liver. It occurs mainly in middle-aged and older women; the onset is mostly hidden. The early symptoms of the disease are not specific, and it is easy to miss or misdiagnose (1). The pathogenesis of PBC needs to be clarified. The interaction among environmental, genetic, and hormonal factors led to congenital and adaptive immune disorders and loss of self-tolerance (2), leading to antibody and T cell-mediated specific immune attacks against the liver, progressive inflammatory necrosis, and fibrosis of the liver (3, 4). The activation of inflammatory mediators such as type I interferon (IFN-I) and tumor necrosis factor (TNF), innate liver lymphocytes, and natural killer T cells played a crucial role in the pathogenesis of the disease (5). In addition, due to the existence of the 'gut-liver axis', when the intestinal mucosa was damaged, Lipopolysaccharides (LPS), the cell wall component of Gram-negative bacteria, entered the liver through the portal vein, which would cause activation of Toll-like receptors 4 (TLR4) and nuclear factor kappa-B (NF-κB) signaling pathway, increased the expression of inflammatory factors, and aggravated the liver from autoimmune damage (6).

CD47 is a transmembrane protein expressed in different cell types, such as thymocytes, T and B cells, monocytes, erythrocytes and nerve cells. It belongs to the immunoglobulin superfamily (7) and is a supramolecular complex composed of integrin, G protein, and cholesterol (8). It interacts with corresponding ligands and mediates cell proliferation, migration, phagocytosis, apoptosis, immune homeostasis, and inhibition of nitric oxide signaling (9, 10). signal-regulatory protein alpha (SIRPα) binds to CD47 to initiate an inhibitory signaling pathway, resulting in weakened phagocytosis of macrophages to malignant cells (11).

The binding of CD47 on erythrocyte to the SIRPα on macrophages prevented erythrocyte phagocytosis by suppressing phagocytic activity. The polycythemia phenotype was restored by intercepting CD47-SIRPα through either anti-CD47 treatment or loss of the inhibitory SIRPα-signal in a PV mouse model (12). Both solid and hematologic malignancies expressed higher levels of CD47, which bound with SIRPα to protect the tumor cell against macrophage-mediated phagocytosis (13–15). The CD47 enabled cancer cells to escape innate and adaptive immune surveillance leading to metastatic spread, which could be restricted by the administration of anti-CD47 antibodies through affecting tumor growth and tumor microenvironment signaling (16, 17). Anti CD47 therapy promoted T cell secretion of pro-inflammatory cytokines in an undifferentiated pleiomorphic sarcoma which expressed highly CD47 (18).

CD47-SIRP α signaling pathway is related to the development of autoimmune diseases. CD47 deficiency improved ocular autoimmune inflammation (19) and furthered macrophage-mediated phagocytosis in type I diabetes (20). CD47 facilitated autoimmune valvular carditis through damaging macrophage efferocytosis and increasing cytokine production (21). T cell activation was regulated by macrophages through CD47/SIRPA in inflammatory bowel disease (22). Jin et al. (7) reported that the CD47 expression level on monocytes in SLE patients was significantly increased, promoting SLE patients' inflammatory response. However, the effect of CD47/SIRPα on the inflammatory response in PBC patients has not been reported.

Therefore, the present research clarified the expression of CD47/SIRPα on mononuclear cells in PBC patients, investigated the effect of serum inflammatory cytokines on the CD47 expression in PBC patients, analyzed potential autoantibodies against CD47 in PBC patients, and explored the role of CD47 and anti-CD47 antibody in the inflammatory response of PBC, provided laboratory basis for the study of the pathogenesis and targets for non-invasive diagnosis and treatment on PBC.

2 Materials and methods

2.1 Research object

From December 2021 to February 2023, 14 PBC patients and 13 healthy subjects were selected from Hunan Provincial People's Hospital (the First Affiliated Hospital of Hunan Normal

University). Diagnosis of PBC was based on the published American Association for the Study of Liver Diseases (AASLD) criteria, APASL clinical practice guidance: the diagnosis and management of patients with primary biliary cholangitis (2022) (23). Patients were excluded with one or more of the following conditions: (1) severe cardiovascular disease, kidney disease and other serious diseases; (2) acute and chronic infectious diseases and various non-PBC immune system diseases; (3) pregnant or lactating women; (4) liver cancer or other types of malignant tumor. The ethics committee of Hunan Provincial People's Hospital approved the exemption of informed consent, because theresearch used the remaining samples after clinical testing, which did not cause additional harm to patients.

2.2 Peripheral blood experiments

2.2.1 Extraction of peripheral blood mononuclear cells

EDTA anticoagulant venous blood of PBC patients or healthy subjects diluted 2-4 times with PBS was mixed with Ficoll lymphatic separation solution at 1:1, centrifuged at 400 g, 18°C-20°C for 40 min. The PBMC layer was taken and centrifuged twice to leave the PBMC for use.

2.2.2 Detection of CD47/SIRPα expression on the mononuclear cells

After treated with Fixable Viability Dye eF780(#65-0865, eBioscience, USA) and FC receptor blockers (#564765, BD Biosciences, Germany), PBMCs (10^5 cells/mL) were incubated for 30 min at 4°C in the dark with 5ul of fluorescent dyes CD3-Percp-Cy5.5 (#560835, BD Biosciences), CD19-BV421 (#562440, BD Biosciences), CD14-BV510 (#563079, BD Biosciences), CD56-BV650(#564057, BD Biosciences), SIRPα-APC (#17-1729-42, eBioscience), 20ul of CD16-PE (#555407, BD Biosciences) or CD47-FITC (#556045, BD Biosciences). After washing, the cells were detected by CytoFlex V5-B5-R3 Flow cytometer (No: 38385, Beckman USA). The expressions of CD47 and SIRPα in $CD3^+$ T cells, $CD19^+$ B cells, $CD56^+$ NK cells or $CD14^+$ monocytes in PBMC were analysed using flowjo 6.2 software. The gate-drawing strategies for $CD3^+$ T cells, $CD19^+$ B cells, $CD56^+$ NK cells and $CD14^+$ monocytes were shown in Figure 1.

2.2.3 Detection of anti-CD47 antibody level and IFN-α level

According to the instructions, the serum anti-CD47 level and the IFN-α level in the PBC patients and healthy subjects were detected by ELISA kits (#F-111548-A, FANKEW, China) (#F10665-A, FANKEW, China).

2.2.4 Detection of ANA and AMA

Antinuclear antibody (ANA) and antimitochondrial antibody (AMA) were detected by indirect immunofluorescence method [EUROIMMUN Medical Diagnostics (China) Co., Ltd.], autoimmune liver disease antibodies were detected by membrane strip immunoblotting (Suzhou Haobo Co., Ltd.), and serum immunoglobulins by rate scattering turbidimetric assay (Siemens, USA), liver function was detected by the automatic biochemical analyzer (Hitachi Medical Instrument Co., Ltd.).

2.3 *In vitro* experiments

2.3.1 Detection of CD47 expression on the $CD14^+$ monocytes

PBMCs were extracted from healthy subjects. PBMC samples were divided into the Control, PBC, IFN-α, and TNF-α groups. Cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS). According to the grouping, 100 uL of healthy human serum, 100 uL of PBC patient serum, IFN-α (#ab200262, absin, China) (0/10/50) ng/mL, or TNF-α (#ab259410, absin) (0/10/50) ng/mL was added to PBMC (10^5 cells/mL), mixed and incubated at 37°C, 5% CO_2 incubator overnight (12 h). Cells were collected and treated with Fixable Viability Dye eF780(#65-0865, eBioscience, USA) and FC receptor blockers (#564765, BD Biosciences, Germany). After washing, 5 μL of fluorescent dye CD14-BV510 (#563079, BD Biosciences) and 20ul of CD47-FITC (#556045, BD Biosciences) were added and incubated at 4°C in the dark for 30 min. After washing, the cells were detected by CytoFlex V5-B5-R3 Flow cytometer (No: 38385, Beckman USA) Flow cytometer.

2.3.2 Detection of TNF-α level in the $CD14^+$ monocytes

PBMCs were extracted from healthy subjects. PBMC samples were divided into the CD47 isotype control group, anti-CD47 antibody group, LPS group, and anti-CD47 antibody + LPS group. The cells were cultured in RPMI-1640 medium containing 10% FBS. According to the grouping, 1 μg/mL of isotype control (#14-4321-85, eBioscience), 1 μg/mL of anti-CD47 antibody (#16-0471-81, eBioscience), 3 μg/mL of LPS (#abs47014848, absin), 1 μg/mL of anti-CD47 antibody (#16-0471-81, eBioscience) + 3 μg/mL of LPS (#abs47014848, absin) was added to PBMC (10^5 cells/mL), respectively. After mixing, they were cultured in 37°C, 5% CO_2 incubator for 5 h. Cells were collected and treated with Fixable Viability Dye eF780(#65-0865, eBioscience, USA) and FC receptor blockers (#564765, BD Biosciences, Germany). After washing, 5 μL of fluorescent dye CD14-BV510 (#563079, BD Biosciences) was added and incubated at 4°C for 30 min in the dark. Washing, an intracellular staining antibody (TNF-α-PE-c) (#557647, BD Biosciences) was added. Washing again, the cells were detected by CytoFlex V5-B5-R3 Flow cytometer (No: 38385, Beckman USA) Flow cytometer.

2.4 Statistical analysis

Statistical analysis was performed using Graph Prism 8.3.0 and SPSS Statistics 25 software. Flow data were analysed using the flowjo 6.2 software. The measurement data of normal distribution were

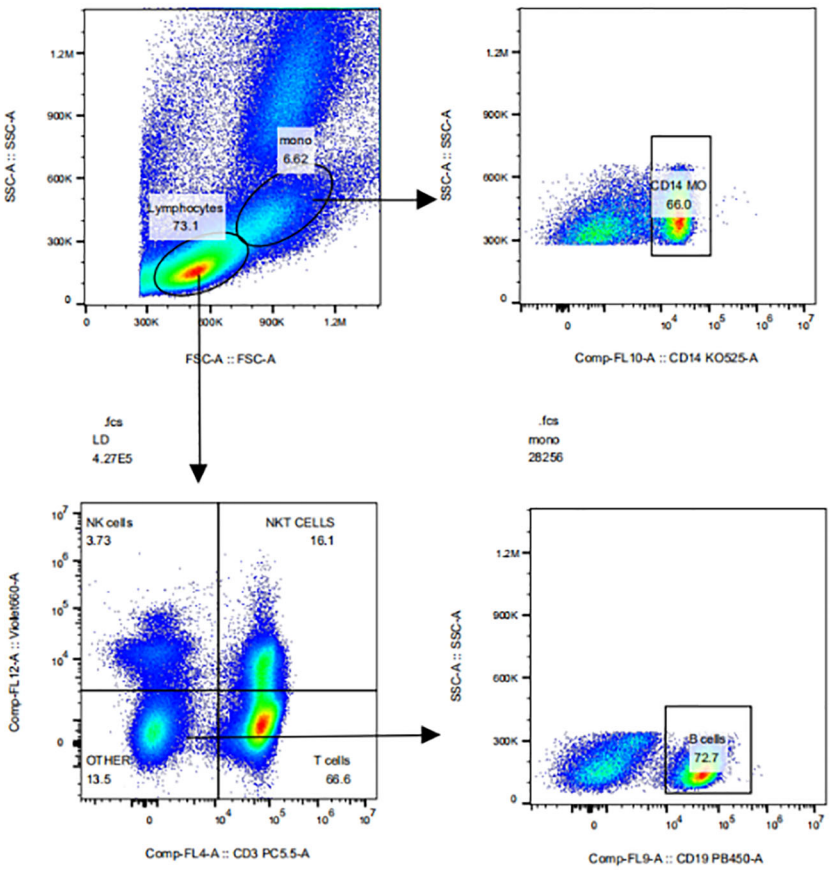

FIGURE 1
The gate-drawing strategies of CD3+T cells, CD19+B cells, CD56+NK cells and CD14+ monocytes.

expressed as mean ± standard deviation. One-way ANOVA was used to compare the differences among multiple groups, and *Post Hoc post hoc* test was used for pairwise comparison. The difference between the two groups was analyzed by independent sample t-test or paired t-test. Non-normal distribution data were expressed as median (quartile). Mann-Whitney U test was used for comparison between two groups and multiple groups. $P<0.05$ indicated a statistically significant difference.

3 Results

3.1 Clinical indicators of patients

A total of 14 patients with PBC and 13 healthy subjects were collected. Among the enrolled 14 PBC patients, 12 cases were positive for ANA, 11 cases were positive for AMA, 1 case was positive for anti-soluble liver antigen/hepatopancreatic antigen antibody (SLA/LP), 1 case for anti-smooth muscle antibody (SMA) and 1 case for anti-hepatocyte cytoplasmic type 1 antibody (LC-1). There was no significant difference in gender and immunoglobulin G between the two groups ($P>0.05$). The levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL), direct bilirubin (DBIL), immunoglobulin M (IgM) and immunoglobulin A (IgA) in PBC patients were higher than those in the control group ($P<0.05$). In contrast, complement 3 (C3) and complement 4 (C4) were opposite ($P<0.05$). The clinical indicators of PBC patients and healthy subjects enrolled in this research were shown in Table 1.

3.2 The expression levels of CD47 and SIRPα on mononuclear cells in PBC patients

The mean fluorescence intensity (MFI) of CD47 and SIRPα expression on the PBMC surface of PBC patients was shown in Figure 2. The expression levels of CD47 on $CD14^+$ monocytes (81484 ± 31179), $CD56^+$ NK cells (28982 ± 10467), $CD3^+$ T cells (25124 ± 7565) and $CD19^+$ B cells (22639 ± 5596) in PBC patients were statistically different ($F=24.705$, $P<0.001$). The CD47 expression level on $CD14^+$ monocytes was higher than that on $CD56^+$ NK cells, $CD3^+$ T cells or $CD19^+$ B cells in PBC patients ($t=5.812$, 6.573, 6.951, $P<0.001$, respectively), there were no significant difference in the CD47 expression levels among the $CD3^+$ T cells, $CD19^+$ B cells and $CD56^+$ NK cells in PBC patients ($P>0.05$) (Figure 2A).

TABLE 1 Clinical indicators of the study subject.

	patients with PBC (n=14)	Control (n=13)	*P*-value
age (years)	62.00±10.72	50.77±7.73	0.005
Female (n, %)	13(92.86%)	11(84.62%)	0.496
ast (U/L)	56.25(42.53, 92.83)	22.90(21.45, 26.40)	<0.001
alt (U/L)	44.85 (17.03, 92.88)	22.60(15.15, 28.10)	0.033
alp (U/L)	149.66±84.03	-	-
ggt (U/L)	111.60(26.80, 245.95)	-	-
TBIL (umol/L)	37.83(21.79, 57.28)	12.57(10.51, 14.26)	0.001
DBIL (umol/L)	20.09(9.07, 48.68)	3.06(2.13, 3.31)	<0.001
γ-globulin	29.57±10.48	-	-
IgG (g/L)	19.57±9.67	12.71±2.01	0.053
igA (g/L)	3.41(2.59, 4.58)	2.08(1.74, 2.99)	0.011
igM (g/L)	2.07(1.52, 6.16)	0.97(0.82, 1.64)	0.024
C3 (g/L)	0.74±0.28	1.11±0.15	<0.001
C4 (g/L)	0.14±0.09	0.26±0.08	0.002
ANA (n, %)	12(100%)a	-	-
SLA/LP (n, %)	1(7.14%)	-	-
sma (n, %)	1(7.14%)	-	-
LKM-1 (n, %)	0	-	-
LC-1 (n, %)	1(7.14%)	-	-
ama (n, %)	11(78.57%)	-	-

-, No data; [a] Data were collected from only 12 patients with PBC. AST: aspartate aminotransferase, ALT, alanine aminotransferase; ALP, alkaline phosphatase; GGT, glutamyl transpeptidase; TBIL, total bilirubin; DBIL, direct bilirubin; IgG, immunoglobulin G; IgA, immunoglobulin A; IgM, immunoglobulin M; C3, complement 3; C4, complement 4; ANA, Antinuclear antibody; SLA/LP, anti-soluble liver antigen/hepatopancreatic antigen antibody; SMA, anti-smooth muscle antibody; LKM-1, anti-liver kidney microsome type 1 antibody; LC-1, anti-hepatic cytosolic antigen type 1 antibody; AMA, anti-mitochondrial antibody.

The SIRPα expression levels on $CD14^+$ monocytes (47112 ± 20060), $CD56^+$ NK cells (6332 ± 3348), $CD3^+$ T cells (1166 ± 785) and $CD19^+$ B cells (1308 ± 813) in PBC patients were statistically different (F=33.367, $P<0.001$). The SIRPα expression level on $CD14^+$ monocytes was higher than that on $CD56^+$ NK cells, $CD3^+$ T cells or $CD19^+$ B cells in PBC patients (Figure 2B).

The CD47 expression level (Figures 3A–D) on $CD14^+$ monocytes in PBC patients was higher than that in the Control group (81484 ± 31179, 39537 ± 11773; t=3.628, $P<0.05$) (Figure 3A). There was no significant difference in the SIRPα expression levels on $CD3^+$ T cells, $CD19^+$ B cells, $CD56^+$ NK cells or $CD14^+$ monocytes between PBC patients and the Control group ($P>0.05$) (Figures 3E–H).

3.3 Serum IFN-α expression in PBC patients

The serum IFN-α level in PBC patients and healthy examiners were measured by ELISA. PBC patients had higher serum IFN-α levels than that in healthy examiners [(112.57(92.23, 174.78) pg/mL, 77.20(36.51, 116.70) pg/mL; Z=-2.10, $P<0.05$, Figure 4)].

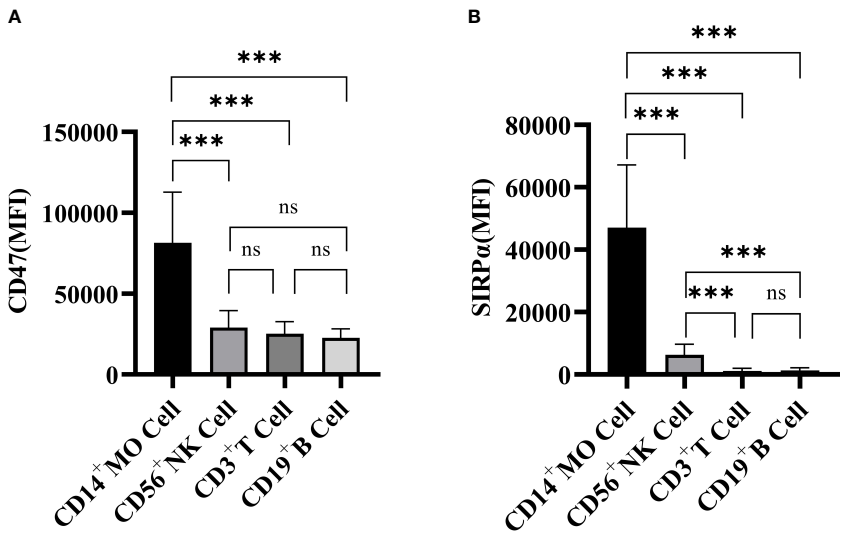

FIGURE 2
The expression of CD47 **(A)** and SIRPα **(B)** on $CD3^+$ T cells, $CD19^+$ B cells, $CD56^+$ NK cells and $CD14^+$ monocytes in PBC patients. ns: no statistical significance; ***$P<0.001$, **$P<0.01$, *$P<0.05$.

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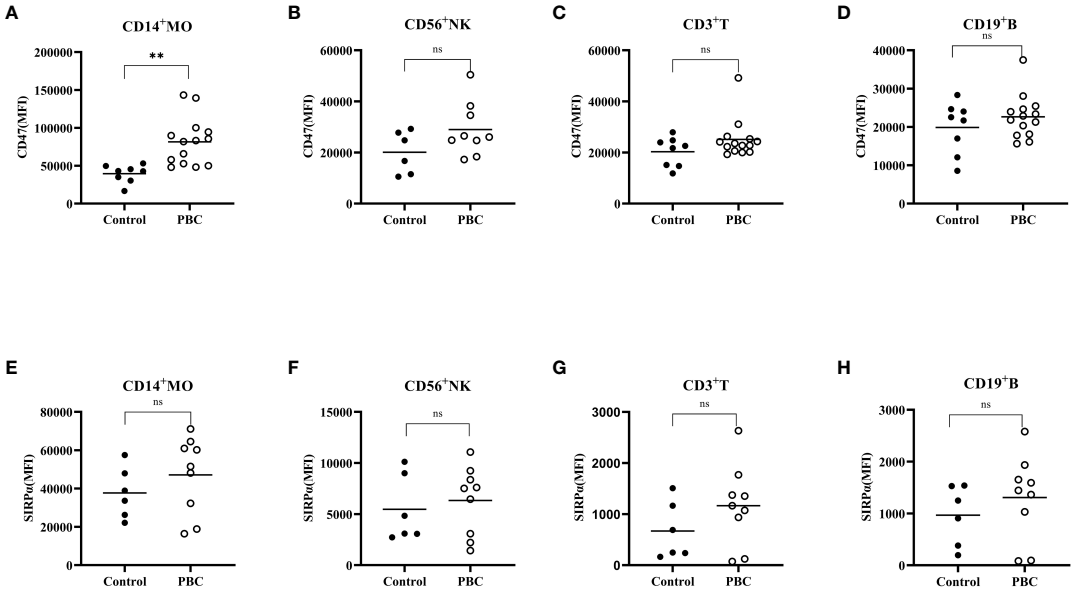

FIGURE 3
The expression of CD47 **(A–D)** and SIRPα **(E–H)** on $CD3^+$ T cells, $CD19^+$ B cells, $CD56^+$ NK cells, and $CD14^+$ monocytes in the Control group and PBC group. ns: There was no statistical significance between the two groups; ***P< 0.001, **P< 0.01, *P< 0.05.

3.4 The CD47 expression level on $CD14^+$ monocytes incubated with PBC patients serum or recombinant IFN-α

PBMCs of healthy subjects were incubated with healthy subjects serum (n=5), PBC patients serum (n=9), different concentrations of recombinant IFN-α (n=4) or recombinant TNF-α (n=4), and the CD47 expression levels on cultured cells were detected. The CD47 expression level on $CD14^+$ monocytes in the PBC group was significantly higher than that in the Control group (86.06% ± 8.98%, 30.09% ± 16.75%; t=6.94, P=0.001, Figure 5).

The CD47 expression levels in the IFN-α 0 ng/mL group, IFN-α 10 ng/mL group and IFN-α 50 ng/mL group were statistically different (F=6.41, P<0.05). The CD47 expression level in IFN-α 50 ng/mL group was higher than that in IFN-α 0 ng/mL group (69.03% ± 6.97%, 37.75% ± 20.21%; t=2.93, P=0.05, Figure 6A). The CD47 expression levels in the different concentrations of recombinant TNF-α groups were not statisticallydifferent (P>0.05), Figure 6B.

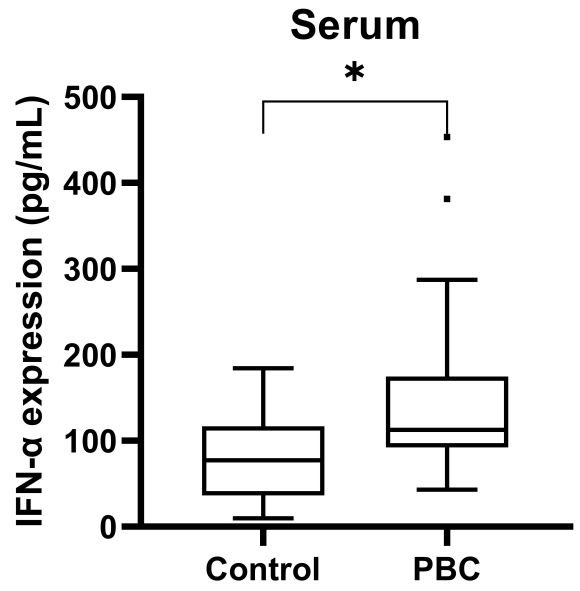

FIGURE 4
The serum IFN-α level in PBC patients and healthy examiners. ***P < 0.001, **P < 0.01, *P < 0.05.

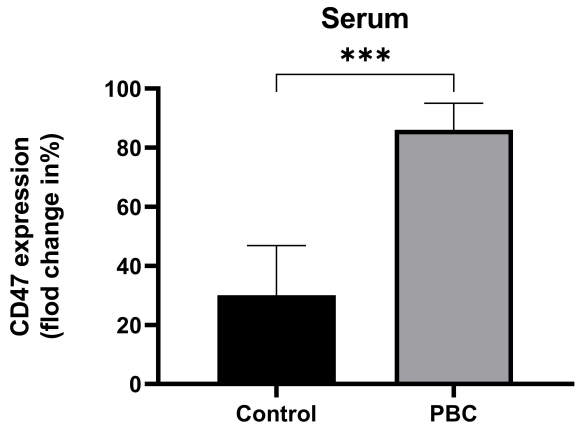

FIGURE 5
The CD47 expression levels on $CD14^+$ monocytes in the Control group and PBC group. ***P ≤ 0.001, **P< 0.01, *P< 0.05.

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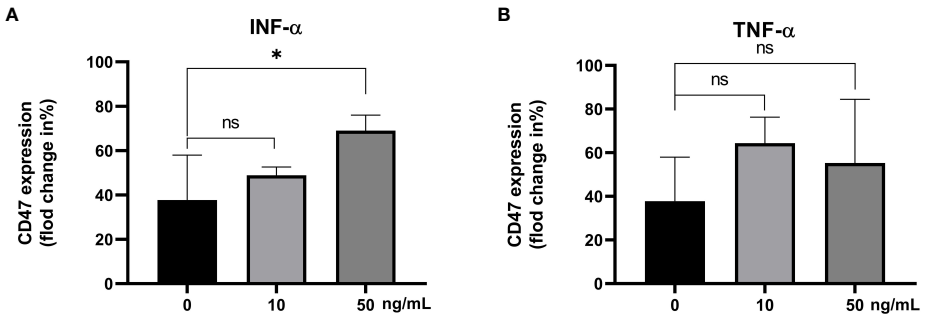

FIGURE 6
The CD47 expression levels on CD14$^+$ monocytes incubated with different concentrations of the IFN-α **(A)** and TNF-α **(B)**. ns: There was no statistical significance between the two groups; ***P< 0.001, **P< 0.01, *P< 0.05.

3.5 Serum anti-CD47 antibody level in PBC patients

The serum anti-CD47 antibody levels in PBC group (n=10) and the Control group (n=9) were detected by ELISA. The level of serum anti-CD47 antibody in PBC patients was significantly higher than that in the Control group [4437.50(3850.00, 10631.25) ng/L,3018.75 (2443.75, 3475.00) ng/L; Z=-3.43, P=0.001, Figure 7)].

3.6 The TNF-α expression level in CD14$^+$ monocytes incubated with the anti-CD47 antibody

PBMC in healthy subjects was incubated with an isotype control antibody, anti-CD47 antibody, LPS or anti-CD47 antibody+LPS. The TNF-α expression levels in CD14$^+$ monocytes in cultured PBMC were detected. There were significant differences in the TNF-α expression in CD14$^+$ monocytes among the isotype control group, anti-CD47 antibody group, LPS group and anti-CD47 antibody + LPS group (F=76.58, P<0.001). The TNF-α expression level in CD14$^+$ monocytes in the anti-CD47 antibody group was not different from that in the isotype control group (3.76% ± 1.51%, 2.19% ± 0.51%; t=1.70, P>0.05), the TNF-α expression level in CD14$^+$ monocytes in the LPS group was higher than that in the isotype control group (35.43% ± 9.85%, 2.19% ± 0.51%; t=5.84, P<0.01), the TNF-α expression level in CD14$^+$ monocytes in the anti-CD47 antibody + LPS group was higher than that in the LPS group (57.80% ± 3.61%, 35.43% ± 9.85%; t=3.70, P<0.05) (Figure 8).

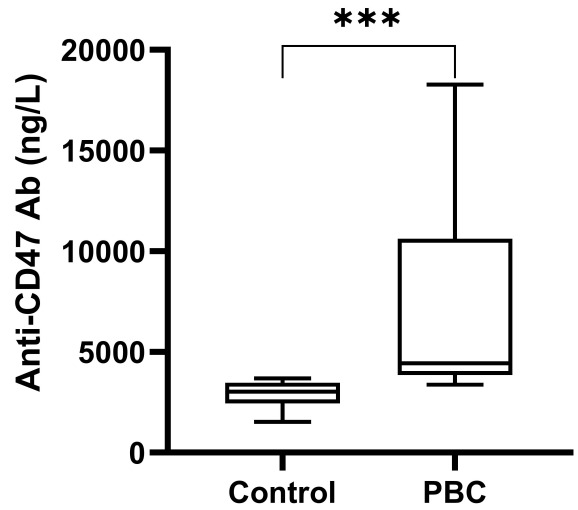

FIGURE 7
The serum anti-CD47 antibody level in the PBC group and the Control group. ***P≤ 0.001, **P< 0.01, *P< 0.05.

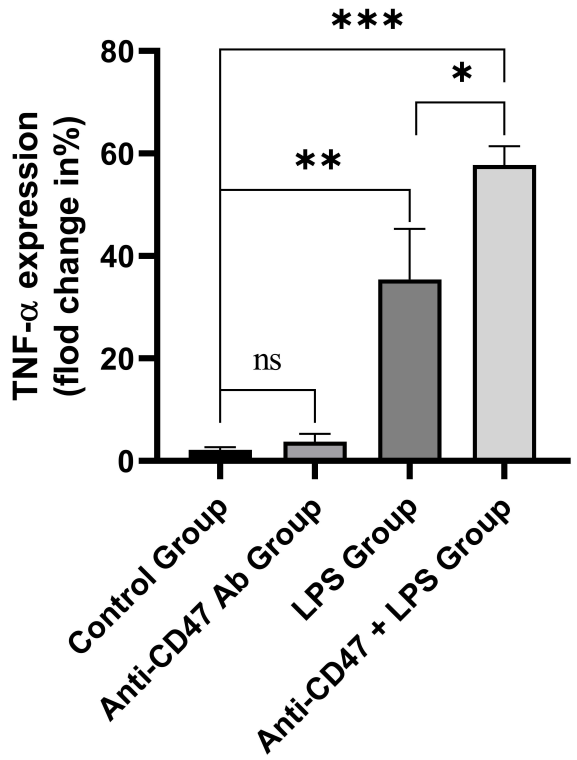

FIGURE 8
The TNF-α expression levels in CD14$^+$ monocytes in the isotype control group, anti-CD47 antibody group, LPS group and anti-CD47 antibody+LPS group. ns: There was no statistical significance between the two groups; ***P< 0.001, **P< 0.01, *P< 0.05.

4 Discussion

Immunosurveillance among normal cells, defective cells and foreign pathogens is regulated by cell surface receptors, which mediate the interaction between immune cells and their targets. These ' self ' signal markers interact with proteins expressed on the surface of phagocytes to inhibit phagocytosis (24). CD47 is a protein expressed in almost all body cells, providing a ' do not eat me ' signal to host phagocytes (including neutrophils and macrophages) (25). Cancer cells escaped the recognition and killing of host phagocytes by expressing high levels of CD47 (26, 27), indicating that pathogenic results will occur in the case of CD47 overexpression. The present research found increased CD47 expression level on $CD14^+$ monocytes in PBC patients, consistent with the report (7).

When the CD47 and SIRPα are expressed on the monocytes at the same time, the sum of the two signals may determine the final effect of CD47 and SIRPα on cell response (28). Our research showed that the CD47 expression level was increased in PBC patients. In contrast, the expression of SIRPα was normal on $CD14^+$ monocytes in PBC patients, suggesting that immune regulation of the CD47/SIRPα signals were imbalanced in PBC patients. The research also showed IFN-β and IFN-γ/TNF-α decreased erythrophagocytosis by human monocytes *in vitro*, which was independent from the increase in SIRP-α or SHP-1 expression (29).

Our research also showed that the PBC patients' serum with increased IFN-α level could improve the CD47 expression on $CD14^+$ monocytes from healthy subjects. Recombinant IFN-α also increased the CD47 expression on $CD14^+$ monocytes in a dose-dependent manner. The data showed enhanced expression of IFN-I and toll-like receptor-3 in PBC (30) and IFN-I signaling as a necessary component of the sex bias in murine autoimmune cholangitis (31). The self-derived IFN inducers and a lack of negative feed-back signals downregulating the IFN response contributed to the continuous IFN production in SLE (32). Therefore, we speculated that increased serum IFN-α caused by self-derived IFN inducers or a lack of negative feed-back signals could promote CD47 expression in $CD14^+$ monocytes in PBC patients.

PBC is an autoimmune disease, which can produce a variety of autoantibodies, which helps to diagnose autoimmune diseases. Our research found that the level of anti-CD47 autoantibodies in PBC patients was higher than that in healthy subjects, suggesting that there were autoantibodies against CD47 in PBC patients. Anti-CD47 antibody enhanced the phagocytosis of macrophages by binding to the Fc receptor on the macrophages, which might destroy the interaction of CD47/SIRPα, increase the phagocytic activity of macrophages, lead to an aggravation of experimental autoimmune encephalomyelitis and autoimmune nephritis (33, 34).

Studies have shown that the immune response of effector $CD4^+$ T cells (Th1, Th17 and follicular helper T cells) and $CD8^+$ T cells to autoantigens (expressed by hepatocytes and biliary epithelium) was related to PBC pathogenesis (35, 36). Activated Th1 and Th17 cells released various inflammatory factors to promote the occurrence and development of PBC (5). When PBC patients were exposed to LPS due to infection, LPS could bind to cell surface receptors (such as TLR 4/CD14) and induced the secretion of pro-inflammatory cytokines (such as TNF-α, IL-1, IL-6, IL-8), promoting inflammation. Our results showed that the anti-CD47 antibody could not promote TNF-α expression in $CD14^+$ monocytes. However, LPS could, and TNF-α expression level was higher when stimulated with the combination of the anti-CD47 antibody and LPS. We assumed that anti-CD47 antibody can enhance the pro-inflammatory effect of LPS, aggravate liver lesions, and form a vicious circle.

In summary, increased inflammatory cytokines promote the expression of CD47 on the $CD14^+$ monocytes in PBC patients. There was anti-CD47 antibody in PBC patients which could enhance the pro-inflammatory effect of LPS, aggravate liver lesions, and form a vicious circle. Blocking the imbalance of CD47/SIRPα signal may contribute to treatment for PBC patients.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by The Medical Ethics Committee of Hunan Provincial People's Hospital. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin because This research utilized samples obtained after clinical testing without causing harm to patients.

Author contributions

CL: Conceptualization, Supervision, Writing – review & editing. XS: Writing – original draft, Data curation, Software. WJ: Writing – original draft, Investigation, Methodology. LL: Writing – original draft, Formal Analysis. ZZ: Data curation, Investigation, Resources, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Primary biliary cirrhosis and psoriasis: a two-sample Mendelian randomization study

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Background: Primary biliary cirrhosis (PBC) and psoriasis are frequently observed to co-occur in clinical settings. However, the causal associations and underlying mechanisms between PBC and psoriasis remain poorly defined.

Methods: In this study, we conducted bidirectional MR analysis to explore the causal relationship between PBC and psoriasis using four MR methods: inverse-variance weighted, MR-Egger regression, weighted median, and weighted mode. Sensitivity analyses were carried out, employing different models and testing methods for comparison to assess the influence of heterogeneity and pleiotropy on our findings and to confirm the robustness of these results.

Results: A causal relationship between the risk of PBC and psoriasis was identified, as confirmed by IVW analysis (OR: 1.081, 95%CI: 1.028~1.137, $P<0.05$). The other three MR methods also produced similar results. However, psoriasis did not have a causal effect on PBC risk (OR: 1.022, 95%CI: 0.935~1.118, $P>0.05$). The intercept of MR-Egger regression was 0.0013 ($P>0.05$), indicating that genetic pleiotropy did not influence the results. Additionally, the leave-one-out analysis demonstrated the robustness of our MR findings.

Conclusion: This study reveals a causal relationship between PBC and psoriasis, with PBC increasing the risk of psoriasis, but not the reverse. This potential causal relationship offers a new perspective on the etiology of PBC.

KEYWORDS

primary biliary cirrhosis, psoriasis, Mendelian randomization study, genome-wide association studies, causal relationship

1 Introduction

Psoriasis is a chronic inflammatory and proliferative skin disorder. Its clinical manifestations differ according to the type, with common forms including plaque, guttate, erythrodermic, and pustular psoriasis. These types share characteristics of skin erythema, thickening, and scaling (1). A systematic analysis and modeling study using the Global Health Database indicate considerable variation in the prevalence of psoriasis among adults across different countries and regions. In Europe, the prevalence is 0.91% (95%CI: 0.29%~3.03%) (2). Psoriasis patients often experience comorbidities such as arthritis, cardiovascular disease, mental disorders, and enteritis (1). The refractory and recurrent nature of psoriasis, combined with the pruritus and pain associated with its lesions, significantly affects the physical and mental well-being and quality of life of patients.

Primary biliary cirrhosis (PBC), also known as primary biliary cholangitis, is an autoimmune condition causing chronic inflammatory damage to the liver. The exact etiology and pathophysiology of PBC remain unclear. It is characterized by chronic non-suppurative destructive cholangitis, primarily affecting the interlobular and septal bile ducts, leading to periductal inflammatory infiltration and necrosis (3). PBC predominantly affects women and individuals over 50 years of age (4). Its prevalence has tended to increase over time, likely due to advancements in diagnostic methods and better access to healthcare resources (5). A systematic review and meta-analysis of primary biliary cholangitis epidemiology in European countries showed that the global prevalence and incidence of PBC vary geographically, with a pooled prevalence of 22.27 per 100,000 population (95%CI: 17.98~27.01) (6). In individuals diagnosed with PBC, there is a notable prevalence of concurrent autoimmune disorders. These include, but are not limited to, Sjögren's Syndrome (3.5–73%), autoimmune thyroid diseases (5.6–23.6%), systemic sclerosis (1.4-12.3%), and systemic lupus erythematosus (0–3.7%) (3). Symptoms such as cholestatic pruritus, abdominal discomfort, and fatigue significantly impair the quality of life of affected individuals (7).

PBC and psoriasis, both immune-related diseases, have an unclear relationship. Psoriasis, with its skin manifestations, adversely affects patients' quality of life and mental health. Therefore, it is crucial for physicians to monitor the skin conditions of patients with PBC, ensuring timely detection and diagnosis of psoriasis, and to optimize treatment regimens. An international multicenter study involving 1,554 PBC patients reported that 23 cases (1.5%) had concurrent psoriasis (8). However, PBC often presents with nonspecific early symptoms, and patients may overlook mild or localized psoriatic lesions, leading to delayed diagnosis and treatment. Certain studies have indicated that both IL-23 and TL1A play roles in the pathogenesis of psoriasis and PBC, suggesting a potential association between these conditions (9, 10). Yet, conclusive evidence establishing a causal link between PBC and psoriasis is lacking, necessitating further investigation.

Traditional observational studies are limited by issues such as confounding, reverse causality, and selection bias. In contrast, Mendelian randomization (MR) is a method in genetic epidemiology that uses genetic variants as instrumental variables (IVs) for causal inference between exposures and outcomes (11). These genetic variants, randomly assorted during meiosis and fixed at conception, are long-term stable exposure factors unaffected by environmental or social factors, thereby overcoming the limitations of observational studies. In this research, we employed a bidirectional MR analysis to investigate whether PBC and psoriasis influence each other genetically.

2 Materials and methods

2.1 Data source

To investigate the causal relationship between PBC and psoriasis, we utilized single nucleotide polymorphisms (SNPs) as instrumental variables, sourced from genome-wide association studies (GWAS) databases. Given the public nature of these databases, additional ethical approval was not required. The PBC-related database encompasses genotype data from 2,861 PBC cases and 8,514 controls, all from European populations, along with association analysis results for 119,756 SNPs (GWAS ID: ebi-a-GCST005581) (12). This database originates from a GWAS published in Nature Genetics, which focused on uncovering genetic susceptibilities and molecular mechanisms of PBC. The database related to psoriasis includes genotype data from 4,510 psoriasis cases and 212,242 controls from Finnish populations, with association analysis results for 16,380,464 SNPs (GWAS ID: finn-b-L12_PSORIASIS).

2.2 SNP selection

To ensure the accuracy and reliability of our research findings, we implemented stringent criteria for SNP selection. SNPs with genome-wide significant associations with the exposure ($P < 5 \times 10^{-8}$) were chosen, and those exhibiting high linkage disequilibrium ($r^2 > 0.001$ and kb < 10,000) were excluded. In cases where the result dataset lacked exposure-related SNPs, we opted for alternative SNPs showing high correlation with the associated SNPs ($r^2 > 0.8$). To maintain the accuracy of the MR analysis, it was necessary to filter out palindromic SNPs, which are SNPs with effect alleles and other alleles as complements. These rigorously screened SNPs were then employed as the final instrumental variables for the Mendelian randomization analysis that followed.

2.3 MR assumption

MR analysis hinges on three core assumptions to minimize bias in the results (13): 1. Relevance Assumption: The selected IVs must be directly associated with the exposure. 2. Independence Assumption: The IVs must be independent of any confounders in the exposure-outcome association. 3. Exclusion Restriction Assumption: The IVs must influence the outcome solely through the exposure (Figure 1). To evaluate the strength of the association between the instrumental variables and the exposure, and to eliminate weak instrumental variables, we utilized the F-statistic (14). This statistic is calculated as

$F = Beta^2/SE^2$, where Beta and SE represent the estimated effect and standard error of the allele on the exposure, respectively. Instrumental variables with an F-statistic less than 10 were excluded to avoid potential genetic confounding or measurement error.

2.4 Statistical analysis

In this research, we conducted a two-sample MR analysis to assess the causal relationship between PBC and psoriasis. The inverse-variance weighted (IVW) method was our primary approach, complemented by three additional methods: MR-Egger regression, weighted median, and weighted mode approaches. These methods collectively provide a comprehensive assessment of the potential relationship. The IVW method, characterized by excluding the intercept term in regression and using the inverse of the outcome variance as the weight for fitting, offers the most accurate reference for causal inference (15). MR-Egger regression, another MR method, can detect and adjust for horizontal pleiotropy by incorporating an intercept term into the IVW method. It is used to evaluate whether the instrumental variables satisfy the exclusion restriction assumption (16). Although its precision is relatively lower, it is useful in understanding the direction and magnitude of the effect. The weighted median approach, with a lower type I error rate and higher causal estimation capability, serves as a sensitivity analysis to verify the reliability of IVW results (17). Lastly, the weighted mode approach leverages the similarity information between SNPs to enhance the precision and robustness of the estimates (18).

2.5 Pleiotropy, heterogeneity, and sensitivity evaluation

We employed Cochran's Q test to examine the presence of heterogeneity among individual genetic variant estimates. In cases of significant heterogeneity ($P<0.05$), we utilized the IVW random effects model, rather than the fixed effects model, to derive the final MR results (19). To assess the impact of horizontal pleiotropy on MR analysis, we calculated the intercept value using MR-Egger intercept analysis. The MR-PRESSO test was also implemented to identify and exclude outliers influenced by horizontal genetic pleiotropy, thereby enhancing the credibility of the Mendelian randomization. Additionally, a leave-one-out analysis was performed to evaluate the influence or bias of each SNP on the combined estimate. All MR analyses and tests were conducted using the "TwoSampleMR" and "MRPRESSO" packages in R software (version 4.3.1).

3 Result

3.1 Causal effect of PBC on psoriasis from MR analysis

We extracted 22 SNPs closely associated with PBC risk, ensuring no linkage disequilibrium ($r^2 < 0.001$) among them, and confirming they were not weak instrumental variables as their F-statistics all exceeded 10, aligning with our earlier selection criteria. Utilizing MR-PRESSO, we identified and removed three anomalous SNPs (rs1646019, rs3135024, and rs34725611). The remaining 19 SNPs were then used as IVs for Mendelian analysis, with their details provided in Supplementary Table S1. Cochran's Q test indicated heterogeneity among the IVs, evidenced by a Q-statistic of 38.15 ($P<0.05$), leading us to employ the IVW random effects model for further MR analysis.

The IVW method revealed a causal relationship between PBC risk and psoriasis (OR: 1.081, 95%CI: 1.028~1.137, $P<0.05$). This finding was corroborated by the weighted model (OR: 1.081, 95% CI: 1.023~1.142, $P<0.05$) and the weighted median (OR: 1.083, 95% CI: 1.031~1.139, $P<0.05$), all indicating a positive causal effect of PBC on the risk of psoriasis. However, the MR-Egger analysis did not show a significant association (OR: 1.078, 95%CI: 0.977~1.190,

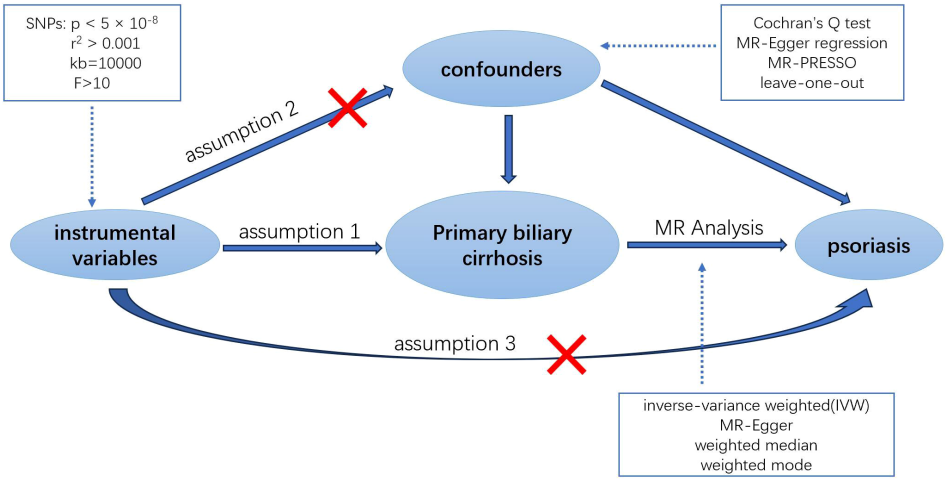

FIGURE 1
An overview of this Mendelian randomization (MR) study design.

P>0.05) (Table 1). Further examination of horizontal pleiotropy through MR-Egger intercept analysis (b-intercept: 0.0013; se: 0.0176; P>0.05) suggested a low probability of genetic pleiotropy effects, thus minimizing the likelihood of confounding bias impacting the analysis results. Sensitivity analysis using the leave-one-out method, which entailed sequential removal and recalculation of causal effects with the remaining SNPs, demonstrated consistent results, affirming the reliability of our findings (Figures 2–5).

3.2 Causal effect of psoriasis on PBC from MR analysis

In our examination of the potential causal effect of psoriasis on the risk of developing PBC, we conducted a two-sample MR analysis with psoriasis as the exposure and PBC as the outcome. The GWAS databases, IV selection methods, analysis approaches, and testing methods were identical to those previously described. Initially, 16 SNPs significantly associated with psoriasis risk ($P<5\times10^{-8}$, $r^2<0.001$) were identified. Of these, 8 SNPs had no corresponding results in the PBC GWAS database. After applying MR-PRESSO, 4 outliers were excluded. The remaining 4 SNPs were then utilized as IVs for MR analysis. Detailed information about these SNPs is provided in Supplementary Table S2. The F-statistics for these SNPs all exceeded 10, indicating an absence of weak IV bias. However, none of the methods used—IVW, MR-Egger, weighted median, and weighted mode—demonstrated evidence of a causal relationship between psoriasis and the risk of PBC (P>0.05) (Table 2). Cochran's Q test revealed no heterogeneity among the IVs, with a Q-statistic of 0.488 (P>0.05). The pleiotropy test using MR-Egger intercept analysis also showed no abnormalities (P>0.05) (Figures 6–9).

4 Discussion

PBC is a chronic autoimmune liver disease, which incidence and prevalence are influenced by various factors, including population, region, socioeconomic status, and environmental conditions. Notably, Europe and North America have higher incidence rates of PBC compared to Asia and Africa. Industrialized or polluted areas, along with certain lifestyle choices such as smoking or using nail polish, are associated with a higher risk of developing PBC (20). Patients with PBC may exhibit various skin symptoms like pruritus, pigmentation, butterfly rash, and xanthoma, which can be linked to skin diseases including vitiligo, psoriasis, and rare sterile pustular dermatosis (21). Research indicates that the prevalence of psoriasis in among PBC patients is higher than that in the general population (22). Therefore, it is crucial for doctors treating PBC patients to also monitor their skin manifestations and assess for concurrent psoriasis, as well as environmental factors that might trigger or exacerbate psoriasis, such as streptococcal infections, stress, smoking, obesity, and alcohol consumption (23). For patients suffering from both PBC and psoriasis, interdisciplinary collaboration among medical professionals is essential for timely diagnosis and optimal treatment. A systematic review and meta-analysis published in 2022 identified several medications effective in alleviating PBC-related pruritus (24), including ursodeoxycholic acid (UDCA), methotrexate (MTX), and GSK2330672, an intestinal bile acid transporter inhibitor. These drugs significantly reduced pruritus scores or provided pruritus relief. A study by Hiromasa Ohira et al. reported on six cases of PBC with psoriasis (25), where three patients had plaque psoriasis and three had palmoplantar pustulosis. Notably, all cases of plaque psoriasis were diagnosed after the onset of PBC, and all six patients were treated with UDCA. Additionally, several other studies (26, 27) have observed that UDCA, the first-line treatment for PBC, also positively impacts psoriasis lesions. These findings highlight the potential for similar etiological factors and therapeutic approaches for both PBC and psoriasis, suggesting a close relationship between these two conditions.

PBC and psoriasis, both chronic immune-related diseases, involve abnormal T cell activation and inflammation. Studies highlight the pivotal role of cytokines such as TNF-α, IL-23, and IL-17 in the development and persistence of psoriasis (1). IL-17, crucial in the psoriasis inflammatory cascade, and IL-23, a key regulator of IL-17A production, influence skin conditions by stimulating keratinocyte overproliferation and T cell-mediated inflammation. Targeted inhibition of these cytokines significantly ameliorates skin symptoms and enhances the quality of life in psoriasis patients (28). As components of the "IL-23/IL-17 axis", these pro-inflammatory cytokines activate inflammatory response effector cells, including neutrophils, macrophages, and fibroblasts. This activation potentially plays a significant role in the pathogenesis of PBC by promoting inflammation (29). However, current literature on the expression differences of IL-23 and IL-17 in PBC patients is limited. Given their roles as key inflammatory factors, IL-23 and IL-17 might impact the skin manifestations

TABLE 1 Mendelian randomization estimates for PBC on psoriasis.

Exposure	Outcome	No. of IVs	Methods	Beta	SE	OR (95%CI)	P
PBC	Psoriasis	19	MR Egger	0.075	0.050	1.078 (0.977~1.190)	0.154
			IVW	0.078	0.026	1.081 (1.028~1.137)	0.002
			Weighted mode	0.078	0.028	1.081 (1.023~1.142)	0.012
			Weighted median	0.080	0.025	1.083 (1.031~1.139)	0.002

IVs, instrumental variables; IVW, inverse variance weighting; SE, standard error; OR, odds ratio; CI, confidence interval.
p < 0.05 was considered statistically significant.

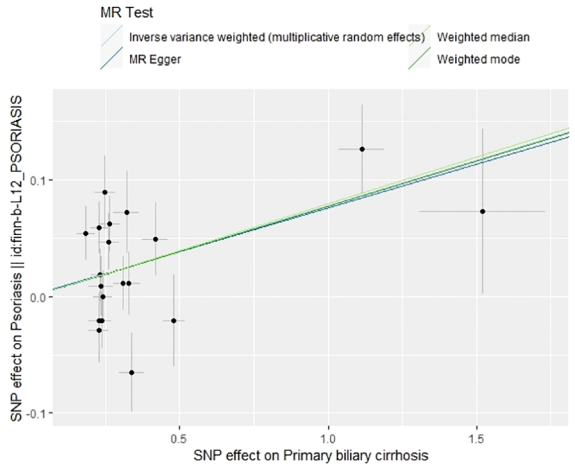

FIGURE 2
Scatter plot of the effect of PBC on psoriasis.

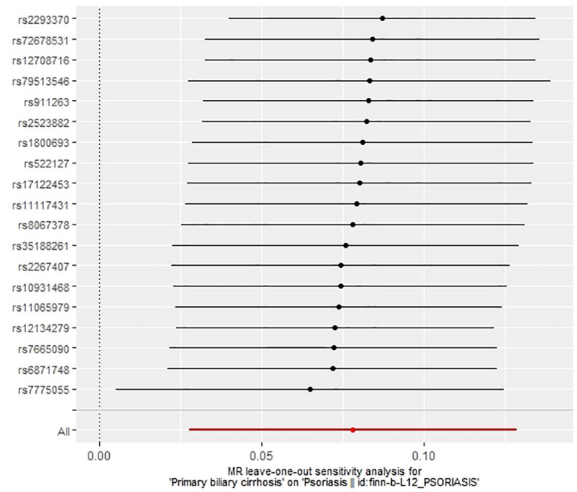

FIGURE 4
Leave-one-out plot of the effect of PBC on psoriasis.

observed in PBC patients. Thus, the specific roles of IL-23 and IL-17 in PBC skin manifestations, and the possibility of shared therapeutic targets between PBC and psoriasis, warrant further experimental research. Even if IL-17 and IL-23 have similar roles in both PBC and psoriasis, other genetic or environmental factors might contribute to the differences observed between these diseases. This asymmetry suggests multiple underlying mechanisms that remain to be investigated through comprehensive research.

PBC and psoriasis, while both chronic immune-related diseases, exhibit distinct pathogeneses and target different organs. PBC primarily affects the liver's small bile ducts, whereas psoriasis predominantly impacts the skin and joints. Additionally, each disease has its unique genetic susceptibilities and risk alleles. Human leukocyte antigen (HLA) alleles are prominent genetic risk factors for autoimmune diseases. PBC's genetic predisposition is influenced by several HLA alleles, including HLA-DRB1, DR3, DPB1, DQA1, and DQB1. In European populations, DQA104:01 is identified as the most significant risk factor, while DQB103:01 serves as the most robust protective factor (30). Conversely, psoriasis's genetic susceptibility is a complex multifactorial issue. Significant alleles include HLA-B57, B37, and C06, with C06 being particularly prevalent in psoriasis patients (up to 57.5%). This allele may influence the onset, severity, and subtypes of psoriasis (31). The environmental factors influencing PBC and psoriasis partially overlap, encompassing various infections and allergens. However, notable differences exist. Psoriasis's development and progression are closely linked to certain drugs, including beta-blockers, lithium salts, synthetic antimalarials, non-steroidal anti-inflammatory drugs, and tetracyclines. These medications can induce or exacerbate psoriasis, or lead to distinct clinical manifestations like psoriatic erythroderma or pustular psoriasis (32). In contrast, PBC's hallmark marker is the anti-mitochondrial antibody (AMA), a crucial diagnostic criterion. AMA may participate in PBC's pathologic process by recognizing mitochondrial antigens on bile duct epithelial cells, triggering an autologous immune response from T and B cells, which

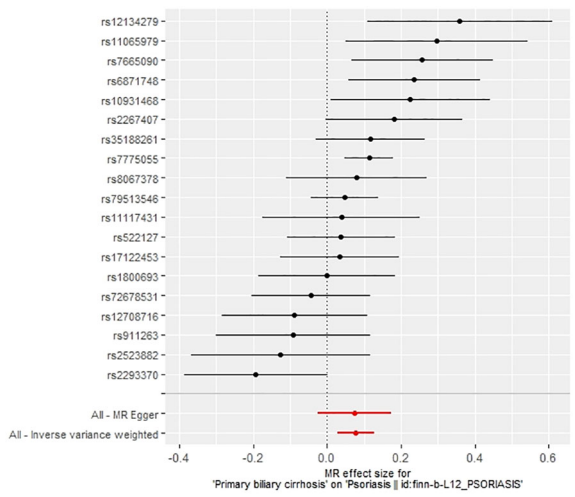

FIGURE 3
Forest plot of the effect of PBC on psoriasis.

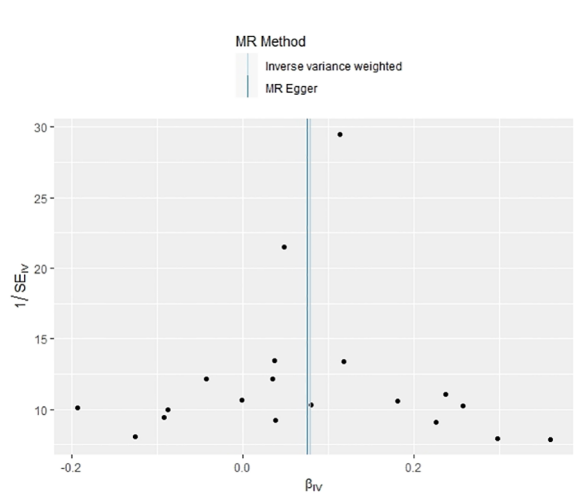

FIGURE 5
Funnel plot of the effect of PBC on psoriasis.

TABLE 2 Mendelian randomization estimates for psoriasis on PBC.

Exposure	Outcome	No. of IVs	Methods	Beta	SE	OR (95%CI)	P
Psoriasis	PBC	4	MR Egger	0.013	0.065	1.013 (0.893~1.150)	0.858
			IVW	0.022	0.046	1.022 (0.935~1.118)	0.633
			Weighted mode	0.015	0.049	1.015 (0.923~1.116)	0.779
			Weighted median	0.016	0.047	1.016 (0.927~1.114)	0.734

IVs, instrumental variables; IVW, inverse variance weighting; SE, standard error; OR, odds ratio; CI, confidence interval.
$p < 0.05$ was considered statistically significant.

leads to bile duct inflammation and liver fibrosis (33). Our study indicates that while psoriasis does not increase the risk of PBC, PBC may elevate the risk of developing psoriasis. This finding suggests that the association between these diseases might not be rooted in autoimmune mechanisms. Instead, it points towards the importance of genetic susceptibility and environmental triggers in their correlation.

A case-control study conducted in two British populations examining risk factors for PBC suggested a potential association between psoriasis and PBC (34). However, this link was not statistically significant in a multivariate analysis and might be influenced by other confounding factors. Notably, the study did not specify the onset or diagnosis time of psoriasis, making it difficult to ascertain whether psoriasis preceded PBC. Consequently, establishing a causal relationship between these two diseases remains uncertain. In an individual case reported by Patricija Tomše et al., a 65-year-old patient first developed psoriatic skin lesions in 2016 and was diagnosed with PBC two years later, after starting biological treatments for psoriasis (35). This patient had a history of slightly elevated liver function indicators for years but had not been tested for autoimmune liver disease, suggesting that her PBC might have been undiagnosed for an extended period. PBC often progresses slowly and may present with subtle or overlooked early symptoms, leading to delayed diagnosis. Based on these observations, it is advisable for patients with psoriasis to undergo regular liver function tests and, if abnormalities persist, to be tested for AMA to ensure timely detection and treatment of PBC. Our study concludes that PBC can causally affect psoriasis, but psoriasis does not cause PBC. This finding underscores the importance of considering skin involvement in PBC management. The implications of this study are manifold: 1. It highlights the necessity for healthcare providers to closely monitor the skin conditions of patients being diagnosed and treated for PBC and to promptly identify and manage

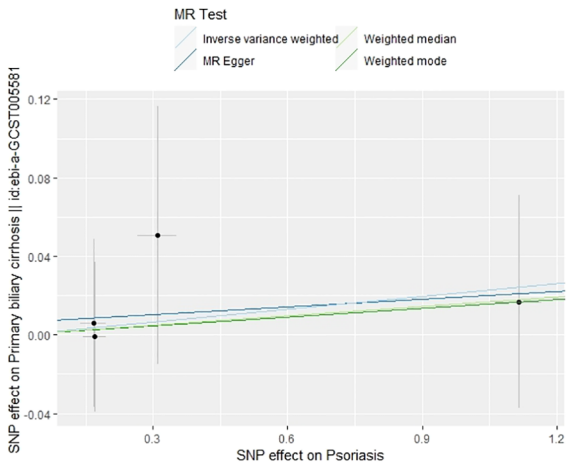

FIGURE 6
Scatter plot of the effect of psoriasis on PBC.

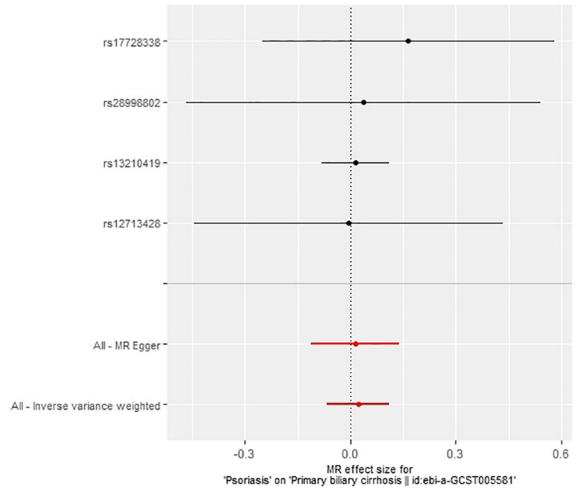

FIGURE 7
Forest plot of the effect of psoriasis on PBC.

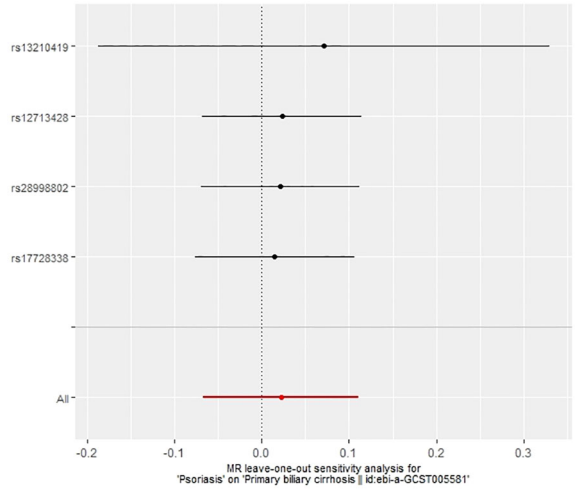

FIGURE 8
Leave-one-out plot of the effect of psoriasis on PBC.

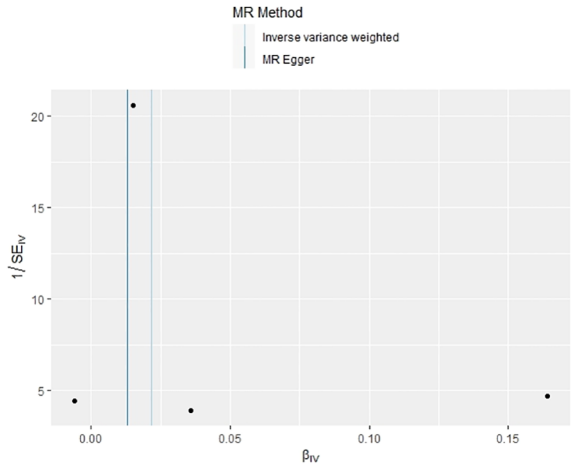

FIGURE 9
Funnel plot of the effect of psoriasis on PBC.

any concurrent psoriasis. 2. The study advises clinicians to be mindful of the potential impact of PBC treatment drugs on psoriasis, steering clear of medications that might exacerbate or trigger psoriatic symptoms. 3. It emphasizes the need to optimize treatment strategies for patients with both PBC and psoriasis, aiming to reduce adverse drug effects on the liver and improve overall quality of life.

However, our study also has limitations that should be addressed in future research. The data we used lacked detailed demographic information, limiting our ability to perform stratified analyses and understand the disease dynamics across different population segments. Our study focused primarily on European populations, which raises questions about the generalizability of our findings to other ethnic groups. Furthermore, the rarity of PBC has led to limited research on its association with psoriasis, indicating a need for more experimental studies, such as animal models or cellular-level investigations, to validate our findings. Notwithstanding these limitations, our study has significant merits. It is the first to use MR analysis to explore the bidirectional causal relationship between PBC and psoriasis. This method reduces the likelihood of confounding bias and reverse causality affecting the results, a common issue in observational studies. Sensitivity analyses were also performed to ensure the consistency and robustness of our causal estimates and findings.

5 Conclusion

Our study reveals that PBC may increase the risk of psoriasis, but psoriasis does not have the same effect on PBC. This identification of a causal association between these disorders provides a new foundation for exploring their common pathogenic mechanisms and potential therapeutic approaches. The insights gained from this study could be valuable in clinical decision-making, potentially improving patient outcomes.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Author contributions

WB: Conceptualization, Formal analysis, Methodology, Software, Supervision, Writing – original draft, Writing – review & editing. DZ: Conceptualization, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. QZ: Data curation, Formal analysis, Methodology, Project administration, Software, Supervision, Validation, Writing – review & editing. FX: Validation, Visualization, Writing – review & editing. FZ: Conceptualization, Formal analysis, Methodology, Software, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1264554/full#supplementary-material

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Clinicopathological and prognostic characteristics of idiopathic membranous nephropathy with dual antigen positivity

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Background: Idiopathic membranous nephropathy (IMN) is the most common pathological type in adults with nephrotic syndrome. Many target antigens have been discovered. However, dual antigen-positive IMN patients are very rare, with only a few such cases being briefly described in various studies. There is no specific study on the clinicopathological and prognostic characteristics of dual antigen-positive IMN patients, and the disease characteristics of such patients remain unclear.

Methods: Immunohistochemical staining of PLA2R, THSD7A, and NELL-1 was conducted on kidney tissue samples obtained from patients diagnosed with IMN. Simultaneously, the presence of corresponding serum antibodies was determined. Patients exhibiting positivity for dual antigens were included in the study, identified either through tissue staining or serum antibody detection. We retrospectively collected their clinical, pathological, and follow-up data and measured their serum antibody levels at multiple time points. Additionally, the same type of dual antigen-positive IMN cases reported in the literature were reviewed to extract clinical, pathological, and prognostic information. We compared the data for all of the above dual antigen-positive and PLA2R single-positive IMN cases at our center.

Results: We identified 6 IMN patients with dual antigen positivity at our center, approximately 0.7% of whole MN series; the previous literature reports 43 IMN patients with dual antigen positivity, the proportion ranged from 0.2% to 2.8%. The IgG1 positivity rate in the renal tissue of the dual antigen-positive patients at our center was significantly lower than that of dual antigen-positive patients previously reported (16.7% vs. 100.0%, p=0.015), but there was no significant difference in clinical or prognostic aspects. Patients with dual antigen positivity reported at our center and in the

literature were combined and compared with PLA2R single-positive IMN reported at our center. Compared with PLA2R single-positive IMN patients, dual antigen-positive IMN patients had a higher renal tissue IgG1 positivity rate (58.3% vs. 22.3%, p=0.016), and the time required to achieve remission was longer [13.5 (3.3,35.0) vs. 3.0 (1.0,8.0), p=0.052]. Overall, The changes in urine protein were consistent with the changes in serum PLA2R antibody levels in dual antigen-positive IMN patients.

Conclusions: For patients with primary membranous nephropathy who did not attain remission following prolonged treatment, multiple target antigen staining should still be actively performed, even with positivity for the PLA2R target antigen.

KEYWORDS

idiopathic membranous nephropathy, PLA2R, THSD7A, NELL-1, dual antigen

1 Introduction

Idiopathic membranous nephropathy (IMN) is an immune-mediated primary glomerular disease and the most common pathological type in adults with nephrotic syndrome (1). Research has confirmed that the main pathogenesis of IMN is specific binding of circulating antibodies to target antigens on the glomerular basement membrane to form immune complexes that are deposited in the subepithelial area, activating the complement cascade, causing podocyte damage, and ultimately leading to proteinuria (2). Since M-type anti-phospholipase A2 receptor (PLA2R), the first specific target antigen in adult IMN, was discovered in 2009 (3) research on IMN target antigens has been growing rapidly. Indeed, many IMN target antigens have been discovered over the past decade, including thrombospondin type 1 domain containing 7A (THSD7A) and neuroepidermal growth factor-like type 1 protein (NELL-1) (4, 5). However, the vast majority of IMN patients reported thus far are single antigen-positive; in contrast, dual antigen-positive IMN patients are very rare, with only a few such cases being briefly described in various studies (6–8). There is no specific study on the clinicopathological and prognostic characteristics of dual antigen-positive IMN patients, and the disease characteristics of such patients remain unclear.

Here, we provide a detailed description of the clinical pathological characteristics and prognosis of dual antigen-positive IMN patients at our center. In addition, we reviewed previous literature on dual antigen-positive IMN cases, extracted patient information, and compared the clinical and pathological data of dual antigen-positive IMN cases reported thus far with those of PLA2R single-positive IMN cases in an effort to help clinicians further understand this rare dual antigen-positive IMN.

2 Materials and methods

2.1 Information collection of dual antigen-positive IMN and PLA2R single-positive IMN patients at our center

We continuously reviewed patients diagnosed with IMN by renal biopsy in the Department of Nephrology, Beijing Anzhen Hospital, Capital Medical University from 2015 to 2019. By staining of PLA2R, THSD7A, NELL-1 antigen in kidney tissue and detection of corresponding serum antibodies, we screened the dual antigen-positive IMN and PLA2R single-positive IMN, diagnosed by positive tissue staining or positive serum antibodies. Their baseline clinical, pathological and prognostic information were retrospectively collected. Follow-up data were obtained by reviewing the medical records and/or from telephone interviews of patients, and they were available on some patients. The treatment and prognostic analyses were performed in patients with complete information on follow-up. The definitions of remission complied with the 2012 Kidney Disease Improving Global Outcomes guideline for glomerular nephropathy (9). Complete remission was defined as urinary protein excretion <0.3 g/d, confirmed by two values at least 1 week apart, accompanied by normal serum albumin and creatinine levels. Partial remission was defined as urinary protein excretion <3.5 g/d and at least a 50% reduction from peak values accompanied by an improvement or normalization of

serum albumin and stable serum creatinine levels. Complete remission and partial remission are collectively referred to as remission. Worsening of renal function was defined as a doubling of creatinine.

2.2 Immunohistochemical staining of renal tissue in dual antigen-positive IMN patients at our center

Paraffin-embedded kidney tissue (4 μm) was obtained. PLA2R and NELL-1 antigens were retrieved with pH 6 citrate solution combined with trypsin; THSD7A was retrieved with pH 9 EDTA solution. The primary antibodies used were monoclonal rabbit anti-human PLA2R1 antibody (Sigma, HPAO12657) (1:800 dilution), monoclonal rabbit anti-human NELL-1 antibody (Sigma, HPAO51535) (1:400 dilution), and monoclonal rabbit anti-human THSD7A antibody (Sigma, HPAO00923) (1:3000 dilution), which were incubated overnight at 4°C. The secondary antibody, alkaline phosphatase-labeled immunohistochemistry reagent (Max Vision, KIT-5103) or horseradish peroxidase-labeled immunohistochemistry reagent (Max Vision, KIT-5004), was added and incubated at room temperature for 30 minutes. Fast-Red reagent (Zhongshan Jinqiao, ZLI-9042) or DAB reagent (Zhongshan Jinqiao, ZLI-9018) was used for color development. Antigen positive is judged as granular brown staining in the glomerular capillary loops, while the renal tubule and other background staining linear reduction (10).

2.3 Other pathological staining and electron microscope examination of renal tissue

The routine fluorescent staining and histochemical staining are carried out in accordance with the standard procedures and the recommended procedures of the reagent specification. Fluorescence intensity criteria: there is no light at both low and high magnification as "-"; negative at low magnification, seemed to be visible at high magnification as "±"; it seems to be visible at low magnification, and blurred at high magnification as "+"; it is obviously visible at low magnification and clearly visible at high magnification as "++"; clearly visible at low magnification and dazzling fluorescence at high magnification as "+++": dazzling at low magnification and dazzling fluorescence at high magnification as "++++". Electron microscope examination of renal tissue was delivered to the testing center.

2.4 Antibody detection in serum of dual antigen-positive IMN patients at our center

Serum samples were collected on the day of kidney biopsy and stored at -80°C until use. Serum anti-PLA2R antibodies were detected using the ELISA (EUROIMMUN, Germany) method. Serum anti-THSD7A and anti-NELL-1 antibodies were detected using an indirect immunofluorescence assay kit (EUROIMMUN, Germany) according to the standard protocol.

2.5 Systematic review of previous reports on IMN patients with dual antigen positivity

We searched for "M-type anti-phospholipase A2 receptor", "thrombospondin type 1 domain containing 7A", and "neuroepidermal growth factor-like type 1 protein" in the three major English databases Medline, Embase, and Cochrane Library and the two major Chinese databases Wan fang and CNKI (the specific search strategies are provided in Supplementary Material). All literature related to dual antigen-positive IMN was screened as of June 6, 2023, and literature information and clinicopathological and prognostic data of dual antigen-positive patients were extracted.

2.6 Statistical methods

The continuous variables of normal distribution were represented by mean ± standard deviation, and the difference between groups was compared by independent sample t test. Continuous variables with non-normal distributions were expressed as median and quartile distances (P25, P75), and non-parametric tests were used to compare group differences. The number and percentage of categorical variables were expressed, and Chi-square test and Fisher exact test were used to compare the differences between groups. SPSS 23.0 software was used for statistical analysis, and bilateral P value <0.05 was considered statistically significant.

3 Results

3.1 Clinicopathological characteristics and prognosis of dual antigen-positive IMN patients at our center

A total of 827 patients diagnosed with IMN by renal biopsy at our center from 2015 to 2019, with an average age of 48 years old, accounted for 63.6% of males. Through tissue antigen staining and serum antibody detection, 6 patients with dual antigen positivity were screened, including 3 PLA2R- and NELL-1-positive patients and 3 PLA2R- and THSD7A-positive patients. Detection of tissue antigens and serum antibodies in the 6 patients is shown in Table 1.

The clinical data of the 6 patients are shown in Table 1. The median age was 55.5 years old, and 4 patients were older than 45 years. Four patients (66.7%) had significant proteinuria, of whom

TABLE 1A Clinicopathological and prognostic characteristics of 6 IMN patients with dual antigen positivity at our center.

		PLA2R		NELL-1		THSD7A	
		tissue antigens	serum antibodies(RU/ml)	tissue antigens	serum antibodies(RU/ml)	tissue antigens	serum antibodies(RU/ml)
PLA2R- and NELL-1-positive							
	MN1	+	12.80	+	–	–	–
	MN2	+	0.00	+	–	–	–
	MN3	+	125.10	+	–	–	–
PLA2R- and THSD7A-positive							
	MN4	+	41.49	–	–	+	–
	MN5	+	0.00	–	–	+	–
	MN6	+	225.50	–	–	+	–

TABLE 1B Clinicopathological and prognostic characteristics of 6 IMN patients with dual antigen positivity at our center.

Clinical features									Pathological features					
Sex	Age (year)	Diabetes	Tumor	24h-urinary protein (g/24h)	ALB (g/L)	Scr (μmol/L)	eGFR(mL/(min·1.73 m^2))	ANA	Stage	Grading of renal interstitial injury	Mesangial hyperplasia	IgA	IgG	IgM
M	68	Yes	No	5.17	28.60	59.20	98.75	weak +	II	1	No	–	3+	–
M	46	No	No	3.70	29.30	71.60	106.59	–	I	1	No	–	3+	–
F	65	No	No	5.18	33.50	57.00	93.71	–	II	1	No	–	3+	–
F	77	Yes	No	4.80	15.70	97.40	48.45	–	I	1	Yes	–	2+	–
M	29	No	No	1.23	41.70	93.00	95.23	–	II	0	No	–	2 +-3+	–
M	44	No	No	2.40	24.50	71.00	108.47	NA	II	0	No	–	3+	–

TABLE 1C Clinicopathological and prognostic characteristics of 6 IMN patients with dual antigen positivity at our center.

Pathological features					
IgG1	IgG2	IgG3	IgG4	C3	Electron dense deposit site
3+	–	–	3+	1+-2+	subepithelial

(Continued)

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TABLE 1C Continued

Pathological features					
IgG1	IgG2	IgG3	IgG4	C3	Electron dense deposit site
–	–	–	2+	–	subepithelial
–	–	–	2+-3+	2+	subepithelial
–	–	–	2+	–	subepithelial
1+-2+	–	–	2+	–	subepithelial, basement membrane
–	–	–	3+	2+	subepithelial

TABLE 1D Clinicopathological and prognostic characteristics of 6 IMN patients with dual antigen positivity at our center.

Prognostic characteristics					
Treatment	Remission	Duration of remission (m)	Relapse	Progress of renal function	Duration of follow-up (m)
CsA	Yes	4	Yes	No	70
CsA	Yes	20	No	No	38
GCs, CsA	Yes	42	NA	No	42
CsA	No	–	–	No	48
NA	No	–	–	No	1
GCs, CTX	Yes	36	NA	No	36

PLA2R, M-type anti phospholipase A2 receptor; NELL-1, Neuroepidermal growth factor like type 1 protein; THSD7A, Thrombospondin type 1 domain containing 7A; ALB, serum albumin; Scr, serum creatinine; eGFR, estimated glomerular filtration rate; TG, triglycerides; TCHO, total cholesterol; M, male; F, female; Renal interstitial injury grading: grade 0, no renal interstitial fibrosis; Grade 1, the range of renal interstitial fibrosis was 1-25%; Grade 2, the range of renal interstitial fibrosis was 26-50%; Grade 3, extent of renal interstitial fibrosis > 50%; Immunofluorescence positive, fluorescence intensity ≥2+; CsA, Cyclosporin; MMF, Mycophenolate Mofetil; CTX, Cyclophosphamide; GCs, Glucocorticoids; RTX, Rituximab; NA, not afford.

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only 3 (50%) presented with nephrotic syndrome (NS); 1 patient (MN4) had a slight increase in blood creatinine. To date, no concomitant tumor diseases have been detected during follow-up of the 6 patients, but the tumor markers of patient MN1 continued to increase during the course of the disease (Supplementary Figure 1).

Typical IMN manifestations under light microscopy were observed for these six patients, with all cases showing subepithelial deposition of fuchsinophilic protein. Only one IMN patient with PLA2R and THSD7A positivity (MN4) exhibited mild mesangial cell proliferation and increased mesangial matrix in the glomerulus. Immunofluorescence showed IgG particles deposited along the basement membrane in all 6 patients, without any other immunoglobulin deposits. IgG subclass staining for all 6 patients mainly showed IgG4; only one PLA2R- and NELL-1-positive patient (MN1) showed the same fluorescence intensity as for IgG1 and IgG4. The results of electron microscopy were consistent with those of light microscopy. In all 6 patients, electronic dense matter was found to be deposited in the subepithelial area of basement membrane area of the glomerulus (Table 1, Figure 1).

The median follow-up time of the 6 patients was 40 months. Except for patient 5, whose treatment regimen we did not track, all patients received immunosuppressive therapy. Four of the patients achieved disease remission after treatment, but only 1 patient (MN1) achieved it within half a year. Of the 2 patients (MN1, MN2) with follow-up data after remission, 1 patient experienced relapse, whereas remission was maintained in the other patient. None of the patients had worsening of renal function during follow-up (Table 1).

3.2 Literature review and information extraction of previous reports on dual antigen-positive IMN patients

We conducted a systematic review of the literature on previous studies involving double antigen-positive IMN. By June 6, 2023, a total of 11 studies (7, 11–20) involving 43 dual antigen-positive IMN patients, were included (Supplementary Table 1, Supplementary Figure 2). Of the 43 patients, highly detailed information was available for only 11, and we extracted clinical, pathological, and prognostic information for these 11 patients (Table 2) and then performed comparisons with the 6 dual antigen-positive IMN patients at our center (Supplementary Table 2).

3.3 Comparison of clinicopathological features and prognosis between dual antigen-positive IMN patients and PLA2R single-positive IMN patients

To explore the difference between dual antigen-positive IMN and PLA2R single-positive IMN, we compared the two groups of patients. However, because the number of PLA2R single-positive IMN at our center is much larger than the number of dual antigen-positive IMN in the same time period, we only continuously enrolled 141 patients with PLA2R single-positive IMN within one year (2018-2019)of this time period. The comparison of

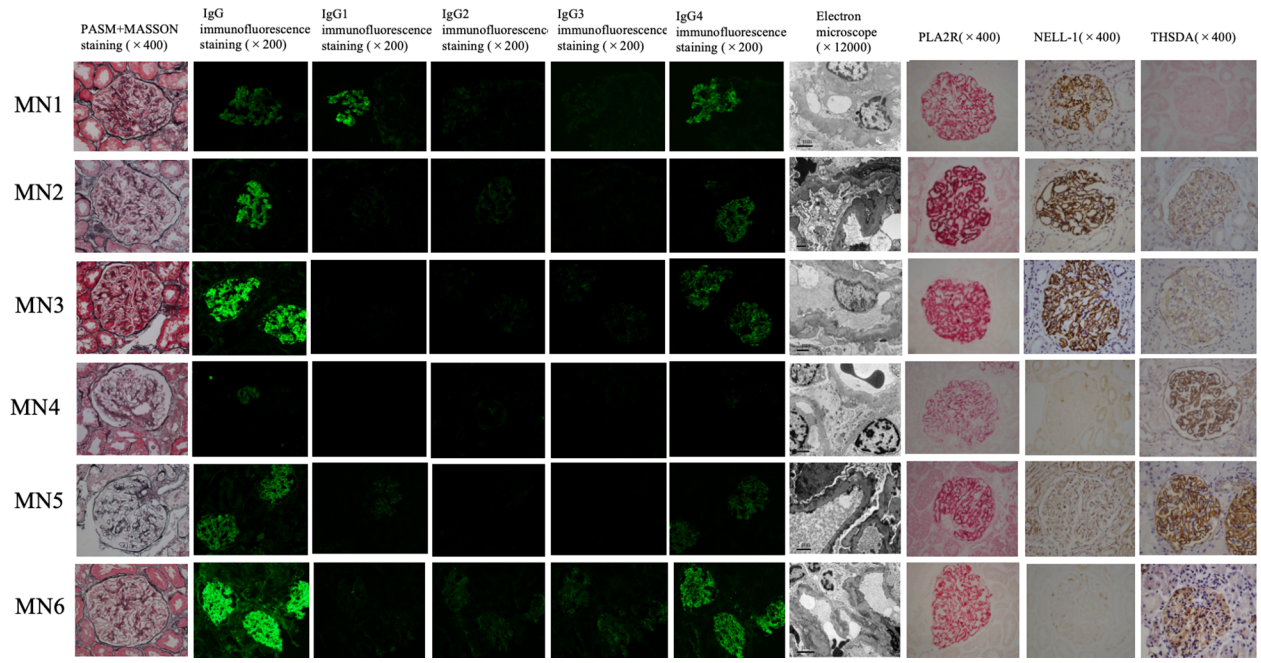

FIGURE 1
Renal tissue staining of 6 dual antigen-positive IMN patients at our center. From left to right, there are PASM+MASSON staining (×400), IgG immunofluorescence staining (×200), IgG1 immunofluorescence staining (×200), IgG2 immunofluorescence staining (×200), IgG3 immunofluorescence staining (×200), IgG4 immunofluorescence staining (×200), Electron microscope (×12000), PLA2R immunohistochemistry staining (×400), NELL-1 immunohistochemistry staining (×400), THSD7A immunohistochemistry staining in renal tissue. Among the 6 cases,MN1– MN3 are positive for PLA2R and NELL-1 staining; MN4-MN6 are positive for PLA2R and THSD7A staining.

TABLE 2 Clinicopathological and prognostic characteristics of 11 cases of dual antigen-positive IMN patients in previous studies.

Study	Case no.	PLA2R		NELL-1		THSD7A		Clinical features								
		tissue antigens	serum antibodies	tissue antigens	serum antibodies	tissue antigens	serum antibodies	sex	Age (year)	Diabetes	Tumor	24h-urinary protein (g/24h)	ALB (g/L)	Scr (μmol/L)	eGFR(mL/(min·1.73 m^2))	ANA
Hara 2019	1	+	NA	NA	NA	+	NA	M	68	NA	NA	11.6	NA	102.54	NA	NA
Zhang 2019	2	+	+	NA	NA	+	–	M	51	NA	+	NA	NA	NA	NA	NA
Zaghrini 2019	3	+	+	NA	NA	+	+	M	65	NA	NA	6	28	52.16	158	NA
	4	+	+	NA	NA	+	+	M	48	NA	NA	4	25	46.85	192	NA
Subramanian 2020	5	+	NA	NA	NA	+	NA	M	50	NA	NA	NA	NA	NA	NA	NA
	6	–	+	NA	NA	+	NA	M	37	NA	NA	NA	NA	NA	NA	NA
Wanderley 2020	7	+	NA	NA	NA	+	NA	M	46	NA	–	4	15	76.02	NA	–
Xue 2020	8	+	+	NA	NA	+	NA	M	35	–	–	4.9	20.7	124	NA	–
	9	+	NA	NA	NA	+	NA	M	72	NA	–	5.3	20.3	135	NA	–
Yeter 2021	10	+	NA	NA	NA	+	NA	M	18	NA	NA	2.5	NA	53.04	147	NA
Inoue 2023	11	NA	+	NA	+	NA	NA	M	70	NA	–	7.65	22	NA	NA	NA

Pathological features												Treatment	Prognostic characteristics				
Stage	Grading of renal interstitial injury	Mesangial hyperplasia	IgA	IgG	IgM	IgG1	IgG2	IgG3	IgG4	C3	Electron dense deposit site		Remission	Duration of remission (m)	Relapse	Progress of renal function	Duration of follow-up (m)
NA	NA	–	–	positive	–	2+	–	–	2+	–	NA	GCs, ARB	Yes	32	No	No	32
NA	NA	NA	–	positive	–	positive	NA	NA	positive	positive	NA	FK506	No	–	–	Yes	22
II	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	ACEI/ARB	Yes	3	No	No	40
II	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	ACEI/ARB	No	–	–	No	3
NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
II	NA	+	positive	positive	NA	positive	NA	NA	positive	positive	subepithelial, mesangial region	ACEI/ARB	No	–	–	No	2
II	1	NA	–	3+	–	2+	–	–	3+	2+	subepithelial	GCs, CTX	Yes	7	No	NA	18
II	1	NA	–	2+	–	2+	–	–	3+	2+	subepithelial	GCs, CTX	NA	NA	NA	NA	NA
NA	0	+	NA	NA	NA	NA	NA	NA	positive	NA	NA	GCs, CsA, MMF,RTX	No	NA	NA	Yes	40
I	NA	–	–	2+	–	2+	–	–	–	–	subepithelial	GCs	Yes	1	No	NA	18

PLA2R, M-type anti phospholipase A2 receptor; NELL-1, Neuroepidermal growth factor like type 1 protein; THSD7A, Thrombospondin type 1 domain containing 7A; ALB, serum albumin; Scr, serum creatinine; eGFR, estimated glomerular filtration rate; TG, triglycerides; TCHO, total cholesterol; M, male; F, female; Renal interstitial injury grading: grade 0, no renal interstitial fibrosis; Grade 1, the range of renal interstitial fibrosis was 1-25%; Grade 2, the range of renal interstitial fibrosis was 26-50%; Grade 3, extent of renal interstitial fibrosis > 50%; Immunofluorescence positive, fluorescence intensity ≥2+; Positive staining in fluorescent staining that did not describe the fluorescence intensity was indicated by us as "positive"; CsA, Cyclosporin; MMF, Mycophenolate Mofetil; CTX, Cyclophosphamide; GCs, Glucocorticoids; RTX, Rituximab; NA, not afford.

clinicopathology and prognosis of the two groups at our center is shown in Supplementary Tables 3, 4. There was no significant difference in clinicopathology and prognosis between the two groups. Since the number of IMN patients with dual antigen positivity is small, meanwhile, we compared the baseline clinical and pathological data of all known dual antigen-positive IMN patients, including the 6 cases from our center and the 11 cases in the literature with complete clinical and pathological data, with 141 PLA2R single-positive patients at our center (Table 3). The results showed that the dual antigen-positive patients had a higher IgG1-positive rate in renal tissue than the PLA2R single-positive patients (58.3% vs. 22.3%, p=0.016). We also conducted a meta-analysis of baseline urine protein and serum albumin comparison results between 17 dual antigen-positive patients and the 141 PLA2R single-positive patients (Supplementary Figures 3, 4), with no significant difference in baseline urine protein and blood

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TABLE 3 Comparison of clinical and pathologic data between all dual antigen-positive IMN patients and PLA2R single-positive IMN patients at our center.

Characteristics	IMN with dual antigen positivity (n=17)	IMN with PLA2R single positivity (n=141)	P-value
Male(%)	15 (88.2)	91 (64.5)	0.050
Age(year)	50.0 (40.5, 68.0)	53.0 (44.3, 63.0)	0.910
Hypertension (%) (n=152)	6 (54.5)	80 (56.7)	1.000
Diabetes(%) (n=147)	2 (28.6)	22 (15.7)	0.708
24h-urinary protein (g/24h)	4.9 (3.4, 5.5)	4.9 (3.0, 8.3)	0.510
ALB(g/L)	24.8 (20.4, 29.1)	25.9 (22.1, 32.1)	0.404
Scr(μmol/L)	71.6 (55.0, 99.9)	68.0 (57.1, 79.1)	0.439
eGFR(mL/(min·1.73 m^2))	106.6 (94.5, 152.5)	102.3 (86.7, 116.0)	0.340
Pathological stage(n=151)			0.264
I	3 (25.0)	35 (26.0)	
II	9 (75.0)	75 (55.5)	
III	0	25 (18.5)	
Crescent body (%)(n=152)	0	4 (2.8)	1.000
Renal interstitial injury grading(n=151)			0.257
0	3 (27.3)	16 (11.4)	
1	8 (72.7)	97 (69.3)	
2	0	24 (17.1)	
3	0	3 (2.1)	
IgG positive(%)(n=153)	12 (100)	140 (99.3)	1.000
IgG1 positive(%)(n=151)	7 (58.3)	31 (22.3)	0.016
IgG2 positive(n=149)	0	8 (5.8)	1.000
IgG3 positive(%)(n=148)	0	9 (6.5)	1.000
IgG4 positive(%)(n=152)	12 (92.3)	117 (84.2)	0.705
Simultaneous positivity of IgG1 and IgG4 (%)	6 (50.0)	29 (20.9)	0.053
IgM positive(%)(n=152)	0	17 (12.1)	0.468
IgA positive(%)(n=153)	1 (8.3)	21 (14.9)	0.847
C3 positive(%)(n=153)	6 (50)	108 (76.6)	0.092
C1q positive(%)(n=152)	0	4 (2.8)	1.000

ALB, serum albumin; Scr, serum creatinine; eGFR, estimated glomerular filtration rate.

albumin between the two groups. Prognosis comparison between the two groups showed a longer time to achieve remission in the patients with dual antigen positivity than in those with PLA2R single positivity, even though this difference did not reach statistical significance (p=0.052) (Table 4).

TABLE 4 Comparison of prognosis between all dual antigen-positive IMN patients and PLA2R single-positive IMN patients at our center.

Characteristics	IMN with dual antigen positivity (n=17)	IMN with PLA2R single positivity (n=62)	P-value
Remission(%)(n=76)	8 (57.1)	43 (69.4)	0.530
Duration of remission(m)	13.5 (3.3, 35.0)	3.0 (1.0, 8.0)	0.052
Remission within six months(%)(n=54)	3 (27.3)	23 (53.5)	0.120
Worsening of renal function(%)(n=74)	2 (16.7)	6 (9.7)	0.608
Immunosuppressive therapy(%)(n=74)	11(78.6)	46(76.7)	1.000

3.4 Follow-up of serum antibodies in dual antigen-positive IMN patients

Serum antibody levels were measured at multiple nodes of disease change for three of the patients at our center (MN1, MN2, and MN4) (Figure 2). The condition of MN1 was protracted, starting with NS. After 4 months of treatment, partial remission was achieved, and the serum anti-PLA2R antibody titer gradually decreased. After 4 years, relapse of NS occurred, and the serum anti-PLA2R antibody titer continued to increase by >1500 RU/ml. Urinary protein remained stable, but his serum anti-NELL-1 antibody remained negative throughout the disease course. MN2 did not achieve complete remission until 21 months of immunosuppressive therapy, and there was no recurrence thereafter. During the course of the disease, the patient's serum anti-PLA2R antibodies and anti-NELL-1 antibodies remained negative. After 4 months of immunosuppressive therapy, the condition of MN4 did not improve. The serum anti-PLA2R antibody titer decreased only slightly from 41.49 RU/ml to 30 RU/ml, and serum was negative for anti-THSD7A antibody.

4 Discussion

This is the first study focusing on dual antigen-positive IMN patients and describes in detail the clinicopathological and prognostic characteristics of dual antigen-positive IMN patients at our center. The clinical, pathological and prognosis of dual antigen-positive IMN patients reported thus far are also summarized. This study provides contributes to our understanding of such IMN patients.

This study showed that the clinical and pathological manifestations of dual antigen-positive IMN patients did not differ significantly from those of general IMN patients. It is currently believed that THSD7A- and NELL-1-positive MN may be associated with tumors (21, 22). Among all reported dual antigen-positive IMN cases, only one patient with PLA2R and THSD7A positivity had concurrent tumors (13). None of the six patients at our center had tumors in the past or during follow-up. However, due to the limited number of dual antigen-positive IMN patients, more cases need to be analyzed. Nevertheless, it is worth

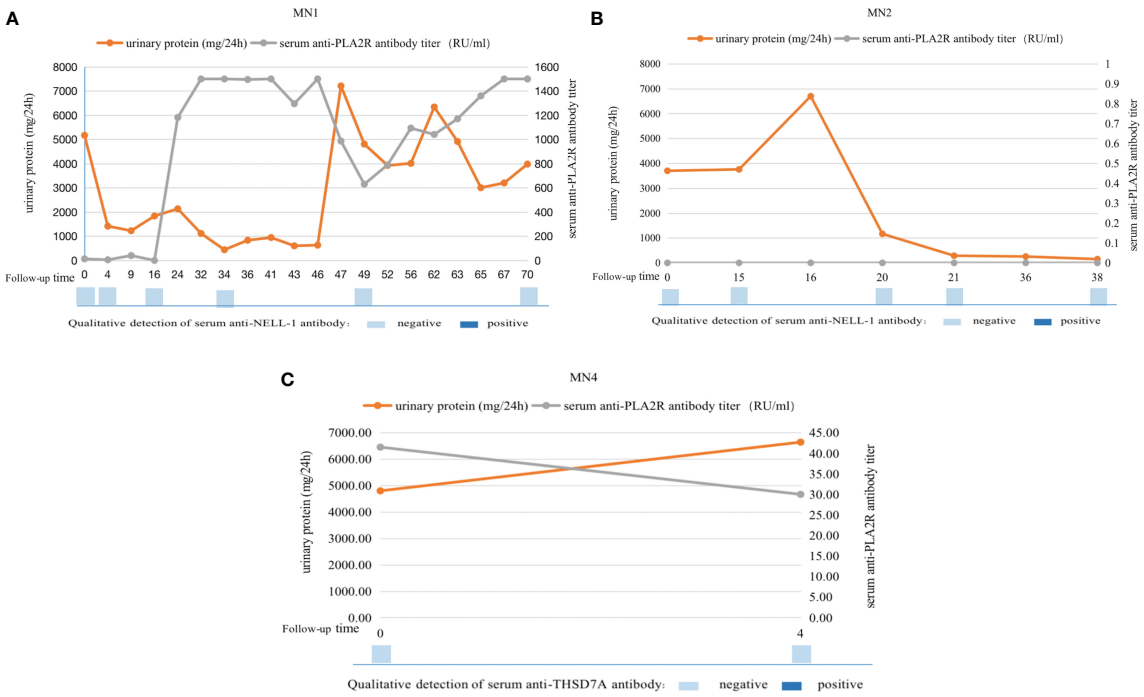

FIGURE 2
Serum antibody levels were measured at multiple nodes of disease change for three of the patients at our center. MN1 patient (PLA2R- and NELL-1-positive) achieved remission after treatment, but relapsed 46 months later. His serum anti-PLA2R antibody titers were consistent with changes in urine protein, but his serum anti-NELL-1 antibody remained negative throughout the disease course **(A)**; MN2 patient (PLA2R- and NELL-1-positive) achieved complete remission after treatment and there was no recurrence thereafter. His serum anti-PLA2R antibody titers continued to be 0R/ml and serum anti-NELL-1 antibodies continued to be negative **(B)**; MN4 patient (PLA2R- and THSD7A-positive) did not improve after 4 months of treatment, with a slight decrease in serum anti-PLA2R antibody titers and continuous negative serum anti-THSD7A antibodies **(C)**.

mentioning that one patient with PLA2R and NELL-1 positivity (MN1) at our center displayed continuous mild increases in CA199 and CEA during follow-up, even though no evidence of tumor was found clinically. After standard treatment, the PLA2R antibody titer remained high, and NS continued. The fluorescence intensity of IgG1 in the renal tissue of this patient was also strong, which suggests that we need to continue to consider other secondary diseases, such as tumors, during follow-up.

In this study, the time to achieve remission after standard treatment was longer in dual antigen-positive IMN than in PLA2R single-positive IMN. Although the difference did not reach statistical significance, it may be related to the small number of cases. Moreover, the positive rate of IgG1 subclasses in the renal tissues of dual antigen-positive IMN patients was higher. Previous studies have shown that IgG4 subclasses predominate in IMN glomeruli with PLA2R or THSD7A single positivity and that IgG1 subclasses predominate in IMN glomeruli with NELL-1 single positivity (23). IgG subclasses are associated with the complement activation pathway. Does the difference in IgG subclasses in the renal tissue of dual antigen-positive IMN correlate with a more refractory prognosis? Is the type and extent of complement activation in the renal tissue of dual antigen-positive IMN different from that of single antigen-positive IMN? Further studies are needed to address these questions.

Based on previous studies of PLA2R-positive MN and THSD7A-positive MN, it is known that the titer of serum antibodies is closely related to the clinical severity and disease change of patients with kidney disease (24, 25). An increase in serum anti-PLA2R antibody may precede aggravation of proteinuria to indicate the possibility of disease recurrence (26, 27). However, are the effects of the two antigens consistent in dual antigen-positive IMN? Which antibodies can help to determine a change in the disease? There are no studies or reports on these issues to date. In this study, 6 patients with dual antigen-positive IMN at our center were tested for serum antibodies. Among them, we only detected PLA2R antibodies (66.7%), whereas THSD7A and NELL-1 antibodies were not detected. The low positive rate of serum antibody detection may be related to the kidney-as-a-sink effect, in which antibodies do not "overflow" the glomeruli into the serum to be detected at the beginning of the disease (28). Literature shows that in a considerable part of IMN patients, serum antibodies are detected later than tissue antigens (29). Three patients (MN1, MN2, MN4) underwent multiple tests of serum anti-PLA2R antibodies during the course of their disease, and levels of serum anti-PLA2R antibodies in MN1 and MN4 were consistent with the progression of their condition. Among the 11 dual antigen-positive cases reported in the literature, only 2 patients with PLA2R and THSD7A positivity underwent serum antibody testing during disease progression (8). In these cases, a gradual decrease in urine protein as the titers of serum anti-PLA2R and THSD7A antibodies declined occurred in one patient; in the other patient, both serum anti-PLA2R antibody titers and urine protein decreased as titers of

serum anti-THSD7A antibodies increased during the disease course. These results suggest that PLA2R antibody levels in dual antigen-positive IMN may correlate well with the condition. A recent study (30) that confirmed that serum anti-PLA2R antibodies can directly induce podocyte damage independently of the complement system also provides some theoretical support for this idea. However, due to the small number of cases, the role of multiple antigens in kidney tissue needs to be clarified in more cases and through further clinical and basic research.

At present, there is no relevant study on the mechanism of the occurrence of dual antigen-positive IMN. The PLA2R, THSD7A and NELL-1 antigens differ in structure and expression position in normal kidneys. Hence, we postulate that the likelihood of an interaction between the two proteins is diminished due to the apparent dissimilarity among the three antigens. However, it has been reported that THSD7A-positive MN and NELL-1-positive MN occur under the action of tumor, drugs and other secondary factors. Is there an underlying cause of THSD7A and NELL-1 positive in kidney tissue? We will also continue to closely monitor these patients and will explore the possible mechanisms of the presence of dual antigens in subsequent basic studies.

This study has certain limitations. Although we reviewed all previous studies that reported dual antigen-positive IMN, there were still very few cases with detailed information. It is necessary to expand the number of cases of multiple antigen-positive patients in larger MN cohorts and with longer follow-up observations.

In summary, this study suggests no significant specificity in clinical and pathological manifestations for patients with dual antigen-positive IMN. Compared with PLA2R single-positive IMN patients, dual antigen-positive IMN patients have a higher IgG1 positivity rate in renal tissue, and a longer is needed to achieve remission after standardized treatment. Among the two antigens, PLA2R may correlate more with the disease, and serum anti-PLA2R antibody levels may be associated with disease progression. For IMN patients who experience poor treatment efficacy, multiple antigen staining should still be actively performed even if PLA2R is positive. For double antigen-positive IMN, continuous tracking of secondary causes and the possibility of tumors is necessary.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human/animal participants were reviewed and approved by the ethics committee of Beijing Anzhen Hospital, with an ethics approval number of 2023168X. Written informed consent was obtained for sampling.

Author contributions

LY: Data curation, Formal Analysis, Software, Writing – original draft, Investigation, Methodology. GW: Writing – review & editing, Conceptualization. NY: Supervision, Writing – review & editing, Methodology. XX: Data curation, Writing – original draft. WC: Data curation, Writing – original draft. LS: Methodology, Writing – original draft, Resources. HD: Methodology, Writing – original draft, Resources. LK: Methodology, Writing – original draft, Resources. XZ: Data curation, Writing – original draft. YG: Data curation, Writing – original draft. HC: Writing – review & editing, Supervision.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1297107/full#supplementary-material

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