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A year in review: Discussions in autoimmune and autoinflammatory disorders

Edited by
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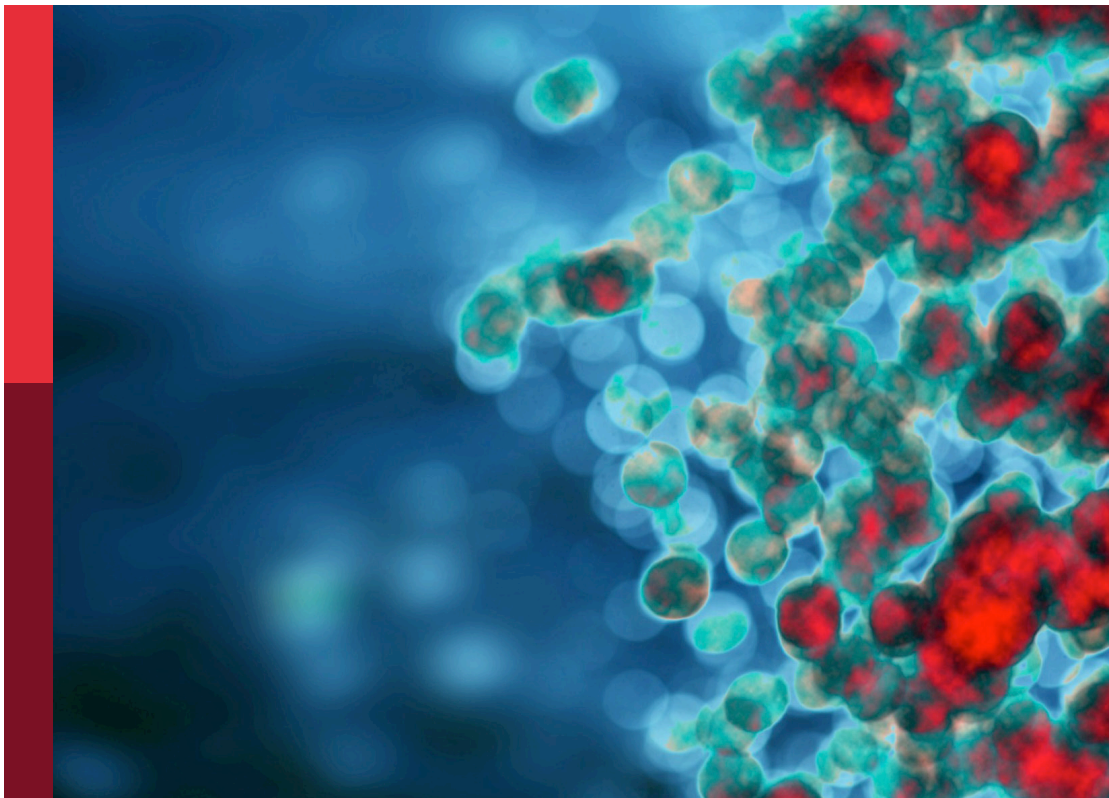
Published in
Frontiers in Immunology



ISSN 1664-8714
ISBN 978-2-83251-938-7
DOI 10.3389/978-2-83251-938-7

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A year in review: Discussions in autoimmune and autoinflammatory disorders

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Citation

Goldbach-Mansky, R., Diamond, B., eds. (2023). *A year in review: Discussions in autoimmune and autoinflammatory disorders*. Lausanne: Frontiers Media SA.

doi: 10.3389/978-2-83251-938-7

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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 22 July 2022
DOI 10.3389/fimmu.2022.907729

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Autoimmune and Autoinflammatory Disorders, a section of the journal Frontiers in Immunology

RECEIVED 30 March 2022
ACCEPTED 27 June 2022
PUBLISHED 22 July 2022

CITATION
Li B, Su R, Yan H, Liu J, Gao C, Li X and Wang C (2022) Immunological risk factors for nonalcoholic fatty liver disease in patients with psoriatic arthritis: New predictive nomograms and natural killer cells.
Front. Immunol. 13:907729.
doi: 10.3389/fimmu.2022.907729

Immunological risk factors for nonalcoholic fatty liver disease in patients with psoriatic arthritis: New predictive nomograms and natural killer cells

Baochen Li[1], Rui Su[1], Huanhuan Yan[1], Juanjuan Liu[2], Chong Gao[3], Xiaofeng Li[1] and Caihong Wang[1]*

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Objective: To search for the immunological risk factors of Psoriatic arthritis (PsA) combined with nonalcoholic fatty liver disease (NAFLD), development and assessment of predictive nomograms for NAFLD risk in patients with PsA, and to further explore the correlation between risk factors and dyslipidemia.

Methds: A total of 127 patients with PsA (46 with NAFLD and 81 without NAFLD) were included in this retrospective study. The clinical and serological parameters of the patients were collected. The percentage and the absolute number of lymphocytes and CD4+T cells were determined by Flow cytometry. Univariate and multivariate binary logistic regression analysis was used to screen independent risk factors of PsA complicated with NAFLD in the model population, and a nomogram prediction model was developed and assessed.

Results: (1) Univariate and multivariate logistic regression analysis of the modeling population showed that the percentage of peripheral blood T helper 1 cells (Th1%) (OR=1.12, P=0.001), body mass index (BMI) (OR=1.22, P=0.005) and triglycerides (TG) (OR=4.78, P=0.003) were independent risk factors for NAFLD in patients with PsA, which were incorporated and established a nomogram prediction model. The model has good discrimination and calibration, and also has certain clinical application value. (2) The number of peripheral blood NK cells in PsA patients was significantly positively correlated with serum triglyceride (TG) (r=0.489, P<0.001),

cholesterol (CHOL) (r=0.314, P=0.003) and low-density lipoprotein (LDL) (r=0.362, P=0.001) levels.

Conclusions: Our study shows that the novel NAFLD nomogram could assess the risk of NAFLD in PsA patients with good efficiency. In addition, peripheral blood NK cell levels may be associated with dyslipidemia in patients with PsA.

KEYWORDS

psoriatic arthritis, nonalcoholic fatty liver disease, nomogram, dyslipidemia, T helper-1 cells, natural killer cells

Introduction

Psoriatic arthritis (PsA) is a chronic, immune-mediated inflammatory joint disease characterized by progressive inflammatory destruction of the axial and peripheral joints. In recent years, the inflammatory process of the pathophysiological changes of PsA has become the focus of attention. Chronic low-grade systemic inflammation associated with skin inflammation is thought to underlie many comorbidities of PsA, including cardiovascular disease, metabolic syndrome, and nonalcoholic fatty liver disease (NAFLD) (1).

NAFLD is a liver disease characterized by hepatic steatosis, with a global incidence of about 25%, and is currently one of the leading causes of liver cirrhosis and liver transplantation (2). A recent study found that even mild fatty liver disease increased a patient's risk of all-cause death by 71%, and the risk was proportional to the severity of fatty liver disease (3). NAFLD is not only associated with increased liver-related mortality or morbidity but is a multisystem disease affecting multiple extrahepatic organs, including the heart and vascular system. Cardiovascular disease (CAD) is considered in some respects to be an inflammatory disease with an inflammatory pathway similar to NAFLD and is the leading cause of death in NAFLD (4, 5). Visceral adipose tissue promotes eotaxin release from atherosclerotic vascular smooth muscle cells by releasing IL-17, so IL-17 may be a key regulator of local inflammation in CAD lesions and a significant risk factor for CVD (6). According to histological form, NAFLD is divided into simple steatohepatitis and nonalcoholic steatohepatitis (NASH). NASH is a potentially progressive disease that causes cirrhosis in 12-25% of cases within 10 years, significantly increasing the risk of advanced liver disease (7). About 65% of patients with PsA have NAFLD (8). Compared with patients with PsA alone, patients with NAFLD tend to have more severe clinical symptoms, and NAFLD is significantly correlated with Psoriasis (PsO) lesions and disease severity index (PASI). In contrast, PsO, especially PsA, is an important predictor of NAFLD severity. When PsO is present, the likelihood of advanced NAFLD increases by approximately 60%, and progression to NASH is more likely. Furthermore, PsA is even a risk factor for advanced liver fibrosis (9). The pathogenesis of PsA combined with NAFLD is not fully understood, and previous studies have attributed it to a metabolic syndrome caused by factors such as dyslipidemia. Recent studies suggest that chronic low-level systemic inflammation is a common cause of both.

Similar to other autoimmune diseases, a variety of CD4+ T cells and their secreted cytokines are involved in the pathogenesis of PsA. Preliminary studies suggest that PsA is a Th1 cell-mediated disease as Th1 cells and their secretion of interferon-gamma (INF-γ) are significantly increased in skin lesions (10). Likewise, Th17 cells and their secreted cytokine interleukin 17A (IL-17A) are not only present in skin lesions (11), but also have an increased frequency in diseased joints (12). In addition to helper T cells, innate immune cells, especially NK cells, are also closely involved in the pathogenesis of PsA. INF-γ produced by NK cells in the skin is a potent factor for Th17 cell migration into skin lesions. IL-17 producing NK cells are also increased in synovial fluid from patients with undifferentiated spondyloarthropathy closely related to PsA (13).

An increasing number of studies have shown that NAFLD is not a simple liver disease and that the immune system is one of the main drivers of disease progression (14), each stage of NAFLD is accompanied by the accumulation of T cells and NK cells with different functions and phenotypes (15). For example, clinical and animal studies have shown that NAFLD patients have significantly increased liver and peripheral blood Th1 cells and their secreted cytokines IFN-γ, IL-12, and TNF-α (16). In addition, the number of Th17 cells in the liver and peripheral blood of NAFLD patients was also significantly increased (17), which not only mediates liver inflammation and liver injury but also has a significant effect on liver fibrosis (18). NK cells account for 30-55% of the total number of hepatic lymphocytes. Recent studies have found that the number of NK cells in NAFLD patients is reduced (19, 20), while the number of NK cells in the liver of NASH patients is significantly increased (21), which promotes the occurrence and development of liver

fibrosis (22, 23), accelerates disease progression and leads to an increased risk of advanced liver disease.

Therefore, PsA and NAFLD share similar underlying inflammatory pathways, and inflammatory cells and pro-inflammatory cytokines associated with PsA may be involved in the occurrence and development of NAFLD through a common chronic inflammatory pathway. However, there is currently no research on the role of inflammatory cells in the pathogenesis of NAFLD and whether they are also involved in abnormal metabolic processes such as lipid disorders. In this study, by looking for the differences in peripheral blood lymphocyte subsets and CD4+T cells in PsA-NAFLD patients, we identified the immunological high-risk factors of PsA patients complicated with NAFLD, development, and assessment of predictive nomograms for NAFLD risk in patients with PsA, and to further explore the correlation between risk factors and dyslipidemia. Early identification and scientific management of risk factors can not only reduce the incidence of NAFLD and the risk of NAFLD-induced advanced liver disease but also effectively control the disease and improve the quality of life of patients.

Materials and methods

Clinical date

Our study collected 127 patients with PsA admitted to the Rheumatology Department of the Second Hospital of Shanxi Medical University from August 2016 to June 2021, including 53 males and 74 females, with an average age of 46.5 ± 13.26 years. All patients were diagnosed according to the CASPAR classification diagnostic criteria revised in 2006 (24). The diagnosis of NAFLD mainly relies on liver ultrasound. In addition, 56 healthy controls were recruited from the physical examination center of our hospital. Patients with other autoimmune diseases, serious infections especially viral hepatitis, tumors, and drug-induced hepatitis were excluded. Exclusion criteria also included alcohol consumption > 20 g/day, recreational drug use, and exposure to environments known to induce hepatic steatosis. Clinical and laboratory data, including blood cell analysis, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), liver enzymes, bilirubin, lipids, and immunoglobulins, were retrospectively collected from patients and healthy controls. All blood samples were collected on an empty stomach on the morning of admission. According to the presence or absence of NAFLD, the patients were divided into two groups, the PsA group (PsA) and PsA combined NAFLD group (PsA-NAFLD). This study was approved by the Ethics Committee of the Second Hospital of Shanxi Medical University (2019YX114). A written informed consent was obtained from every participant.

Flow cytometry for absolute counts of peripheral blood lymphocytes and CD4+T cell subsets

Whole blood samples from patients with PsA were collected in heparin anticoagulant tubes, and the peripheral blood mononuclear cell suspension was prepared by Ficoll-hypaque density gradient centrifugation. Different antibodies were added in turn for the combined staining of peripheral blood lymphocyte subsets and CD4+T cells. The staining protocol for peripheral blood lymphocyte subsets is as follows: anti-CD3-FITC/CD8-PE/CD45-PercP/CD4-APC antibody is used to label T lymphocytes, anti-CD3-FITC/CD16+56-PE/CD45-PercP/CD19-APC antibody is used to label B lymphocytes and NK cells. CD4+T cell subsets were stained and identified by the following protocols: anti-CD4-FITC/IFN-γ-APC (intracellular staining) for Th1 cells and anti-CD4-FITC/IL-4-PE (intracellular staining) for Th2 Cells, anti-CD4-FITC/IL-17-PE (intracellular staining) to detect Th17 cells, anti-CD4-FITC/CD25-APC/FOXP3-PE (intracellular staining) to identify and detect Treg cells. All immunofluorescent antibodies were purchased from BD Biosciences (BD Biosciences, Franklin Lakes, NJ, USA), blood samples were mixed, incubated, and washed according to the manufacturer's recommendations, and stained samples were detected within 24 hours using FACSCalibur flow cytometer and BD Multitest software (BD Biosciences, Franklin Lakes, NJ, USA).

Statistical analysis

Statistical analysis was performed using SPSS software (version 23.0; SPSS Inc., Chicago, IL, USA) and R software (4.1.2, USA). Count data were tested using the chi-square goodness-of-fit test. Measurement data were measured using the Kolmogorov-Smirnov test and Levene t-test; mean ± standard deviation was used to express normality and homogeneity of variance. The independent samples t-test was used for comparison between two groups, and the analysis of variance was used for comparison between groups. Non-normally distributed data are expressed as median (M) and interquartile range and tested using the Kruskal-Wallis H test. Correlation analysis was performed using Pearson's or Spearman's correlation. Univariate and multivariate binary logistic regression analysis was used to screen independent risk factors for PsA combined with NAFLD, and a nomogram prediction model was constructed using these risk factors. The discriminative power of the model was evaluated by the area under the receiver operating characteristic curve (AUROC) and the C-index; the calibration of the model was evaluated by the calibration curve and the Hosmer-Lemeshow test; the DCA curve was used to verify the clinical validity of the model.

Results

Comparison of demographic and clinical features and laboratory data between the PsA group and PsA-NFFLD group

A total of 127 patients with PsA were included in this study, including 53 male patients and 74 female patients. The mean age of the patients was 46.5 ± 13.26 years. The main demographic characteristics, disease characteristics, and serological parameters of the two groups of patients are shown in Table 1. There were no significant differences between the PsA group and the PsA-NAFLD group in terms of sex, age, disease course, and family history. The BMI of PsA-NAFLD patients was significantly higher than that of PsA patients. Diabetes and hypertension are risk factors for NAFLD. The prevalence of hypertension in PsA-NAFLD was 30.43%, which was significantly higher than that in the PsA group (12.35%). In

TABLE 1 Clinical characteristics of PsA group and PsA-NAFLD group.

	PsA (n=81)	PsA-NAFLD (n=46)	*p*
Demographics			
Age (years)[a]	45.62 ± 13.25	48.07 ± 13.27	0.320
Male n (%)	50 (61.72%)	24 (52.17)	0.389
Female n (%)	31 (38.27%)	22 (47.83%)	
BMI [a]	22.63 ± 3.83	25.97 ± 4.57	< 0.001
Disease duration (month) [b]	25.00 (4.00-108.00)	25.00 (6.88-88.50)	0.730
Family history of PsA (%)	18 (22.22%)	10 (21.74%)	1
Risk factors for NAFLD			
Smoking n = (%)	11 (13.58%)	10 (21.74%)	0.347
Drinking n = (%)	7 (8.64%)	6 (13.04%)	0.63
Hypertension n (%)	10 (12.35%)	14 (30.43%)	0.023
Diabetes n (%)	4 (4.94%)	4 (8.7%)	0.647
Current use of medication			
NSAIDs n (%)	69 (85.19%)	38 (82.61%)	0.897
DMARDs n (%)	43 (53.09%)	26 (56.52%)	0.851
Methotrexate n (%)	26 (32.10%)	16 (34.78%)	0.910
Leflunomide n (%)	7 (8.64%)	3 (6.52%)	0.933
Sulfasalazine n (%)	13 (16.05%)	8 (17.39%)	0.998
TNF-α inhibitors n (%)	33 (40.74%)	25 (54.35%)	0.196
Laboratory Characteristics			
ESR (mm/h) [b]	30.50 (18.00-68.00)	26.50 (14.50-58.50)	0.380
CRP (mg/ml) [b]	9.65 (3.33-24.63)	11.50 (3.13-26.00)	0.640
Complete blood count			
WBC ($*10^9$/L) [b]	6.14 (4.78-7.17)	6.83 (5.70-8.14)	0.029
Hb (g/L) [a]	125.00 (116.00-137.50)	135.50 (121.00-151.25)	0.004
PLT ($*10^9$/L) [b]	260.00 (206.50-321.50)	261.00 (211.50-308.00)	0.840
LY ($*10^9$/L) [b]	1.71 (1.37-2.04)	2.01 (1.63-2.20)	0.077
Liver Function Test			
ALT (U/L) [b]	14.50 (10.65-21.05)	17.95 (12.9-25.60)	0.040
AST (U/L) [b]	17.5 (14.20-21.50)	17.65 (13.98-23.33)	0.760
GGT (U/L) [b]	17.80 (12.80-25.40)	24.20 (17.70-45.40)	0.013
TBIL(μmol/L) [b]	10.50 (8.80-13.50)	12.60 (9.75-16.10)	0.040
TG (mmol/L) [b]	1.09 (0.81-1.43)	1.47 (1.11-1.81)	< 0.001
CHOL (mmol/L) [b]	3.82 (3.19-4.68)	4.34 (3.73-4.87)	0.020
HDL (mmol/L) [b]	1.10 (0.95-1.36)	1.04 (0.86-1.25)	0.090
LDL (mmol/L) [b]	2.09 (1.75-2.68)	2.56 (2.16-2.94)	0.005
Immunoglobulin			
IgG (g/L) [b]	12.40 (10.60-14.50)	10.10 (11.70-13.80)	0.150

(Continued)

TABLE 1 Continued

	PsA (n=81)	PsA-NAFLD (n=46)	*p*
IgA (g/L) [b]	2.94 (2.12-3.69)	2.51 (1.99-3.38)	0.140
IgM (g/L) [b]	0.95 (0.75-1.31)	0.91 (0.73-1.21)	0.095

[a]Results are expressed as the mean ± standard deviation.
[b]Results are expressed as the median and 25th and 75th percentiles.
BMI, body mass index; DMARDs, disease-modifying antirheumatic drugs; NSAID, nonsteroidal anti-inflammatory drug; ESR: erythrocyte sedimentation rate; CRP, C-reactive protein; WBC, white blood cells; Hb, Hemoglobin; PLT, platelets; LY, lymphocytes; ALT, Alanine transaminase; AST, Aspartic transaminase; GGT, Gamma-glutamyltransferase; TBIL, Total bilirubin; TG, Triglycerides; CHOL, Cholesterol; LDL, Low-density lipoprotein; HDL, High-density lipoprotein; IgG, Immunoglobulin G; IgA, Immunoglobulin A; IgM, Immunoglobulin M.

addition, there were no significant differences in medication use between the two groups. The use rate of tumor necrosis factor inhibitor (54.35%) was higher in the PsA-NAFLD group, but the difference was not statistically significant.

By comparing the differences in blood cell analysis between the two groups, it was found that the peripheral blood white blood cell count and hemoglobin content in the PsA group were significantly lower than those in the PsA-NAFLD group. In addition, there were significant differences in liver function-related indicators between the two groups. First, the partial liver enzymes (Alanine transaminase, Gamma-glutamyltransferase) and total bilirubin in the PsA-NAFLD group were significantly higher than those in the PsA group. Since the main diagnosis of NAFLD in this study is based on abdominal ultrasound, it is difficult to accurately determine the degree of NAFLD lesions, so we speculate that the reason for the elevated liver enzymes in PsA-NAFLD patients may be that some patients progress to early NASH. Second, the blood lipid levels in the PsA-NAFLD patients were higher, especially the levels of triglycerides, cholesterol, and low-density lipoprotein were significantly higher than those in the PsA group.

Peripheral Th1 cells and NK cells were elevated in PsA-NAFLD patients

We compared the numbers and percentages of peripheral blood lymphocyte subsets and CD4+ T cell subsets between the two groups of PsA patients and healthy controls. Compared with the healthy control group, the number of total T cells (P=0.001) and percentage (P<0.001), the number of total B cells (P=0.050), the number of CD4 +T cells (P<0.001), and the percentage (P<0.001), Th1 cell number (P<0.001) Th17 cell number (P<0.001) and percentage (P=0.001), Th17/Treg (P<0.001) all increased in different degrees. The percentage of NK cells (P<0.001) and Treg cells (P<0.001) in the healthy control group were significantly higher than those in the two groups of PsA patients (Tables 2A, B, Figure 1A, B).

In addition, we compared the number and percentage of peripheral blood lymphocyte subsets and CD4+T cell subsets in the two PsA groups and found that the number (P<0.001) and percentage (P=0.001) of peripheral blood Th1 cells, Th17 cells (P=0.037), Th1/Th2 ratio (P=0.021), NK cell numbers (P=0.007) and percentages (P=0.041) were significantly increased in the PsA-NAFLD group (Tables 2A, B, Figures 1A, B).

Development of the nomogram for prediction of nonalcoholic fatty liver disease in psoriatic arthritis

The 127 patients with PsA included in this study were divided into the modeling population (102 cases) and the validation population (25 cases) according to the ratio of 8:2 by the random number table method. The auxiliary examination results and other data were compared (P>0.05), the difference was not statistically significant, and the two groups were comparable. Univariate Logistic regression was used to screen the risk factors of NAFLD in PsA patients. The clinical indicators with the statistical difference (P<0.05) are shown in Figure 2. Risk factors with P<0.01 in the univariate logistic regression were included in the multivariate logistic stepwise regression analysis. The results showed that in addition to traditional risk factors such as BMI and TG, peripheral blood Th1% was also an independent risk factor for NAFLD in patients with PsA (Table 3). The above three independent risk factors were incorporated into the prediction model, and the individualized nomogram prediction model of PsA complicated with NAFLD was established (Figure 3A). Considering that peripheral blood Th1% is not a traditional risk factor for NAFLD, we removed Th1% from the above prediction model, re-established a new prediction model 2, and compared the test performance of the two models (Table 3).

Validation of the nomogram

First, we compared the discriminability between the two models. The AUC of Model 1 was 0.83 (95%CI: 0.76~0.90), and the C-index was 0.83. Model 2 had an AUC of 0.79 (95% CI: 0.70–0.87) and a C-index of 0.79, which was worse than the aforementioned models (Table 3, Figure 3B). This suggests that peripheral blood Th1% in PsA patients plays an important role in predictive models of NAFLD onset.

The discriminability of the model was verified by plotting the ROC curves of the two groups of people. The AUC of the modeling population was 0.83 (95% CI: 0.76-0.90), the C-index was 0.83 (Figure 3E), and the AUC of the validation population was 0.81 (95% CI: 0.57-0.87), and the C-index was 0.81 (Figure 3F), the C-index of the prediction model in both populations is > 0.75, and the model has good discriminative power.

TABLE 2 Absolute counts and proportions of lymphocytes in the peripheral blood in the study participants.

(A)	PsA (n=81)	PsA-NAFLD (n=46)	Health (n=56)	*p*
Total T (cells/μL)	1308.00 1099.51-1659.50	1401.34 1135.48-1675.87	1144.22 1011.73-1357.42	0.001
T%	76.32 71.81-79.38	74.16 69.31-78.49	71.00 64.35-75.17	< 0.001
Total B (cells/μL)	198.11 145.29-278.52	209.50 163.22-323.48	168.00 133.65-220.00	0.050
B%	11.86 9.15-14.14	11.85 7.98-16.27	10.61 8.15-13.91	0.463
NK (cells/μL)	180.00 109.97-238.39	226.01 146.96-371.80	257.00 185.48-377.56	< 0.001
NK%	10.57 6.97-13.19	11.81 7.00-18.68	15.56 12.00-21.29	< 0.001
CD4+T cells (cells/μL)	788.00 640.12-980.00	841.57 694.48-1034.15	613.86 508.65-703.90	< 0.001
CD4+T%	45.40 40.47-49.45	43.28 36.95-47.51	36.63 31.57-41.75	< 0.001
CD8+T cells (cells/μL)	454.16 364.17-639.76	533.21 402.30-687.92	432.11 326.66-555.43	0.090
CD8+T%	26.78 22.65-31.36	26.54 21.88-31.75	26.33 22.00-31.09	0.920
CD4+T/CD8+T	1.72 1.33-2.17	1.62 1.27-1.95	1.39 1.02-1.88	0.039
(B)	**PsA (n=81)**	**PsA-NAFLD (n=46)**	**Health (n=56)**	***p***
Th1 (cells/μL)	114.07 79.16-157.78	163.00 119.66-226.69	90.09 51.45-124.76	< 0.001
Th1%	14.63 10.21-22.00	20.48 13.99-25.07	15.44 8.65-17.56	0.001
Th2 (cells/μL)	7.81 5.29-13.02	7.65 4.81-12.18	6.17 3.79-9.68	0.040
Th2%	1.06 0.73-1.42	0.82 0.65-1.33	0.99 0.72-1.55	0.538
Th17 (cells/μL)	7.93 4.86-11.74	11.40 5.10-19.24	4.56 3.42-7.15	< 0.001
Th17%	1.13 0.66-1.47	1.41 0.68-2.11	0.68 0.52-1.21	0.001
Treg (cells/μL)	27.02 19.76-39.96	26.31 20.66-40.73	30.46 24.43-41.79	0.354
Treg%	3.60 2.48-4.67	3.06 2.35-4.60	5.28 4.20-6.35	< 0.001
Th1/Th2	13.90 8.25-23.95	19.79 12.26-31.22	12.74 7.78-19.96	0.011
Th17/Treg	0.29 0.19-0.46	0.47 0.18-0.73	0.14 0.10-0.25	< 0.001

T, T lymphocyte; B, B lymphocyte; NK, natural killer cell.
Th1, T-helper 1 cells; Th2, T-helper 2 cells; Th17, T-helper17 cells; Treg, regulatory T cells.

Next, we plotted the calibration curve of the diagnostic model. The prediction curve of the model is close to the actual observation curve, indicating that the calibration ability of the model is good. The Hosmer-Lemeshow test was further performed on the diagnostic model, and its P-value was 0.658 (P>0.05). The difference was not statistically significant (Figure 3C). Furthermore, by plotting the clinical decision curve (DCA), it was shown that the Cutoff value (37.0%) obtained by the ROC analysis (Figure 3E) was within the threshold probability range of the DCA curve. Further analysis showed that when 37.0% was set as the threshold probability of diagnosing PsA with NAFLD, 21 out of 100 PsA patients at risk for NAFLD diagnosed using this model could benefit from it without harming others (Figure 3D). Therefore, the nomogram

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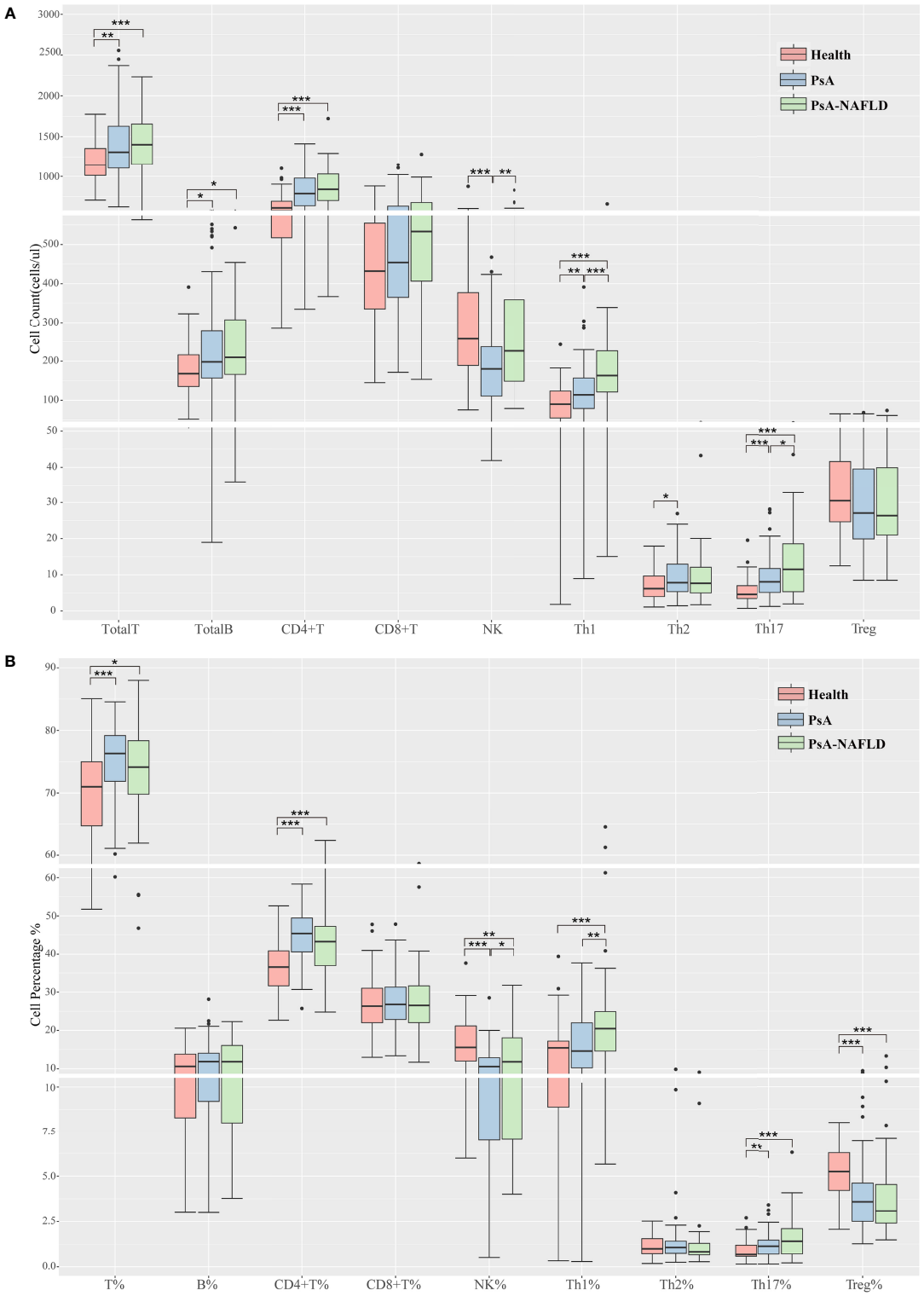

FIGURE 1
(A). Comparison of peripheral blood lymphocyte subsets and CD4+T cell counts among each study group. **(B)**. Comparison of the proportion of peripheral blood lymphocyte subsets and CD4+ T cells among each study group. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

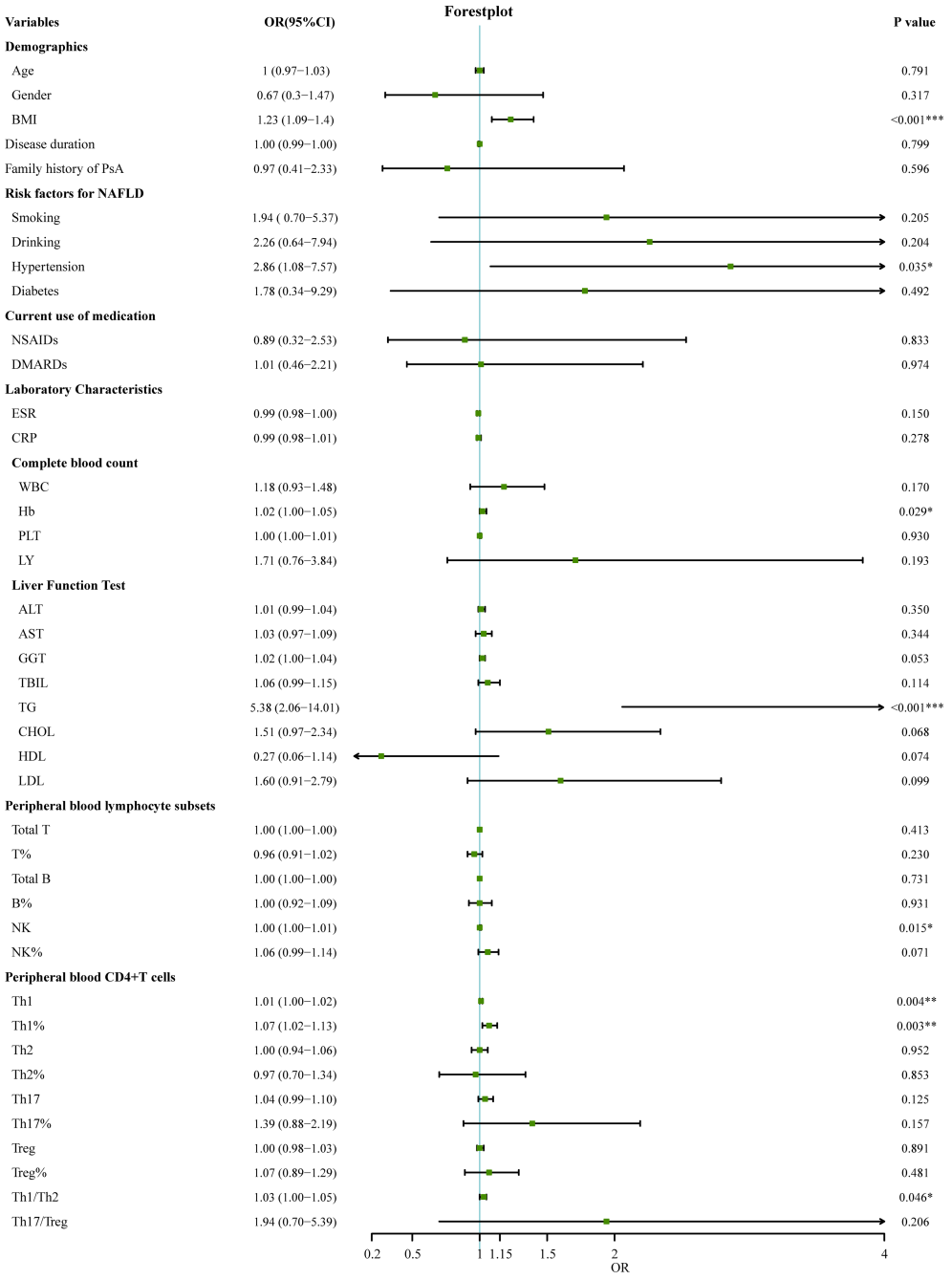

FIGURE 2
Univariate logistic regression analyses for factors associated with the presence of NAFLD in PsA patients. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). OR, odds ratio; 95%CI:95% confidence interval; BMI, Body Mass Index; DMARDs, disease-modifying antirheumatic drugs; NSAID, nonsteroidal anti-inflammatory drug; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; WBC, white blood cells; Hb, Hemoglobin; PLT, platelets; LY, lymphocytes; ALT, Alanine transaminase; AST, Aspartic transaminase; GGT, Gamma-glutamyltransferase; TBIL, Total bilirubin; TG, Triglycerides; CHOL, Cholesterol; LDL, Low-density lipoprotein; HDL, High-density lipoprotein; T, T lymphocyte; B, B lymphocyte; NK, natural killer cell; Th1, T-helper 1 cells; Th2, T-helper 2 cells; Th17, T-helper17 cells; Treg, regulatory T cells.

TABLE 3 Univariate logistic regression analyses for factors associated with the presence of NAFLD in PsA patients.

	Model1 Odds Ratio (95% CI)	*P*	Model2 Odds Ratio (95% CI)	*P*
BMI	1.22 (1.07-1.42)	0.005**	1.18 (1.05-1.35)	0.008**
TG	4.78 (1.84-14.41)	0.003**	4.08 (1.66-11.91)	0.004**
Th1%	1.12 (1.04-1.19)	0.001**	–	–
AUC	0.83 (0.76-0.90)		0.78 (0.70-0.87)	
C-Index	0.83		0.79	

BMI, Body Mass Index; TG, Triglycerides; Th1, T-helper 1 cells; AUC, Area Under the ROC Curve. (**p < 0.01).

of the prediction model has a better prediction effect on the risk of NAFLD in PsA patients and has a certain clinical application value.

Peripheral blood NK cell levels are associated with dyslipidemia in patients With PsA

Dyslipidemia is a traditional risk factor for NAFLD. Our study also showed that the levels of some blood lipids (TG, CHOL, LDL) were significantly higher in the PsA-NAFLD group than in the PsA group. Further analysis of the correlation between common lipids in serum of PsA patients and peripheral blood lymphocyte subsets revealed that the number and percentage of peripheral blood NK cells were positively correlated with CHOL, TG, and LDL levels. Among them, the correlation between the number of NK cells and the level of TG is more obvious (Figures 4A, B). This suggests that NK cells may be involved in the occurrence of dyslipidemia in PsA patients.

In addition, we also found that the numbers of Th1 cells, Th2 cells, Th17 cells, and Treg cells in the peripheral blood of PsA patients were all correlated with certain blood lipids, but the correlation was weak (Figure 4A).

Discussion

Previous research on PsA has focused on the skin and joints. In the past ten years, with the in-depth study of the comorbidities of PsA patients, the pathophysiological relationship between PsA and cardiovascular disease, metabolic syndrome, insulin resistance, and mental illness has gradually been recognized. The occurrence of PsA-related comorbidities and their impact on mortality have received increasing attention from scholars (9, 25). Many recent studies have shifted the focus of PsA complications to the liver, especially NAFLD (26, 27). Because NAFLD is not only highly prevalent in patients with PsA, but more importantly, when PsA and NAFLD co-occur, the severity of both diseases may increase significantly. Therefore, early identification and intervention of NAFLD in PsA patients are particularly important.

This retrospective study analyzed the characteristics of peripheral blood lymphocyte subsets and CD4+T cells in patients with PsA and PsA-NAFLD and used univariate and multivariate Logistic regression to find high-risk immunological factors for NAFLD. The results of the analysis showed that Th1% was an independent risk factor for NAFLD. Combined with other risk factors BMI and TG, a nomogram prediction model of NAFLD risk in PsA patients was established and validated, hoping to predict and identify NAFLD early. Furthermore, the relationship between lymphocyte subsets in peripheral blood and serum lipid profile was further discussed to seek the correlation between immune cells and lipid metabolism abnormalities.

PsA is a chronic inflammatory arthritis associated with PsO. Many studies have shown that both PsA and PsO are chronic inflammations mediated by abnormal T cells, and various pro-inflammatory cytokines play key roles in their development (28). Interactions between T-cell subsets, stromal cells, and cytokines in the local microenvironment determine disease characteristics, including colitis, synovitis, bone and cartilage loss, and new bone formation in the axial and peripheral musculoskeletal systems (29). PsA was originally thought to be a Th1 cell-mediated disease. Since the level of Th1 cells and their secreted INF-γ in skin lesions was significantly increased, *in vitro* experiments showed that IFN-γ could enhance the proliferation of keratinocytes (30). And in successfully treated PsO patients, the levels of Th1 cells in skin lesions and peripheral blood were significantly reduced (31). Recent studies suggest that Th17 cells and their secreted IL-23/IL-17 play a central role in the pathogenesis of PsA, similar to other autoimmune diseases (32). The levels of Th17 cells and IL-17 in the skin lesions of PsA patients were much higher than those in healthy skin, especially IL-17A played a key role in maintaining PsO plaque inflammation, and IL-17A mRNA levels correlated with disease activity. Th17 cells and IL-17 levels are also elevated in the synovial fluid of patients with PsA (33). Randomized clinical trials show that IL-17A and IL-17F antibodies have good efficacy and are potential therapeutic targets (34). Notably, these two types of CD4+T cells promote and activate each other during the pathogenesis of PsA. IFN-γ secreted by Th1 cells can induce Th17 cells through IL-1 and IL-23, and the overactivity of Th17 cells can lead to the exacerbation of Th1 immune responses and the development of chronic inflammatory states (33). Thus, Th1 cells

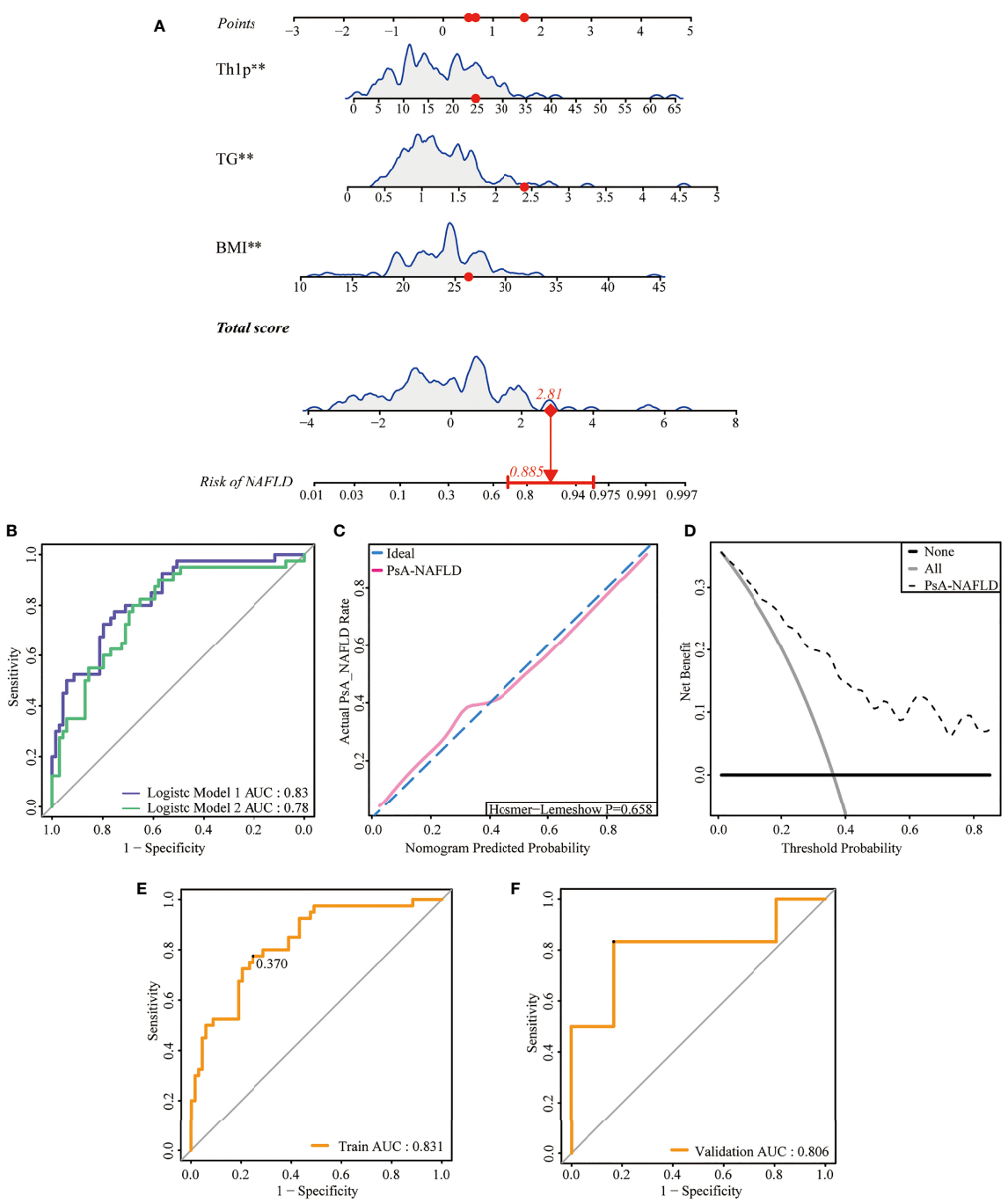

FIGURE 3
Development and assessment of a nomogram for prediction of NAFLD in PsA. **(A)**. Example of prediction nomogram for risk of NAFLD in PsA patients. The nomogram incorporates the risk factors of Th1%, TG, and BMI. **(B)**. The receiver operating characteristic (ROC) curve for the discrimination of the nomogram to predict the risk of NAFLD in PsA patients. Model 1: The nomogram incorporating the risk factors of Th1%, TG, and BMI; Model 2: The nomogram incorporating the risk factors of TG and BMI. **(C)**. Calibration curve for predicting the risk of NAFLD in PsA patients. The red line along the dashed line indicates that the predicted prevalence is close to the actual prevalence. **(D)**. Decision curve analysis for predicting the risk of NAFLD in PsA patients. **(E)** The receiver operating characteristic (ROC) curve for the discrimination of the nomogram to predict the risk of NAFLD in modeling population. **(F)** The receiver operating characteristic (ROC) curve for the discrimination of the nomogram to predict the risk of NAFLD in validation population **p < 0.01.

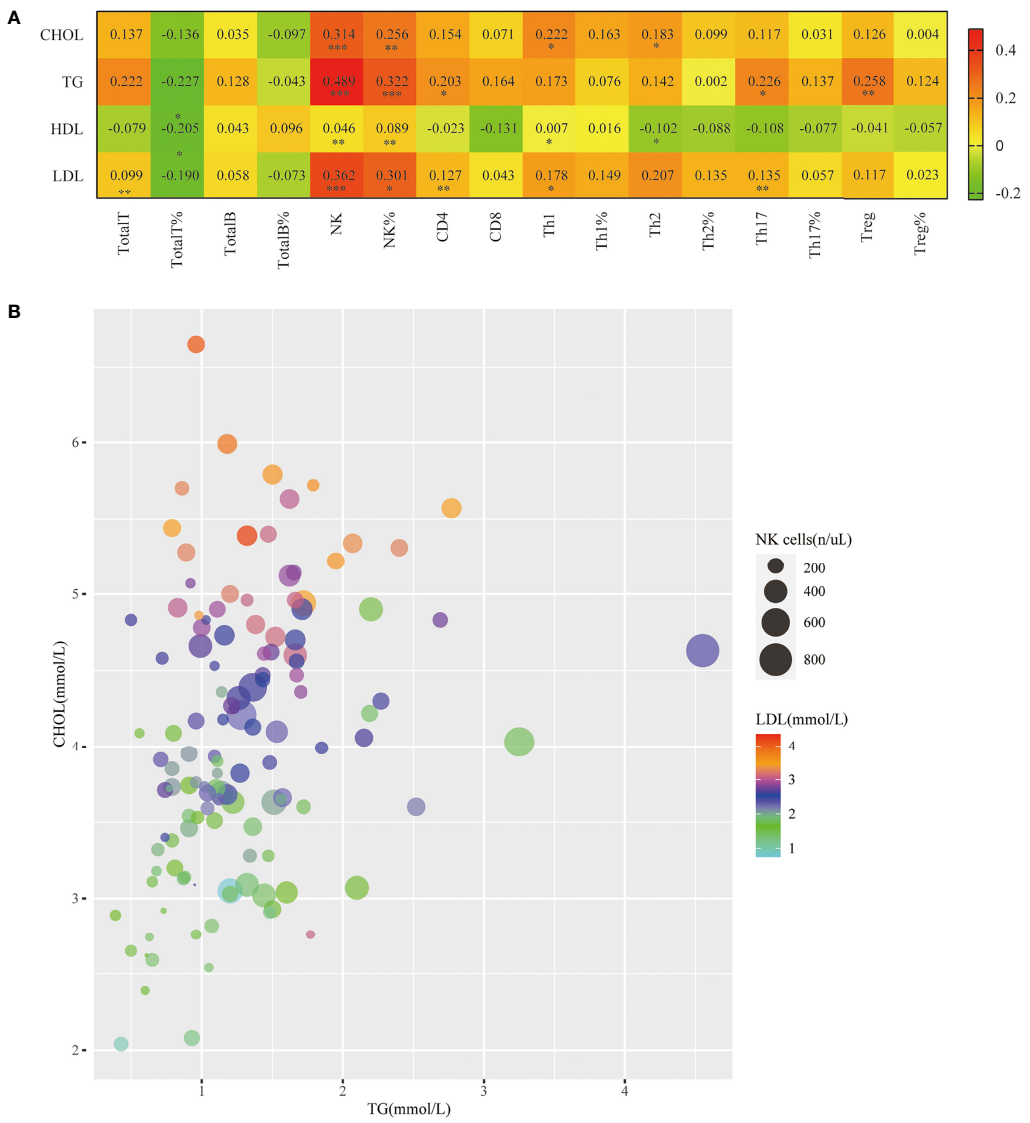

FIGURE 4
Correlation of peripheral blood NK cell levels with hyperlipidemia in patients with PsA. **(A)**. Heatmap of correlation of the serum lipid levels (TG, CHOL, LDL, HDL) with Total T, Total T%, Total B, Total B%, NK, NK%, CD4+T, CD8+T, Th1, Th1%, Th2, Th2%, Th17, Th17%, Treg, Treg%. (*p < 0.05, **p < 0.01, ***p < 0.001; by *Spearman correlation test*). **(B)**. Correlation bubble plot of TG, CHOL, LDL, and peripheral blood NK cell count.

and Th17 cells play a central role in systemic chronic inflammation in PsA. Our study also showed that the levels of Th1 cells and Th17 in the peripheral blood of PsA patients were significantly higher than those of healthy people, suggesting that their abnormal levels may be involved in the pathogenesis of PsA.

Treg cells, a subset of CD4+T lymphocytes, can suppress effector T cells and inflammation, and play an important role in maintaining autoimmune tolerance. Existing studies on the level of Treg cells in the lesional skin and peripheral blood of PsO are still controversial, but most studies have shown that the number and function of Treg cells in peripheral blood are defective (35), and the ratio of Th17/Treg was significantly positively correlated with PASI score (36). Our study also demonstrated decreased Treg% and Th17/Treg cell imbalance in peripheral blood of PsA patients.

In addition to CD4+T cells, NK cells also play a role in the skin lesions of PsA. Studies have shown that cellular infiltrates in acute psoriatic plaques include 5-8% NK cells and are primarily confined to the middle and papillary dermis. NK cells also function by secreting cytokines such as INF-γ, TNF, and IL-22. Once NK cells enter the diseased skin, they will produce INF-γ, which is an effective factor for Th17 cells to migrate to the skin lesions (37). It is unclear whether peripheral blood NK cells are involved in the pathogenesis of PsO, but multiple studies have shown that peripheral blood NK cells are reduced in PsO patients.

Our findings also showed that both the number and percentage of NK in the peripheral blood of patients with PsA decreased.

Notably, chronic systemic inflammation caused by abnormal levels of CD4+T cells and NK cells in PsA patients promotes the occurrence and development of hepatic steatosis, especially NAFLD, and significantly increases the risk of advanced liver disease. Because the two diseases share a common inflammatory pathway, which plays a bidirectional promoting role in the course of both diseases. Therefore, identifying key links in the inflammatory pathway and intervening early can not only prevent the occurrence of NAFLD but also help control the disease of PsA. Our study showed that the level of CD4+T cells in peripheral blood of PsA-NAFLD patients was significantly different from that of PsA patients, especially the number and percentage of Th1 cells, and the number of Th17 cells and the ratio of Th1/Th2 were significantly increased. In addition, the number and percentage of NK cells also showed a significant upward trend.

CD4+T cells are involved in the pathological process of NAFLD and promote the occurrence of liver fibrosis and even liver cancer. A recent study explored the role of CD4+T cells in a high-fat diet-induced (HFD) mouse model of humanized NAFLD and found that it has an important role in promoting liver fibrosis. In HFD mice, marked infiltration of CD4+T cells was found and the levels of proinflammatory cytokines IFN-γ, IL-6, IL-17A, and IL-18 were elevated. However, depletion of CD4+T cells in HFD mice not only prevented liver steatosis but also significantly reduced immune infiltration and liver fibrosis (38). Th1 cells, as an important part of CD4+T cells, play an important role in the pathogenesis of NAFLD. Levels of hepatic Th1 cells and related cytokines IFN-γ, IL-12, and TNF-α are elevated in steatotic mice on a choline-deficient diet, a process associated with increased STAT4 and T-bet expression (39). In addition, elevated Th1 cells may be involved in the development of liver fibrosis in the advanced stages of the disease. Human studies also found elevated levels of Th1 cells in peripheral blood and liver in NAFLD patients, with no significant difference between NAFLD and NASH (16).

It has been confirmed that Th1 cells are involved in the pathological process of obesity-related adipose tissue inflammation. Th1 cells are abundant in subcutaneous and visceral adipose tissue in an HFD-fed obese mouse model (40, 41), and blocking Th1 function can reduce adipose tissue inflammation and alleviate symptoms of impaired glucose tolerance (42). Furthermore, in morbidly obese adult patients, genes involved in T cell activation and Th1 cell phenotype switching were already upregulated in the liver before the characteristic leukocyte infiltration occurred (43), this series of evidence suggests that Th1 cells may be involved in the early stages of NAFLD. Through multivariate logistic regression analysis, our study found that peripheral blood Th1% in PsA patients was an independent risk factor for NAFLD. Combined with other risk factors BMI and TG, a nomogram prediction model of NAFLD risk in PsA patients was constructed. Considering that Th1% is not a traditional risk factor for NAFLD, a new predictive model was reconstructed after removing Th1% from the model. By plotting the ROC curve, it is found that the discriminative power of the new model is worse than the original model, so Th1% plays an important role in the prediction model. When we used the cutoff value of the ROC curve of the prediction model (37.0%) as the threshold of the DCA curve, the clinical net benefit rate of patients was higher than the two extreme methods of no intervention and all intervention. This suggests that patients will benefit from immediate intervention when the model predicts risk of NADLD higher than 37.0%, and if the risk is lower than 37.0%, the intervention is temporarily withheld. Therefore, this model is beneficial to the formulation of clinical decision-making programs for PsA patients.

The role of Th17 cells in NAFLD has been extensively studied, and relevant animal and human studies have shown that Th17 cells are elevated in peripheral blood and liver tissue (14), especially in NASH patients (44). IL-17 secreted by Th17 cells is an important factor in promoting hepatic steatosis. Numerous animal and *in vitro* studies have found that hepatic steatosis increases when IL-17 is present but decreases after blocking IL-17 function (39, 45). In addition, Th17 cells also have obvious effects on liver fibrosis, and the possible mechanism is that IL-17 directly acts on hepatic stellate cells in a JNK- and STAT3-dependent manner to induce collagen production (46).

To date, the level and function of NK cells in NAFLD remain controversial. Recent studies have shown that NAFLD patients have a lower frequency of CD56dim NK cells and lower expression of the activating receptor NKG2D compared to healthy individuals (22). Furthermore, NK cell function is impaired, resulting in reduced granzyme/perforin and INF-γ production, ultimately reducing cytotoxicity and tumor lethality (19). However, in NASH patients, the level of NK cells in the liver parenchyma is increased, which directly activates hepatic stellate cells through the activation of receptors NKG2D, NKp46, and p38/PI3K/AKT pathways, and promotes the development of liver fibrosis (23, 47, 48). Our study also showed that the percentage of peripheral blood NK in PsA-NAFLD patients was lower than that in healthy people, but it is worth noting that the number and percentage of peripheral blood NK increased in the PsA-NAFLD group compared with the PsA group. We speculate that it may be associated with higher levels of systemic inflammation in patients with PsA-NAFLD. Considering the role of NK cells in NASH and liver fibrosis, the occurrence of NASH and advanced liver disease in PsA-NAFLD patients should be monitored in clinical practice.

Dyslipidemia is a common risk factor for both diseases, in addition to the common chronic low-grade systemic inflammation. PsA patients are often accompanied by a variety of lipid metabolism abnormalities, such as increased TG and decreased High-density lipoprotein cholesterol (HDL-C), which are more prevalent during disease activity (49), suggesting a

potential link between inflammation and lipid profiles. Abnormal lipid metabolism is also closely associated with NAFLD, manifested by increased levels of TG and low-density lipoprotein cholesterol (LDL-C) and decreased high-density lipoprotein cholesterol (HDL-C) levels (50), therefore PsA and NAFLD have similar lipid profiles. However, the lipid profile of PsA-NAFLD patients has not been reported. In our study, serum TG, CHOL, and LDL levels were significantly higher in the PsA-NAFLD group than in the PsA group. It is worth noting that the number and percentage of NK cells in peripheral blood were positively correlated with these three lipids, especially the correlation between the number of NK cells and TG was more significant. At present, the research on the relationship between NK cells and lipid metabolism such as TG is still relatively limited. Recent studies have found that in the NASH mouse model, liver NK cells are significantly increased, and after NK cell depletion, liver TG content is reduced, and the symptoms of hepatitis in mice are significantly relieved, suggesting that NK cells are involved in the process of liver lipid accumulation (51). Therefore, given the correlation of NK cell levels with differential lipids, we speculate that NK cells may be involved in the occurrence of dyslipidemia in PsA patients, thereby increasing the susceptibility to NAFLD. The exact relationship between peripheral blood NK cells and dyslipidemia in PsA patients still requires epidemiological investigations with large samples.

Of course, our study has certain limitations. First, the modeling data came from the same medical center and lacked validation from external data. If patient data from multiple centers can be incorporated at a later stage, with simultaneous external validation, the model can be further adjusted and strengthened. Second, the independent risk factors in the nomogram prediction model are all continuous measurement variables, which are not clinically concise. In the later stage, the method of optimal scale regression can be considered to group the continuous measurement data variables and convert them into categorical variables, which is convenient for the clinical application of the nomogram prediction model. Finally, because this study was a retrospective clinical analysis, an accurate assessment of PsA disease activity and NAFLD severity was lacking.

In conclusion, NAFLD is a common complication of PsA. If not detected and intervened early, it may lead to the progression of PsA and even increase the risk of advanced liver disease. Our study found that Th1% in peripheral blood of PsA patients was an independent risk factor for NAFLD. Combined with the traditional risk factors of hyperlipidemia and obesity, an individualized nomogram prediction model was constructed to predict the risk of NAFLD in PsA at an early stage. The prediction model has good discrimination, calibration, and clinical validity, and the visualized prediction model is convenient for clinical operation. In addition, the level of NK cells in peripheral blood is related to dyslipidemia in patients with PsA, and may also be involved in the pathogenesis of NAFLD. Therefore, Th1 cells and NK cells may be potential biomarkers to detect NAFLD in PsA patients. In the future, we will analyze the levels of inflammatory cytokines downstream of Th1 and NK cells to explore their molecular biological mechanisms.

Data availability statement

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

Ethics statement

This study was approved by the Ethics Committee of the Second Hospital of Shanxi Medical University (2019YX114). A written informed consent was obtained from every participant.

Author contributions

Individual authors' contributions are as follows: BL performed the data analyses and wrote the manuscript. HY and JL participated in the collection of samples and clinical data. RS participated in the performance of the research and statistical analysis. CG and XL participated in the study design and revising of the manuscript. CW provided intellectual input and supervision throughout the study and made a substantial contribution to manuscript drafting. All authors contributed to the article and approved the submitted version.

Funding

This study was funded by the National Natural Science Foundation of China (No. 81971543), National Natural Science Foundation of China (No. 81471618), and Key Research and Development (R&D) Projects of Shanxi Province (201803D31119).

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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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 01 August 2022
DOI 10.3389/fimmu.2022.911730

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Autoimmune and Autoinflammatory Disorders, a section of the journal Frontiers in Immunology

RECEIVED 03 April 2022
ACCEPTED 13 July 2022
PUBLISHED 01 August 2022

CITATION
Cheng H, Zhang X-y, Yang H-d, Yu Z, Yan C-l, Gao C and Wen H-y (2022) Efficacy and safety of belimumab/low-dose cyclophosphamide therapy in moderate-to-severe systemic lupus erythematosus.
Front. Immunol. 13:911730.
doi: 10.3389/fimmu.2022.911730

Efficacy and safety of belimumab/low-dose cyclophosphamide therapy in moderate-to-severe systemic lupus erythematosus

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Objectives: We have reported previously that Belimumab, a human monoclonal antibody that inhibits B-cell activating factor(BAFF) could be an effective and safe option to treat Neuropsychiatric manifestations of SLE (NPSLE). To avoid inadequate efficacy of Belimumab and significant adverse events of often-used dose of cyclophosphamide (CYC) for SLE, we evaluated the efficacy, safety, and possible immune mechanisms of Belimumab treatment in combination with intermittent low-dose intravenous CYC for moderate-to-severe SLE.

Methods: In this non blinded and parallel-group trial, we collected 82 cases of moderate-to-severe SLE patients, 40 received Belimumab treatment and 42 received conventional treatments as historical controls for 24 weeks. The demographic features, clinical manifestations, and laboratory indicators including peripheral blood lymphocyte subgroups or subsets were compared before and after the treatments.

Results: Compared with the baseline, 6 months post Belimumab group treatment, disease activity score SLEDAI (13.78 to 3.82, $P<0.05$) and BILAG scores (16.40 to 5.48, $P<0.05$) were reduced; C3 (0.19 to 1.14, $P<0.05$) and C4 (0.04 to 0.22, $P<0.05$) increased; the absolute numbers of B and T cells were the first decreased and then significantly increased, tended to balance. Moreover, Belimumab group treatment significantly reduced the serum levels of IL-6, the ratio of B and T cells, and the proportion of infections and menstrual disorders.

Conclusion: Compared with conventional treatment, Belimumab with low-dose intravenous CYC significantly reduced disease activity scores and maintained the B/T cell balance for SLE patients at 24 weeks. It was more efficacy and safe (adverse events such as infection were significantly lower). It should be the mechanism that Belimumab combined with low-dose intravenous CYC therapy restores the balance of T and B cells, which proposes a potential treatment strategyfor SLE.

KEYWORDS

belimumab, low cyclophosphamide (CYC), systemic lupus erythematosus (SLE), B cells, T cells, IL-6

Introduction

Systemic lupus erythematosus (SLE) is a complex chronic autoimmune disease with highly variable disease manifestations and organ involvement (1–3). At present, corticosteroids and conventional immunosuppressive agents are the main treatment methods. However, they are not always effective and tolerable and they may cause the adverse events in some patients. So, active SLE with infection remains an important cause of relapse and mortality (4).

Current treatments for moderate-to-severe SLE patients include cytotoxic immunosuppressive agents, including usually middle (modified NIH regimen) and high-dose (NIH regimen) cyclophosphamide (CYC), and high-dose steroids. Remission occurs following treatment in 70-90% of patients. However, disease relapse, persistent disease activity, and treatment-associated toxicity contribute to mortality and chronic incapacity. Most deaths are associated with the treatment side effects (4, 5). Emerging biological agents have also been used for treatment of SLE. Rituximab is a chimeric monoclonal antibody targeted against CD20 which is a surface antigen present on B cells. As Rituximab (RTX) has been shown to increase BAFF levels following B cell depletion, repeated RTX treatments may result in more severe flares driven by BAFF (6). Thus, high BAFF levels post RTX could limit its effectiveness in some patients with SLE (7). Furthermore, a large, phase III, and randomized placebo-controlled trial failed to meet their primary endpoints (4, 8). Therefore, treatments with less toxic and more effective are urgently needed.

Since clinical response are extremely variable in SLE patients, no single mediator or pathway can account for the complex pathogenesis. Some studies have showed that immune dysregulation at the level of T cells, B cells, macrophages, and cytokines are closely related to SLE pathogenesis (9, 10). B cells are implicated in SLE pathogenesis by production of autoantibody, presentation of autoantigen to T cells, T cell activation, and cytokine production. Meanwhile, T cell also are critically in SLE, infiltrating widely into target organs to cause inflammation and organ damage. On the other hand, many studies of SLE have shown that T cells have many abnormalities of cytokine production and cell signaling transduction, which not only determines the abnormal differentiation of T cells, but also the overactivation of B cells (11–14).

The treatment regimen of CYC and glucocorticoids widely acted on B and T cells and significantly increased long-term efficacy (nearly 10-year period), leading to less mortality in SLE (4, 9, 15, 16). However, infection is one of the leading causes of morbidity and mortality in SLE, which has intrinsically increased risks that are expanded by immunosuppressive therapy. Belimumab, a human monoclonal antibody that inhibits B-cell activating factor (BAFF), reduces the number of circulating B cells (8–10). Although Belimumab has demonstrated efficacy both in clinical trials and in real-world settings and has a safe long-term side-effect profile, it is not a panacea for all SLE patients. In clinical trials, at least 40% of SLE patients did not demonstrate a clinically meaningful response to Belimumab that only targets BAFF (10). Also, we reported the effect of Belimumab on five patients who were unresponsive to conventional therapy. Our case reports suggest that Belimumab could be an effective and safe option to treat NPSLE, even in refractory cases, allowing to spare glucocorticoids and immunosuppressants (15). A combination therapy targeting multi-pathways and/or cells could be more effective. Therefore, we developed a novel therapy: Belimumab with a low-dose intravenous CYC for moderate-to-severe SLE.

This study is to investigate whether Belimumab with low-dose intravenous CYC is more effective and safe for SLE patients and effects of T cells and cytokines besides B cells in the peripheral blood of moderate-to-severe SLE patients. To our knowledge, we described, for the first time, the changes in disease activity, absolute numbers of peripheral lymphocyte subgroups, and serum cytokines after Belimumab (10mg/kg, the first three doses were administered once every 2 weeks and then once every 4 weeks total 8) and low-dose intravenous CYC

(the first doses was 400mg and then a fixed dose of 200mg/3week) treatment in patients with SLE over a 24 weeks period.

Methods

Study design and participants

To assess the efficacy and safety of Belimumab combined with low-dose intravenous CYC in patients with moderate-to-severe SLE, we did a retrospective study at the Department of Rheumatology, Second Hospital of Shanxi Medical University (Taiyuan, China), with approval from the Second Hospital of Shanxi Medical University Ethics Committee (ethics number: 2019YX140). This trial was registered at the Chinese Clinical Trial Registry (ChiCTR2200055471).

We collected 40 cases of moderate-to-severe SLE patients in the Second Hospital of Shanxi Medical University from January 2021 to December 2021 into the study, who received Belimumab treatment. A total of 42 moderate-to-severe SLE patients who met the same criteria from April 2014 through June 2015 and received conventional treatments as historical controls.

All patients eligible participants were age ≥18 years and had to fulfill ≥4 of the 11 American College of Rheumatology 1997 classification criteria for SLE (17, 18), and were required to have serum positivity for antinuclear antibodies (ANAs) and/or anti-double-stranded DNA (anti-dsDNA) antibodies at the time of screening (17). All had active severe disease: defined as Systemic Lupus Erythematosus Disease Activity Index 2000 (SLEDAI-2K) score of ≥6, and either ≥1 organ system (include renal or central nervous system) with a British Isles Lupus Assessment (BILAG) A score (severe disease activity) or ≥2 organ systems with a BILAG B score (moderate disease activity). At least 1 of the BILAG A grades or ≥2 of the BILAG B grades must have been in the mucocutaneous, musculoskeletal or cardiorespiratory BILAG-2004 index domains (19–21), and coexists with another autoimmune diseases were excluded. Any known intolerance or contraindications to CYC and others DMARDs, acute infection, cancer or other malignant disease, or other connective tissue diseases were excluded from the study.

Procedures

The patients continued to receive corticosteroid and any of concomitant immunosuppressants at the same dose used before study entry. For those with high disease activity at screening, a dose increase in corticosteroid was allowed at the investigator's discretion. Belimumab at 10 mg/kg was given intravenously at 2-week intervals for the first 3 doses and combined with low-dose intravenous CYC at the first doses was 400mg and then 3-week intervals 200mg until week 24. The CYC group included patients who received induction with monthly intravenous CYC (0.5–1.0 g/m^2) (Figure 1).

The clinical follow-up assessment mainly included laboratory assessment (including serum cytokine), disease activity, occurrence of infection, and any other adverse reactions at baseline, 4, 8, 12, 24 weeks after treatment. Data for each patient was assessed by the same investigator throughout the study using the following outcome measures.

Assessment of disease activity

The BILAG index was used to assess response, and scores were converted to numeric values (A = 9, B = 3, C = 1, D = 0, E = 0) to enable evaluation of fluctuating global summary scores (20, 21).

Laboratory assessment

The absolute numbers of lymphocyte subpopulations in peripheral blood of these individuals were determined by flow

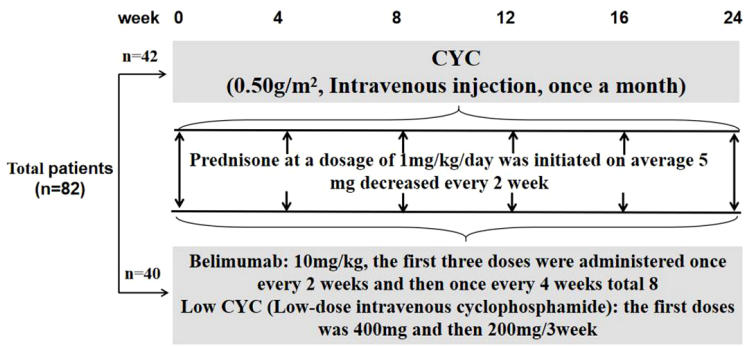

FIGURE 1
Flow chart of the study design and patient disposition.

cytometry combined with a known number of fluorescent beads (21). Anti-double-stranded DNA (anti-dsDNA) antibody titers were measured by enzyme-linked immunosorbent assay, and C3 and C4 complement levels and total serum immunoglobulin titers and Erythrocyte Sedimentation Rate (ESR) were measured by nephelometry (20, 22–24).

Measurement of serum cytokine

The levels of serum cytokines [IL-6, IL-10, IL-17, and tumor necrosis factor (TNF)] in SLE (serums were kept at −80°C until analysis) were detected by magnetic bead-based multiplex immunoassay using Human Th1/Th2/Th17 subgroup test kit. The Bio-Plex 200 reader was used to acquire the data of cytokines, which was output as Median Fluorescence Intensity (MFI) and concentration (pg/mL) using the BioPlex Manager software (25, 26).

Safety assessments

Liver dysfunction was defined as more than twice the upper limit of normal (AST, ALT ≤ 40 U/L). Renal dysfunction was defined as an increase in serum creatinine concentration of more than 30% above baseline levels at any study time point. Blood system toxicity refers to bone marrow suppression, such as leukopenia. Gastrointestinal reactions were nausea, vomiting, abdominal pain and other discomfort, and menstrual disorders. Serious adverse events (SAEs), infusion-related AEs (an AE occurring during or within 24 hours following completion of the infusion of a study drug), and infection-related AEs were summarized independently. Infection refers to a fever occurring, and evidence of a pathogenic infection is found (16, 27–29).

Statistical analysis

Data were statistically significant at a value of $P<0.05$. Statistical analyses were performed by SPSS version 23.0 (IBM Corp, Armonk, NY, USA) and GraphPad Prism version 8.01. Normal distributed variables' descriptive data were presented as mean and standard deviation (SD) and non-normal distributed variables were presented as median with range. Categorical variables were reported as numbers. Paired-samples t-test or paired-samples Wilcoxon test was used for comparison of changes before and after treatment. Independent-samples t-test or Mann-Whitney U test was used to compare the differences between two groups. Spearman's rank correlation test was used to evaluate the correlation. The correlation coefficient of 0.1 to 0.3 was weak correlation, 0.3 to 0.5 was moderate correlation, and 0.5 to 1.0 was strong correlation.

Results

Clinical and laboratory characteristics of 82 SLE patients were collected and analyzed. Among them, 42 were received formal dose of CYC treatment (CYC group), and 40 were received Belimumab with low-dose intravenous CYC (Belimumab group). The demographic and disease characteristics of the patients are presented in Table 1. The mean age of the patients was 30.79 ± 11.68 years. Between the two groups, the differences were not statistically significant in the general conditions and disease indicators, including SLEDAI-2K and BILAG scores, and C3, C4, as well as laboratory tests ($P>0.05$). The two groups were comparable.

Equal effects of belimumab/low-dose CYC and conventional CYC therapy

First, we analyzed the efficacy of Belimumab co-treated with low-dose intravenous CYC for 6 months. Compared with the baseline, Belimumab group reduced significantly SLEDAI-2K and BILAG scores (Figure 2B) of SLE patients, and low complement (C3 and/or C4, < lower limit of normal) or high ESR demonstrated an improvement (Figure 2E). Similarly, immunoglobulin IgG decreases, but the difference was not statistically significant (Figure 2).

At the same time, we used conventional therapy alone (CYC group) as controls, and compared the efficacy Belimumab group. Compared with the baseline, after 24 weeks of treatment, both two treatments reduced the SLEDAI-2K and BILAG scores significantly (Figure 3B), but the difference between the two groups was no statistical significance. There were significant improvements in C3, C4 after both treatments ($P <0.05$), but no statistically significant differences between the two groups (Figure 3D). However, ESR in the CYC group was significantly higher than that in Belimumab group at 8 and 12 weeks. And there was no statistical difference in other follow-up nodes between the two groups (Figure 3). Immunoglobulin IgG was no significant difference between the 2 groups of patients (Figure 3). All above suggest that Belimumab with low-dose CYC can achieve the equal effects of conventional CYC therapy.

Changes of peripheral blood lymphocyte levels

First, we analyzed the changes of peripheral blood B cells, T cells and their ratios before and after the treatment of the Belimumab group. We found that the absolute number of B cells decreased from the baseline at 4-8 weeks of the treatment, and then gradually increased to 12 weeks after the treatment, with significant differences (Figure 4A–F). At the same time, T cells decreased slightly after 4 weeks of treatment, with no

TABLE 1 Baseline characteristics of 82 patients with SLE.

Variables	Belimumab (N=40)	CYC (N=42)	*P* (value)
Age, mean ± SD (years)	30.57 ± 11.68	31.02 ± 12.04	0.619
Female, n (%)	39(97.5)	40(95.2)	0.341
History of smoking, n (%)	1(2.50)	1(2.38)	0.862
Disease duration, mean ± SD (years)	2.6 ± 2.36	2.7 ± 2.86	0.563
SLEDAI, mean ± SD	13.78 ± 2.21	12.63 ± 1.96	0.813
BILAG, mean ± SD	16.4 ± 6.52	16.0 ± 6.84	0.613
C3, mean ± SD	0.19 ± 0.11	0.21 ± 0.10	0.374
C4, mean ± SD	0.04 ± 0.09	0.05 ± 0.11	0.416
ESR, mean ± SD (mm/h)	61.81 ± 12.68	58.15 ± 11.00	0.599
CRP, mean ± SD (ng/ml)	32.57 ± 7.84	34.21 ± 10.11	0.674
ANA, n (%)	40(100)	42(100)	1.000
Anti-ds-DNA, n (%)	40(100)	42(100)	1.000
Anti-Sm, n (%)	28(70.0)	34(81.0)	0.085
	Background prednisone, n (%)		
Daily prednisone use	40(100)	42(100)	0.875
>7.5 mg/d at baseline	40(100)	42(100)	0.875
Prednisone, mean ± SD (mg/d)	41.4 ± 16.2	42.7 ± 15.6	0.762
	Background immunosuppressive drug, n (%)		
CYC, n (%)	40(100)	42(100)	0.875
MMF, n (%)	36(90.0)	37(88.1)	0.657
HCQ, n (%)	38(95.0)	42(100)	0.375
LEF, n (%)	2(5.0)	4(9.5)	0.215
AZA, n (%)	1(2.5)	0(0)	0.425

Except stated otherwise, values are reported as mean ± SD or number (%). ANA, Antinuclear antibodies; Anti-ds-DNA, anti-double-stranded DNA; CYC, cyclophosphamide; MMF, Mycophenolate mofetil; HCQ, Hydroxychloroquine; LEF, leflunomide; AZA, azathioprine.

statistical difference from baseline. Subsequent treatment up to 24 weeks led to a gradual increase in T cells, and a statistical difference from baseline and 4 weeks (Figure 4). The B/T ratio tended to balance at 24 weeks, but compared with the baseline, there were statistical differences when compared with the B/T ratio at 8 week (Figure 4). Second, we analyzed and compared to the effect of two treatment regimens on B/T cell balance. Our research suggested that, the absolute numbers of B and T cells of CYC group of patients decreased significantly as compared to those before with treatment, there was a continuous decrease, especially in T cells. However, the Belimumab group showed a slight decrease in B and T cells in the early stage of treatment (4-8 weeks) and gradually increased as treatment continued. After 8 weeks, B and T cells were statistically different between the two groups (Figure 4E). The ratio of the two is maintained in a relative equilibrium state. The imbalance of B and T cells was obvious at 8 weeks after CYC group treatment (Figure 4).

Changes of cytokines Levels

In this study, after Belimumab treatment for 24 weeks, the serum levels of IL-6 and IL-10 were significantly decreased comparing with the baseline. Serum levels of IL-6 of CYC group showed the most rapid and prominent reduction, but increased again at week 8 of treatment and was significantly different from Belimumab. Similarly, Serum levels of IL-10 increased again after 8 weeks of treatment, but there was no statistical difference between the two groups. After 24 weeks of treatment, IL-6 and IL-10 were significantly decrease than baseline in both groups, with significant differences (Figures 5A, B). However, serum levels of IL-17 and TNF showed no difference after treatment (Figures 5C, D). We analyzed the correlation of the serum levels of these cytokines with B cells, T cells and their ratios in the groups. And we found that the serum levels of IL-6 were significantly correlated T cells in Belimumab group. It is suggested that our therapeutic regimen may affect the absolute T cells through IL-6. But other indicators were not significant correlation (Figure 5E).

Safety

The safety of Belimumab has been demonstrated in the treatment of SLE (25, 26). In this study, it had no serious adverse events. However, the incidence of adverse events of infection and

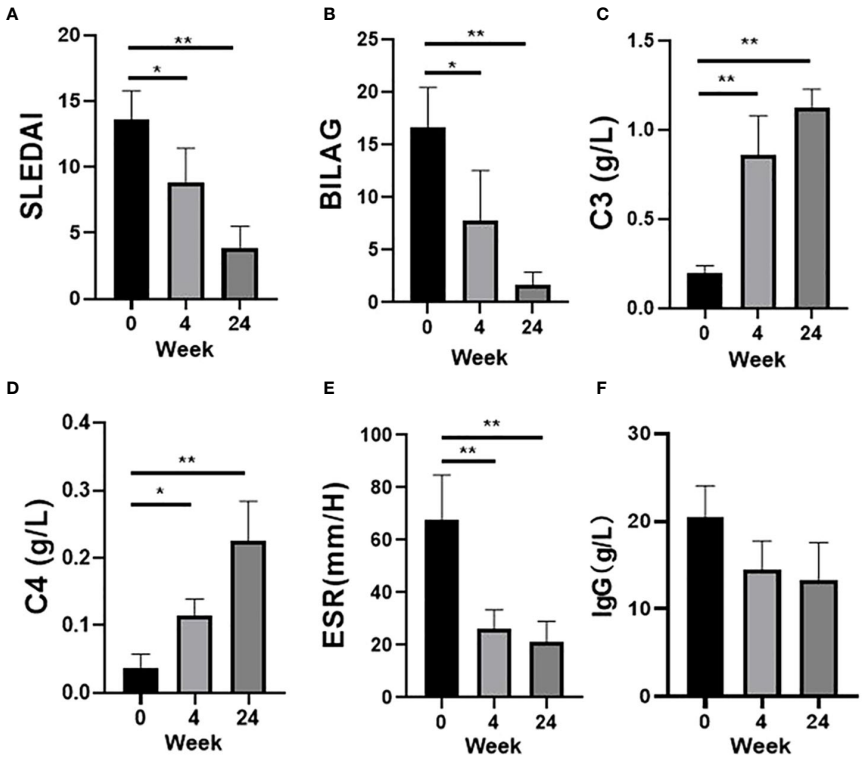

FIGURE 2
Effects of Belimumab after 6 months as compared to the baseline occurring before treatment. **(A, B)** Belimumab significantly improved SLEDAI and BILAG score. **(C, D)** C3 and C4 showed a significantly increased. **(E, F)** ESR and IgG show a significantly downward (*p<0.05, **p<0.01).

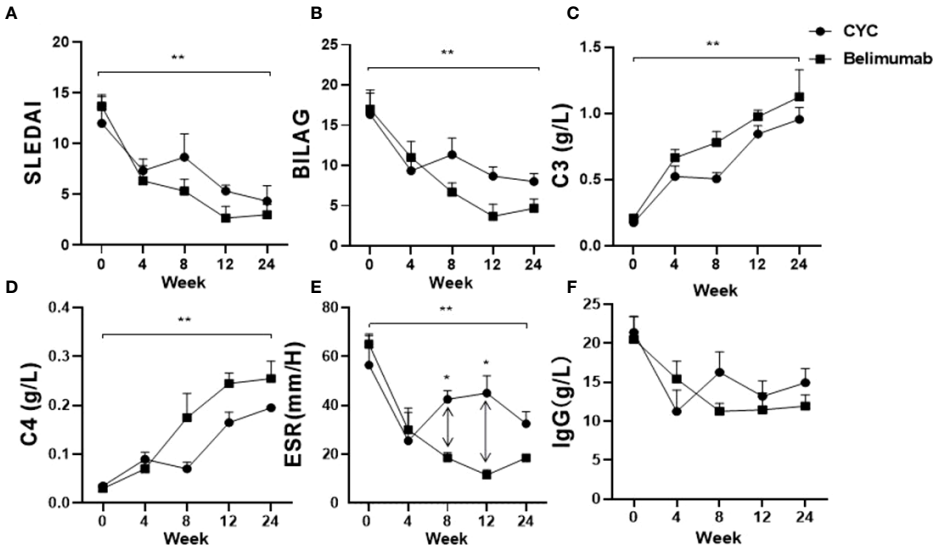

FIGURE 3
Effects of Belimumab and CTX treatment after 6 months as compared to the baseline occurring before treatment. **(A, B)** Two groups significantly improved SLEDAI and BILAG score. **(C, D)** C3 and C4 of both groups showed a significantly increased. **(E, F)** ESR and IgG show a significantly downward (*p<0.05, **p<0.01). Belimumab Group: Belimumab combined with low-dose CTX; CTX Group: Standard-dose of CTX.

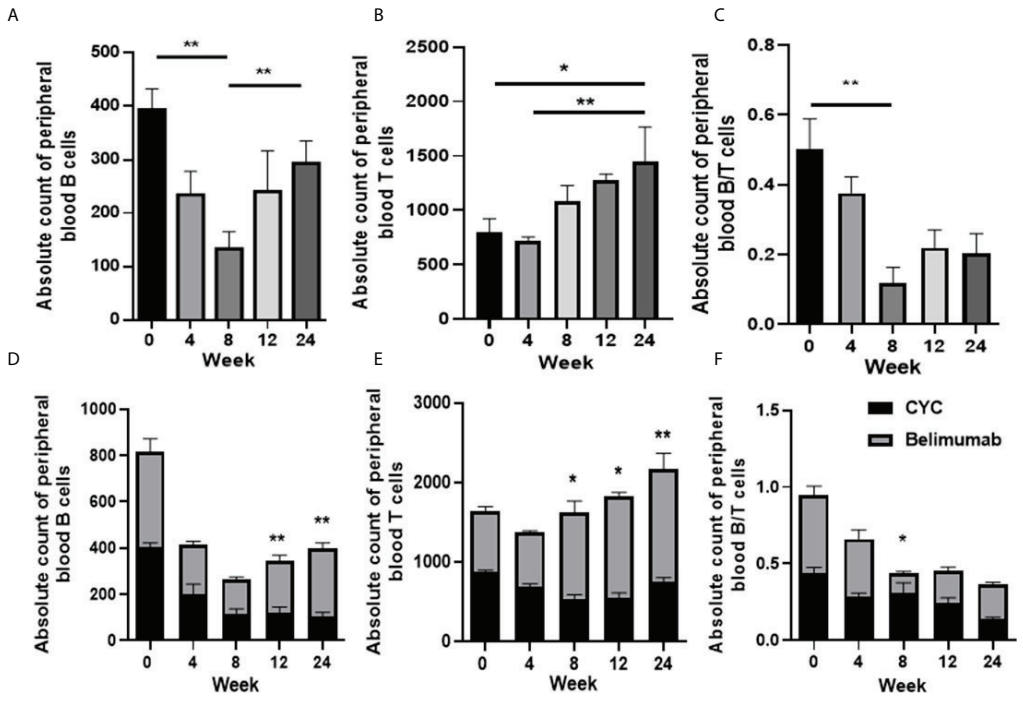

FIGURE 4
Absolute numbers of B and T cell in SLE patients before and after therapy was analyzed by flow cytometry (FCM). **(A–C)** The absolute number of B, T cells and the B/T ratio of the Belimumab group. **(D-F)** The absolute number of B, T cells and the B/T ratio of two treatment regimens. *p<0.05 and ** p<0.01 are significantly compared to the baseline. Belimumab Group: Belimumab combined with low-dose CTX; CTX Group: Standard-dose of CTX.

menstrual disorders were significantly increased in the CYC group, which was significantly higher than that in Belimumab group. In the CYC group, 2 patients did not complete follow-up treatment due to severe infection; 10 patients were re-hospitalized due to infection; the remaining infected cases were cured successfully by antibiotic treatment in outpatient. Menstrual disorders, gastrointestinal reactions, leukopenia, and liver damage were improved after treatment by a specialist (Table 2).

Discussion

This is the first retrospective study to analyze the efficacy and safety of standard-dose CYC versus Belimumab combined with low-dose CYC in the treatment of moderate to severe SLE. Although the study was not powerful enough to fully assess efficacy and was primarily designed to assess safety, the overall efficacy was consistent with those in many published literatures, and our therapy was no significant different from conventional treatment (4, 10, 16). The Belimumab with low-dose CYC significantly reduced the risk of infection and menstrual disorders, as well as hematological toxicity and gastrointestinal reactions compared with the standard dose. In addition, decrease in IL-6 may be a key step in keeping B/T cell homeostasis.

The pathogenesis of SLE involves abnormalities of several components of the immune system, including B cells, T cells, cytokines and growth factors (11, 14). Intermittent intravenous impulse therapy with high-dose CYC combined with glucocorticoid has been the classic treatment for moderate and severe SLE (16, 30). While SLE patients applied CYC to induce often require high-dose and long-term treatment for disease remission. However, this treatment often leads to many adverse reactions, including leukopenia, infection, reproductive toxicity, hair loss and gastrointestinal reactions (11). In recent years, a better understanding of the pathogenesis of SLE has led to the introduction of several biologics, such as rituximab (RTX) and Belimumab, which are available in clinical practice (4, 9, 31). Belimumab is the only targeted biologic approved for the treatment of lupus. Based on the results of randomized clinical trials (RCTs) and real-life experience, Belimumab is particularly effective in patients with active, serologically active disease, and early use leads to a better clinical response (10, 32). But the addition of Belimumab to CYC, rituximab, and glucocorticoid treatment regimens targeting different mechanisms of action of B cells did not achieve better efficacy and resulted in an increase in serious infectious adverse events (7, 33).

In recent years, many clinical studies have shown that low-dose CYC combined with intermittent intravenous impulse

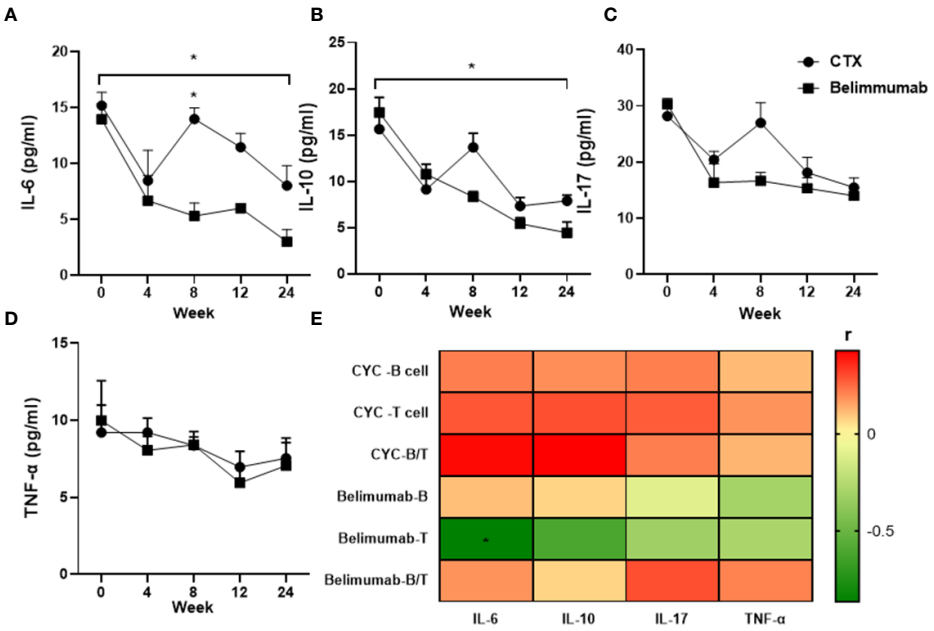

FIGURE 5
Decrease in levels of serum pro-inflammatory cytokines of SLE patients in both groups after 6-months treatment. Mean concentrations (pg/mL) of serum cytokines were quantified by ELISA: **(A–D)** IL-6, IL-10, IL-17, TNF. **(E)** Heatmap showed that the serum levels of IL-6, IL-10, IL-17, TNF have significant correlation with the peripheral blood lymphocyte (B cell, T cell) and B/T (*r>0.5 or<-0.5). Spearman's correlation test was used. T: Total CD3+T cells, B: Total B cells. B/T the radio of B cell versus T cell. *p<0.05 is statistically significant compared to the baseline. Belimumab Group: Belimumab combined with low-dose CTX; CTX Group: Standard-dose of CTX.

therapy of glucocorticoid is equivalent to that of high-dose regimen in the treatment of low-moderate SLE, with fewer adverse reactions (16). According to the lymphocyte cell cycle, our research group gave a low dose of CYC 200mg every 3 weeks, but the disease might be poorly controlled for moderate- severe SLE (34). Therefore, our research protocol applied a low dose of CYC combined with Belimumab for the first time.

In this study, we retrospectively analyzed that both treatment regimens were effective, especially in reducing disease activity scores and lupus indicators. We found that there was no difference between the two groups. Similarly, we found that both treatment regiments affected T cells besides B cells. Importantly, B cell function requires a combination of antigen presentation, cytokine production, and T cell activation and polarization. Studies have shown that B cell depletion therapy has a significant effect on diseases, which was previously thought to be primarily driven by T cells (35, 36). T lymphocytes play a fundamental role and are believed to trigger SLE disease, especially enhancing autoantibody production by B cells. Dysregulation of transcription factors and cytokines in B

TABLE 2 Adverse events occurred in both groups.

Variables	Belimumab (N=40)	CYC (N=42)	*P (value)*
Death	0(0)	0(0)	1.000
Increased serum creatinine	0(0)	0(0)	1.000
Abnormal liver function tests	2(5.0)	13(31.0)	0.0903
Infection	4(10.00)	27(67.5)	<0.001
serious infection	0(0)	2(4.7)	0.778
menoxenia	3(7.5)	21(50.0)	<0.05
leukopenia	0(0)	2(4.7)	0.778
Gastrointestinal reaction	6(15.0)	16(38.1)	0.0681
hemorrhagic cystitis	0(0)	0(0)	1.000

cells can lead to abnormal maturation of B cells and the production of autoantibodies (36). Targeted blocking of B-cell-related cytokines have an obvious effect on down-regulating the strong inflammation immune response (17, 26). During the treatment of Belimumab combined with low-dose CYC, B and T cells decreased slightly from baseline after 4-8 weeks of treatment, but increased from baseline after 24 weeks of treatment, and the ratio of the two cells tended to balance. It is suggested that the treatment scheme acts on both B cells and T cells, and their equilibrium and interaction mechanism may be affected by some key cytokines.

IL-6 levels are found to be raised in the serum of SLE patients and it has both inflammatory and anti-inflammatory effects (17). IL-6, IL-10, TNF, IL-17, and immunoglobulin levels were reduced by our therapy. Interestingly, serum levels of IL-6 and IL-10 showed the most rapid and significantly earlier decline and serum levels of TNF and IL-17 also had moderate and slower reduction. A decrease in IL-6 levels most likely reflects a general reduction in inflammatory activity and in clinical markers of disease activity, which is known to play an important role in T-helper cell differentiation, as shown here and in previous observational studies (37, 38). And there is evidence that IL-6 can induce and magnify the production of autoantibodies in autoimmune diseases (37). So, it is important that an early decrease in IL-6 levels within 6 months of Belimumab/low-dose CYC treatment is associated with sustained remission of SLEDAI, BILAG scores, and clinical indicators. The consistency of these associations supports the role of IL-6 as a useful marker of inflammation in SLE patients, and early decline may thus indicate suitability for continued therapy. Tocilizumab, which also targets the IL-6 receptor, is already used to treat severe active RA, systemic and polyarthritis JIA, and giant cell arteritis (GCA) (39–41). Tocilizumab has been shown to be effective in the treatment of juvenile systemic lupus erythematosus (JSLE), especially in patients with SLE involving the central nervous system (15, 42). In the study of Shirota et al. Tocilizumab was used in 15 SLE patients with mild-moderate disease activity with a reduction in the activity of T and B cells (43). A phase II randomized trial of CNTO 136 in patients with active lupus nephritis is completed and the results are awaited. Successful cases of tozizumab and tacrolimus in the treatment of patients with rheumatoid arthritis complicated by SLE have also been reported (44). Belimumab combined with IL-6 inhibitor of IL-6 receptor may be a novel treatment for SLE.

Despite the retrospective nature, our results indicate that both treatments can induce remission of the disease. Two patients in the standard-dose CYC group stopped follow-up treatment due to severe infection. No patients in the Belimumab combined with low-dose CYC group were interrupted by the drug side effects of this treatment. Most of the adverse events in patients who received were low grade. Importantly, it demonstrated multidimensional effects on patients with SLE. Therefore, Belimumab combined with low-dose CYC is safe, feasible and may become the optimal regimen for the treatment of SLE, especially for the moderate-to-severe SLE.

There are limitations in our study. This was small sample size study that enrolled a limited number of patients with SLE. More clinical and laboratory studies using a large size samples and long-term observation are needed to confirm importance of the B/T balance in SLE. From a clinical point of view, treatment is controlled by organ involvement and predictors in different disease subgroups need to be identified to facilitate an individualized management approach. We were unable to analyze concomitant and prior medications other than glucocorticoids. Observing design can also be considered a disadvantage, for example, decisions directed by the treating physician rather than the purpose of the study may hinder standardization of background treatment. However, patient cases represent real life scenarios and follow-up represents current clinical practice, both of which can also be considered as strengths of the study. Furthermore, we only retrospective selected patients with conventional immunosuppressants as comparators, specific csDMARDs combined with Belimumab should be optimized in the future. This was a pilot study designed based on short-term efficacy and safety. To provide preliminary data that could drive more conclusive testing. Therefore, high-quality, large-scale, multicenter randomized controlled trials with longer follow-up are needed to further compare safety and efficacy.

Conclusions

Our study shows at first time that Belimumab combined with low-dose CYC treatment can improve disease activity and clinical performance without severe adverse events. In particular, the adverse events of infection were far less than those of conventional treatment. In addition to targeting B cells, Belimumab also appears to affect T cells and their subsets *via* IL-6, acting as an immune-balancing therapy. However, further study should be done using large-size samples and a well-designed, double-blind, randomized controlled trial is needed.

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 participants were reviewed and approved by the Second Hospital of Shanxi Medical University Ethics Committee (ethics number: 2019YX140). The patients/

participants provided their written informed consent to participate in this study.

Author contributions

HC and X-YZ performed the data analyses and wrote the manuscript. H-DY, ZY, and C-LY participated in the collection of samples and clinical data. CG participated in the study design and revising of the manuscript. H-YW provided intellectual input and supervision throughout the study and made a substantial contribution to manuscript drafting. All authors contributed to the article and approved the submitted version.

Funding

This study was supported from Key Scientific Research Project of Medical Science of Shanxi Province (2021XM08), Basic Research Youth Project of Shanxi Province (202103021223442) and 2020 Shanxi Province Emerging Industry Leadership Project (2020-15).

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.2022.911730/full#supplementary-material.

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frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 23 August 2022
DOI 10.3389/fimmu.2022.952954

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Autoimmune and Autoinflammatory Disorders, a section of the journal Frontiers in Immunology

RECEIVED 25 May 2022
ACCEPTED 29 July 2022
PUBLISHED 23 August 2022

CITATION
Zheng J, Duan H, You S, Liang B, Chen Y and Huang H (2022) Research progress on the pathogenesis of Graves' ophthalmopathy: Based on immunity, noncoding RNA and exosomes. *Front. Immunol.* 13:952954. doi: 10.3389/fimmu.2022.952954

Research progress on the pathogenesis of Graves' ophthalmopathy: Based on immunity, noncoding RNA and exosomes

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Graves' ophthalmopathy (GO), also known as thyroid-associated ophthalmopathy, is a common potentially vision-threatening organ-specific autoimmune disease and the most common extrathyroidal manifestation of Graves' disease. It can happen to those who have hyperthyroidism or euthyroidism. At present, the pathogenesis of GO has not been fully elucidated, and the majority of clinical treatments are symptomatic. Therefore, we are eager to discover any new therapeutic strategies that target the etiology of GO. To provide fresh ideas for the creation of new therapeutic techniques, this study primarily discusses the research state and progress of GO-related pathogenesis from the perspectives of GO's cellular immunity, autoantigens, non-coding RNAs, and exosomes.

KEYWORDS

Graves' ophthalmopathy, cellular immunity, autoantigen, noncoding RNA, exosomes, biologics

1 Introduction

Graves' ophthalmopathy (GO), also known as thyroid-associated ophthalmopathy, is an organ-specific autoimmune disease that is vision-threatening and cosmetically damaging. GO is an ocular abnormality in Graves' disease (GD) and occurs in approximately 30% to 50% of GD patients (1). GO can occur before, during or after the onset of thyroid disease. Signs and symptoms of GO include eyelid retraction, diplopia, proptosis, exposure keratitis, corneal clouding and ulceration, and in severe cases, vision-threatening compressive optic neuropathy (2). Corneal scarring caused by

exposure or optic nerve compression can result in vision loss or possibly blindness, impacting the patient's quality of life significantly (3). Because the pathogenesis of GO has not yet been elucidated, there is no cure and treatment is limited, and symptomatic treatment is still the conventional treatment in clinical practice. According to recent research, GO is a multifactorial disease involving cellular immunity, autoantigens, non-coding RNAs, and exosomes. In this paper, we will review the pathogenesis of GO through several of these factors.

2 Immune factors

2.1 Cellular immunity

In the orbital tissues and extraocular muscles of GO patients, there are numerous localized and diffuse monocyte infiltrates and mucopolysaccharide deposits, with the infiltrating cells primarily $CD4^+$ T lymphocytes, but also modest numbers of neutrophils and plasma cells (2). This shows that the main pathophysiology of GO is cellular immunity (Figure 1). $CD4^+$ T lymphocytes can be divided into helper T cells1 (Th1 cells), Th2 cells, Th17 cells and regulatory T cells (Treg cells) (4), all of the cells listed above play a crucial part in GO pathogenesis. The target cells involved in GO autoimmunity include: infiltrating auto-reactive T cells and orbital fibroblasts (OFs) located between the extra-orbital muscles (2). T lymphocytes interact with OFs through CD40-CD154 costimulatory pathway (2). When OFs are activated, they release a considerable number of cytokines and extracellular matrix (ECM), causing severe orbital inflammation and tissue remodeling. Also, the heterogeneity of OFs determines the outcome and clinical presentation of orbital tissue remodeling. CD90 can be expressed on the surface of OFs, and $CD90^+$ facilitates the fibrosis of OFs, while $CD90^-$ tends to cause adipogenesis (5). During the active phase of orbital tissue changes, the volume of tissue around the eye increases due to inflammatory cell infiltration and orbital tissue edema, which in turn results in an increase in intraocular pressure (6), and the eye moves beyond the bony rim of the orbit, leading to exophthalmos. The major pathological changes in GO include cytokine production and inflammation, adipogenesis, hyaluronan synthesis and myofibrillogenesis (6).

According to Aniszewski et al., orbital tissue in patients with less than two years of hyperthyroidism mostly invaded Th1 cells, whereas orbital tissue in patients with more than two years of hyperthyroidism predominantly infiltrated Th2 cells (7). It can

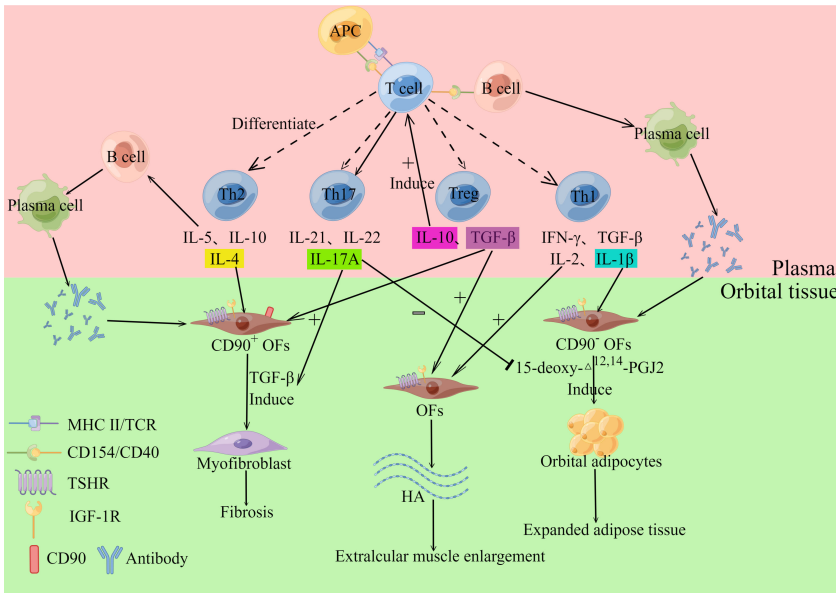

FIGURE 1
Cellular immunity. T cells can differentiate into Th1, Th2, Th17 and Treg cells, which can secrete various cytokines. Th1 cells can secrete IL-1β, IL-2, IFN-γ and TNF-α, and the above cytokines acting on Orbital fibroblasts (OFs) can induce their synthesis and release of hyaluronan (HA). IL-1β also stimulates OFs to differentiate into adipocytes. Th2 cells release IL-5, IL-10, and IL-4, which stimulate B cells and participate in humoral immunity. IL-4 also stimulates OFs to produce collagen, which participates in the GO fibrosis response. Th17 cells mainly secreted IL-17A, IL-21, and IL-22, among which IL-17A promoted TGF-β-induced fibrosis of $CD90^+$ OFs while inhibiting 15-deoxy-$\triangle^{12,14}$-PGJ2-induced adipogenesis of $CD90^-$OFs. Treg cells mainly secrete IL-10 and TGF-β. IL-10 induces T cells to differentiate into pathogenic Th17 cells. TGF-β induces OFs to produce HA and induce OFs to differentiate into myofibroblasts. The inflammatory mediator (Il-1β) that promotes adipogenesis activates $CD90^-$ OFs to differentiate into adipocytes. In contrast, $CD90^+$ orbital fibroblasts were activated by TGF-β and differentiated into myofibroblasts. By Figdraw (www.figdraw.com).

be hypothesized that cell-mediated (Th1-type) immune responses predominate in the early phases of GO, whereas humoral immunity (Th2-type) plays a larger role in the latter stages.Th1 cells produce cytokines including interferon (IFN)-γ, interleukin (IL)-1, IL-2, and tumor necrosis factor (TNF)-α, which cause OFs to create and release significant amounts of glycosaminoglycans like hydrophilic hyaluronan, resulting in swelling of orbital tissues (especially the extraocular muscles) (8). Among others, it has also been shown that IL-1β is associated with adipogenesis in GO (9). The association between elevated circulating levels of TNF-α in GO patients and the severity of GO suggests that monoclonal antibodies against TNF-α, such as Infliximab and Etanercept, could be used to treat GO (10). Studies have shown that both of these drugs have a positive effect on the treatment of GO, reducing the inflammatory response to GO and improving ocular symptoms (11, 12). Th2 cells normally produce IL-4, IL-5 and IL-10. These cytokines are mainly involved in humoral immunity and can stimulate the activation of B cells, leading to the production of autoantibodies (13). Similar to IFN-γ, IL-4 stimulates the proliferation of OFs. Unlike IFN-γ, IL-4 stimulates type IV collagen synthesis, and high doses of IL-4 (>1ug/L) inhibit hyaluronan production (14). Since type IV collagen production is associated with fibrosis and high doses of IL-4 inhibit hyaluronan production, we can speculate that high IL-4 production is a marker of late GO and one of the main causes of orbital tissue fibrosis in late GO.

The above orbital inflammatory state combined with the hyperthyroid state allows excessive production of reactive oxygen species (ROS), mainly superoxide anion and hydrogen peroxide. As with many inflammatory diseases, GO is associated with oxidative stress because of its reduced antioxidant capacity to cope with increased ROS production, which consequently leads to oxidative stress (15). Many studies support the pathogenic role of oxidative stress in the pathogenesis of GO, and researchers have detected ROS in the OFs of GO patients (16). Regemorter et al. reported that an increase in ROS in GO may eventually lead to an increase in pro-inflammatory cytokines (e.g. IL-6), resulting in an inflammatory response in GO (15). Kim et al. reported that ROS production was measured during GO adipocyte differentiation and that the increase in ROS levels was maximal on day 1 and then ROS levels remained at approximately 200% of day 0 levels (17). Therefore, we can conclude that ROS can induce GO to generate adipocytes. Bartalena et al. reported that ROS stimulated the proliferation and differentiation of OFs and produced hyaluronan (18). Meanwhile, ROS up-regulated the expression of human leukocyte antigen-DR and heat shock protein-72, which contributed to the process of GO inflammatory response by participating in T-lymphocyte activation. In summary, we can conclude that oxidative stress is involved in the pathogenesis of GO.

The early studies of GO focused on cytokines produced by Th1 and Th2 cells and their pathogenic effects. Recent researches have shown that Th17 cells are also involved in the disease development process of GO. Several studies have shown that in studies examining the relationship between Th17 cells and GO, higher levels of Th17 cells were detected in GO patients compared to healthy controls, especially those with a clinical activity score (CAS) ≥3 (5). Th17 cells mainly secrete IL-17A, IL-21, and IL-22, which can cause tissue inflammation and autoimmune. They also found that IL-17A also promoted transforming growth factor (TGF)-β-induced fibrosis in $CD90^+$ OFs while inhibiting 15-deoxy-$\triangle^{12,14}$-PGJ2 induced adipogenesis in $CD90^-$ OFs (5). It has been shown that Th17 cells produce high levels of IL-17A acting on OFs, causing OFs to secrete more inflammatory cytokines and ECM and express fibrosis-related proteins, which is one of the causes of the inflammatory response and the development of fibrosis (5). Since then, further insights on the investigation of Th17 cells in GO patients have been presented by researchers. Fang et al. found that most GO patients had $CCR6^+$ Th17 cells expressing IL-17A and that IL-17A production by $CCR6^+$ Th17 cells gradually decreased from active GO patients to inactive GO patients (19). Meanwhile, the increase in CAS of GO patients was synchronized with the increase in the number of $CCR6^+$Th17 cells. This suggests that $CCR6^+$ Th17 cells are associated with GO activity and that they may maintain the development of GO orbital inflammation. We can also speculate that $CCR6^+$ Th17 cells, similar to Th1 cells, have a role in the early phases of GO. The symptoms of GO can manifest not only in the orbital connective tissue, but also in the lacrimal glands, such as ocular surface inflammation and xerophthalmia (2). Huang et al. found that IL-17A produced by Th17 cells in the lacrimal glands promoted the differentiation of TGF-β-initiated myofibroblasts in GO lacrimal fibroblasts and 15-deoxy-$\triangle^{12,14}$-PGJ2-initiated adipocytes (20). They concluded that the above process could be one of the major reasons for GO lacrimal glands fibrosis. Since IL-17A has similar pro-inflammatory, pro-fibrotic and pro-adipogenic mechanisms in lacrimal and orbital connective tissues, targeting Th17 cells may have therapeutic effects on both tissues. SHR-1314 is a recombinant humanized monoclonal antibody against IL-17A that has been demonstrated to be effective in clinical trials for a variety of autoimmune illnesses (21). Therefore, we speculate that it may also be effective in the treatment of GO, and we can carry out clinical studies on the efficacy and safety of SHR-1314 for the treatment of GO in the future to provide a new approach for the treatment of GO. In recent years, researchers have also proposed the concept of Th17 cell plasticity, which means that Th17 cells can transform into other $CD4^+$ T cell subsets, such as Th1 and Th2 cells (22). We envisioned whether pathogenic Th17 cells could be selectively depleted while normal cells were preserved by identifying particular surface markers of pathogenic Th17 cells. Or study the mechanism by which Th17 cells are transformed into other cells, and stop the disease by

intervening to transform pathogenic cells into cells that are beneficial to the body.

Treg cells are immunosuppressive T-cell subtypes, and it has been shown that reduced numbers or functional defects of Treg cells are related to the development of various autoimmune diseases (23). However, the mechanism of Treg cells in the development of GO is unclear. The majority of Treg cells are predominantly Foxp3$^+$, which mainly produce TGF-β, IL-10 and other anti-inflammatory cytokines, and they can exert their immune effects through a variety of different mechanisms. Hyaluronan is a potential regulatory substance that plays a role in the differentiation of orbital fibroblasts into myofibroblasts induced by TGF-β. It has been shown that TGF-β induces the production of hyaluronan by OFs (24). Ma et al. found that TGF-β1 can induce OFs to differentiate into myofibroblasts *via* HA-CD44 signaling, characterized by α-smooth muscle actin up-regulated (24). The same results were reported by Wang et al. who noted that TGF-β1 stimulation of OFs from GO patients produced higher expression of fibrosis and extracellular matrix production markers (such as α-SMA, FN1 and COL1A1) than stimulation of OFs from GO-free subjects (25). It also indicates that OFs from GO patients have differentiated into myofibroblasts. Wu et al. showed that TGF-β1 can induce the transdifferentiation of OFs into myofibroblasts through the MAPK signaling pathway, characterized by enhanced expression of fibrotic proteins such as α-smooth muscle actin, connective tissue growth factor, and fibronectin (26). In addition, their study showed that TGF-β1 induces the up-regulated of type I collagen with the accumulation of ECM proteins. The fibrosis and the accumulation of ECM together lead to remodeling of GO extraocular muscles. In conclusion, TGF-β plays an important role in orbital tissue fibrosis in GO patients. TGF-β can also promote the proliferation, differentiation and survival of lymphocytes and other immune cells to maintain immune tolerance (27). IL-10 induces the differentiation of T cells into pathogenic Th17 cells (28), and also interferes with the migration of Th1 cells to sites of intestinal inflammation (29). Another study suggests that the number of Treg cells may be related to the severity of the inflammatory response in GO, and that GO with a higher frequency of Treg cells tends to exhibit a decreased clinical course (30). Muñoz A et al. found that CD69$^+$ Foxp3$^-$ Treg cells were significantly increased in the peripheral blood of GO patients and that there was a positive correlation with GO activity (31). Therefore, the number of Treg cells in the peripheral blood of GO patients can be used as a predictor of the clinical course. Furthermore, in recent years, it has also been reported that dysregulation of Treg/Th17 balance is also involved in the pathogenesis of GO (32). Therefore, we hypothesized that inflammation and fibrosis in GO orbital tissues may be attenuated when the Treg/Th17 ratio is elevated. Conversely, disease symptoms may develop and/or worsen when Treg/Th17 is decreased.

2.2 GO-associated autoantigens

2.2.1 TSHR

The thyroid stimulating hormone receptor (TSHR) is a G protein-coupled receptor that is a glycoprotein hormone receptor. It is the major autoantigen of GO and can promote cAMP synthesis and activate the PI3K cascade. In 1976, Kriss et al. first proposed the concept of a common antigen between thyroid and orbital affected tissues, in which TSHR expressed on OFs is a cross-cutting antigen causing autoimmune reactions in GO patients (33). Several studies have confirmed that TSHR mRNA can be detected in the orbital connective tissue of GO patients, and the expression of TSHR in the orbital adipose tissue of GO patients is higher than that of normal tissue (34). Therefore, we can speculate that the activation of TSHR can induce adipogenesis in orbital tissue. The production of new adipose in the eye frame of GO patients also enhances the expression of TSHR in this tissue (6). Lu et al. demonstrated that TSH can stimulate adipogenesis even without adipogenic factors by using the *in vitro* model of adipogenesis of mouse embryonic stem cells (35). GO patients have circulating autoantibodies against TSHR, and M22 is a monoclonal antibody against TSHR. M22 has been shown to increase cAMP production as well as the production of three hyaluronan synthases (HAS1, HAS2 and HAS3), thus activating OFs and increasing hyaluronan production (36). In addition, M22 increases the expression and secretion of IL-6 in pre-adipose fibroblasts and adipocytes of GO patients, while IL-6 promotes the expression of the autoantigen TSHR by OFs, and also drives B-cell immunoglobulin production, plasma cell development (Figure 2), aggravating the autoimmune response and influencing the disease's clinical activity (37). IL-6 is negatively related to GO duration, and positively related to CAS (38). The level of anti-TSHR antibodies relates to the severity and clinical activity of GO. The production of adipose and hyaluronan in OFs reflects the remodeling of GO orbital tissue.

Tocilizumab is a humanized monoclonal antibody against IL-6R, which can block IL-6 activity and signal transduction. Tocilizumab significantly improved disease activity and severity in patients with corticosteroid-resistant GO, and its effects have been shown in a multicenter, randomized, double-blind trial conducted in Spain (39). Human monoclonal autoantibody K1-70 is a particular TSHR antagonist with therapeutic potential for patients who block the effect of thyroid stimulants on TSHR. After administering K1-70 to a female patient with GD, severe GO and follicular thyroid cancer, it was reported that the patient's CAS and proptosis improved significantly (40). Results from a recent phase I clinical trial of K1-70^{TM} in the treatment of patients with GD and GO showed that K1-70^{TM} was safe and well-tolerated by patients and produced the expected pharmacodynamic effects without immunogenic reactions (41). As a new drug, K1-70^{TM} shows considerable promise for application with the ability to block the effect of thyroid-stimulating agents on TSHR.

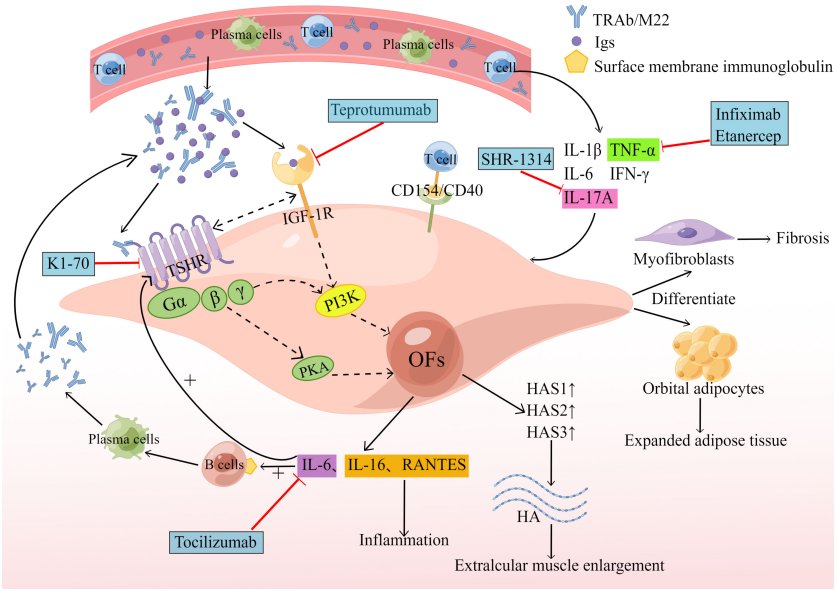

FIGURE 2
Humoral immunity and biologics. The circulating TRAb and M22 act on TSHR to induce cAMP production, which activates the PI3K cascade reaction and promotes the production of HAS1, HAS2 and HAS3, resulting in increased production of hyaluronan, leading to extraocular muscle hypertrophy. M22 also increases the expression and secretion of IL-6. Subsequently, the increased expression of IL-6 promotes the expression of the autoantigen TSHR by OFs and also drives B-cell immunoglobulin (Ig) production, and plasma cell development. Serum Igs from GO patients bind to IGF-1R and produce IL-16 and RANTES, promoting an inflammatory response. TSHR and IGF-1R can form physical and functional protein complexes that can interact to increase signaling when activated by their respective ligands, resulting in increased hyaluronan (HA) and IL-6 production. The generated HA and IL-6 then repeat the above reaction, aggravating the GO pathological process. New therapeutic drugs are indicated by blue boxes and red lines. By Figdraw (www.figdraw.com).

2.2.2 IGF-1R

It has recently been shown that the insulin-like growth factor-1 receptor (IGF-1R) is overexpressed in GO (42). We speculate that it may be another important autoantigen of GO. The serum immunoglobulins (Igs) from GO patients act on OFs to produce T-cell chemotactic agents such as IL-16 and chemokine RANTES (43)(Figure 2), which regulate the expression and secretion of activated, normal T cells and also facilitate the transport of $CD4^+$ T cells (44), allowing for a sustained GO autoimmune response. This response can be weakened by blocking autoantibodies to IGF-1R or by transfecting fibroblasts with dominant-negative mutant IGF-1R (6), suggesting that IGF-1R signaling mediates this process. IGF-1R can also interact with autoantibodies against the receptor and participate in adipogenesis and hyaluronan synthesis (45). However, the effect of IGF-1R on hyaluronan synthesis appears to be indirect, as IGF-1 itself does not increase HAS2 transcription.

As the study progressed, researchers found that IGF-1R can also be involved in GO pathogenesis through the IGF-1R/TSHR crosstalk pathway, where GO Igs activate crosstalk by binding to TSHR on OFs (46). Kriege et al. proposed that IGF-1R and TSHR must be physically adjacent to each other to make their signaling pathways the same, and that arrestin-β-1 can provide a scaffold for the TSHR/IGF-1R protein complex, which provides the basis for crosstalk between the two receptors (47). Thus, TSHR and IGF-1R can form physical and functional protein complexes. It has been shown that IGF-1R and TSHR can interact to increase signaling when activated by their respective ligands, and that inhibition of IGF-1R activity blocks signaling downstream of TSHR (46). IGF-1R/TSHR signaling also boosts the production of hyaluronan in GO OFs and other glycosaminoglycan components in the ECM (48) (Figure 2). Increased deposition of ECM, especially high molecular weight hyaluronan, can promote enlargement, inflammation, edema and congestion of GO orbital tissue (49). Activation of the IGF-1R/TSHR protein complex also increases the secretion IL-6 and IL-8, which are involved in the autoimmune response to GO. Teprotumumab is the only drug approved by the FDA for the treatment of GO. Teprotumumab is a human monoclonal IGF-1R blocking antibody that can achieve therapeutic effects for GO by blocking IGF-1R/TSHR crosstalk-mediated increase in hyaluronate synthesis and adipogenesis on the surface of OFs (50, 51). A recent randomized, multicenter, placebo-controlled, double-blind trial showed that treatment with Teprotumumab in GO patients was more effective than placebo in reducing ocular proptosis and CAS (52).

3 Non-coding RNA

Epigenetics is a research hotspot in recent years, which refers to heritable changes in gene function without changes in the DNA sequence of genes, and ultimately leads to changes in phenotype, such as histone modifications, DNA methylation, and non-coding RNA (ncRNA). Here we mainly elaborate the relationship between ncRNA and GO.

NcRNA refers to a class of RNA transcripts lacking protein-coding functions, which can be classified into microRNA (miRNA), circular RNA (circRNA) and long non-coding RNA (lncRNA). It is reported that their abnormal expression is importantly associated with the pathophysiology of autoimmune thyroid disease (53) (Table 1).

3.1 MiRNA and GO

MiRNA is an endogenous short non-coding RNA molecule, 18 to 23 nucleotides in length, which can effectively mediate the degradation of mRNA or restrict its translation by binding to the structural domain of the 3' untranslated region of the target gene mRNA, thereby exerting a strong negative regulatory influence on post-transcriptional levels (70). It has been shown that miRNAs play a great role in a wide range of biological activities, including immune function, apoptosis, cell differentiation and development, cell proliferation and metabolism (59). It is reported that abnormal miRNA expression has been linked to the pathogenesis of GO, and because miRNAs are easily detectable in blood and tissue samples, they are expected to be early biomarkers for evaluating disease risk and severity (71).

3.1.1 MiRNA in GO circulation

It has been suggested that some circulating miRNAs have been linked to the development of GO and are closely associated with the differentiation or activation of immune cells, and the regulation of the immune response. One of the most studied miRNAs is miR-146a, which is a typical multifunctional miRNA characterized by regulatory roles in immune regulation, cell proliferation, differentiation, apoptosis and ECM (72). Several studies have found that GO patients' peripheral blood miR-146a levels are considerably lower than healthy controls (72). Meanwhile, CAS and IL-17 levels are negatively related to down-regulated miR-146a expression, whereas IL-17 levels are positively related to CAS (55). The down-regulated of miR-146a may result in an unexpected increase in IL-17 levels, promoting the development of GO ocular inflammation. MiR-146a inhibits Th1 differentiation and cell proliferation process of $CD4^+$ T lymphocytes by targeting NUMB protein (72). A recent study reported that the down-regulated miR-146a expression in $CD4^+$ T lymphocytes from GO patients attenuates the above inhibitory effect, thereby promoting ocular inflammation in GO (54). In conclusion, the down-regulated circulating miR-146a expression may indirectly contribute to the development of GO by influencing the production of different inflammatory factors. The nuclear factor κB (NF-κB) gene is an important immune and inflammatory response gene. MiR-146a and miR-155 are both reported to be trans targets of NF-κB, which can constitute a negative feedback pathway through the involvement of certain genes (73). Therefore, we speculate that miR-146a and miR-155 can have the same target gene in NF-κB signaling and regulate each other's immune response. Li et al. hypothesized that, in contrast to miR-146a, miR-155 could augment GO ocular inflammation by boosting inflammatory T cell production (74). In addition, we are speculated that miR-155 may have other functions in the pathophysiology of GO. In macrophages, miR-155 promotes the expression of TNF-α and IL-6 by targeting the SOCS1 gene (75). In systemic sclerosis, MiR-155 induces persistent collagen expression in fibroblasts and plays a critical role in fibrosis (76). In 3T3-L1 cells, however, miR-155 inhibits adipogenesis and adipocyte development (77). From this, we hypothesized that miR-155 may play an important role in GO inflammatory response, fibrosis and adipogenesis.

Rebeca et al. concluded that other miRNAs, such as miR-Let7-5p, miR-142-3p, miR-21-5p, miR-301a-3p and miR-95-5p are also differentially expressed in autoimmune thyroid disease and that in GD patients (including GO), the above miRNAs correlate with disease severity (59). When compared to GD patients who don't have GO, miR-Let7-5p was significantly lower in GO patients. MiR-Let7-5p was negatively related to CAS, and miR-Let7-5p levels were lower in GO patients with more severe diseases (59). This is because miR-Let7 has a Treg cell-mediated activity that suppresses IFN-γ secretion and inflammatory responses by Th1 cells (59), so the low levels of miR-Let7-5p discovered in GO might be linked to the previously observed defective Treg cellular function in individuals with autoimmune thyroid disease (23). It has been reported that miR-21 has been linked to the regulation of immune function as well as the development of numerous autoimmune disorders (78, 79), from which we can speculate that miR-21 may have a common mechanism of action in different immune cells. The reality that miR-21 expression can affect T cell activation, including Th1/Th2 balance (80), and Th17 cell differentiation (81), could explain the above findings.

Thiel et al. reported that miR-96 and miR-183 were abundantly expressed in $CD4^+$T cells from GO patients' peripheral blood and in human and mouse T cells activated *in vitro* (60). They proposed that these two miRNAs boost $CD4^+$ T cell proliferation by influencing the expression of EGR1 associated with the PTEN-PI3K-AKT signaling pathway, leading to their expansion *in vivo* and thus promoting the development and progression of GO.

Shen et al. reported that low levels of miR-224-5p in serum may be associated with insensitivity to glucocorticoid (GC) therapy in GO patients. MiR-224-5p may serve as a valid biomarker to predict the efficacy of GC therapy in GO

TABLE 1 The role of non-coding RNAs in the pathogenesis of GO.

Noncoding RNAs	Samples/cells	Expression change	Function	Effects in GO	References
miR-146a	peripheral blood	Down-regulated	Inhibition of Th1 differentiation and cell proliferation processes in CD4+ T lymphocytes	Promotes inflammation	(54)
	Plasma/Serum	Down-regulated	Promotes Th17 cell differentiation	Promotes inflammation	(55)
	Orbital adipose/connective tissue	Up-regulated	Inhibition of TGF-β-induced fibrosis marker production	Inhibition of fibrosis process	(56)
	Orbital tissue	Up-regulated	Inhibition of IL-1β-induced IL-6 protein production and ICAM-1 expression	Relief of inflammation	(57)
miR-146a and miR-155	OFs	Up-regulated	Reduced expression of ZNRF3 and PTEN	Enhancement of OFs proliferation	(58)
miR-Let7-5p	Serum	Down-regulated	Prevention of Th1 cell-mediated IFN-γ secretion and inflammatory response	Relief of inflammation	(59)
miR-183 and miR96	peripheral blood	Up-regulated	Promotes proliferation of CD4+ T cells	Promotes inflammation	(60)
miR-224-5p	Serum	Up-regulated	Increased GC sensitivity and GR expression *via* GSK-3β	Increase GC treatment sensitivity	(61)
miR-885-3p	Plasma exosomes	Up-regulated	Inhibition of AKT/NFκB signaling pathway, up-regulated of GR levels and down-regulated of inflammatory factor levels	Increase GC treatment sensitivity and alleviate inflammatory response	(62)
miR-27a-3p	Plasma/Serum	Up-regulated	——	As a potential biomarker for predicting the progression of GD to GO	(63)
miR-22-3p	Plasma/Serum	Down-regulated	——	As a potential biomarker for predicting the progression of GD to GO	(63)
miR-29	OFs	Up-regulated	Inhibition of TGF-β-mediated synthesis of the ECM of OFs	Inhibition of fibrosis process	(64)
miR-21	Orbital adipose tissue	Up-regulated	Inhibits PDCD4 expression, thereby promoting the proliferation of OFs	Promotes the fibrosis process	(65)
miR-27a and miR-27b	Orbital adipose tissue	Down-regulated	Inhibition of PPARG and C/EBP expression is involved in adipogenesis	Promotes adipogenesis	(66)
miR-130a	OFs	Up-regulated	Targeting AMPK and attenuating AMPK activity to promote lipid accumulation	Excessive accumulation of adipose tissue	(67)
circRNA_14940	Orbital adipose/connective tissue	Up-regulated	Involved in Wnt signaling pathway with up-regulated CCND1, involved in PI3K-Akt signaling pathway with down-regulated TNXB, interacts with ECM receptor	Involvement in GO pathogenesis	(68)
circRNA_10135	Orbital adipose/connective tissue	Up-regulated	Interacts with PTGFR through calcium signaling pathway and plays a role in adipogenesis	Promotes adipogenesis	(68)
LINCO1820:13	Orbital adipose/connective tissue	Up-regulated	RNA-induced silencing complexes that competitively bind miR-27b, thereby upregulating FPR2 expression	Involvement in GO pathogenesis	(69)
ENST0000499452	Orbital adipose/connective tissue	Up-regulated	Competitive inhibition of miR-27a, thereby weakening its inhibitory effect on CXCL1	Involvement in GO pathogenesis	(69)

The red values provided in Table 1 indicate the references cited in the row where each non-coding RNA is located.

patients, and they hypothesized that circulating miR-224-5p overexpression may increase GC receptor and GC sensitivity expression by targeting glycogen synthase kinase-3β (61). Sun et al. found that miR-885-3p was up-regulated in the plasma of GO patients with improved symptoms after intravenous GC treatment (62), miR-885-3p inhibits the AKT/NFκB signaling pathway and up-regulated GRE luciferase reporter gene plasmids and GC receptor levels to improve GC sensitivity and down-regulated inflammatory factor levels in OFs to alleviate GO autoimmune responses. Therefore, miR-885-3p can also be used as a biomarker to assess intravenous GC sensitivity in GO patients. Zhang et al. found by high-throughput proteomics and miRNA sequencing experiments that the up-regulated miR-27a-3p and down-regulated miR-22-3p in expression levels from GO serum/plasma compared to healthy controls, can be employed as possible biomarkers to predict the progression of GD to GO (63).

In summary, we hypothesized that detection of various miRNAs differentially expressed in GO patients could be employed as potential biomarkers to predict the progression of GO.

3.1.2 MiRNA in GO orbital tissue

In addition to being expressed in the peripheral circulation, miRNAs from GO patients can also be differentially expressed in orbital tissue. OFs play a role in the pathophysiology of GO as both target and effector cells. Several recent researches have demonstrated that some miRNAs may be link to GO orbital tissue fibrosis, autoimmune response and adipose tissue formation.

It has been shown that miR-146a in orbital tissue is associated with fibrotic, inflammatory responses in GO orbital tissues. Sun et al. found significantly higher miR-146a expression levels in the orbital adipose tissue of GO patients than in non-GO orbital adipose tissue (56). And then they found experimentally that miR-146a may become connected to the production of TGF-β-induced fibrosis markers such as fibronectin, collagen type Iα and α smooth muscle actin as negative regulators, i.e. miR-146a may be regulated the antifibrosis in OFs of GO patients (56). However, it has also been reported that TSHR signaling in GO OFs can enhance the proliferation of GO OFs by inducing the production of miR-146a and miR-155 to reduce the expression of target genes that block cell proliferation ZNRF3 and PTEN (58). The pathological process of partial fibrous hyperplasia reported in GO could be explained by the TSHR-dependent expression of miR-146a and miR-155. However, Sun et al. discovered that miR-146a is also connected to the anti-inflammatory process of GO in prior work. By analyzing miR-146a mimics, they discovered that miR-146a mimics inhibited IL-1β-induced IL-6 protein synthesis and intercellular adhesion molecule-1 expression (57). This finding supports a previous study that found miR-146a inhibits the NF-κB pathway by downregulating its target genes, such as IRAK1 and TRAF6 (82), resulting in the relief or termination of the inflammatory response. Comparing the relationship between miR-146a and inflammatory response in peripheral blood, we can conclude that different levels of miR-146a play different roles in the inflammatory response of OFs by regulating the NF-κB pathway. The down-regulated of miR-146a levels promotes the development of inflammation, while the up-regulated of its levels allow the remission or termination of the inflammatory response. It has been proposed that miR-155 plays a crucial role in the development of Th17 cells during autoimmunity (69). Also, miR-155 has an essential role in fibrosis by mediating TGF-1β1 signaling to drive collagen synthesis (83). However, the precise mechanism of miR-155 in GO is yet unknown, and more research is needed.

In addition to miR-146a, miR-29 and miR-21 have also been reported to be associated with OFs fibrosis. Similar to miR-146a, miR-29 overexpression significantly inhibited TGF-β-mediated synthesis of the ECM of OFs (64). In contrast to miR-146a, PDGF-BB significantly inhibits PDCD4 expression by upregulating miR-21 in OFs, thereby promoting the proliferation of OFs (65).

MiR-27a and miR-27b inhibit adipose differentiation in GO patients' OFs. Sun et al. discovered that GO patients' orbital adipose tissue had considerably lower levels of miR-27a and miR-27b than non-GO tissue by real-time enzyme chain polymerization reaction, and reduced levels of adipogenesis-induced PPARG, C/EBP-α and C/EBP-β proteins and miRNA expression in miR-27a and miR-27b mock-transfected OFs (66). These findings imply that down-regulated miR-27a and miR-27b expression may be implicated in adipocyte formation by inhibiting PPARG and C/EBP expression (66). We thus speculate that miR-27a and miR-27b, *via* modulating the formation of adipocytes, may provide potential therapeutic targets for the treatment of GO-induced ocular proptosis. In addition to miR-27a and miR-27b, miR-130a also plays a role in GO orbital tissue adipose tissue formation. Hammond et al. first found that miR-130a was elevated in patients with OFs prone to lipogenesis and promoted lipid accumulation, and that miR-130a also targeted and attenuated adenosine monophosphate-activated protein kinase activity and promoted lipid accumulation (67). This new mechanism may lead to new treatments for patients with GO.

In summary, miRNAs play a crucial role in the development of GO, with miR-146a being a promising target. However, we do not fully understand the mechanism of miRNA action in GO, so additional research is needed to elucidate the application of miRNA in GO.

3.2 CircRNA and GO

CircRNA is an RNA molecule with a closed-loop structure, which is a characteristic structure generated by covalent bonds at

the 3' and 5' ends after reverse splicing (84). Based on the biogenesis pattern of genomic regions, circRNAs can be classified into four categories: intronic circular RNA, exonic circular RNA, exon-intron circular RNA, and intergenic circular RNA (85). CircRNAs provide a variety of biological functions, including serving as miRNA sponges for adsorption, attachment to various RNA-binding proteins, and engagement in protein translation (86).

But current research on the role of circRNAs in the pathophysiology of GO is in its early stages. Wu et al. derived from RNA sequencing of orbital adipose/connective tissue samples from GO patients that 1631 circRNAs were differentially expressed in GO samples (68). Analysis of circRNA-miRNA co-expression and circRNA-miRNA interactions showed that circRNA_14940 may be involved in GO pathogenesis by participating in the Wnt signaling pathway with up-regulated CCND1, interaction with down-regulated TNXB involved in ECM receptor, focal adhesion and PI3K-AKT signaling pathway (68). Therefore, differentially expressed circRNAs may have a role in GO pathogenesis, with the circRNA_14940-CCND1-Wnt signaling pathway being a key regulatory axis. Wu et al. hypothesized that circRNA_10135 may interact with the up-regulated PTGFR through the calcium signaling pathway and play a role in adipogenesis in GO (68). In addition, they hypothesized that hsa-miR-10392-3p may function in GO by regulating circRNA_14936, which is associated with TNFRSF19 and thus affects cytokine-cytokine receptor interactions associated with B-cell survival and, in turn, the development of GO (68). Therefore, circRNA may play a key role in the development of GO.

However, there have been few studies on the role of circRNAs in the pathogenesis of GO, and there are many circRNAs whose functional analysis is not comprehensive, so more experiments are needed for functional validation.

3.3 LncRNA and GO

LncRNAs are a type of long-stranded multifunctional RNAs with a length of more than 200 that have been reported to act at many different levels of gene expression, including epigenetic regulation, transcriptional regulation, post-transcriptional regulation, and miRNA regulation (87). Salmena et al. suggested the competitive endogenous RNA hypothesis for lncRNA-miRNA interactions (88), which is characterized as mutual interference between coding and non-coding RNAs *via* miRNA response elements, resulting in an extensive regulatory network in the transcriptome (69).

Yue et al. derived by quantitative real-time PCR that there were differences in the expression of four mRNAs (CHRM3, THBS2, FPR2, CXCL1) and two lncRNAs (LINC01820:13, ENS0000499452) between GO patients and control patients (69). They hypothesized that LINCO1820:13 up-regulated FPR2 expression by competitively binding to the RNA-induced silencing complex of miR-27b, resulting in autoimmune responses and inflammation in GO patients (69). ENST0000499452 suppresses miR-27a through the same mechanism, thereby impairing its inhibitory effect on CXCL1 and enhancing immune response, inflammation, and fibrosis in GO patients' OFs (69).

Wu et al. found that co-expression interactions between differentially expressed lncRNAs and 52 ECM-related mRNAs (e.g., COL12A1, TNXB, COL6A3, KAZALD1, FBN1 and SPON1) in GO patients' orbital adipose/connective tissue samples suggest that lncRNAs may regulate ECM function in GO patients' orbital adipose/connective tissue from (89). For example, co-expression interactions of lncRNA with COL12A1 and negative co-expression of lnc-PTP4A2-3:7 with TNXB may be associated with altered ECM in GO orbital adipose/connective tissue (89). Wang et al. shown that the combination of upregulated lncRNA LPAL2 and downregulated miR-1287-5p in orbital tissues of GO patients induced increased levels of cell adhesion factors and activation of GO OFs by TGF-β1 through EGFR/AKT signaling (90). Meanwhile, EGFR has been reported to increase the proliferation of OFs, which may contribute to the fibrosis of extraocular muscles (91).

In conclusion, lncRNAs have a significant role in the pathophysiology of GO, and we need to conduct more studies in the future to find the relationship between lncRNAs and the pathogenesis of GO.

4 Exosomes and GO

Exosomes are extracellular vesicles that are 30-150 nm in size and can transport specific compounds such as proteins, lipids, miRNA, DNA, etc (92). It can be found in many different body fluids, including blood, tears urine, breast milk, saliva, etc. (93). Exosomes contain a wide range of bioactive molecules, including chemokines, inflammatory factors, signal transduction factors, different RNAs, etc., and proteins with specialized roles on their surface, such as adhesion molecules, co-stimulatory molecules, ligands, receptors, and so on (92). Exosomes regulate the immunological response through two main mechanisms: direct action of exosomes on target cells to activate downstream signals and exosomal regulation of the immune response mediated by exosomal miRNAs (94) (Figure 3). The former works in three ways: surface signaling molecules act directly, bioactive substances are released extracellularly, and signaling molecules are modulated intracellularly during membrane fusion (95). The immunomodulatory effects of exosomes include T cell activation, antigen presentation, inflammatory response, immunosuppression and intercellular communication (96). Exosomes have been implicated in the initiation and progression of GO in several studies, but research into them is still in its early stages.

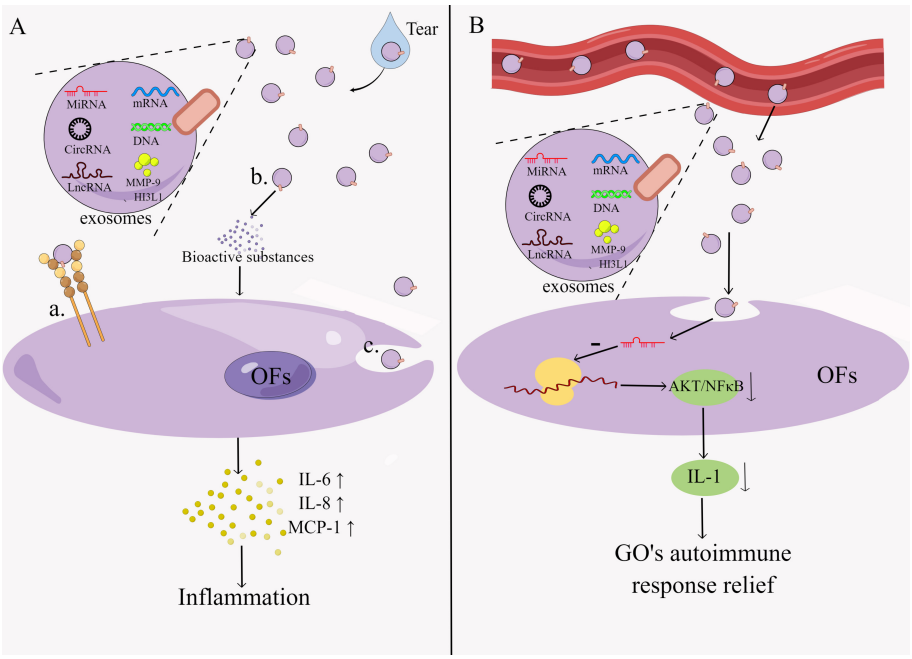

FIGURE 3
Pathogenesis of exosomes in GO.Exosomes regulate the immune response by two main mechanisms: **(A)** exosomes act directly on target cells thereby activating downstream signals. This process is characterized by: a. The direct action of surface signaling molecules. b. Extracellular release of bioactive substances. c. Intracellular regulation of signaling molecules during membrane fusion. **(B)** Exosomes mediate regulation of immune responses through exosomal miRNA. By Figdraw (www.figdraw.com).

Han et al. demonstrated that exosomes in tears of GO patients were 2.3-fold higher than in healthy controls and that exosomes triggered the release of inflammatory cytokines IL-6, IL-8 and monocyte repellent protein-1 from OFs in an *in vitro* experiment. These data imply that the abundance of specific proteins (e.g., MMP-9, CHI3L1) in exosomes can activate the inflammatory response, orbital tissue remodeling, and fibrosis in OFs of GO patients (97). MMP are enzymes that play a key role in fibrosis, inflammatory processes and tissue remodeling, and previous reports have shown increased serum MMP-9 concentrations in GO patients and an association with CAS in GO patients (98). CHI3L1 increases the synthesis of inflammatory cytokines like IL-1β, IL-6, TNF-α, and IFN-γ, connective tissue growth and fibrosis (99).

Sun et al. discovered that differences in miRNA levels in GO patients were caused by changes in miRNA content in exosomes rather than changes in exosome concentrations per unit volume of plasma, and that exosome-delivered miR-885-3p could inhibit the AKT/NFκB signaling pathway, affecting OFs in GO patients (62). This suggests that plasma exosomes' miRNAs can be transferred to recipient cells *via* exosomes and bind to target cells, which then regulate cellular functions.

Based on the current evidence, exosomes have great potential as diagnostic biomarkers and therapeutic approaches for GO. However, exosome research is still in its infancy, and their potential mechanisms in GO have yet to be fully elucidated. Therefore, we need to continue our research to determine the specific mechanism of exosomes in GO, which will contribute to a better understanding of the diagnosis and prognosis of the disease.

5 Conclusion and prospect

GO is an autoimmune disease with multiple factors involved in its pathogenesis, and its pathogenesis is still being explored. Cellular immunity plays a key role in orbital inflammation in GO, where T cells interact with OFs through the production of various cytokines, as well as induce the propagation of multiple intracellular signaling cascades, resulting in hyaluronan secretion, adipogenesis, and the persistence of orbital inflammation. TSHR, one of the major autoantigens of GO, is associated with immune response and adipogenesis in orbital tissues, and also induces IL-6 expression and secretion, thereby exacerbating the autoimmune response. IGF-1R on OFs is involved in adipogenesis and hyaluronan synthesis, as well as mediating some aspects of orbital changes. The researchers found that IGF-1R and TSHR can form physical and functional protein complexes that are involved in the development of GO. With the advent of high-throughput gene sequencing technology, the importance of ncRNA in the pathogenesis of GO has been

gradually revealed, and current studies have found that it is mainly related to cell differentiation, immune regulation, and adipogenesis. Exosomes have received a lot of attention in recent years, mainly through direct action on target cells to trigger downstream signals and through exosomal miRNAs to regulate GO immune responses, orbital tissue remodeling and fibrosis. However, these studies are still in the exploratory stage, and revealing the molecular mechanisms behind GO is expected to provide insights for formulating new treatment plans, developing new therapeutic strategies, and optimizing our clinical management of the disease.

Currently, we most commonly use GC for the treatment of GO, which have immunosuppressive and anti-inflammatory effects and can be used to alleviate the clinical symptoms of GO. However, long-term or high-dose GC treatment can lead to many complications, such as medically induced Cushing's syndrome, diabetes, hypertension and osteoporosis, etc. Unfortunately, a small percentage of GO patients are resistant to GC treatment, making treatment more challenging. Some biologics developed for immune mechanisms, such as Infliximab, Etanercept, Tocilizumab and Teprotumumab, have shown promising results in the treatment of GO, but because their drugs are expensive and require multiple intravenous treatments, they can impose a significant financial burden on patients. The current treatment methods are difficult to meet the patient's treatment requirements. Therefore, it is imperative to conduct more effective clinical studies to explore more effective and safer drugs for the long-term treatment of GO.

Author contributions

JZ, HD and HH wrote the manuscript. JZ and HD wrote the first draft of the manuscript. HH revised the manuscript and edited. SY, BL and YC assisted in manuscript preparation. All authors contributed to the article and approved the submitted version.

Funding

This research was supported by the Second Affiliated Hospital of Fujian Medical University horizontal scientific research project (NO: HX202201 and HX202202), the Second Affiliated Hospital of Fujian Medical University Doctoral Research Project (NO: 2021GCC03).

Acknowledgments

Thanks to Figdraw (www.figdraw.com) for their support in drawing the figure.

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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frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 12 September 2022
DOI 10.3389/fimmu.2022.954129

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Autoimmune and Autoinflammatory Disorders, a section of the journal Frontiers in Immunology

RECEIVED 27 May 2022
ACCEPTED 22 August 2022
PUBLISHED 12 September 2022

CITATION
Zhang M, Zou Y, Zhou X and Zhou J (2022) Inhibitory targeting cGAS-STING-TBK1 axis: Emerging strategies for autoimmune diseases therapy. *Front. Immunol.* 13:954129. doi: 10.3389/fimmu.2022.954129

Inhibitory targeting cGAS-STING-TBK1 axis: Emerging strategies for autoimmune diseases therapy

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The cGAS-STING signaling plays an integral role in the host immune response, and the abnormal activation of cGAS-STING is highly related to various autoimmune diseases. Therefore, targeting the cGAS-STING-TBK1 axis has become a promising strategy in therapy of autoimmune diseases. Herein, we summarized the key pathways mediated by the cGAS-STING-TBK1 axis and various cGAS-STING-TBK1 related autoimmune diseases, as well as the recent development of cGAS, STING, or TBK1 selective inhibitors and their potential application in therapy of cGAS-STING-TBK1 related autoimmune diseases. Overall, the review highlights that inhibiting cGAS-STING-TBK1 signaling is an attractive strategy for autoimmune disease therapy.

KEYWORDS

CGAS, STING, autoimmune disease, inhibitor, TBK1

1 Introduction

Autoimmune diseases including various chronic inflammatory illnesses have affected the health of around 3%-10% of people in the world (1). The aberrant responses of the immune system to self are thought as the major factor leading to autoimmune diseases. Although the innate immune system which detects and responds to the pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) serves as the organism's first line of defense against foreign invasion, the dysregulation and over-activation of the innate immune system will lead to various inflammatory illnesses (2, 3). Moreover, the autoinflammation induced by the abnormal innate immune signaling can achieve the establishment of adaptive immune responses, thus leading to the progress of autoimmunity. The endosomal or cytosolic nucleic-acid sensing involved in innate immunity is one of the initial triggers of autoimmunity. The nuclear acid recognition receptors, including retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5), Toll-like receptors (TLR3, 7, 8, and

9), and the cyclic GMP-AMP synthase–stimulator of interferon genes–tank-binding kinase 1 (cGAS-STING-TBK1) axis, have been directly related to the pathogenesis of various autoimmune diseases (4–9).

The cGAS-STING signaling pathway combines DNA sensing with the induction of a strong innate immune defense program, playing a crucial role in the host immune response (10). Through the recognition of the exogenous DNA from virus and bacterial or own damaged DNA, cGAS catalyzes the synthesis of cyclic GMP-AMP (cGAMP) from adenosine triphosphate (ATP) and guanosine triphosphate (GTP). cGAMP further interacts with STING and activates downstream pathways to induce the expression of type I interferons (IFNs), interferon-stimulator genes (ISGs), and other pro-inflammatory cytokines, thus extensively activating the host immune system and further inhibiting and eliminating tumors or viruses (7, 11–14). The dysregulation of this broad and powerful recognition system (cGAS-STING) can also disrupt the dynamic homeostasis of cells and organs by inducing aberrant innate immune responses and a variety of inflammatory triggers (15, 16). The persistent or chronic inflammatory signaling that links to the activation of cGAS-STING signaling is prone to developing the autoimmune disease including Aicardi-Goutières syndrome (AGS), systemic lupus erythematosus (SLE), STING-associated vasculopathy of infancy (SAVI), and amyotrophic lateral sclerosis (ALS), etc. (17–21) Interestingly, the cGAS-STING pathway inhibitors such as H-151 effectively improved the symptoms of the autoimmune diseases ALS and psoriasis by decreasing the inflammatory signaling in animal models, thereby becoming a promising therapeutic agent for autoimmune diseases (17, 22). Moreover, Ablasser's lab reported that H-151 inhibited the inflammation in SARS-COV-2 driven disease COVID-19 (23). Herein, we focus on the key pathways mediated by cGAS-STING and various cGAS-STING-TBK1 signaling related autoimmune diseases, as well as the recent development of cGAS, STING, or TBK1 selective inhibitors.

2 Basic structural features of cGAS-STING-TBK1 axis

2.1 The structural features of cGAS

Human cGAS is a 60 kD protein from the nucleotidyl transferase (NTase) family, consisting of a non-conserved N-terminal structural domain (1-160) and a highly conserved C-terminal NTase structural domain (161-522) (Figure 1A) (24). The N-terminal domain is identified to play a role in stabilizing or suppressing cGAS protein (25).While the NTase domain contains three dsDNA binding sites and is essential for dsDNA recognition and the synthesis of the second messenger 2'3'-cGAMP. The cGAS can bind non-sequence-dependently to dsDNA through the phosphate backbone, leading to significant conformational changes in the NTase structural domain of cGAS and a structural switch in the catalytic pocket, which initiates the catalytic synthesis of GTP and ATP to 2'3'-cGAMP (Figures 1B, C) (10, 15, 26). The synthesis of 2'3'-cGAMP is a critical step in the triggering of the STING-mediated immune system (14, 27). The binding of long-stranded dsDNA to cGAS which forms a ladder-like network is thought necessary to the activation of the cGAS-STING signaling pathway, thus becoming a pattern that effectively prevents the activation of STING by the short-stranded DNA (28, 29). cGAS binds to the negatively charged acidic patch formed by histones H2A and H2B through its DNA binding site. High-affinity nucleosome binding prevents dsDNA recruitment and keeps cGAS in an inactive conformation (30–34).

2.2 The structural features of TBK1

The human TBK1 kinase consists of 729 amino acids, including an N-terminal kinase domain (KD), a ubiquitin-like domain (ULD), an alpha-helical scaffold dimerization domain (SDD), and a C-terminal adaptor-binding domain (CTD) (Figure 2A) (35, 36). Extensive interactions between KD, ULD, and SDD form the dense TBK1 dimer. The KD of TBK1 consists of N-terminal and C-terminal leaflets with an active ATP binding site at the interface. Ser172 residue on the activation loop is the phosphorylation site for TBK1 kinase (37). When TBK1 is phosphorylated, the αC-helix of the kinase structural domain rotates to the inward active position, facilitating the formation of a critical salt-bridge interaction between Glu55 of the αC-helix and Lys38 at the active site. However, when TBK1 is in the inactive conformation, the activation loop is disrupted and the αC-helix is positioned in an inactive position outside the ATP-binding structural domain (35). In the structure of the TBK1 dimer, the activation of TBK1 is mainly controlled by trans-autophosphorylation, in which two KDs limit the cis-autophosphorylation activity of TBK1 (38). A highly conserved PLRT/SD motif in the C-terminal tail (CTT) of STING mediates the recruitment of TBK1 by binding directly to the dimeric interface of TBK1. Further analysis of the crystal structure of STING and TBK1 showed that the dimeric TBK1 binds to two monomers of the CTT of STING, with each STING monomer simultaneously binding to two TBK1 monomers to form a 2:2 complex (Figures 2B, C) (39). The 2',3'-cGAMP binding initiates the STING activation by forming a stable oligomer, and the conserved PLPLRT/SD protein motif in STING-CTT can dimerize the TBK1 interface to induce the phosphorylation and activation of STING and TBK1 through hydrophobic binding. Further recruitment and phosphorylation of interferon regulatory factor 3 (IRF3) and TBK1 leads to the involvement of downstream signaling components and the

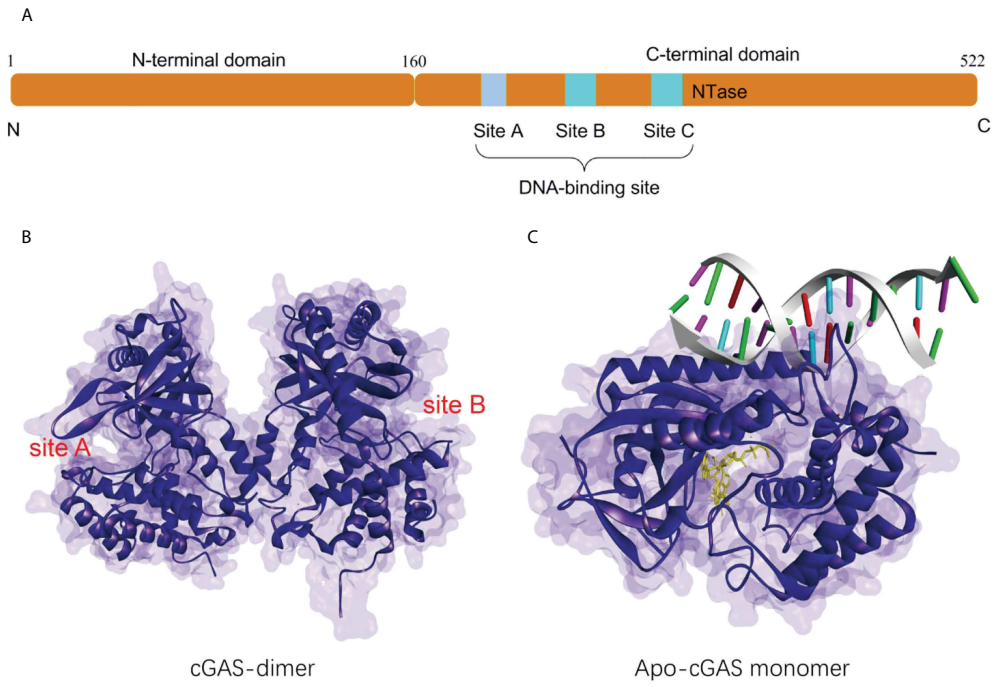

FIGURE 1
(A) Schematic organization of the structural domain of human cGAS; **(B)** Structure of the human cGAS dimer (PDB-ID: 4LEV); **(C)** Structure of porcine cGAS (blue ribbon, apo-cGAS monomer) in complex with DNA, ATP and GTP (yellow ribbon, PDB ID: 4KB6).

inducible regulation of IFN-I transcription, which is the hallmark signal for the initiation of the cGAS-STING-TBK1 signaling pathway (35, 39–42).

2.3 The structural features of STING

Human STING (MITA) is a transmembrane protein located on the endoplasmic reticulum (ER) and consists of an N-terminal transmembrane structural domain (NTD) containing four transmembrane helices TM1 (residues 21-41), TM2 (residues 47-67), TM3 (residues 87-106), TM4 (residues 116-136) and a globular C-terminal structural domain (CTD, residues 157-379) (Figure 3A). STING is highly expressed in immunomodulatory-related cells and tissues such as bone marrow, spleen, and peripheral blood leukocytes (43–46). The STING-CTD is comprised of a ligand-binding domain (LBD, residues 157-335), an IRF3-binding domain (residues 362-366), and a TBK1-binding motif (TBM, residues 369-377) (47–49). Through biophysical technology, especially X-ray crystallography, the STING-CTD is identified as a butterfly-like dimer with a ligand binding site located at the groove of the interface (Figure 3B).

The endogenously produced 2'3'-cGAMP is detected by STING, which in turn binds to STING CTD in a dimeric form (45). Subsequently, STING performs extensive conformational changes, with an inward 180°C flip of the V-type STING LBD dimer, an "open" to "closed" transition of conformation to form a "lid" covering the 2'3'-cGAMP binding site (Figure 3C) (45, 50). The formation of STING polymers via a C148-mediated disulfide bond is essential to the activation of STING, while the "opening" or "closing" of the LBD regulates its activity by affecting the affinity with the ligand to the protein (50). It is important to note that cyclic-di-GMP (CDG) binding does not produce the conformational changes induced by 2'3'-cGAMP or cyclic-di-AMP (CDA), while CDG-bound STING may also lead to the activation of the cGAS-STING-IRF3 pathway (Figure 3B). What is more, recent research indicated that the potent STING agonist diABZI did not promote the closure of the lid region of STING either (51).

The subsequent translocation of STING from the ER to the Golgi apparatus is mediated primarily by coat protein complex II (COPII) vesicles (52), which is dependent on GTPase SAR1A and COPII complex components, including SEC24C and ARF-GTPase ARF1. After transporting to the Golgi apparatus, STING is palmitoylated at two cysteine residues Cys88 and Cys91, which is necessary for the recruitment of TBK1 and IFNs transcription (53, 54). However, TBK1 recruiting to STING alone does not induce the activation of IRF3 at the CTT of STING (residues 342-379) (Figure 3A). The residues Leu333 and Arg334 at STING-CTD play critical roles in c-GAMP-induced autophagy and phosphorylation of TBK1 and IRF3 (52). TBK1

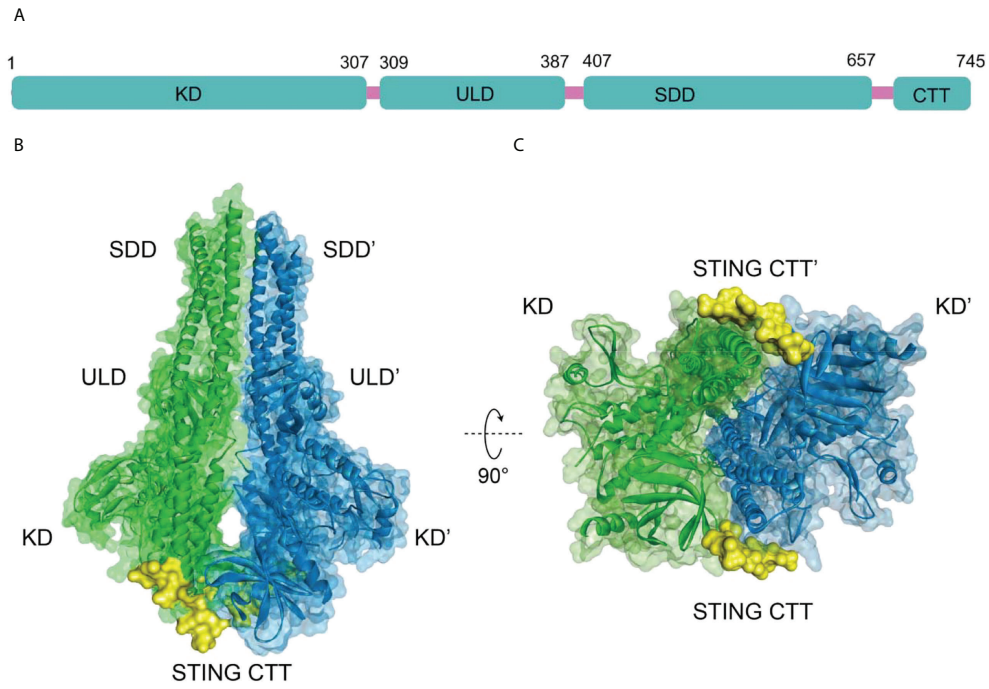

FIGURE 2
(A) Schematic organization of the structural domain of human TBK1, **(B)** Structure of human TBK1 in complex with chicken STING CTT (PDB-ID: 6NT9); **(C)** Bottom view of TBK1 structure.

phosphorylates IRF3, which subsequently induces the dimerization and translocates into the nucleus, thereby driving the transcriptional expression of IFNs (55). After the initiation of downstream signaling, STING is degraded in endolysosomes, and the residues 281-297 are required for the transport-mediated STING degradation (56).

3 cGAS-STING-mediated signaling pathways

3.1 cGAS-STING-IRF3 pathway

The activation of the cGAS-STING axis will induce the modification of IRF3 and its translocation to the nucleus, thereby driving the transcriptional expression of IFNs (57–59). Simultaneously, the binding of IFNs to its receptor activates Janus kinases (JAKs), including JAK1 and tyrosine kinase 2 (TYK2), which in turn phosphorylate the receptor (20). This process allows DNA-binding protein signal transducer and activator of transcription 1 (STAT1) and 2 (STAT2) to bind to the receptor, thereby phosphorylating and dimerizing them. The dimer then translocates to the nucleus where it upregulates the transcription of IFN-responsive genes, including the transcription of IFNs dependent on interferon regulatory factor 9 (IRF9) (Figure 4). The synthesis and release of IFN and its binding to the IFN receptor further upregulated the interferon genes in a positive feedback loop (20).

To avoid a severe inflammatory response to the induced transcription of excess IFN, there is also an associated negative feedback mechanism. When the cGAS-STING-mediated immune response is continuously activated, STING is induced to be degraded in the endosome. Following the activation and translocation of STING, it is phosphorylated by serine/threonine protein kinase 1/autophagy-associated protein 1 (ULK1/ATG1) to inhibit the sustained induction into IFNs and inflammatory disease (60). ULK1-2 function can be regulated by AMP-activated protein kinases (AMPK) or mammalian targets of rapamycin (mTOR) which is activated under cellular stress conditions. Cytoplasmic dsDNA and/or CDN are found to activate ULK1-2, which initiates a negative feedback loop controlling STING overexpression through restricting STING translocation from the Golgi apparatus by autophagy-associated protein 9a (ATG9a) and decreasing the association of STING and TBK1 (61). NOD-like receptor C3 (NLRC3) binds to STING and prevents its translocation from the ER to the Golgi apparatus, thereby reducing the IFNs response (62). Also, the movement of STING outside the ER facilitates its recruitment to LC3 autophagic vesicles through a WD repeat structural domain phosphoinositides interacting protein 2 (WIPI2)-dependent

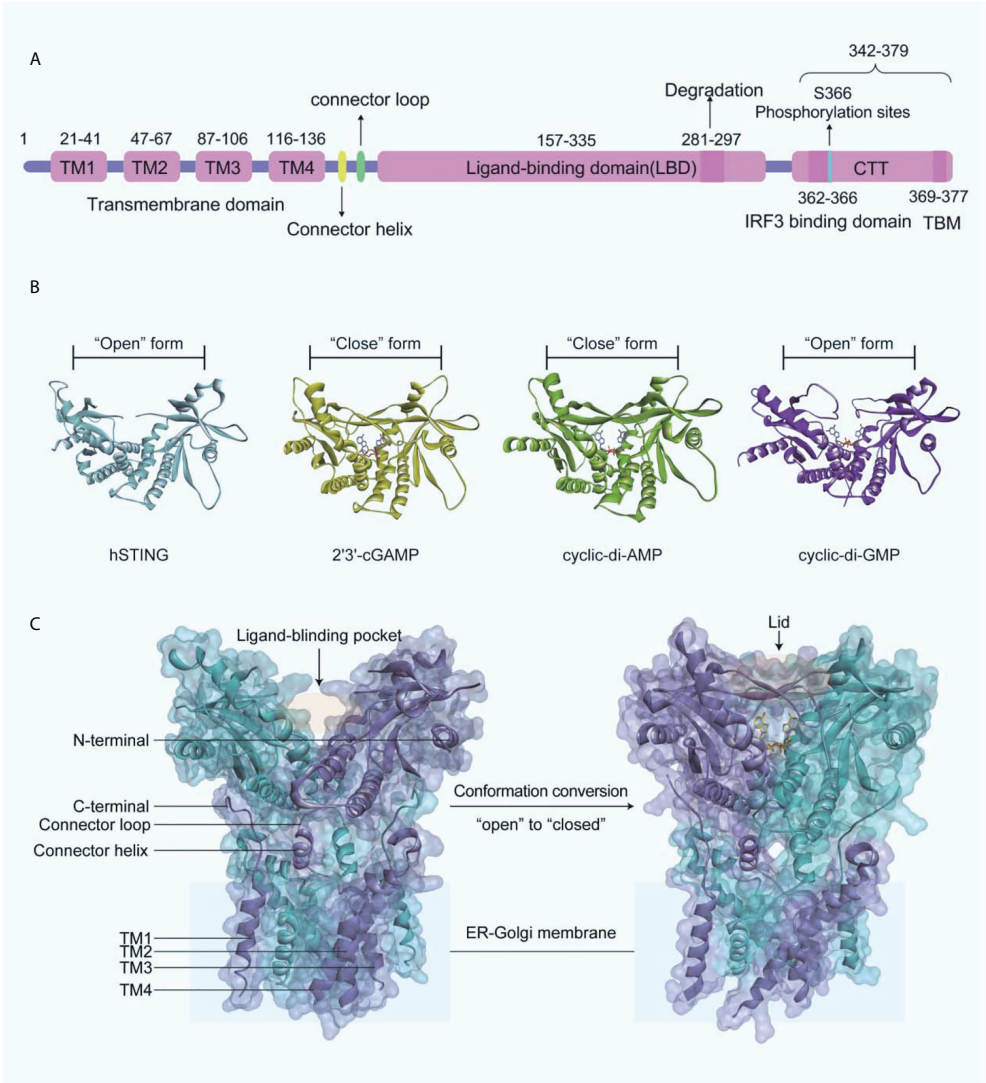

FIGURE 3
(A) Schematic organization of the structural domain of human STING; **(B)** Crystal structures of human STING CTD (blue ribbon, apo-STING, PDB-ID: 4EMU; yellow ribbon, STING bound 2'3'-GAMP, PDB-ID: 4LOH; green ribbon, STING bound CDA, PDB-ID: 4F5D; purple ribbon, STING bound CDG, PDB-ID: 4F5Y); **(C)** On the left is the crystal structure of full-length human STING in open conformation (PDB-ID: 6NT5), and on the right is the crystal structure of chicken STING and 2'3'-cGAMP in closed conformation (PDB-ID: 6NT7).

mechanism (52). LC3 coordinates the negative regulation of STING by transporting STING complexes, DNA, and pathogens to autophagy vesicles for lysosomal dependent degradation, a process that requires the RAS-associated protein Rab-7a (RAB7) GTPase (52, 63). Moreover, recent findings have revealed a mechanism that moves STING from the Golgi to the ER to downregulate the cellular activation of STING. Specifically, the adaptor protein SURF-4 interacts with STING on the Golgi apparatus to promote STING encapsulation into coat protein complex I (COPI) vesicles for retrograde transporting STING from the Golgi apparatus to the ER, thereby inhibiting sustained STING activation (64, 65). Upon the entry of IRF3 into the nucleus and the activation of ISGs, the STING-TBK1-IRF3 complex is dissociated and drives E3 ubiquitin ligase RNF5/TRIM30α mediated K48-linked polyubiquitinated STING, which promotes the degradation of STING via the proteasome pathway (66, 67).

3.2 cGAS-STING mediated NF-κb pathway activation

Another major signaling module involved in the regulation of STING is nuclear factor kappa B (NF-κB)-mediated

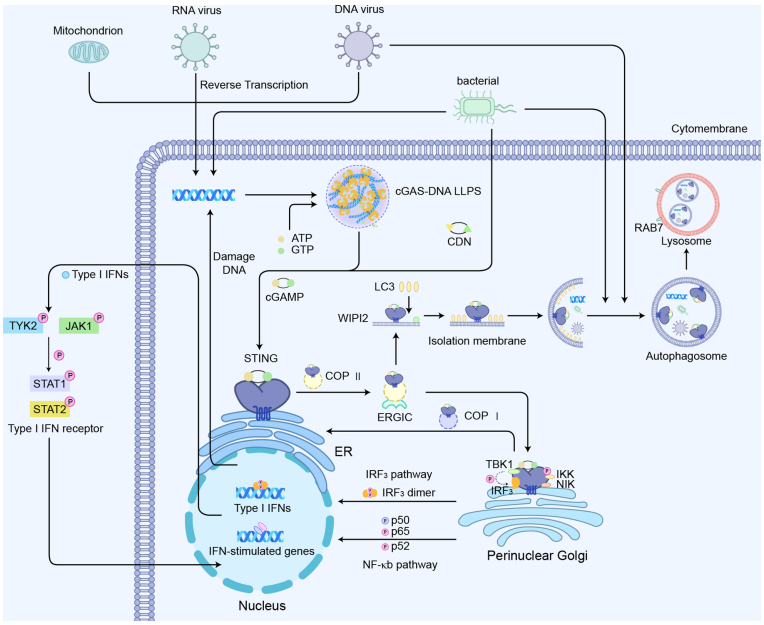

FIGURE 4
cGAS-STING-mediated signaling pathways:1) cGAS-STING-IRF$_3$ pathway; 2) cGAS-STING mediated NF-κb pathway activation; 3) cGAS -STING induced autophagy process.

transcriptional activation, which promotes the expression of several pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 (Figure 4) (43). The CTT motif of STING is necessary for triggering the IRF3-dependent transcription of IFNs, whereas the STING-dependent NF-κB pathway is not entirely relied on the CTT of STING (68). STING-mediated NF-κB activation indicates much less sensitivity to the knockout of TBK1 (69). TBK1 alone is dispensable for STING-induced NF-κB responses in human immune cells, while acts redundantly with IκB kinase ϵ (IKKϵ) to drive NF-κB upon STING activation (68, 70). Consistently, the ancestral STING homologs in insects and early postlarvae completely lack CTT signaling, but could still achieve a host defense by promoting NF-κB responses. Using a tamoxifen-induced TBK1 deficiency model in adult mice, it was observed that TBK1 deficiency had little effect on cytokines of NF-κB following the administration of the mouse STING agonist DMXAA (69). Interestingly, the nuclear DNA damage would induce the non-canonical activation of STING by ATM and IFI16, leading to the activation of NF-κB signaling (71). Also, the genotoxic DNA damage induced by camptothecin drove IL-6 production through non-canonical STING signaling in the STING-expressing cancer cells (72). The stimulation of cGAS-STING also promotes a non-canonical NF-κB response by triggering p52 nuclear translocation (Figure 4) (73, 74). This signaling restricts IFN-I and the classic NF-κB pathway as regulators of the negative feedback mechanism of STING (75). Therefore, the explicit mechanisms of STING interacting with NF-κB pathway components are still required to be verified.

3.3 cGAS -STING-induced autophagy process

Previous work suggested that autophagy induction via STING trafficking is a primal function of the cGAS-STING pathway (52). The cGAS–STING pathway can induce canonical autophagy through liquid-phase separation of the cGAS–DNA complex, the interaction of cGAS and Beclin-1, and STING-triggered ER stress–mTOR signaling. Moreover, both cGAS and STING can trigger non-canonical autophagy via LC3-interacting regions and binding with LC3. What is more, autophagy induced by the cGAS–STING pathway plays crucial roles in balancing innate immune responses, maintaining intracellular environmental homeostasis, and restricting tumor growth (76). Conventional autophagy dependent on the ULK complex and TBK1 is involved in STING-mediated LC3 autophagy vesicle formation, and the activation of STING can also trigger non-canonical autophagy responses mediated by the PI3P effector WIPI2 and the ATG5-12-16L1 complex (Figure 4) (52, 60, 61, 77, 78). STING activation is indispensable for autophagic induction LC3 interacting regions (LIRs), and mutants of STING abolish its interaction with LC3 and its activation of autophagy (78). Of note, autophagy components also feedback

on the regulation of STING activity through assisting STING intracellular trafficking capabilities as well as its lysosomal degradation (79). The direct interaction between Beclin-1 autophagy protein and cGAS not only inhibits STING signaling and decreases IFNs expression but also promotes the autophagy-mediated degradation of cytosolic DNA to avoid excess cGAS-STING activation (80). Similarly, key genes involved in the mechanism of autophagy, such as ULK-1 and Atg9, have appeared to suppress the STING/TBK1/IRF3 pathway, effectively inhibiting sustained immune response and excessive inflammation (60, 61). Moreover, autophagy receptor CCDC50 modulates STING-directed IFNs signaling activity by delivering the K63-polyubiquitinated STING to autolysosomes for the degradation (81).

4 cGAS-STING related autoimmune diseases

4.1 Monogenic autoinflammatory syndromes

4.1.1 Aicardi-goutières syndrome

AGS is an early-onset systemic inflammatory disorder that manifests clinically as neurological dysfunction and frostbite-like skin lesions. The nuclei acid exonuclease TREX1 was the first gene found to be related to AGS (Figure 5A) (82). TREX1 prevents excessive accumulation of endogenous auto-DNA and prevents aberrant activation of DNA-mediated cGAS-STING signaling, while structurally inactivated TREX1 leads to the IFN-dependent autoimmune disease AGS (19). In addition, it has also been reported that mice with mutations in three RNaseH2 enzyme complexes (RNaseH2 A, RNaseH2 B, and RNaseH2 C) exhibit increased IFN signaling and inflammation, and ultimately cause AGS-like symptoms (83).The failure of mutated RNaseH2 to degrade RNA/DNA hybrids led to the excessive activation of cGAS-STING signaling, which induced AGS. The lethality of some mice with dysfunctional mutated RNaseH2 was rescued by the knockout of STING (84). Similarly, sterile α motif and histidine-aspartate domain-containing protein 1 (SAMHD1) promotes the degradation of nascent DNA in human cell lines by stimulating the exonuclease activity of meiotic recombination 11 homolog A (MRE11A), and the deletion of SAMHD1 lead to the accumulation of genomic DNA in the cytoplasm and triggers AGS (85).

4.1.2 STING-associated vasculopathy of infancy

Mutations in exon 5 of STING lead to functional activation of STING, resulting in the excessive STING-induced IFN signaling, causing a disorder termed SAVI including recurrent fever, ulcerative skin lesions, vasculitis, and interstitial lung disease (Figure 5B) (18, 20). Mutant residues are located in two separate regions on STING, the connector helix loop (N154S, V155M, G158A, G166E, H72N, and V147M/L) and the polymerization interface (C206Y/G, G207E, F279L, R281Q/W, and R284G/S) (86–89). Mutations in the regions can spontaneously rotate around the connected helix loop by inducing the LBD allosteric activation, or by promoting the STING polymerization, thereby triggering the ligand-independent activation of STING (50, 58). An obvious feature of the mouse SAVI model is the severe lymphopenia and immunodeficiency due to the abnormal lymphocyte development and aberrant intrinsic T cells (90). However, it has likewise been reported that the inhibition of IFN signaling did not affect disease pathogenesis in the N153S STING mouse model of SAVI. Instead, the T-cell depletion protected N153S mice from lung disease progressions, which may explain why JAK inhibitors targeting the IFN-α receptor (IFNAR) are not always successful in the treatment of SAVI patients (91).

The Ca2+ sensor stromal interaction molecule 1 (STIM1) has been reported to be a promising target for the treatment of SAVI, where STIM1 directly interacts with STING and inhibits the transport of STING from the ER to the Golgi apparatus (92). A peptide ISD017 has been reported to block the activity of STING in vivo and improve the disease progression of a mouse model of lupus in a STIM1-dependent manner (93). The activity of three disease-associated STING variants, V147L, N154S, and V155M, also can be inhibited by STIM1 in part by blocking their translocation to the ERGIC. In the SAVI model, the activation of STING leads to cellular T-cell defects by modulating T cell proliferation and differentiation (90, 94), and plays a key role in the initiation and progression of SAVI (95).

4.1.3 COPA syndrome

Pathogenic COPA variants can lead to immune dysregulation in Mendelian syndrome. COPA is a subunit of COPI that mediates STING from the Golgi apparatus to ER transport, and the dysfunction of the target thereby leads to the structural activation of STING (Figure 5C) (96). In a mouse model of COPA syndrome (CopaE241K/+), IFN-driven inflammation of the mice could be rescued through crossing with STING-deficient mice (STING1gt/gt). In addition, the embryonic lethality in Purex COPAE241K/E241K mice could be rescued by the knockout of STING (64). JAK inhibitors can improve clinical performance and IFN levels, but the effect is very limited (97). In mouse models with functionally acquired STING mutations, the development of lung lesions is dependent on T cells instead of IFN-I (98). This result may explain the poor therapeutic effect of JAK inhibitors in human COPA syndrome. In addition, the small molecule STING inhibitor H-151 has also been reported to improve the inflammation in COPA syndrome (99). The inhibition of STING has emerged as an efficient way for the treatment of COPA syndrome.

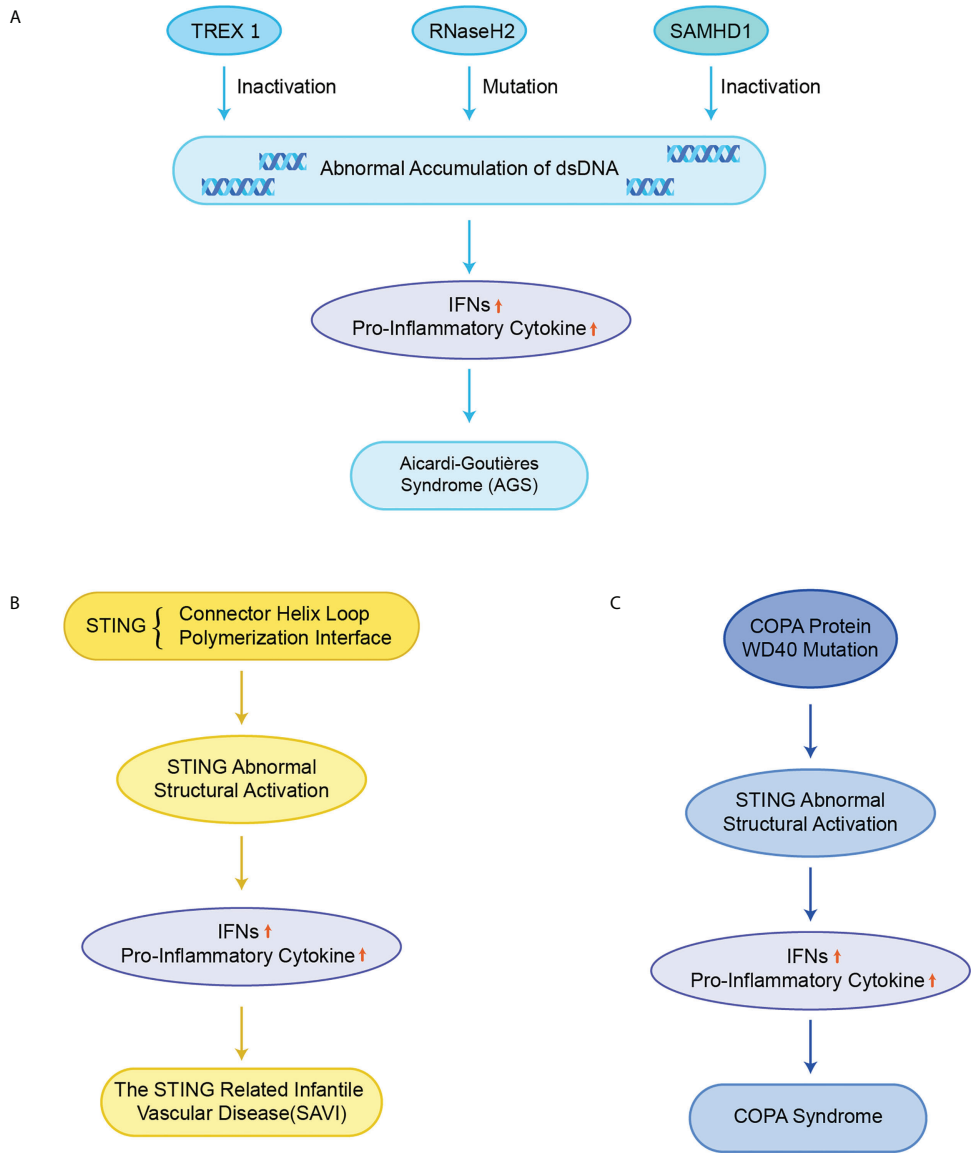

FIGURE 5
STING in monogenic autoinflammatory syndromes **(A)** Inactivation of TREX1, RNaseH2 and SAMHD1 leads to abnormal accumulation of dsDNA in normal cells, which over-activate the cGAS-STING signaling pathway, upregulates the expression of IFNs and pro-inflammatory cytokines, and ultimately triggers AGS; **(B)** Mutations in STING in the connector helix loop (N154S, V155M, and V147L) and the polymerization interface (G207E, R281Q, R284G, and R284S) lead to structural activation of STING, upregulating the expression of IFNs and pro-inflammatory cytokines, and ultimately causing SAVI; **(C)** Missense mutations in the structural domain of COPA WD40 impair endoplasmic reticulum binding and target protein sequencing, leading to structural activation of STING, upregulating the expression of IFNs and pro-inflammatory cytokines, and ultimately causing COPA syndrome.

4.2 Autoimmune neurodegenerative diseases

While the association of STING with neurodegenerative diseases has been poorly investigated in previous studies, the activation of the immune system is a prominent feature of several neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), frontotemporal dementia (FTD), multiple sclerosis (MS), and ALS. Several recent studies have revealed the relationship between STING and autoimmune neurodegenerative diseases (100). Although IFNs are also produced by neurons and astrocytes, STING is mainly expressed in microglia to elicit the IFN responses in the brain (101, 102). In chronic neurodegenerative disease states, aberrantly activated STING signaling induces the expression of IFNs and increases the

phenotype of microglia and astrocytes, thereby accelerating the development of neuroinflammation (103, 104).

4.2.1 Amyotrophic lateral sclerosis

The cytoplasmic accumulation of TDP-43 is a hallmark of ALS (Figure 6A) (105). TDP-43 induces mtDNA release via voltage-dependent anion channel 1 (VDAC1), which subsequently induces IFNs and inflammatory cytokine expression in a cGAS-STING dependent manner (17). STING gene deletion or the use of small molecular inhibitors of STING significantly improved ALS symptoms and prolonged the life span of mice. Besides, the expansion of the hexanucleotide repeat sequence (GGGGCC) in the mice lacking chromosome 9 open reading frame 72 (C9orf72) gene is the most common cause of familial ALS (106). Dendritic cells isolated from mice lacking the C9orf72 protein showed marked early activation of IFN-I responses, and mice showed age-dependent lymphoid hypertrophy and autoinflammation. The C9orf72-deficient mice were more likely to develop experimental autoimmune encephalitis. Also, bone marrow cells lacking C9orf72 showed signs of hyper-activation upon being exposed to STING agonists and reduced autolysosomal degradation of STING. The C9orf72- deficient ALS patients had higher levels of IFN-I signaling than patients with sporadic ALS and could improve symptoms with STING inhibitors treatment.

4.2.2 Niemann–pick disease type C

NPC is a chronic neuroautoimmune disease caused by a deficiency of Niemann-Pick C1 (NPC1), which leads to the impaired metabolism of neurospherin phospholipids (Figure 6B) (107). NPC1 is an auxiliary protein for transporting STING to the lysosome for its degradation. NPC1 deficiency leads to the accumulation of cholesterol and other lipids in the lysosome, resulting in the decrease of ER cholesterol levels and the activation of SREBP2-SCAP translocation from the ER to the Golgi apparatus (108). The STING protein is recruited by the SREBP2-SCAP complex, which triggers 2'3'-cGAMP-independent STING activation by hijacking STING to transport from the ER to the Golgi apparatus in NPC1 KO cells, thereby leading to a progressive loss of Purkinje in NPC1-/- mice, thus resulting in impaired motor function and reduced survival (63, 109).

4.2.3 Multiple sclerosis

MS is considered a progressive autoimmune disease which is caused by inflammation and neurological damage from immune system attacks on myelin (110). Early studies reported that the antiviral drug ganciclovir induced the suppression of MS experimental autoimmune encephalomyelitis (EAE) model in a STING-dependent manner (109). STING acts as a regulator of microglial cell reactivity and neuroinflammation, which improves the pathology of EAE in mice by reducing immune cell infiltration and inhibiting the proliferation of microglia or the immune cells of the central nervous system. Besides, The oral administration of Bowman-Birk inhibitor (BBI), a serine protease inhibitor derived from soy was reported to inhibit EAE (111). The inhibition is dependent on STING

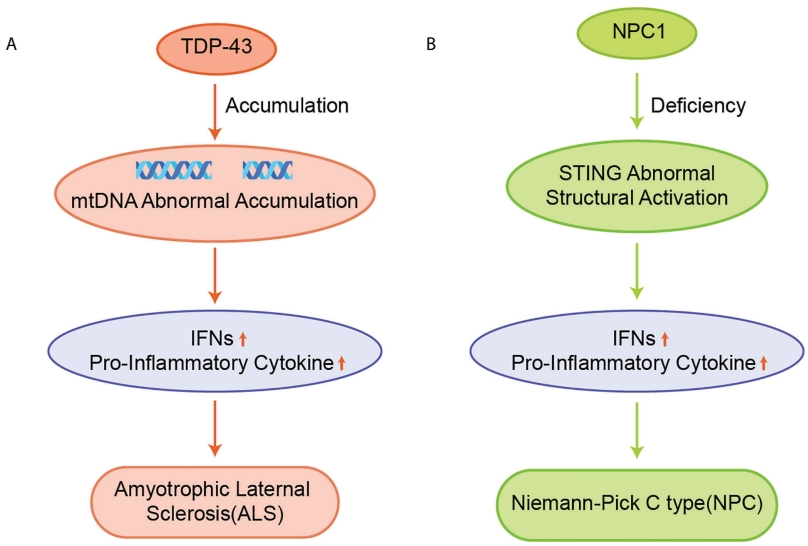

FIGURE 6
(A) Accumulation of TDP-43 leads to aberrant accumulation of mtDNA, which induces the expression of IFNs and pro-inflammatory cytokines in a cGAS/STING dependent manner and promotes the development of ALS; **(B)** NPC1 leads to the 2'3'-cGAMP-independent activation of STING, upregulating the expression of IFNs and pro-inflammatory cytokines, and ultimately causing NPC.

and IFN-β secreted by macrophages, and the absence of IFNAR in bone marrow cells restricts the inhibition of EAE by BBI.

While it is true that neurodegenerative diseases occur after aberrant activation of the cGAS-STING pathway, the role of this pathway in different neurological diseases is still needed to be further investigated. The inhibition of the STING signaling may be a potential way in therapy of STING-associated neurodegenerative diseases. However, we must also note that activation of cGAS-STING signaling is not a single facilitator in neurodegenerative disease. IFNs, the downstream expression products are also negative regulators of some inflammation in the peripheral or central nervous system (109, 112). Therefore, how to effectively regulate the cGAS-STING signaling using a cGAS or STING modulator is key to the treatment of neurological autoimmune diseases.

4.3 Other cGAS-STING related autoimmune diseases

4.3.1 Systemic lupus erythematosus

SLE is also an autoimmune disease that has been reported to be related to STING. The elevated serum 2'3'-cGAMP levels in SLE, leading to the redundant STING activation, have been reported in approximately 15% of all SLE patients (21). The exact cause of SLE is not clear yet, but elevated dsDNA levels were identified in cells from SLE patients, and apoptosis-derived membrane vesicles (AdMVs) in the serum of SLE patients had high inducing ISGs (Figure 7A) (113). Defective clearance of apoptotic cells produces dsDNA-containing AdMVs, which in turn induces ISGs via the cGAS-STING pathway. Next, ISGs activate an immune response that leads to tissue damage in various organs, resulting in further production of AdMVs and a positive feedback loop of ISGs.

Besides, a comprehensive genetic analysis has identified FCGR2B as a susceptibility gene in patients with SLE, and the mutation of the FCGR2B gene leads to the induction of SLE (Figure 7A) (114). The disruption of STING signaling relieves the lupus development in FCGR2B-deficient mice, and the transplantation of STING-activated bone marrow-derived dendritic cells into the mice with both FCGR2B and STING defects restores the lupus phenotype (115). MYSM1 interacts with STING and cleaves the ubiquitination of the STING at Lys63 to inhibit cGAS-STING signaling (Figure 7A) (116). In PBMCs from patients with SLE, the expression of MYSM1 was reduced, while the level of IFN-I and pro-inflammatory cytokines were increased.

During the development of SLE, the mTOR signaling is activated, and blocking the mTOR pathway using rapamycin has emerged as a new strategy for treating SLE in animal models and patients (117–119). A phase 1/2 clinical trial of rapamycin showed the improvement in disease in SLE patients over a 12-month treatment period (120). Inhibition of mTOR by rapamycin prevented IFN-I production by SLE monocytes and promoted autophagy-mediated degradation of STING (121). Transmembrane protein 203 (TMEM203) is an intracellular regulator of STING-mediated signaling that interacts with STING to activate the cGAS-STING signaling pathway (122). The signaling of TMEM203 is elevated in T cells isolated from SLE patients and correlates with disease severity, and inhibiting TMEM203 may also be a potential therapeutic option for the treatment of SLE (Figure 7A) (123).

Although both SLE elicitation and development appear to be associated with STING and IFN-I upregulation, there are contradictory results in different mouse models of lupus. It suggested that STING could also be a negative regulatory factor in SLE. The deficiency of STING failure to constrain aberrantly activated TLR signaling cascades responsible for the disease (124). In addition, the knockdown of IFNAR in MRL/LPR mice exacerbates lymphocyte proliferation, autoantibody production, and organ damage (125, 126). Therefore, the role of STING in the pathogenesis of SLE still needs to be further investigated.

4.3.2 Rheumatoid arthritis

The pathogenesis of RA is associated with dsDNA accumulation (127). Deoxyribonuclease II (DNase II) can degrade DNA by hydrolyzing its phosphodiester bonds to prevent its abnormal accumulation. The lack of DNase II prevents this process and promotes STING activation and IFN-dependent systemic auto-inflammation, such as collagen-induced arthritis (CIA) (Figure 7B) (128, 129). STING gene-deficient mice had significantly higher levels of anti-collagen antibodies and showed better survival rates than wild-type (WT) mice (128). STING promotes the expression of IFN-inducible genes and the expansion of dendritic cells in CIA. In the CIA model, STING plays a negative regulatory role in B cells when BCR is involved. The inhibition of STING promoted anti-collagen antibody production and B-cell survival, and STING-deficient mice did not spontaneously develop similar autoimmune symptoms (7).

4.3.3 Sjögren's syndrome

SS is a chronic autoimmune disease affecting multiple organ systems and is characterized by elevated IFN-I levels, which have likewise been reported to be associated with STING (130). Subcutaneous administration of DMXAA to female C57BL/6 mice induced features similar to those of SS patients, such as hypoglandular function and autoantibody production. Activation of STING induced an increase in the expression of IFN-β, IL-6, TNF-α, and IFN-γ in salivary glands and the recruitment of type 1 innate lymphoid cells (ILC1) to the lungs, thereby causing persistent inflammation in the lung (131).

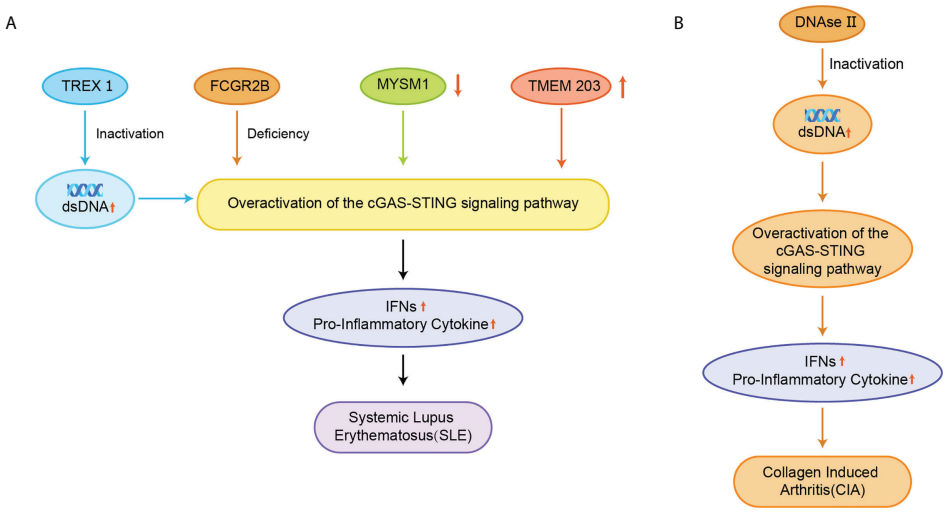

FIGURE 7
(A) Inactivation of TREX1 resulted in the accumulation of AdMVs in serum, deletion of the FCGR2B gene, decreased MYSM1 production in PBMC, and elevated TMEM203 signaling in T cells were associated with overexpression of the cGAS-STING signaling pathway, upregulation of IFNs and pro-inflammatory cytokine expression, and induction of SLE; **(B)** Inactivation of DNase II causes abnormal accumulation of dsDNA, which over-activates the cGAS-STING signaling pathway and triggers CIA.

5 Inhibitors targeting cGAS-STING-TBK1 axis

5.1 Inhibitors targeting cGAS

5.1.1 Catalytic site cGAS inhibitors

Hall et al. performed a saturation transferred differential 1H NMR screening for the Pfizer fragments library using the cGAS crystallized structure, and a low-affinity fragment tetrazolo[1,5-a] pyrimidine (Kd = 171 μM) was identified with weak inhibition of cGAS (IC50 = 78 μM) (132–134). Further optimization for this compound led to compound 1 (Kd = 0.2 μM, IC50 = 4.9 μM) (Figure 8). However, compound 1 lacked inhibitory activity in cellular assays for high levels of intracellular ATP and GTP.

Moreover, Vincent et al. performed a high-throughput screen of 123306 compounds and identified four compounds that exhibited good activities (135). The compounds occupied the active center of mouse cGAS and formed key stacking interactions with Agr376 and Try436 at the catalytic site. Based on the binding mode, they subsequently obtained the high-affinity cGAS ligand 2 (Kd = 36 nM) with the best inhibitory activity in cellular assays (IC50 = 0.70 μM) (Figure 8). Meanwhile, the tested results in other signaling pathways showed that compound 2 was a selective inhibitor of cGAS and reduced the mRNA level of IFN-β in bone marrow derived macrophages (BMDM) of AGS model TREX1-/- mice, thus indicating the potential for the treatment of autoimmune diseases.

Lama et al. performed an ATP-coupled high-throughput assay for the identification of small molecule inhibitors of h-cGAS (136). Two cross-species active compounds, 3 and 4 (G chemotype backbone), were obtained after multiple rounds of screening (Figure 8). Their analogue compound 5 exhibited the good inhibitory activity against both THP-1 cells (IC50 = 1.96 μM) and primary human macrophages (IC50 = 0.62 μM) (Figure 8). Compound 5 showed selective inhibition of cGAS in a series of inhibition tests of other innate immune pathways. The structural biology data identified its analogs binding to the cGAS active site. However, the G backbone compounds do not fully occupy the ATP and GTP binding pockets of cGAS and fail to give a clear structure-activity relationship (SAR), and further optimization studies on this backbone are still required.

The crystal structures of cGAS have been solved, which provides quite useful information for structural-based drug design. Based on the high-resolution crystal structure (1.8 Å) of cGAS and compound 1 complex (Figure 9), four effective fragments were identified by virtual screening and thermal shift analysis by Zhao et al. (137) Subsequently, the inhibitory activity of 59 compounds was evaluated using PPiase-coupled assays. One of these compounds did not show any activity in the thermal shift assay and was found to have better inhibitory activity. A similarity search based on this compound was performed and compound 6 (IC50 = 4.9 μM) was identified by PPiase coupling assay (Figure 8).

5.1.2 cGAS inhibitors interpret DNA-cGAS interaction

Anti-malarial drugs such as compound 7 and compound 8 have been reported to disrupt the binding of cGAS to dsDNA

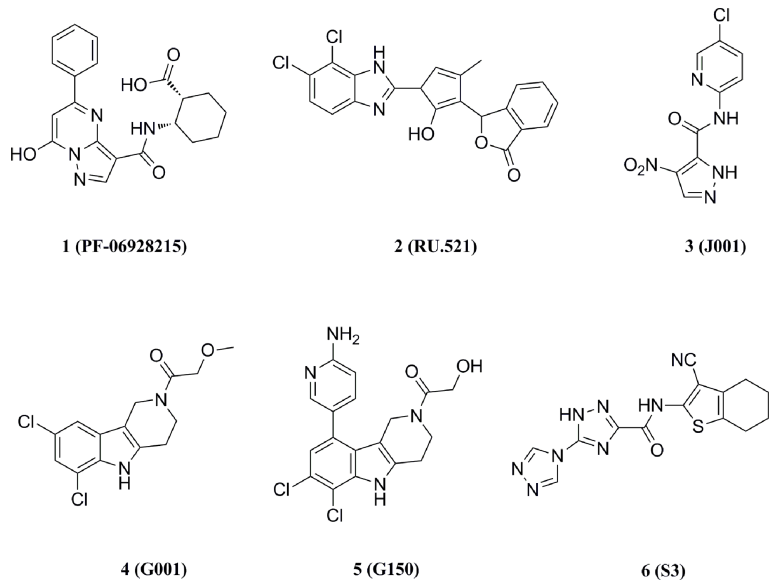

FIGURE 8
Structures of cGAS inhibitors targeting catalytic site.

and selectively block cGAS-double-stranded β interactions to inhibit the IFN expression (Figure 10) (138, 139). In turn, a second-generation compound 9 was obtained and the potential for this type of compounds in therapy of autoimmune diseases such as AGS or SLE was validated in the AGS model TREX1-/- mice (Figure 10) (140).

Wang et al. screened a library of 268 compounds to obtain the cGAS inhibitor Suramin, which interfered with the formation of the cGAS-dsDNA complex by competing with the dsDNA binding site of cGAS (Figure 10) (141). It was also proposed that the potential mechanism of action of compound 10 was that its anionic sulfate acted as a phosphate mimetic, binding to the positively charged region on cGAS (138). Besides, Dai et al. found that acetylation of any of the three cGAS residues K384, K394, or K414 affected the binding of cGAS to DNA (142). Further studies revealed that compound 11 which might acetylate the residues significantly reduced ISGs in peripheral blood mononuclear cells of AGS patients and attenuated the auto-DNA-induced autoimmune symptoms in TREX1-/- mice (Figure 10).

5.1.3 cGAS inhibitors with the undisclosed mechanism

Padilla-Salinas et al. performed a virtual screen towards a potentially druggable pocket around Lys347 and Lys394 in h-cGAS to develop protein-protein interface inhibitors of the cGAS dimer itself (143). Only one hit (12) was active to inhibit h-cGAS in vitro with the IC50 of 100 μM (Figure 10). Subsequent optimization resulted in a highly potent inhibitor 13 (IC50 = 0.24 μM) which selectively inhibited the activity of cGAS (Figure 10). Molecular docking suggested that this series of compounds might bind to a binding pocket other than the dsDNA binding site or the catalytic site, while the crystal structure of compound 13 and the cGAS complex could not be solved to verify the precise binding mode. In addition, Aduro Biotech has disclosed several classes of cGAS inhibitors which have shown a good inhibitory activity at both protein and cell levels. However, the underlying mechanism of cGAS inhibition by these compounds is still required for further elucidation (144–146).

In addition, an oligonucleotide A151 was reported to inhibit cGAS activity (147). A151 contains four TTAGGG motif repeats that can act as an inhibitor of cGAS by interacting with the dsDNA binding domain. In cellular experiments, A151 effectively abolished the activation of cGAS by cytoplasmic DNA, thereby inhibiting the production of IFN-I by human monocytes and preventing endogenous DNA accumulation in TREX1-deficient monocytes. The inhibitory activity of A151 is dependent on the nucleotide sequence and phosphate backbone structure, but its specific binding site to cGAS remains to be further explored (148). Through a screen of 2'OMe ASOs and further sequence mutant, Valentin et al. recently characterized key features within the 20-mer ASOs regulating cGAS and TLR9 inhibition and identified a highly potent cGAS inhibitor, which exhibited more potently than A151 (149).

As a potent inhibitor of AMPK, Lai et al. found that compound 14 also inhibited dsDNA-dependent induction of IFN-I (150–152). Further experiments showed that IFN-β was

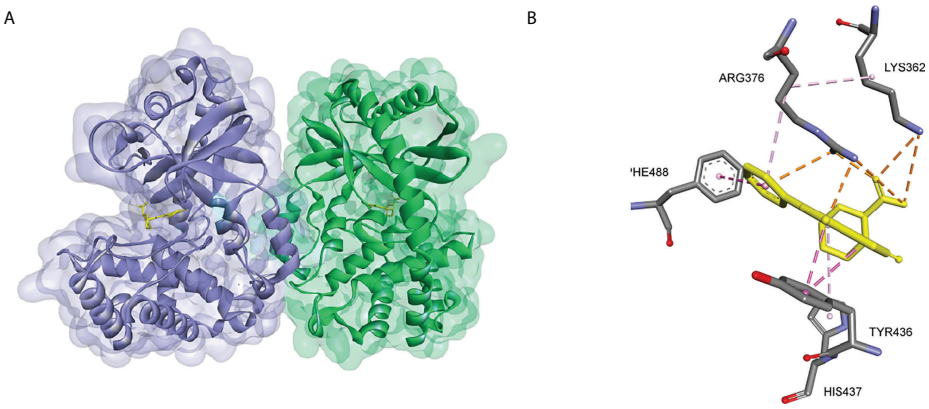

FIGURE 9
(A) Crystal structure of the cGAS inhibitor PF-06928215 bound to a cGAS dimer (PDB-ID: 4LRC); **(B)** Residues of the cGAS active center interacting with PF-06928215 and the interactions (PDB-ID: 4LRC).

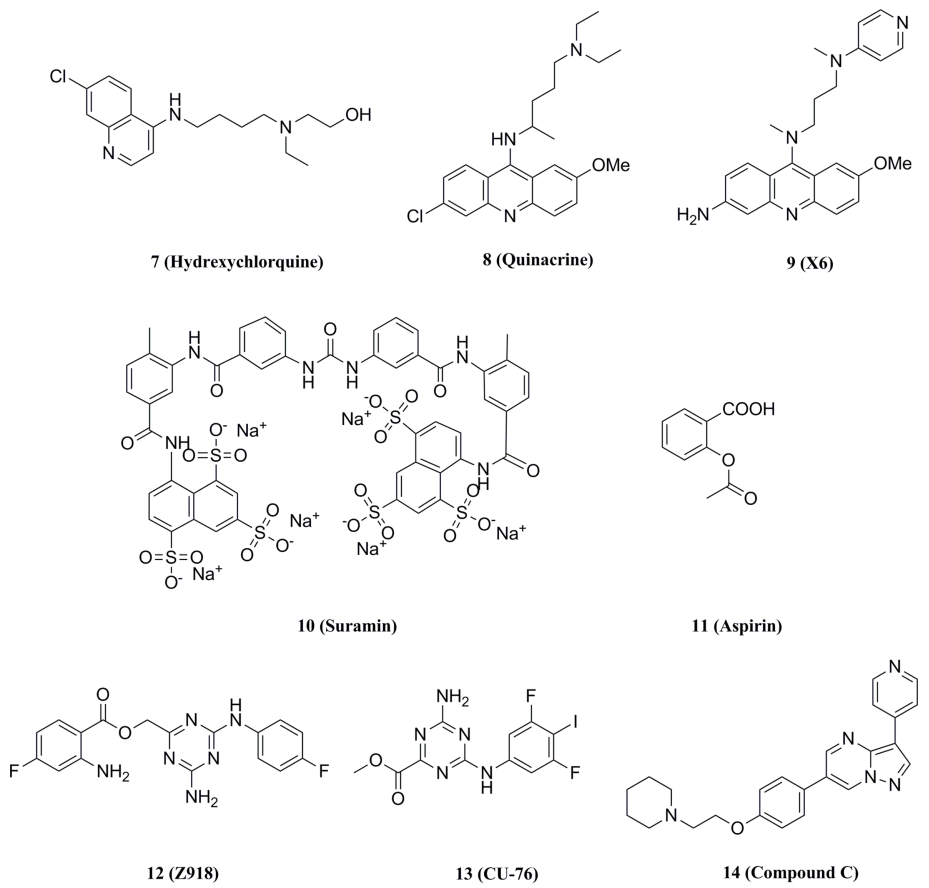

FIGURE 10
Structures of other types of cGAS inhibitors.

down-regulated by compound 14 through the inhibition of cGAS rather than the inhibition of STING or TBK1. The IFN-β expression was also inhibited by compound 14 in knockdown AMPK cell lines. Subsequent experiments showed that compound 14 improved the autoimmune phenotype of TREX-/- cells. However, they did not resolve the crystal structure of the complex formed by compound 14, cGAS, and dsDNA. They suggested that compound 14 did not bind directly to the cGAS active site, but rather inhibited the upstream genes of the cGAS-mediated pathway. Besides, Huffman et al. develop a stereoselective butyrolactone coupling with the rapid construction of C-C bonds (153). By this method, four inhibitors for chemical screening of cGAS-STING pathway-targeted cell phenotypes were identified based on a 250,000 compound library.

Besides, cGAS binds DNA in a sequence-independent manner through multivalent interactions mediated by its catalytic core and its positively charged disordered N-terminal domain and induces liquid-liquid phase separation (LLPS) of cGAS-DNA bimolecular condensates (154, 155). Recent works indicated that the natural product epigallocatechin gallate (EGCG) directly impacted DNA-induced cGAS-LLPS in vitro, which might represent a novel opportunity to control some self-autoimmune diseases driven by cGAS (156, 157).

5.2 Inhibitors targeting TBK1

5.2.1 BX795 aminopyrimidine-like small molecular TBK1 inhibitors

15 (IC50 = 6.0 nM) was the earliest TBK1 inhibitor reported in 2009 (Figure 11) (158). This compound was originally developed as an inhibitor of 3 phosphoinositide-dependent protein kinase 1 (PDK1, IC50 = 111 nM), but has also shown strong inhibition of several other kinases (136). Biological assays had shown that 15 inhibited the inflammatory response induced by gram-positive bacteria and the infection of cells with multiple drug-resistant strains of herpes simplex virus type 1. In addition, 15 inhibited the proliferation of oral squamous cell carcinoma (OSCC) by inducing apoptosis and M-phase blockade (159). However, off-targeting effects of 15 on other kinases limited its further development. Further optimization of 15 resulted in 16 (TBK1 IC50 = 19.0 nM, IKKε IC50 = 160.0 nM), which showed good selectivity for IKKα, IKKβ, etc (Figure 11) (160). The co-X-ray crystal structure of TBK1 with 16 shows that it binds to TBK1 in a similar pattern but forms fewer interactions with the kinase compared to 15, resulting in reduced potency and off-target effects (37).

Likewise, the JAK1/2 kinase inhibitor 17 for the treatment of myelofibrosis exhibited inhibitory activity for TBK1 (IC50 = 58 nM, Figure 11) (161, 162). 18 is also a highly selective TBK1 inhibitor (pIC50 = 6.8), and this compound effectively inhibits TBK1-mediated IRF3 phosphorylation and IFNα/β production in addition to its high water solubility and cell permeability (Figure 11) (163). Since all of these early TBK1 inhibitors carry a central aminopyrimidine backbone, SAR studies based on this backbone will further deepen the understanding of the pharmacophores for such type of TBK1 inhibitors.

5.2.2 Amlexanox and its derivatives

19 (TBK1 IC50 = 0.8 μM, IKKε IC50 = 5.8 μM) is a drug approved for the treatment of mouth sores and asthma. Biological studies have shown that 19 increases energy expenditure by increasing thermogenesis, improving insulin sensitivity, and reducing body weight and steatosis in mice (Figure 12) (164, 165). In addition, 19 has been found to alleviate acetaminophen-induced liver fibrosis and acute liver injury in mice by inhibiting TBK1/IKKε (166). However, the low solubility and moderate potency of 19 limited its further development. Further structural modifications were performed to the C3-carboxylic acid and C7-isopropyl substituents of 19. Among the analogs, only the tetrazole-substituted compound 21 containing C3-carboxylic acid showed strong inhibition of TBK1 (IC50 = 0.4 μM) and IKKε (IC50 = 0.2 μM), but the cellular activity of this compound was low (Figure 12). Among the other analogs, C7-cyclohexyl analog 22 produced the highest levels of

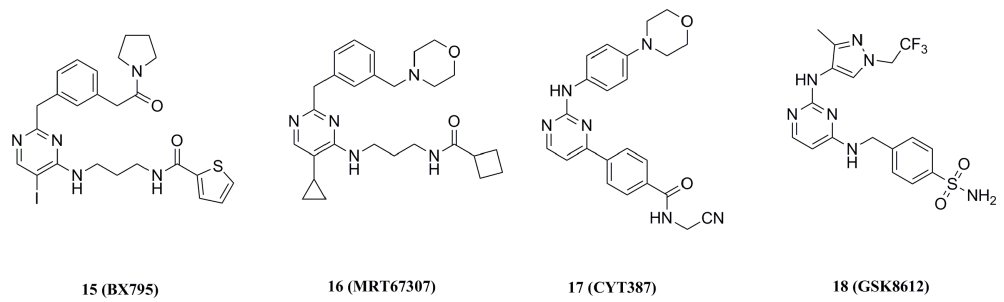

FIGURE 11
Aminopyrimidine structural TBK1 small molecular inhibitors.

IL-6 secretion in 3T3-L1 cells, but none of these compounds had a synergistic effect.

5.2.3 TBK1 inhibitor based on PROTAC technology

PROTAC (Proteolysis Targeting Chimeras) is an emerging and popular technology in the field of drug discovery in recent years (167). Based on this technique, the Crews group selected a TBK1 bound inhibitor 2,4-diaminopyrimidine-like structure and VHL (Von Hippel Lindau) ligand as a linkage model for PROTAC construction (168). After the optimization, the highly efficient TBK1 inhibitor 23 (TBK1 DC50 = 12 nM, Dmax=96%) was obtained, with good selectivity for the related kinase IKKε. (Figure 13) The ability of PROTACs to display high potency and selectivity towards TBK1 was revealed by changing the linker length and modulating the binding affinity. The potential for PROTACs was further confirmed in several cancer cells, where TBK1 was almost completely degraded and had no effect on the proliferation of tested cancer cells.

5.2.4 Other small molecular TBK1 inhibitors

Wang et al. reported a series of imidazopyridines as TBK1 inhibitors, of which the representative compound 24 (IC50 = 9 nM) showed enhanced efficacy and good kinase selectivity (Figure 14) (169, 170). The structurally similar imidazopyridine derivative 25 (IC50 = 5 nM) synergized with the MEK inhibitor AZD6244 to induce apoptosis in drug-resistant NRAS-mutant melanoma cells (Figure 14) (171). In contrast, 26 (IC50 = 13 nM), also with an imidazopyridine backbone, was found to be a potent, low toxicity inhibitor of TBK1 with promising therapeutic effects in mice against autoimmune diseases such as systemic lupus erythematosus (Figure 14) (172). The compound also inhibited the growth of cancer cell lines in non-small cell lung cancer by inhibiting TBK1, thereby leading to a reduction in downstream AKT signaling. The benzimidazole compound 27 (IC50 = 2 nM) reported by Bayer is a highly selective TBK1 inhibitor, but its poor pharmacokinetic properties led it to exhibit poor anti-tumor activity in melanoma mice (Figure 14).

Recently, idronoxil 28 is found to be effective in inhibiting the STING signaling pathway. 28 was reported to disrupt the complex formed by TBK1 and STING, blocking the phosphorylation of Ser172 and leading to dual inhibition of the IRF3 and NF-κB transcriptional programs (Figure 14) (173). 28 has shown promising results in models for the treatment of COVID-19, providing a potential drug with direct access to the clinic for the treatment of inflammatory diseases.

5.3 Inhibitors targeting STING

5.3.1 Competitive inhibitors for CDN binding site

In 2018, Siu et al. used the symmetry of the CDN binding domain to design small molecular inhibitors that were able to bind to the STING protein. Using mass spectrometry-based ligand screening techniques, they found a low-affinity hit (compound 29, R71H-G230A-R293Q HAQ STING IC50 = 7.3 μM) (Figure 15). Co-crystal of determination showed that STING adopted an inactive open conformation, with two molecules occupying the CDN ligand pocket (Figure 16). Based on the identification of several major hydrophobic interactions and polar contact between compound 29 and STING protein, compound 30 (HAQ STING IC50 = 0.08 μM) was identified by further SAR studies, which bound to STING similarly and could inhibit 2'3'-cGAMP-induced the secretion of IFN-β with an IC50 of 11 μM (Figure 15).

Li et al. identified the natural product 31 from a composite-type cyclopeptides screen based on a reporter gene assay. Further experiments using biotin-labeled compound 31 and h-STING demonstrated the competitive binding of compound 31 to the CDN site, and the addition of high concentrations of CDN (10-fold) abolished the binding of biotin-labeled compound 31 with STING (Figure 15) (174). Subsequent mechanistic studies showed that compound 31 locked the recruitment of IRF3 to STING signaling vesicles without affecting the DNA sensing and TBK1 recruitment, thereby preventing the downstream signaling in the cGAS-STING pathway. Notably, GlaxoSmithKline disclosed a series of N-methylamide-based benzimidazole-like STING antagonists in a patent (Figure 15) (175). The analogs are derived from the previously reported agonist diABZI, which occupies the CDN site at the STING dimer interface. Notably, compounds in this group, such as compound 32, have shown

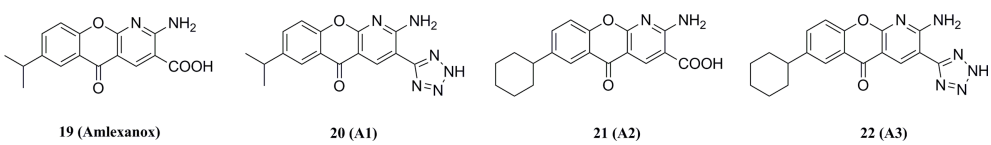

FIGURE 12
Amlexanox and its derivatives.

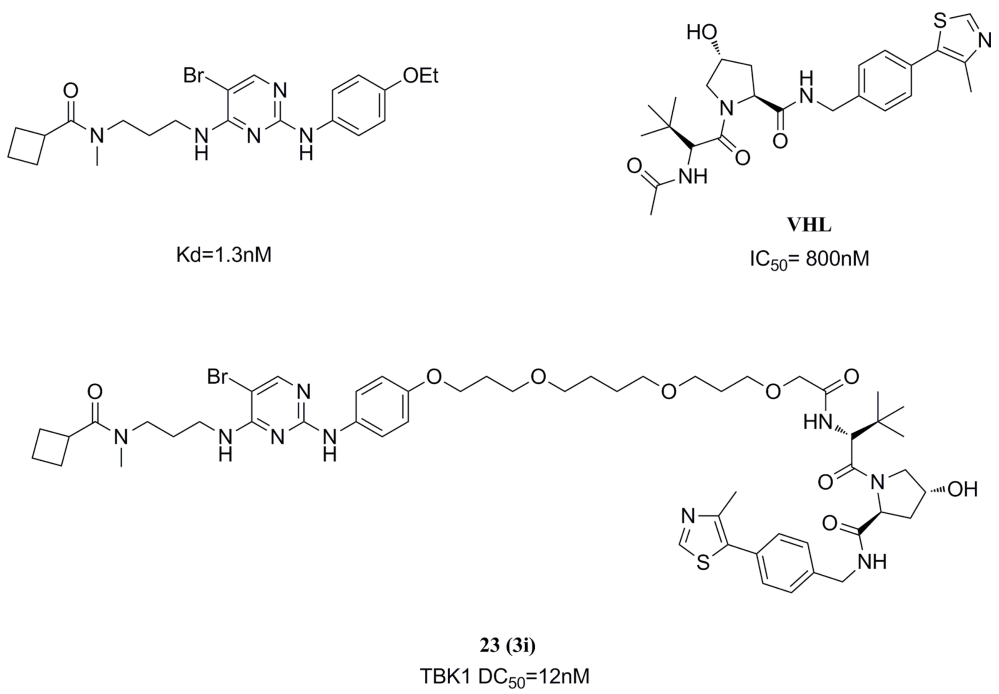

FIGURE 13
TBK1 targeting PROTAC molecule.

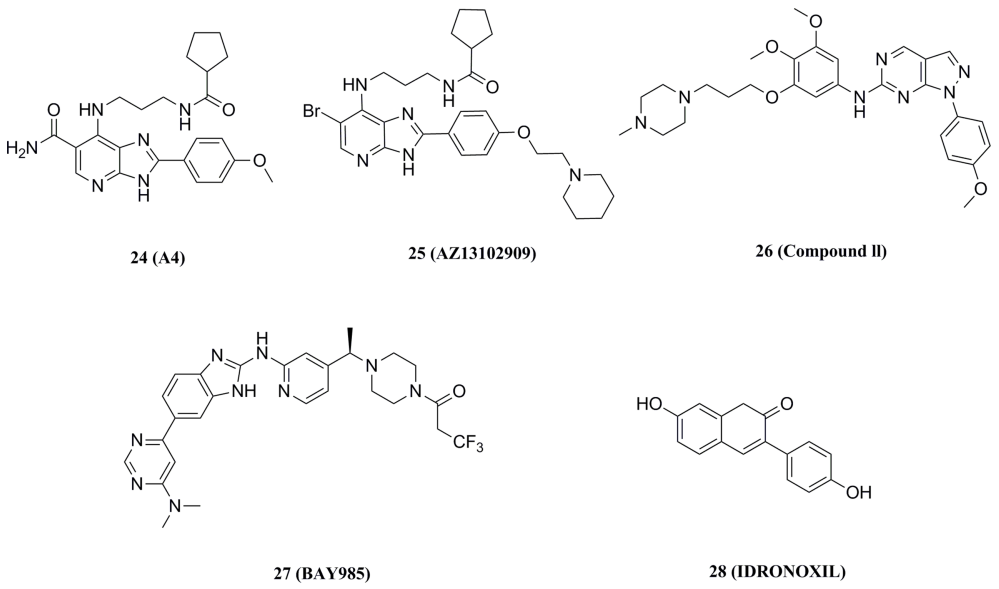

FIGURE 14
Other TBK1 small molecule inhibitors.

promising inhibitory properties in both binding assays and cellular experiments (FRET pIC50 > 9.9, THP-1 pIC50 = 8.9%, hPBMC pIC50 = 7.1%) (51).

In 2021, Hong et al. obtain the STING small molecular inhibitor compound 33 (IC50 = 0.076 μM) by the virtual screening towards the STING CDN site and following SAR studies (Figure 15) (176). Compound 33 has a higher affinity with binding to the upper CDN binding pocket compared to endogenous 2'3'-cGAMP and locks the STING dimer in an open inactive conformation. This process prevents STING from the oligomerization, translocation, and activation of cytoplasmic DNA, thereby significantly reducing STING-driven IFN-I and pro-inflammatory cytokine expression. Subsequent cellular and animal experiments showed that the compound not only inhibited the over-activation of STING mutants from SAVI patients but also significantly alleviated auto-inflammatory symptoms and prevented the death in TREX1-/- mice. Meanwhile, this compound exhibited comparable inhibitory activity to the previously reported STING covalent inhibitor compound 36 without cytotoxicity, which provides strong support for the development of STING inhibitors for the treatment of STING-related autoimmune diseases.

5.3.2 Covalent inhibitors

In 2018, Haag et al. discovered the covalent STING inhibitor compound 36 through structural optimization based on the structures of compound 34 and compound 35, the mouse STING covalent inhibitors which were obtained through high-throughput screening (Figure 17) (177). Compound 36 and the analogs blocked the activated palmitoylation of STING by covalently binding to Cys91, thus preventing STING from assembling into a multimeric complex in the Golgi apparatus, thus inhibiting its downstream signaling (Figure 18). Importantly, compound 36 shows great potential for the treatment of autoimmune diseases. Compound 36 significantly reduced the systemic cytokine response in CMA-treated mice. Treatment with compound 36 in ALS-model mice effectively ameliorated the inflammatory signal caused by the accumulation of TDP-43, restoring neuronal number and motor function (17, 178). Subsequently, compound 36 was found to reduce the

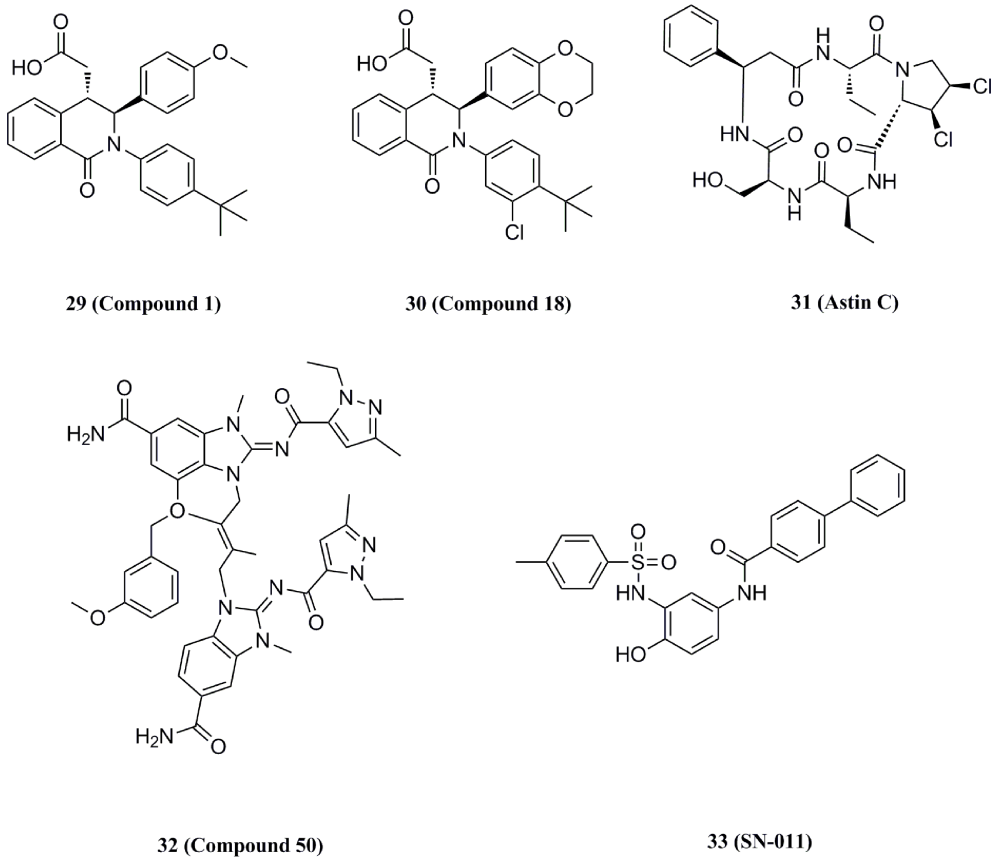

FIGURE 15
Structures of STING competitive binding antagonists.

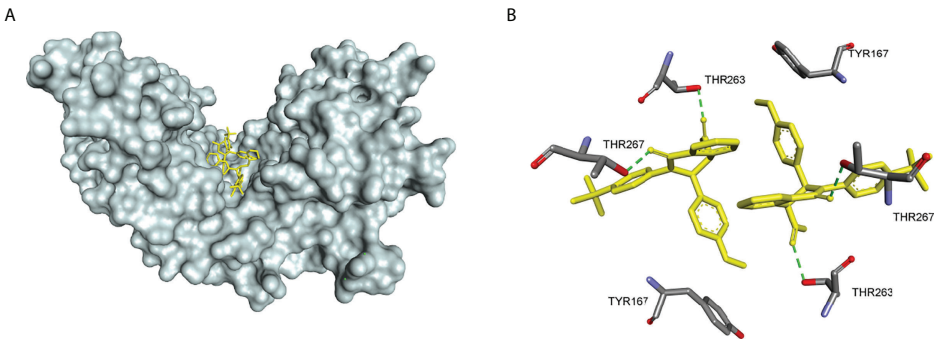

FIGURE 16
(A) X-ray structure of high-affinity ligand bound to STING protein (PDBID: 6MX3); **(B)** the interactions between compound 1 and Thr-263 and Thr-267, green dashed line indicating selected hydrogen bonding interactions.

symptoms of the chronic inflammatory disease psoriasis by decreasing protein levels of the pro-inflammatory cytokines IL-17A, IL-23, and IL-6 in serum and skin lesions (22).

Besides, Hansen et al. found that host infection with HSV results in the formation of nitro fatty acids in vivo and that endogenous nitro fatty acids (NO2-Fas) can covalently modify STING by Michael addition reaction to adjacent cysteines at positions 88 and 91 (Cys88/91) or N-terminal histidine (His16), thereby inhibiting the palmitoylation of STING and subsequent production of IFNs in host cells (Figure 17) (179). Similarly, the lipid peroxidation during viral infection leads to an increase in one of the major products, 4-hydroxynonenal (38), which promotes the carbonylation of STING, thereby inhibiting the transport of STING from the ER to the Golgi apparatus and suppressing STING activation (Figure 17) (180).

5.3.3 PROTAC target STING

Based on the PROTAC technique, Liu et al. selected the previously reported STING inhibitor C-170 linked to pomalidomide (CRBN ligand) as the PROTACs targeting STING (Figure 19) (181). Among them, compound 40 (DC50 = 3.2 μM) induced the degradation of STING via the CRBN-dependent ubiquitin-proteasome pathway, and dose-dependently downregulated the levels of IFN-β, IL-6, and CXCL10 triggered by 2'3'-cGAMP in THP-1 cells. A partial biological evaluation of this compound as an anti-inflammatory agent was also performed. PROTAC has the advantages of reduced drug exposure, low toxicity, and overcoming drug resistance compared to conventional drugs, and this compound has been reported to provide an alternative strategy for the development of new STING inhibitors.

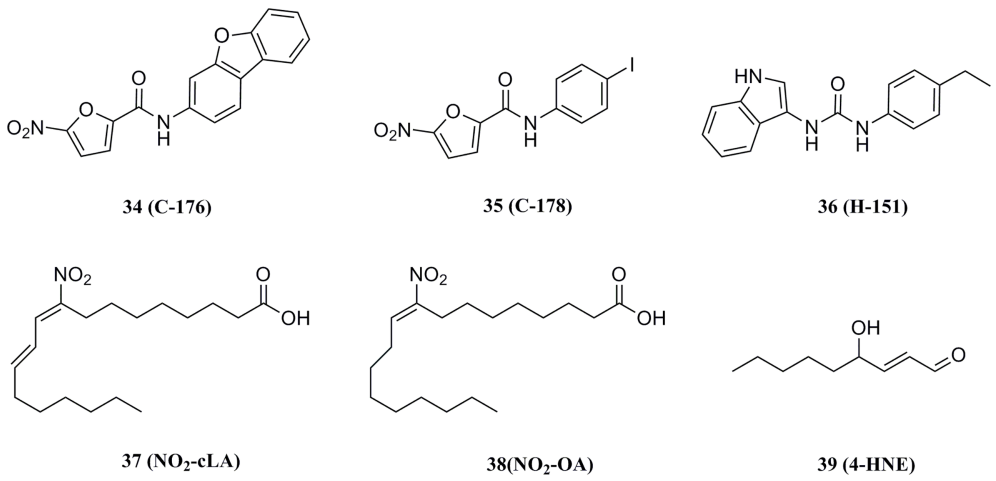

FIGURE 17
Structures of STING covalent binding antagonists.

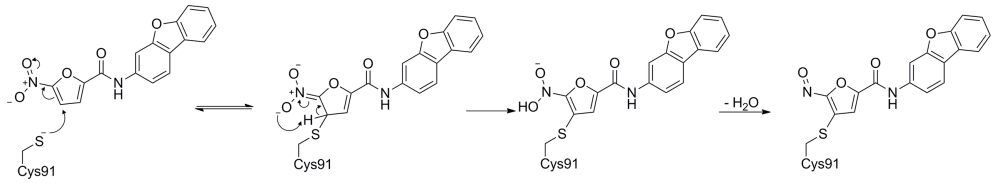

FIGURE 18
Possibly reaction mechanism of covalent small molecular inhibitors and STING proteins (using C-176 compound as an example).

6 Discussion and Perspective

The GAS-STING signaling plays a critical role in the innate immune response, and the abnormal activation of GAS-STING is linked to various autoimmune diseases (182). The genetic mutants which induce the continuous activation of STING or the cytoplasmic dsDNA accumulation contribute to several STING-relevant autoimmune diseases. While for some autoimmune diseases like SLE which are regarded as the systemic disease for multifactorial complex pathogenesis, the GAS-STING signaling activation is only one of the multiple factors. Moreover, quite a few neurodegenerative diseases including ALS and NPC belong to STING-relevant autoimmune diseases. Importantly, the knock-outing of the STING gene would ameliorate the pathological features of the STING-relevant autoimmune disease, which indicate that the cGAS-STING-TBK1 axis is a promising therapeutic target for various autoimmune diseases.

The inhibitors of cGAS-STING-TBK1 signaling were reported to decrease the protein levels of the inflammatory cytokines and the inflammatory signaling at the cellular and animal levels. The covalent STING inhibitor compound 36 significantly decreased systemic cytokine responses in CMA (the STING agonist) treated mice, thereby attenuating symptoms of autoinflammatory disease in vivo. Moreover, another CDN binding STING inhibitor compound 33 treatment shows the comparable suppression of IFN-β and ISGs expression in the TREX1−/−mice. Therefore, the STING antagonists have become the potential therapeutic agents for STING-relevant autoimmune diseases. Besides the direct inhibition of the STING signaling, targeting the upstream and downstream nodes of the STING activation pathway is also an alternative way to develop the drug against autoimmune

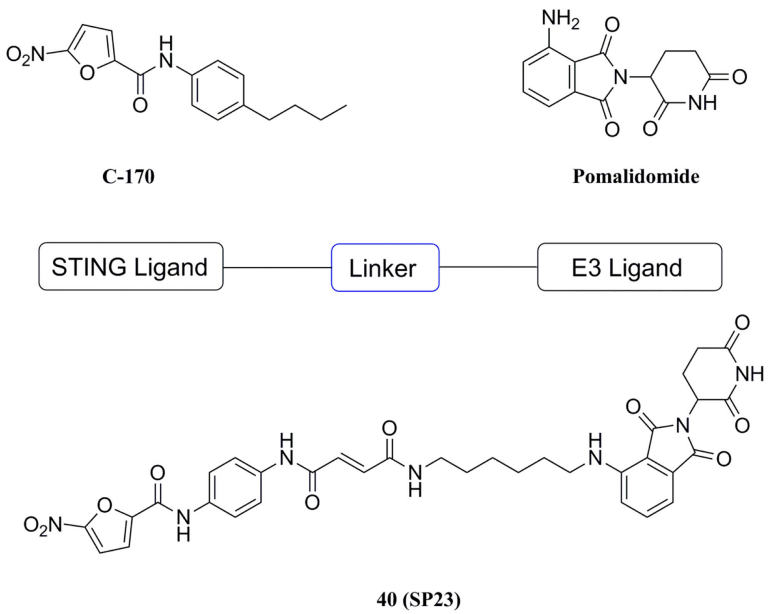

FIGURE 19
PROTAC molecule target STING.

diseases. For instance, inhibitors targeting cGAS significantly downregulated IFN expression in TREX1-/- PMBCs in AGS model mice, showing excellent potential for drug development in the treatment of autoimmune diseases caused by DNA accumulation such as AGS and SLE. However, drugs targeting cGAS did not show promising results in the SAVI model, and treatment of cGAS-independent autoimmune diseases may still have to focus on small molecular inhibitors targeting STING. Nerveless, cGAS inhibitors still can be a good complement to therapeutic regimens for the treatment of DNA-dependent autoimmune diseases. Besides, it was reported that TBK1 inhibitor BX795 could downregulate IFN-I activation in PBMCs of SS, SLE, and MS patients (183, 184). Compared to blocking common natural immune targets, such as TBK1 or IFNs, inhibition of cGAS-STING has less risk of immunosuppression and opportunistic infections without keeping the other PRR systems intact. Moreover, STING inhibitors may also be more potent than existing therapeutic agents (e.g., JAK inhibitors and IFN receptor antibodies) because the latter do not limit the maladaptive effects of other cytokines such as TNF-α and IL-6 (185).

Three types of STING inhibitors have been reported including covalent inhibitors forming specific covalent linkage to Cys91, Cys88/91, or His16, with compound 36 as the representative compound (177). The second of type inhibitors just like 28 can disrupt STING/TBK1 interactions (173). The third type of STING antagonists compete with 2'3'-cGAMP at the STING CDN binding site such as 33 (176). Currently, both the first type and the third type show much more potent activity (the IC50 is at the nanomolar level) than the second type inhibitor (the IC50 is at the micromolar level). It was reported that 33 had lower cytotoxicity and higher specificity than compound 36, which indicated that the competed inhibitor at CDN binding site would lead to better specificity and less toxicity. Interestingly, compound 32 which is derived from the STING agonist diABZI exhibits the potent inhibitory of cGAS-STING signaling. The molecular dynamics studies should be performed to investigate how the structural modification in such a scaffold affects the conformational changes of STING CTD and lead to the agonistic or antagonistic activity of the diABZI analogs, which would shed light on the modulation mechanism of STING activation. Moreover, the crystal structures of the complexes of STING and the antagonists have been solved (PDB code: 6MX3), which would facilitate rational drug design based on the complex structure. In fact, the discovery of compound 33 was achieved through the optimization of the active hit which was obtained via the molecular docking towards the CDN site using the virtual chemical database (ZINC).

Author contributions

MZ, YZ, XZ collect and analysis the reference data. MZ designed the experiments and wrote the original manuscript. JZ reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version. All authors contributed to the article and approved the submitted version.

Funding

This work was in part supported by the National key research and development program (2018YFE0107600, ZJM), Nature Science Foundation of China (22077115, 81672559). National Mega-Project for Significant new drug discovery (2018ZX09711003-002-002, ZJM).

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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10.3389/fimmu.2022.954129

Glossary

PAMPs	pathogen-associated molecular patterns
DAMPs	damage-associated molecular patterns
RIG-I	retinoic acid-inducible gene I
MDA5	melanoma differentiation-associated gene 5
TLRs	Toll-like receptors
2'3'-cGAMP	2'3'-cyclic GMP-AMP
ATP	adenosine triphosphate
GTP	guanosine triphosphate
ISGs	interferon-stimulator genes
AGS	Aicardi-Goutières syndrome
SLE	systemic lupus erythematosus
SAVI	STING-associated vasculopathy of infancy
ALS	amyotrophic lateral sclerosis
ER	endoplasmic reticulum
KD	N-terminal kinase domain
ULD	ubiquitin-like domain
SDD	alpha-helical scaffold dimerization domain
NTD	N-terminal transmembrane structural domain
CTD	C-terminal structural domain
LBD	ligand-binding domain
TBM	TBK1-binding motif
CDG	cyclic-di-GMP
CDA	cyclic-di-AMP
COPII	coat protein complex II
TBK1	tank-binding kinase 1
IFNs	type I interferons
IRF3	interferon regulatory factor 3
CTT	C-terminal tail
JAKs	Janus kinases
TYK2	tyrosine kinase 2
STAT1	signal transducer and activator of transcription 1
IRF9	interferon regulatory factor 9
AMPK	AMP-activated protein kinases
mTOR	mammalian targets of rapamycin

(Continued)

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ATG9a	autophagy-associated protein 9a
NLRC3	NOD-like receptor C3
WIPI2WD	repeat structural domain phosphoinositides interacting protein 2
RAB7	RAS-associated protein Rab-7a
COPI	coat protein complex I
NF-kB	nuclear factor kappa B
LIRs	LC3 interacting regions
SAMHD1	histidine-aspartate domain-containing protein 1
MRE11A	meiotic recombination 11 homolog A
IFNAR	IFN-αreceptor
STIM1	stromal interaction molecule 1
AD	Alzheimer's disease
PD	Parkinson's disease
HD	Huntington's disease
FTD	frontotemporal dementia
MS	multiple sclerosis
VDAC1	voltage-dependent anion channel 1
C9orf72	chromosome 9 open reading frame 72
NPC1	Niemann-Pick C1
EAE	experimental autoimmune encephalomyelitis
BBI	Bowman-Birk inhibitor
AdMVs	apoptosis-derived membrane vesicles
TMEM203	Transmembrane protein 203
DNase II	Deoxyribonuclease II
CIA	collagen-induced arthritis
WT	wild type
ILC1	type 1 innate lymphoid cells
SAR	structure activity relationship
LLPS	Liquid-liquid phase separation
EGCG	epigallocatechin gallate
PDK1	3 phosphoinositide dependent protein kinase
MLK1-3	mixed lineage kinase 1-3
MARK1-4	AMP-activated protein kinase 1-4)
OSCC	oral squamous cell carcinoma
NSCLC	nonsmall-cell lung cancer
PDAC	pancreatic ductal adenocarcinoma
BMDM	bone marrow derived macrophages

frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 15 September 2022
DOI 10.3389/fimmu.2022.940122

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Autoimmune and Autoinflammatory Disorders, a section of the journal Frontiers in Immunology

RECEIVED 10 May 2022
ACCEPTED 24 August 2022
PUBLISHED 15 September 2022

CITATION
Corona-Sanchez EG, Martínez-García EA, Lujano-Benitez AV, Pizano-Martinez O, Guerra-Durán IA, Chavarria-Avila E, Aguilar-Vazquez A, Martín-Márquez BT, Arellano-Arteaga KJ, Armendariz-Borunda J, Perez-Vazquez F, García-De la Torre I, Llamas-García A, Palacios-Zárate BL, Toriz-González G and Vazquez-Del Mercado M (2022) Autoantibodies in the pathogenesis of idiopathic inflammatory myopathies: Does the endoplasmic reticulum stress response have a role?
Front. Immunol. 13:940122.
doi: 10.3389/fimmu.2022.940122

Autoantibodies in the pathogenesis of idiopathic inflammatory myopathies: Does the endoplasmic reticulum stress response have a role?

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Idiopathic inflammatory myopathies (IIMs) are a group of rare, acquired autoimmune diseases characterized by profound muscle weakness and immune cell invasion into non-necrotic muscle. They are related to the presence of antibodies known as myositis-specific antibodies and myositis-associated antibodies, which are associated with various IIM phenotypes and the clinical prognosis. The possibility of the participation of other pathological mechanisms involved in the inflammatory response in IIM has been proposed.

Such mechanisms include the overexpression of major histocompatibility complex class I in myofibers, which correlates with the activation of stress responses of the endoplasmic reticulum (ER). Taking into account the importance of the ER for the maintenance of homeostasis of the musculoskeletal system in the regulation of proteins, there is probably a relationship between immunological and non-immunological processes and autoimmunity, and an example of this might be IIM. We propose that ER stress and its relief mechanisms could be related to inflammatory mechanisms triggering a humoral response in IIM, suggesting that ER stress might be related to the triggering of IIMs and their auto-antibodies' production.

KEYWORDS

endoplasmic reticulum stress, idiopathic inflammatory myopathies, myositis specific antibodies, autophagy, myositis associated antibodies

1 Introduction

Idiopathic inflammatory myopathies (IIMs), also known as myositis, are a group of conditions characterized by chronic inflammation of the musculoskeletal system that leads to proximal or distal muscle weakness, although other organs such as the skin, joints, heart, lungs, and gastrointestinal tract can also be affected (1). The IIM pathogenesis includes genetic, environmental, and immune factors (2, 3). To date, the immunopathological mechanisms of this group of conditions remain incompletely understood; however, they are related to inflammatory responses characterized by the infiltration of T- and B-cells in muscle tissue, the presence of myositis-specific antibodies (MSAs) myositis-associated auto-antibodies (MAAs),

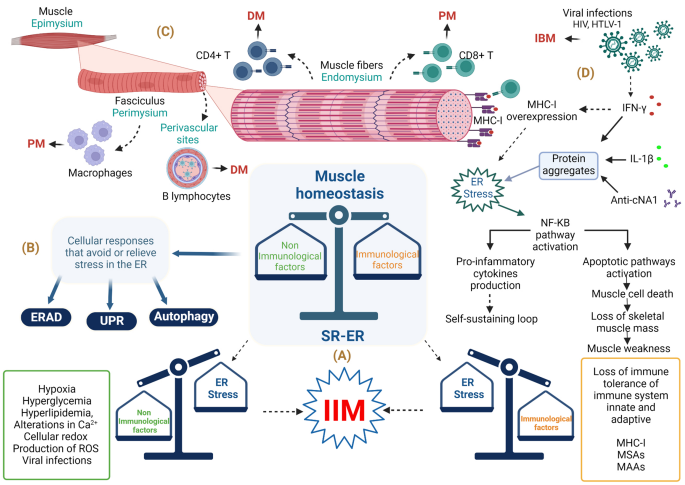

FIGURE 1
Involvement of endoplasmic reticulum stress in the musculoskeletal system in idiopathic inflammatory myopathies. **(A)** Immunopathological and non-immunopathological factors with possible repercussions on homeostasis of the musculoskeletal system. **(B)** ER stress relief mechanisms. **(C)** Histological importance in the diagnosis of IIM. **(D)** Involvement of immune and non-immune responses in IBM. *Abbreviations:* anti-cN1A, anti-cytosolic 5'-nucleotidase 1A antibodies; DM, dermatomyositis; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum–associated protein degradation; HIV, human immunodeficiency virus; HTLV-1, human T lymphotropic virus; IBM, inclusion body myositis; IFN-γ, interferon gamma; IIM, idiopathic inflammatory myopathy; IL-1β, interleukin-1 beta; MHC-1, major histocompatibility complex class I; MSAs, myositis-specific antibodies; MAAs, myositis-associated auto-antibodies; NF-KB, nuclear factor κB; PM, polymyositis; ROS, reactive oxygen species; SR-ER, sarcoplasmic reticulum–endoplasmic reticulum; UPR, unfolded protein response. The figure was created with BioRender.com (agreement no. ZO24BHA4GB).

and ubiquitous abnormal overexpression of major histocompatibility complex class I (MHC-I) in myofibers (2) (Figure 1A). However, one of the clinical observations is that the level of muscle inflammation does not correspond to the severity of the disease or the alterations in muscle fibers in patients with IIM, so non-immunological mechanisms are involved (4). Among the non-immunological mechanisms involved in the pathogenesis of IIM are endoplasmic reticulum (ER) stress and the responses that avoid or relieve this stress, such as the unfolded protein response (UPR), ER-associated protein degradation (ERAD), and autophagy (Figure 1B). Specifically, the UPR increases the capacity of the ER to fold proteins efficiently and attenuates the general translation of proteins to reduce the load on the ER, while proteins that cannot be repaired are removed by ERAD and autophagy (5). It is important to emphasize that the ER is very sensitive to challenges that can compromise its structure, integrity, and function; such challenges include calcium (Ca^{2+}) depletion, protein glycosylation, disulfide-bond formation, hypoxia, redox conditions, and viral infection, which can result in unfolded or misfolded protein accumulation, generating ER stress that triggers an inflammatory response (6–8) (Figure 1A). Currently, it is known that ER stress is involved in the pathogenesis of different diseases, such as obesity, diabetes, atherosclerosis, inflammatory bowel disease, Alzheimer's disease, breast cancer, rheumatoid arthritis, Sjögren's syndrome, and myopathies, among others (9, 10). This review will focus on current state-of-the-art research seeking to understand ER stress, focusing on UPR, ERAD, and autophagy as trigger factors in the IIM clinical phenotype pathogenesis and the possible link with MSAs.

2 Idiopathic inflammatory myopathies: Classification, diagnosis, and treatment

In 2017, the European League Against Rheumatism and the American College of Rheumatology (EULAR/ACR) published the most recent criteria for myositis classification for adult and juvenile IIM, which covered the following conditions: dermatomyositis (DM), amyopathic DM (ADM), juvenile DM (JDM), polymyositis (PM), immune-mediated necrotizing myopathy (IMNM), juvenile myositis (JM), and inclusion body myositis (IBM) (11). The clinical characteristics of IIM are proximal and distal muscle weakness, fatigue, fever, cutaneous features including pathognomonic rashes in DM (heliotrope, Gottron's sign), dysphagia, increased serum muscle enzyme levels (of, e.g., aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, and aldolase), muscle biopsy with histopathological features related to inflammation in the perimysium or perivascular areas, necrotic fibers between other characteristics, detection of conduction abnormalities by electromyography, and the presence of MSAs and MAAs (12). MSAs are used as a diagnostic tool in IIM; such auto-antibodies include anti-aminoacyl tRNA synthetase antibodies (anti-ARS), anti-nucleosome remodeling deacetylase antibodies (anti–Mi-2), anti-melanoma differentiation–associated protein 5 (anti-MDA5/CADM140), anti-nuclear matrix protein (anti-MJ/NXP2), anti-transcription intermediary factor-1 γ/α (anti-TIF-1 γ/α or p155/140, anti-hydroxymethylglutaryl coenzyme A reductase (anti-HMGCR), anti-small ubiquitin-like modifier activating (anti-SAE), anti-cytosolic 5'-nucleotidase 1A (anti-cN1A), and anti-signal recognition particle (anti-SRP) (13, 14) (Table 1). However, the anti-histidyl tRNA synthetase antibody (anti–Jo-1), an anti-ARS auto-antibody, is considered only by the EULAR/ACR 2017 guideline as a criterion for IIM classification (15). IIM treatment aims to reduce muscle inflammation and improve extra muscular manifestations, allowing patients to have a better quality of life, and it consists of high doses of oral glucocorticoids as initial treatment in combination with other conventional disease-modifying anti-rheumatic drugs or biological therapy (1).

3 Clinical features in idiopathic inflammatory myopathies

3.1 Dermatomyositis

This phenotype is characterized by the presence of heliotrope, a pathognomonic cutaneous manifestation that involves the presence of a bilateral and symmetrical edema with violaceous coloration in the upper eyelids (16) as well as the presence of other cutaneous rashes, including Gottron's sign, V sign, and Shawl's sign (17). Histopathology shows predominant $CD4^+$/T-cell perivascular infiltration (18). In classical adult DM, the enzyme muscle level rises significantly, but it usually carries a good clinical prognosis. This phenotype of IIM has been associated with the presence of anti–Mi-2 and anti-MJ/NXP2 (1, 13).

3.2 Amyopathic dermatomyositis

These patients present the same cutaneous manifestations observed in classic DM; however, there is no clinical or laboratory evidence of muscle disease (19). This phenotype of IIM has been broadly associated with anti-MDA5/CADM140 auto-antibodies and the development of rapidly progressive interstitial lung disease (13).

3.3 Juvenile dermatomyositis

This DM variant includes the same features as those of classic DM; however, it develops during childhood or youth.

TABLE 1 Stratification of Idiopathic Inflammatory Myopathies patients according to myositis-specific antibodies.

MSAs	Target	IIM
Anti-ARS	Aminoacyl tRNA synthetase	ASS
Anti-Jo-1	Histidyl tRNA synthetase	ASS
Anti-EJ	Glycyl tRNA synthetase	ASS
Anti-PL-7	Threonyl tRNA synthetase	ASS
Anti-OJ	Isoleucyl tRNA synthetase	ASS
Anti-PL-12	Alanyl tRNA synthetase	ASS
Anti-YRS/HA	Tyrosyl tRNA synthetase	ASS
Anti-KS	Asparaginyl tRNA synthetase	ASS
Anti-Zo	Phenylalanyl tRNA synthetase	ASS
Anti-cN1A	Cytosolic 5'-nucleotidase 1A	IBM
Anti-HMGCR	3-hydroxy-3-methylglutaryl coenzyme A reductase	IMNM
Anti-MDA5 (anti-CADM140)	Melanoma differentiation-associated protein 5	DM, ADM
Anti–Mi-2	Nucleosome remodeling deacetylase (Mi-2α/β)	DM
Anti–NXP-2 (anti-MJ)	Nuclear matrix protein 2	DM
Anti-SAE	Small ubiquitin-like modifier activating enzyme (SAE1/2)	DM
Anti-SRP	Signal recognition particle	PM, IMNM
Anti-TIF1γ/α (anti-p155/p140)	Transcription intermediary factor-1 γ/α	DM

ADM, amyopathic dermatomyositis; anti-ARS, anti-aminoacyl tRNA synthetase antibodies; anti–Jo-1, anti-histidyl tRNA synthetase antibodies; anti-EJ, anti-glycyl tRNA synthetase antibodies; anti–PL-7, anti-threonyl tRNA synthetase antibodies; anti-OJ, anti-isoleucyl tRNA synthetase antibodies; anti–PL-12, anti-alanyl tRNA synthetase antibodies; anti-YRS/HA, anti-tyrosyl tRNA synthetase antibodies; anti-KS, anti-asparaginyl tRNA synthetase antibodies; anti-Zo, anti-phenylalanyl tRNA synthetase antibodies; anti-cN1A, anti-cytosolic 5'-nucleotidase 1A antibodies; anti-HMGCR, anti-hydroxymethylglutaryl coenzyme A reductase antibodies; anti-MDA5/CADM140, anti-melanoma differentiation–associated protein 5 antibodies; anti–Mi-2, anti-nucleosome remodeling deacetylase Mi-2 α/β antibodies; anti-MJ/NXP2, anti-nuclear matrix protein 2 antibodies; anti-SAE, anti-small ubiquitin-like modifier activating antibodies; anti-SRP, anti-signal recognition particle antibodies; anti-TIF-1γ/α or p155/140, anti-transcription intermediary factor 1 α/γ antibodies; ASS, anti-synthetase syndrome; DM, dermatomyositis; IBM, inclusion body myositis; IIM, Idiopathic Inflammatory Myopathies; IMNM, immune-mediated necrotizing myopathy; JDM, juvenile dermatomyositis; JM, juvenile myositis; MSAs, myositis-specific antibodies; PM, polymyositis.

This phenotype has a greater association with calcinosis, pericarditis, and gastrointestinal ulcers (20, 21).

3.4 Polymyositis

PM is observed as a kind of proximal muscle weakness without dermatologic manifestation found usually in the adult population. Histopathology shows predominant $CD8^+$/T-cell perivascular infiltration and upregulation of MHC-I molecules in muscle biopsy (18). PM patients are usually seronegative for MSAs or MAAs, and they are now considered a rare IIM subgroup; in a study of a U.K. IIM cohort of 255 patients, PM was diagnosed in 37/255 (14.5%) patients, but reclassification using the EULAR/ACR 2017 criteria led to the final diagnosis of only 9/255 (3.5%) patients, with the rest meeting criteria for IMNM, DM, overlap syndromes, etc. (22). This phenotype of IIM might be associated with anti-synthetase syndrome, and patients are usually positive for anti-ARS auto-antibodies, especially anti–Jo-1 (13, 23).

3.5 Immune-mediated necrotizing myopathy

The necrotizing terminology in this context is used to describe muscle fiber necrosis with minimal leukocyte infiltrates, which could be due to genetic causes or statin-induced (24). The muscular weakness found in these patients progresses more rapidly and tends to be more severe. Another important features are the serum levels of creatine phosphokinase, which are remarkably high in this phenotype of IIM compared to other types of myositis, and the presence of anti-SRP or anti-HMGCR auto-antibodies (25, 26).

3.6 Juvenile myositis

This myositis phenotype is diagnosed according to the EULAR/ACR 2017 guideline when the first symptom occurs at <18 years of age and there are no cutaneous manifestations (the major differential feature of JDM) (15).

3.7 Inclusion body myositis

IBM patients experience a slow progression of skeletal–muscle disease, usually after 50 years of age, with a higher prevalence in men and a predominance of distal muscle weakness. In addition, dysphagia occurs in >50% of IBM patients (1). Serum detection of anti-cN1A is highly specific for IBM (90%–95%) compared to DM or PM (5%–10%); however; this auto-antibody has also been detected in patients with systemic lupus erythematosus (0%–20%) and Sjögren's syndrome (0%–36%) (27).

4 Pathological mechanisms in idiopathic inflammatory myopathies

The common denominator in all IIMs is muscle inflammation (28). Muscle fibers have a cylindrical structure and consist of intercalating thick and thin filaments (myofilaments) that are organized longitudinally in sarcomeres that allow the contraction of muscle fibers (29). In particular, muscle fibers are arranged in three layers of connective tissue, as follows (a) the endomysium, which surrounds the muscle fibers; (b) the perimysium; and (c) the epimysium, which is composed of fasciculi surrounding the entire muscle (30). There are no conclusive data on the pathophysiological mechanisms related to muscle inflammation in IIM, where genetic factors such as human leukocyte antigen classes I and II (HLA-I and HLA-II) and environmental factors such as ultraviolet radiation and viral infections play a role (31–33). However, these do not fully explain the triggering of IIM, so the participation of immunopathological mechanisms related to the innate and adaptive immune systems are probably linked to IIM development (1). Nevertheless, as we have mentioned in previous paragraphs, the level of muscle inflammation does not correspond to the severity of the disease or to alterations in muscle biopsy in IIM; therefore, these pre-suppose the contribution of non-immunological mechanisms such as ER stress and altered proteins responses (UPR, ERAD, and autophagy). Based on this information, it is impossible to rule out the idea that the immunological mechanisms do not have repercussions on the non-immunological ones.

5 Immunopathological mechanisms in idiopathic inflammatory myopathies

The involvement of both the innate and the adaptive immune systems has been reflected in histopathological evidence characterized by the infiltration of inflammatory cells in the muscle and skin, microvasculature affection, auto-antibody production against nuclear and cytoplasmic proteins, and inflammatory responses by interferon signature (34). For example, in DM patients, a complement-mediated micro-angiopathy that affects blood vessels of muscle tissue has been described; resulting in complement activation and membrane attack complex formation that leads to perivascular inflammation, capillary necrosis, and ischemic damage of myofibers; along with pro-inflammatory cytokines, macrophages, a high percentage of $CD4^+$ T and B lymphocytes in perivascular sites, and dendritic plasmacytoid cells (18, 35, 36). The immune response in PM is mediated by lymphocytic infiltrates with a predominance of $CD8^+$ T lymphocytes related to perforin and granzyme release in the endomysium as well as a lower proportion of macrophages in perivascular sites; increased expression of MHC-I; the roles of interleukin (IL)-1 and interferon-γ (IFN-γ) in myotoxicity; chronic inflammation and fibrosis *via* the involvement of transforming growth factor-β (TGF-β); and T-cell extravasation in muscle tissue through the involvement of chemokines like C-X-C motif chemokine ligand 8 (CXCL8), C-X-C motif chemokine ligand 9 (CXCL9), C-C motif chemokine ligand 2 (CCL2), and C-C motif chemokine ligand 9 (CCL9) (18, 36, 37) (Figure 1C). It has been observed that muscle cells also express human leukocyte antigen-G (HLA-G) in IIM due to stimulation by IFN-γ, which is synthesized locally by inflammatory cells of the disease-specific immune micro-environment, and the increase of HLA-G can interfere with cytotoxic effector functions of $CD8^+$ T- and natural killer (NK) cells (38) because this molecule is a ligand for killer cell Ig-like receptor 2DL4 (KIR2DL4, also known as CD158d), an inhibitor receptor expressed in these cells (39). The immunopathological mechanisms involved in IMNM are mainly associated with statin prescription and the presence of anti-SRP and/or anti-HMGCR auto-antibodies; further ectopic expression of the respective auto-antigens in the myofiber surface has been reported (35, 40, 41). In addition, these auto-antibodies can induce muscle atrophy incrementally in the transcription of genes related to atrophy—such as muscle atrophy F-box (*MAFbx*) and tripartite motif containing 63 (*TRIM63*)—and reactive oxygen species (ROS) and can decrease the production of anti-inflammatory cytokines such as IL-4 and IL-13 (26, 42). Particularly, IBM is considered a complex disorder involving inflammatory and cytotoxic responses mediated by $CD8^+$ T-cells with vacuole formation, accumulation of tubulofilamentous inclusions, and cytoplasmic accumulations of amyloid filaments, which could trigger or exacerbate ER stress responses (43). The IBM pathogenesis includes mitochondrial dysfunction as reflected by high levels of differential growth factor 15 (GDF15), a mitochondrial disease marker (44). Furthermore, in myoblasts of IBM patients, reduced adenosine triphosphate production, cellular vulnerability to oxidative stress, and reduced mitochondrial size have been documented (44). The mitochondrial damage in IBM is also associated with impaired autophagy mechanisms, and

abnormal autophagy causes autophagosome accumulation with vacuole formation (27).

6 Non-immunopathological mechanisms

The cell has an integrated and interconnected signaling system that avoids or relieves ER stress through processes such as UPR, ERAD, and autophagy, which are considered to be non-immunological mechanisms associated with IIM pathogenesis (45, 46). The fundamental goal of these mechanisms is to recover ER homeostasis to preserve ER functions that are important for cell survival (47). Briefly, we describe some important mechanisms to achieve this goal.

6.1 Endoplasmic reticulum

An organelle associated with skeletal muscle homeostasis is the sarcoplasmic reticulum (SR), which is part of compartmentalization in the cytoplasm of the eukaryotic cell by an endomembrane system (ES) (48–50). The functioning of the ES can be represented as a factory, where proteins assembly begins; components are then delivered to the Golgi apparatus (GA), where the protein assembly ends, and the proteins are modified, labeled, classified, and finally packaged to be sent to their destination. The cell nucleus coordinates this entire manufacturing process in communication with ES through transport vesicles that bud from the membrane (donor) to merge with the membrane of the next compartment (acceptor), giving rise to what is known as the secretory pathway (51–53). Because there are multiple actors in the production of proteins, coordination between them is crucial to ensure protein synthesis occurs with an adequate structure and function, and one of these main actors is the ER (54, 55). The ER is involved in many cellular functions such as synthesis and processing (folding, maturation, and post-translational modification) of proteins (56–58). The ER is divided into three domains that are functionally and structurally different (56). The first domain includes a nuclear envelope, which forms using inner and outer membranes, which are continuous to the nuclear pores and function as a site where the proteins of the membrane are diffused through the nucleus and cytoplasm (56, 59). The second domain represents a smooth and rough ER (with attached ribosomes) (56). The third domain involves numerous heterotypic membrane contact sites with membranes of other organelles, such as the plasma membrane ER–plasmatic membrane, which is a classic example of an heterotypic membrane contact site and the first such example described in muscle cells related to muscle contraction through a massive influx of Ca^{2+} (60), ER–mitochondria, ER–peroxisomes, ER–lipid droplets, and ER–Golgi (61, 62). The secretory pathway can be explained in the following steps: (a) synthesis, anchoring, and translocation of the protein to ER lumen; (b) protein folding and quality control; and (c) protein sorting (63).

6.1.1 Synthesis, anchoring, and translocation of the protein to the endoplasmic reticulum lumen

When the proteins are synthesized by ribosomes attached to the ER membrane, they are translocated to the ER while their translation is in progress (co-translational manner), and these proteins will either follow the conventional secretory pathway or exist inside the ER. If free ribosomes synthesize proteins in the cytoplasm, they can be directed to different organelles, such as the nucleus, mitochondria, or peroxisomes, and can also enter the ER when their translation is complete in a process called post-translational translocation (64). The ribosomes attached to the ER membrane are responsible for synthesis in approximately 1/3 of total cellular proteins, and it is well known that this attachment is promoted by the SRP complex, which consists of 7SL RNA and six different polypeptides that are named according to their molecular mass in kilodaltons (kDa), as follows: SRP9, SRP14, SRP19, SRP54, SRP68, and SRP72 (65–68). The SRP complex recognizes a sequence of hydrophobic amino acids in the N-terminal region of the nascent protein known as a signal sequence or leader sequence through SRP54 kDa (65, 69–74). When protein translation begins in the cytoplasm, the signal sequence is exposed, and the SRP complex recognizes it through SRP19 kDa, preventing the continuation of the translation (arrest elongation) (65, 67, 75–77). Finally, the SRP complex is attached to its receptor through a heterodimer formed by SRP68 and SRP72 kDa, allowing the nascent protein–ribosome complex to be coupled to the translocation site, which is a protein channel known as translocon (74, 76, 78–80). This event permits the continuation of protein translation with the subsequent translocation of the polypeptide to the ER lumen through translocon while the ribosome is still synthesizing it or with insertion in the ER membrane (65, 67, 81–84) (Figure 2A).

6.1.2 Protein folding and quality control

Once in the ER, the folded protein is modified and exposed to quality control in order to achieve its native structure (proper three-dimensional conformation) through co-translational modifications such as N-glycosylation and disulfide bond formation (68, 82, 85, 86). Importantly, those proteins that undergo quality control in the ER to confirm their correct folding and assembly are packaged in a coat complex protein (COP)II–coated vesicle, which allows its transport through the secretory pathway by an anterograde movement (forward transport) to the next compartment of this pathway, i.e., the GA (48, 87). Furthermore, traffic

through the secretory pathway is bidirectional; transportation from the GA to the ER is a retrograde movement allowing immature or ER-resident proteins to go back to ER through COPI-coated vesicles produced in the trans-Golgi network (TGN) and involved in traffic between GA compartments (88) (Figure 2B). In this way, the ER is not only responsible for the synthesis of proteins that follow the secretory pathway but also for their processing and maturation and is essential for shipment to its destiny (89–91).

Multiple factors assist in the folding and maturation process of newly synthesized proteins, such as ER chaperone proteins and folding enzymes (92, 93). The best-recognized ER chaperones are the glucose-regulated/immunoglobulin-binding protein of 78 kDa (BiP/GRP78), calnexin (CNX), and calreticulin (CRT) (93–101).

6.1.3 Protein sorting

Within the GA, proteins continue their maturation; the N-linked glycan chains (preformed oligosaccharides) added to the peptides in the ER are structurally modified through a series of reactions that occur in a sequential manner *via* multiple trimming and elongation steps (102). The labeled proteins with their specific N-linked glycan chains, when they arrive to the TGN, are classified and packaged in transport vesicles to be sent to their final destination in the cellular surface, extracellular medium, or any of the compartments of the secretory pathway (53, 101, 103) (Figure 2C).

6.2 Endoplasmic reticulum stress

The ER is very sensitive to cellular disturbances that can disrupt the efficiency of its function, including a loss of Ca^{2+} homeostasis, impaired redox balance and endogenous ROS production, nutrient deprivation, virus infection, changes in protein glycosylation and autophagy defects, protein folding defects, or an increase in protein synthesis level (104). These cellular disturbances can influence protein synthesis and folding, including post-translational modifications, and lead to an accumulation of unfolded and/or misfolded proteins in the ER lumen, generating a cellular stress situation known as ER stress (6, 8, 57, 105).

6.3 Unfolded protein response

The best-known response to relieve ER stress caused by the detection of unfolded and/or misfolded proteins in the ER lumen is the UPR pathway (106) (Figure 1B). UPR transduces the stress signal from the ER to the cell nucleus through three sensors, which are the transmembrane proteins inositol-requiring kinase 1 (IRE1), double-stranded RNA–activated protein kinase (PKR)-like ER kinase (PERK), and activating transcription factor 6 (ATF6) (68, 107, 108). The N-terminal luminal domain of these three sensors is responsible for detecting the accumulation of unfolded/misfolded proteins in the ER lumen (109). BiP/GRP78

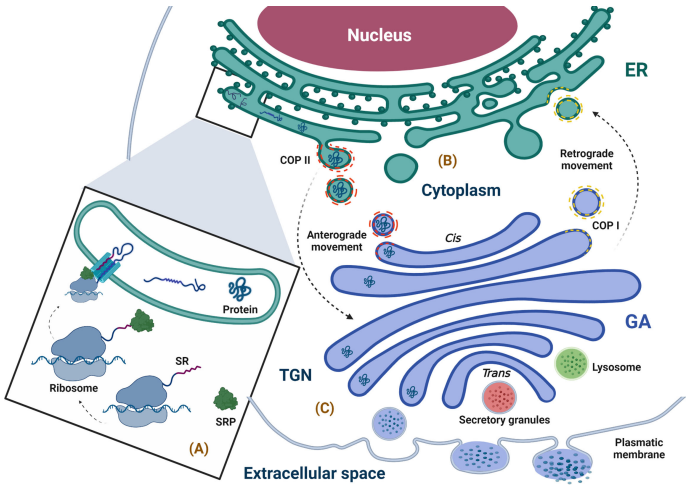

FIGURE 2
Physiological overview of the secretory pathway. **(A)** Synthesis, anchoring, and translocation of protein to the ER (ribosome binding to the ER membrane is promoted by the SRP complex, which recognizes the SR; once in the ER lumen, the ribosome–nascent protein complex, coupled to the translocon, translates the polypeptide into the lumen of the ER), **(B)** Protein folding and quality control (the newly synthesized proteins with correct folding and assembly continue their forward movement in COPII-coated vesicles that fuse with the GA for cargo delivery; however, the secretory pathway is bidirectional, and the circulatory pathway may stretch from the GA to ER, moving retrogradely through COPI-coated vesicles). **(C)** Protein sorting (when proteins arrive at the TGN, they are classified and packaged in transport vesicles to be sent to their final destination, i.e., the extracellular space). ER, endoplasmic reticulum; GA, Golgi apparatus; SR, signal recognition; SRP, signal recognition particle; TGN, trans-Golgi network. The figure was created with BioRender.com (agreement no. AM24BH3QGW).

is a repressor of UPR when it forms a stable complex with the luminal domain of the three sensors in unstressed cells; however, high concentrations of unfolded/misfolded proteins compete for BiP/GRP78, blocking its attachment to the three sensors (competition model) (110–114).

IRE1 is a type I transmembrane protein resident in the ER; PERK is also an ER resident type I transmembrane protein with serine/threonine kinase activity (115). Under conditions of ER stress, PERK can activate nuclear factor (NF)-κB in a manner dependent on the control of the translation mediated by the phosphorylation of eIF2α and independent of I kappa B kinase (IKK) activation, respectively (116). Finally, ATF6 is a type II transmembrane protein whose C-terminal domain is pointed toward the lumen of the ER and the N-terminal domain to the cytosol (117–119). Following stress-induced dissociation of BiP/GRP78 from ATF6 in the ER, it travels through COPII transport vesicles to the GA, where it undergoes proteolytic processing (120, 121).

6.4 Endoplasmic reticulum-associated protein degradation

ERAD is a crucial mechanism for maintaining protein quality control in the ER. It is responsible for removing misfolded proteins and redirecting them to the cytosol to be removed by the proteasome (122–124). Given the above, it is clear that the proteasome cannot process protein aggregates, so the cell has another important intracellular proteolytic system called autophagy (125).

6.5 Autophagy

Autophagy is an important catabolic process of cell degradation and macromolecule recycling (mainly damaged/old organelles and protein aggregates) (126). It is constitutively carried out at basal levels to maintain cellular homeostasis, but the level of autophagy can markedly increase under stress conditions as a cytoprotective response (127). Three types of autophagy are known to exist, including micro-autophagy, macro-autophagy, and chaperone-mediated autophagy, and all culminate in lysosome-mediated cargo degradation but differ in the mechanisms through which they deliver cargo to lysosomes (128). Macro-autophagy is the most studied process for the degradation of large loads, such as damaged organelles and protein aggregates; when speaking of autophagy, macro-autophagy is typically what is being referred to (129). The cellular hallmark of autophagy is the formation of double-membrane structures called autophagosomes that are responsible for transporting cargo to lysosomes (130).

To deliver cargo to the lysosome, the autophagosome fuses with the lysosome through its outer membrane, leading to the formation of an autolysosome; lysosomal enzymes first break down the inner membrane of the autophagosome to release the autophagic cargo and then break it down into its components, which are returned to the cytoplasm *via* the lysosomal membrane, allowing for their re-use by the cell (131). It has been reported that autophagosomes are composed of membranes from multiple sources, so the ER could be one of several sources (132).

Autophagy is mediated by a group of approximately 30 evolutionary conserved autophagy-related genes (ATGs), many of which have been detected in mammalian homologs. Among them, ATG1–10, 12–14, 16–18, 29, and 31 are essential for autophagosome formation (133). Autophagy can be selective or non-selective. Selective autophagy consists of labeling the cargo to be sequestered using ubiquitin (134). Subsequently, autophagy receptors such as ubiquitin-binding protein p62, also called sequestosome 1 (SQSTM1), recognize the marked charge and function as adapters that, by associating with certain specific proteins of the inner membrane (proteins of the ATG8 family) of the phagophore (early stage of autophagosome formation), can connect the cargo labeled with the inner membrane of the growing phagophore to allow for phagophore formation (135).

6.6 Endoplasmic reticulum/endoplasmic reticulum stress in idiopathic inflammatory myopathies

The IIM pathogenesis is associated with immunological and non-immunological mechanisms, which involve the participation of autoreactive T lymphocytes and auto-antibody production. Despite these, the literature has not explained the origin of muscle fiber damage or muscle weakness (136). To date, the most well-described example is upregulation of the expression of MHC-I in myofibers (sarcolemma surface), whose expression is not usual in normal muscle tissue (endomysial capillaries), thus stimulating the activation of ER stress responses such as an accumulation of misfolded glycoproteins and the activation of NF-κB, causing an inflammatory response *via* IRE1, tumor necrosis factor (TNF)-α, and TRAF2 (137, 138). It has been demonstrated that BiP/GRP78, CRT, and heat shock protein 90-α2 expression levels are augmented according to intermediate or high MHC-I expression in myositis muscle (139). In addition, elevated expression of several genes, including *PERK* and *ATF3*, in myositis, has been observed. Furthermore, the PERK signaling *via* transcription factor CCAAT/enhancer binding protein δ (C/EBPδ) has been associated with tumors by the action of chemokines such as CXCL8, and C-C motif chemokine ligand 20 (CCL20), which have been related to tumor-promotion with immunosuppressive properties and are triggered by activated oncogenes, nutrient deprivation, and hypoxia (140). In addition, the increased

expression of PERK has been correlated with macrophage and dendritic cell infiltration into the tumor micro-environment (141). At this time, the link between IIM and cancer development is not well understood (142), although factors strongly associated with cancer development are anti-TIF-1γ and anti-MJ/NXP2 (143). In addition, our research group reported the presence of MSAs, particularly anti–TIF-1γ, in a group of women with breast cancer without clinical evidence of myositis (144). We will next address the three points of this section.

As mentioned above, the ER stress response pathway activates NF-κB. Interestingly, it has been shown that caspase-12, the main caspase associated with the apoptosis pathway, is highly expressed in mouse skeletal muscle tissues, so the activated apoptotic pathways in muscle cells could induce cell death, a loss of skeletal muscle mass, and muscle dysfunction and weakness (4).

It has also been reported that TRE-H-2Kb x mck-Tta (HT) mice with conditional overexpression of H-2Kb in muscle presented an upregulation of ER stress markers and molecular targets of UPR such as BiP/GRP78, CRT, CNX, and ATF6, in addition to pathologic features of non-specific myopathy with variations in myofiber size, numerous centronucleated fibers, and endomysial fibrosis (139). In that work, the authors also reported that an intracellular accumulation of MHC-I in the muscle of IBM patients correlated with UPR (139). Such findings suggest that MHC-I involvement in myositis is pathological because of the triggering of UPR responses instead of the facilitation of antigen presentation to $CD8^+$ T lymphocytes. Therefore, the detection of MHC-I by immunostaining may be a diagnostic approach in IIM (145). However, although the positive detection of MHC-I has a high sensitivity for IIM, it has a low specificity, hence, it has been suggested that it can be combined with the positive detection of MHC-II (146).

In a study of our work group, we reported that recombinant human IL-1β induced phosphorylation and upregulation of SRP72 in Jurkat cells (147). Importantly, this cytokine and IL-18 are overexpressed in muscle biopsies of patients with DM and PM (148, 149). IL-1β and IL-18 are proteolytically matured by the NLRP3 inflammasome and allow the recruitment and maturation of caspase-1 (150). Another mechanism linked to the NLRP3 inflammasome is pyroptosis, a kind of cellular death, in addition to apoptosis and NETosis, which are also sources of auto-antigens related to the inflammatory response in the immunopathogenesis of autoimmune diseases such as IIM (151). In IMNM, an isolated report showed a correlation of BiP/GRP78 in muscle biopsy assessed by immunohistochemistry and serum lactate dehydrogenase along with a negative correlation with the Manual Muscle Testing–8 score (152).

Aggregates of p62/SQSTM1 protein, which normally degrade during autophagy, can be detected in the muscles of IBM and IMNM patients, suggesting dysregulation of the autophagic process of this protein (153, 154). It is known that dysregulation or excessive autophagy could lead to cell death and has been associated with several human diseases, including IBM (155). Although autophagy has not been extensively studied in other IIM phenotypes, Cappallettic et al. (156) found that, in addition to IBM, autophagy processes are also activated in PM, DM, and JDM. Girolamo et al. (157) reported a higher proportion of myofibers correlated with the presence of autophagy markers such as microtubule-associated protein light chain 3b (LC3b) and p62/SQSTM1 in muscle biopsies from patients with IMNM compared to those from patients with DM and PM.

Recent study was reported that a selected group of autophagy-related genes, such as *CCL2*, cyclin-dependent kinase inhibitor 1A (*CDKN1A*), *FOS*, myelocytomatosis (*MYC*), and TNF superfamily member 10 (*TNFSF10*)—whose functions are related to IFN-I signaling pathways—significantly influenced the infiltration of multiple immune cells, including B-cells, macrophages, and NK cells, in samples from DM patients compared to controls, suggesting that these genes may be potential diagnostic biomarkers for DM (158).

7 Idiopathic inflammatory myopathies and myositis-specific antibodies: A role for endoplasmic reticulum stress?

In addition to the detection of MHC-I by immunostaining, another diagnostic tool in IIM is the presence of MSAs. Although a direct association between MSAs production and ER stress has not been defined, some reports have touched on the subject. The SRP auto-antigen might be an example of antigen released from tissue damage (159), a target of anti-SRP antibodies (160). It has been reported that auto-antibodies against SRP54 exist in IMNM and PM patients with dilated cardiomyopathy, disease severity, and remarkably high levels of muscle enzymes (160–162). One study of DM patients documented the presence of anti-SRP72 antibodies (163). Recently, a possible association between anti-calreticulin (anti-CRT) antibodies and malignancy in IIM was reported (164).

In patients seropositive for anti-cN1A, an auto-antibody commonly associated with IBM, colocalization of the cN1A auto-antigen with p62/SQSTM1 (an autophagy-related protein considered a pathological hallmark of IBM) in perinuclear regions of myofibers was found (165). Furthermore, *in vitro* and *in vivo* passive immunization models with immunoglobulin G extracted from anti-cN1A–seropositive IBM patient serum samples have been found to exhibit

higher p62/SQSTM1 expression and abundant aggregations in the cytoplasm of supplemented rhabdomyosarcoma cells. Likewise, anti-cN1A–positive IBM immunoglobulin G–injected mice showed p62/SQSTM1 aggregates in myofibers (165). Concerning the involvement of ER stress as a potent inducer of autophagy, it has been hypothesized that other MSAs, such as anti-SRP, in IMNM could alter the function of SRP in the proper elongation of polypeptide chains, the induction of ER stress, and chaperone-assisted selective autophagy of defective polypeptides (154). Interestingly, both anti-SRP and anti-HMGCR auto-antibodies might have shown a pathogenic role *in vitro* because they are involved in muscle fiber atrophy associated with the increase of IL-6, TNF, and ROS as well as impaired muscle regeneration by a defect of myoblast fusion due to decreased levels of IL-4 and IL-13 (166).

Inflammatory mediators such as cytokines have been reported in the muscles of IBM patients, with a correlation between the messenger RNA expression of IL-1β and amyloid precursor protein, a protein frequently observed in rimmed vacuoles associated with the IBM phenotype. Upregulation of amyloid precursor protein and β-amyloid expression in skeletal muscle after IL-1β and IFN-γ stimulation, as well as co-localization of IL-1β and β-amyloid, has also been reported (167). Additionally, IFN-γ induces ubiquitylated inclusions in a manner independent of cell type in mouse and human cells (168). Considering that viral infections, especially by human immunodeficiency virus or human T lymphotropic virus 1, are related to IBM immunopathogenesis (169), we could infer that they facilitate an autoimmune process because of the secretion of cytokines such as IFN-γ, which promotes, in turn, the MHC-I upregulation and ubiquitylated inclusions that finally trigger ER stress responses (Figure 1D).

It is interesting that protein aggregates have also been reported in other autoimmune diseases, e.g., Sjögren's syndrome, which has also been associated with the presence of the auto-antibody anti-cN1A in up to 36% of patients (27, 170). In addition to all these mechanisms, the presence of auto-antibodies can also probably cause ER stress because in patients with lupus nephritis, the anti–double-stranded DNA antibodies (anti-dsDNA) bound to a human mesangial cell induce ER stress and activation of NF-κB *via* PERK/eIF2α/ATF4 (171). Finally, it is important to recall that MSAs have a pathogenic role or are an epiphenomenon in IIM (172).

8 Conclusions and perspectives

Taking into account the importance of the ER for the maintenance of homeostasis of the musculoskeletal system in the regulation of proteins, there is probably a relationship between immunological, non-immunological, and infectious pathophysiological processes for the activation of ER stress and autoimmunity. An example of this might be IIMs, and, although this process is not fully understood, there are indications of the participation of cells of the immune system, auto-antibodies, viral processes, involvement of MHC-I, and cytokine signaling pathways in the activation of ER stress. However, ER stress responses have also been observed in other autoimmune diseases, as discussed above. Another special issue, which was not mentioned in this review and is important to consider in future studies, is the design of possible targeted therapies to attenuate, modulate, or eliminate ER stress in addition to the classical therapies used in IIM. Finally, it is possible to suggest that ER stress is related to the origin of autoimmune diseases and their possible consequences on auto-antibody production in IIM.

Author contributions

Conceived and designed the idea: EC-S, EM-G, AL-B and MV-M. Conducted the bibliographic search: EC-S, EM-G, AL-B, OP-M, IG-D, EC-A, AA-V, BM-M, KA-A, JA-B, FP-V, GT-G and MV-M. Analysis and discussion of the information: EC-S, EM-G, AL-B, OP-M, IG-D, EC-A, AA-V, BM-M, KA-A, JA-B, FP-V, IG-T, AL-G, BP-Z, GT-G and MV-M. Wrote the paper: EC-S, EM-G, AL-B, OP-M, EC-A, AA-V, and MV-M. Figure editing: EM-G, and AL-B. All authors contributed to the article and approved the submitted version.

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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frontiers | Frontiers in Immunology

TYPE Brief Research Report
PUBLISHED 20 September 2022
DOI 10.3389/fimmu.2022.926087

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Autoimmune and Autoinflammatory Disorders, a section of the journal Frontiers in Immunology

RECEIVED 22 April 2022
ACCEPTED 24 August 2022
PUBLISHED 20 September 2022

CITATION
Zheng W, Fan X, Yang Z, Shangguan Y, Jin T, Liu Y, Huang J, Ye X, Zhou Q and Li X (2022) Strong inflammatory signatures in the neutrophils of PAMI syndrome.
Front. Immunol. 13:926087.
doi: 10.3389/fimmu.2022.926087

Strong inflammatory signatures in the neutrophils of PAMI syndrome

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PSTPIP1 (proline-serine-threonine phosphatase-interactive protein 1)–associated myeloid-related proteinemia inflammatory (PAMI) syndrome is a rare autoinflammatory disease caused by heterozygous gain-of-function mutation in PSTPIP1. As one of the PSTPIP1-associated inflammatory diseases (PAIDs), neutropenia is a distinct manifestation to separate PAMI syndrome from other PAIDs. This study aimed to investigate the potential role of neutrophils and inflammatory signatures in the pathogenesis of PAMI. PAMI neutrophils displayed markedly increased production of interleukin-1β (IL-1β) and IL-18 by enzyme linked immunosorbent assay (ELISA) assay and intracellular cytokine staining. ASC speck formation and lactic dehydrogenase (LDH) release are also increased in patient neutrophils suggesting elevated pyrin inflammasome activation followed by upregulated cell death in PAMI neutrophils. RNA sequencing result showed strong inflammatory signals in both nuclear-factor kappa B (NF-κB) pathway and interferon (IFN) pathway in patient neutrophils. This study highlighted that elevated proinflammatory cytokines IL-1β and IL-18, increased pyrin inflammasome activation, and upregulation of NF-κB and IFN signaling pathways in neutrophils play important roles in pathogenicity of PAMI syndrome.

KEYWORDS

inflammation, neutrophil, PAMI syndrome, PSTPIP1, pyrin inflammasome

Introduction

PSTPIP1 (proline-serine-threonine phosphatase-interactive protein 1)–associated myeloid-related proteinemia inflammatory (PAMI) syndrome is a rare disease combined autoinflammatory and immunodeficiency. It is caused by heterozygous mutation p.E250K or p.E257K in PSTPIP1 (1). PAMI syndrome shares three main

clinical features with PAPA (pyogenic arthritis, pyoderma gangrenosum and acne) syndrome (1). Neutropenia, hepatosplenomegaly, high myeloid-related protein 8 (MRP8) and MRP12 concentration are distinct manifestation to separate PAMI syndrome from PAPA syndrome, which is also caused by PSTPIP1 mutation (1).

PSTPIP1 is a cytoskeleton-associated adaptor protein involved in regulation of the actin cytoskeleton. It could bind with pyrin and promote the interaction of pyrin and apoptosis-associated speck-like protein containing a CARD (ASC) to facilitate pyrin inflammasome formation (2, 3). Compared with p.E250Q, the most common mutation in PAPA syndrome, p.E250K or p.E257K mutation of PSTPIP1, shows increased interaction with pyrin due to charge reversal in the y-domain (1). As a consequence, pyrin inflammasome was assembled to activate caspase-1 and process proinflammatory cytokines pro–interleukin-1β (IL-1β) and pro–IL-18 into mature IL-1β and IL-18 (4–6).

IL-18 is expressed by epithelia cells and macrophages. It has an endogenous antagonist called IL-18 binding protein that is induced by IFN-γ. Although serum IL-18 elevation has been previously reported associated with macrophage activation syndrome (MAS) in systemic juvenile idiopathic arthritis, adult-onset Still's disease, and NLR family CARD domain containing 4 (NLRC4) inflammasomopathy autoinflammation (7, 8), recent study revealed that PAPA syndrome is associated with chronic and unopposed elevation of serum IL-18 levels without risk of MAS (9). Studies have shown that neutrophils play a potential pathogenic role in PAPA syndrome. Enhanced neutrophil extracellular trap formation has been detected in neutrophils, low-density granulocytes, and skin biopsies of patients with PAPA (10), which was related to the elevated inflammatory cytokines. Although caused by mutations in the same gene, PAMI syndrome is a more refractory autoinflammatory disease compared with PAPA syndrome, and the disease mechanisms of PAMI syndrome are still poorly understood. This study aimed to investigate the potential role of neutrophils and inflammatory signatures in the pathogenesis of PAMI syndrome.

Materials and methods

Patient and sample

Patient and patient control (P2) were recruited under protocols approved by the Institutional Review Board and the Medical Ethics Committee of The Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University (number 2021-K-349-02). The parents of the patients provided written informed consent. Patient control (P2) was a 9-year-old girl who presented with anemia at the age of 3 years and then developed with skin ulceration, pancytopenia, and splenomegaly. Whole exome sequencing (WES) revealed a *de novo* heterozygous c.748G>A, p.E250K, pathogenic variant in the PSTPIP1 gene, so PAMI was diagnosed. Her condition was partially controlled with steroid and cyclosporine. All of the following laboratory investigations in neutrophils were performed after immunosuppressive therapy.

Whole exome sequencing

DNA from whole blood was extracted using the Maxwell RSC Whole Blood DNA Kit (Promega, AS1520). One microgram of DNA was used for WES. WES and data analysis were performed as previously described (11–13). The identified variant was then confirmed by Sanger sequencing.

Cell preparation

Peripheral blood mononuclear cell (PBMCs) and neutrophils were separated by lymphocyte separation medium (LSM) and dextran according to the manufacturer's instructions, respectively. PBMCs and neutrophils were grown in RPMI-1640 (Gibco) supplemented with 10% fatal bovine serun (FBS) and penicillin/streptomycin.

Intracellular cytokine staining

Intracellular cytokine staining for IL-1β was measured in neutrophils at baseline. Cells were washed twice with phosphate belanced solution (PBS), then treated with Golgi plug (BD Biosciences) for 6 h at 37°C, with 5% CO_2, and then permeabilized with Perm/Fix for 30 min at 4°C. Cells were stained by antibodies IL-1β (BioLegend, cat. no. 508208). All events were acquired on BD LSRFortessa (BD Biosciences) and analyzed by FlowJo (TreeStar).

ASC speck formation

Neutrophils (5×10^4) of patient were seeded on each well of 24-well plate with one poly-L-lysine–coated 12-mm glass coverslip (Shanghai Jing An Biology, J24002). The plate was incubated in a humidified incubator (37°C, 5% CO_2) for 3 h. The coverslips were fixed with 500 µl of 4% Paraformaldehyde (PFA) (15 min, 37°C) and rinsed three times with 1× PBS. The coverslips were blocked with blocking buffer (1× PBS/5% normal serum/0.3% Triton™ X-100) for 60 min. The coverslips were incubated with the diluted primary antibody [ASC/TMS1 (E1E3I) rabbit mAb, #13833] overnight at 4°C and then were rinsed with the 1 × PBS. The coverslips were incubated with diluted fluorochrome-conjugated secondary antibody for 1–2 h at room

temperature in dark and rinsed with 1× PBS. Nuclei were stained with 4,6-diamino-2-phenyl indole (DAPI) for 10 min. The coverslips were visualized using zeiss LSM 710 laser scanning confocal microscope, and images were acquired using ZEN-Blue. Fiji-ImageJ software was used to analyze the images. The percentage of ASC-specks containing cells was calculated as the fraction of ASC-positive specks containing specks.

Cytokine measurement and LDH detection

Cytokine concentrations for IL-1β and IL-18 in serum were measured by Human IL-1β/IL-1F2 Duoset ELISA (R&D, DY201) and Human Total IL-18 Duoset ELISA (R&D, DY318) according to the manufacturer's instructions. LDH released in supernatant was measured with the LDH Cytotoxicity Assay Kit (Beyotime, C0017) according to the manufacturer's instructions. The concentrations of IL-6 in serum were measured by the BD Cytometric Bead Array (BD FACSCanto) according to manufacturer's instructions (P010001-111). Data were analyzed by FCAP (3.0.1) software (BD FACSCanto).

RNA sequencing

RNA libraries were generated using the NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs) and then sequenced on Illumina NovaSeq to get 150–base pair paired-end reads. featureCounts was used to count the reads numbers mapped to each gene. Differential expression analysis was performed using the DESeq2 R package.

Results

A 6-year-old female patient born to Chinese healthy parents presented with mild to moderate anemia (HGB, 80–100 g/L) from the age of 6 months. At 1 year old, she presented with swelling and pain of the left ankle, fever, and hepatosplenomegaly accompanied with notable increase in inflammatory indexes. Joint incision and drainage was done as pyogenic arthritis in local hospital. Then, she developed hypotension and pancytopenia after operation. Serum ferritin was high at 1,634 ng/ml. Laboratory investigation showed elevation of liver enzymes (alanine transaminase, 151 U/L; aspartate aminotransferase, 312 U/L), hypertriglyceridemia (2.2 6 mmol/L), and hypofibrinogenemia (0.85 g/L). A small number of phagocytes were found in bone marrow cytology, and bone marrow biopsy revealed myelofibrosis. She was diagnosed with septic shock and treated with imipenem, vancomycin, and high-dose dexamethasone (about 1.1 mg/kg/day). Her condition was relieved, and dexamethasone and antibiotic were withdrawn in 3 weeks. At the age of 4 years, she was hospitalized because of fever and swelling and pain of her right elbow and left knee joint. Joint fluid routine revealed white blood cell count (WBC; 100–150 $\times 10^9$/L) with > 90% neutrophils but negative gram stain and culture. Joint ultrasound showed thickened synovium and joint effusion of elbow and knee joint. Magnetic resonance imaging (MRI) of the elbow and knee indicated diffuse bone marrow edema, thickened synovium, and massive joint effusion (Figure 1A). Laboratory testing showed elevated WBC (10.44×10^9/L), absolute neutrophil count (ANC; 3.8×10^9/L), platelet count (690×10^9/L), decreased hemoglobin (69 g/L), as well as increased acute phase reactants such as serum amyloid A (SAA; 152 mg/L), C-reactive protein (CRP; 173 mg/L), and erythrocyte sedimentation rate (ESR, 64 mm/h). Ibuprofen was initiated with the dose of 28.6 mg/kg/day. One week later, ESR and CRP decreased to 38 mm/h and 31 mg/L, respectively. However, leukopenia and neutropenia (WBC, 2.88×10^9/L; ANC, 0.67×10^9/L) were observed while the inflammatory episode was partially controlled.

Considering the recurrence of the disease, autoinflammatory disease was suspected. Prednisone tablets (about 0.71 mg/kg/day) combined with IL-6 inhibitor tocilizumab of 160 mg (11.4 mg/kg) every 4 weeks were initiated, but ESR, CRP, and SAA were not controlled well. Then recombinant human tumor necrosis factor–a (TNF-a) receptor fusion protein etanercept (0.8 mg/kg every week), cyclosporine (3 mg/kg/day) combined with methotrexate (9.8 mg/m2)were initiated, and the disease was controlled efficiently. ESR, CRP, and SAA gradually returned to normal, and hepatosplenomegaly gradually subsided. Reexamination of the left knee and right elbow joint MRI indicated that the effusion in the articular cavity was obviously reduced and synovitis was significantly improved (Figure 1A). Without arthritis and fever, the remission state had been maintained for a year until now, and the amount of prednisone has been reduced to 5 mg daily (about 0.26 mg/kg/day). No pyoderma gangrenosum and acne were found during follow-up. Neutropenia persists despite being inactive status (minimum, 0.61×10^9/L), but no recurrent infections were observed. The genotype was a de novo mutation of p.E250K (c.748G>A) in the PSTPIP1 gene and was also validated by Sanger sequencing (Figures 1B–D), so the diagnosis of PAMI syndrome was confirmed.

Neutropenia is a common symptom in PAMI syndrome, and our patient showed low ANC as well. The reduction of neutrophil happened after inflammation, but the relationship between inflammation and neutropenia is still unclear in patients with PAMI syndrome. We first measured ASC speck formation, the hallmark of pyrin inflammasome activation (14, 15), in the patient neutrophils. We observed higher ASC speck compared with healthy controls (Figures 2A, B); the similar level of increased ASC specks was also confirmed in the second patient with PAMI syndrome (P2), which indicated increased pyrin inflammasome assembly and activation in neutrophils of

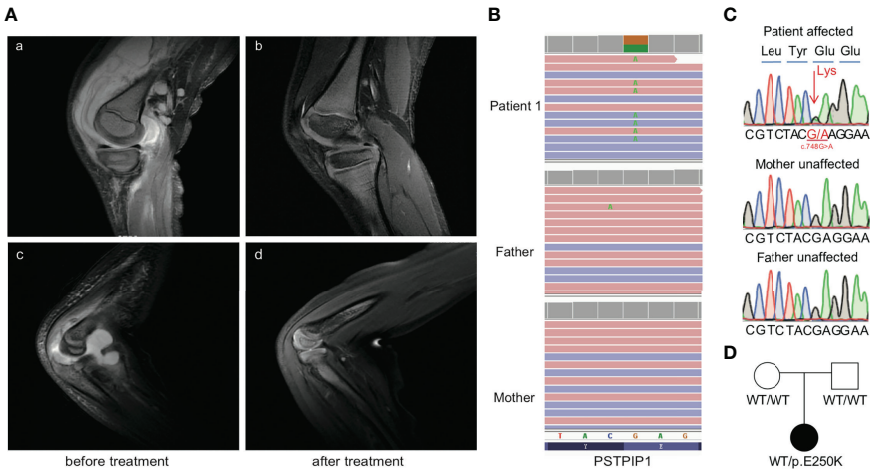

FIGURE 1
Clinical manifestation of pre- and post-treatment and confirmation of the PSTPIP1 mutation in the patient with PAMI. **(A)** T2WI MRI signals of knee and elbow joints pretreatment **(A, C)** and post-treatment **(B, D)**. **(B)** Exome sequencing reads covering the p.E250K variant in patient, displayed by the integrative genomics viewer. **(C)** Sanger sequencing confirmed the PSTPIP1 p.E250K variant. **(D)** Pedigree of the family with p.E250K variant in PSTPIP1.

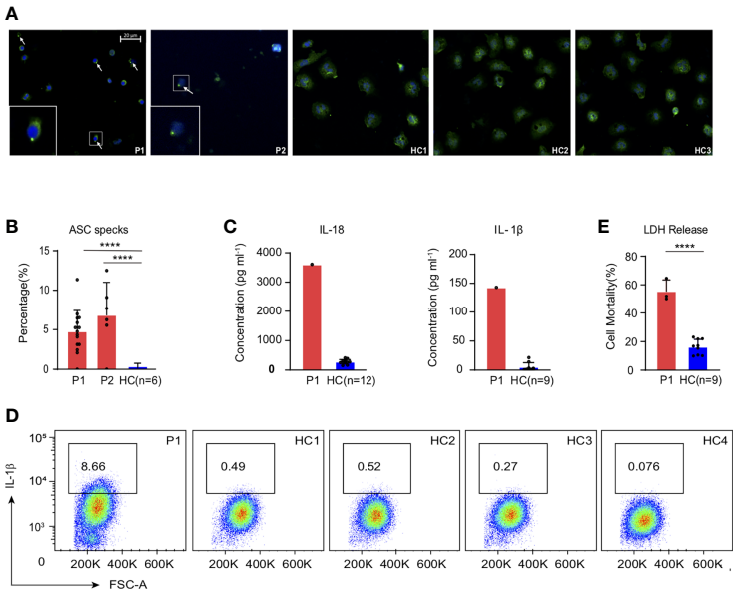

FIGURE 2
Strong inflammatory signatures in the patient with PAMI compared with healthy controls. **(A, B)** Increased ASC speck formation in the neutrophils of the patients (P1 and P2) compared with six healthy controls. **(C)** Excess levels of IL-18 and IL-1β in the serum of the patient1 compared with 12 and 9 healthy controls, respectively. **(D)** Higher IL-1β level in the neutrophils of the patient1 compared with four healthy controls. **(E)** LDH release in the neutrophils of the patient1 compared with three healthy controls. ****p<0.0001.

the patients with PAMI. Then, we measured IL-1β and IL-18 concentration released in serum by ELISA assay, and the patient showed significant increased cytokines compared with healthy controls (Figure 2C). In addition, the results were consistent with the excess serum IL-18 observed in patients with PAPA (9), and it also established the association of IL-18 with PAMI syndrome. To confirm whether neutrophils contributed the cytokine release in serum, we analyzed the IL-1β production in neutrophils with intracellular cytokine staining. We detected elevated level of IL-1β in patient neutrophils at basal level compared with four healthy controls (Figure 2D). The excess production of IL-1β in neutrophils of the patient with PAMI further confirmed that pyrin inflammasome was over activated to promote downstream inflammatory cascade reaction in the patient.

Moreover, the activation of pyrin is one of the prerequisites for triggering pyroptotic cell death (pyroptosis) (16, 17). Activated caspase-1 can specifically cleave Gasdermin D

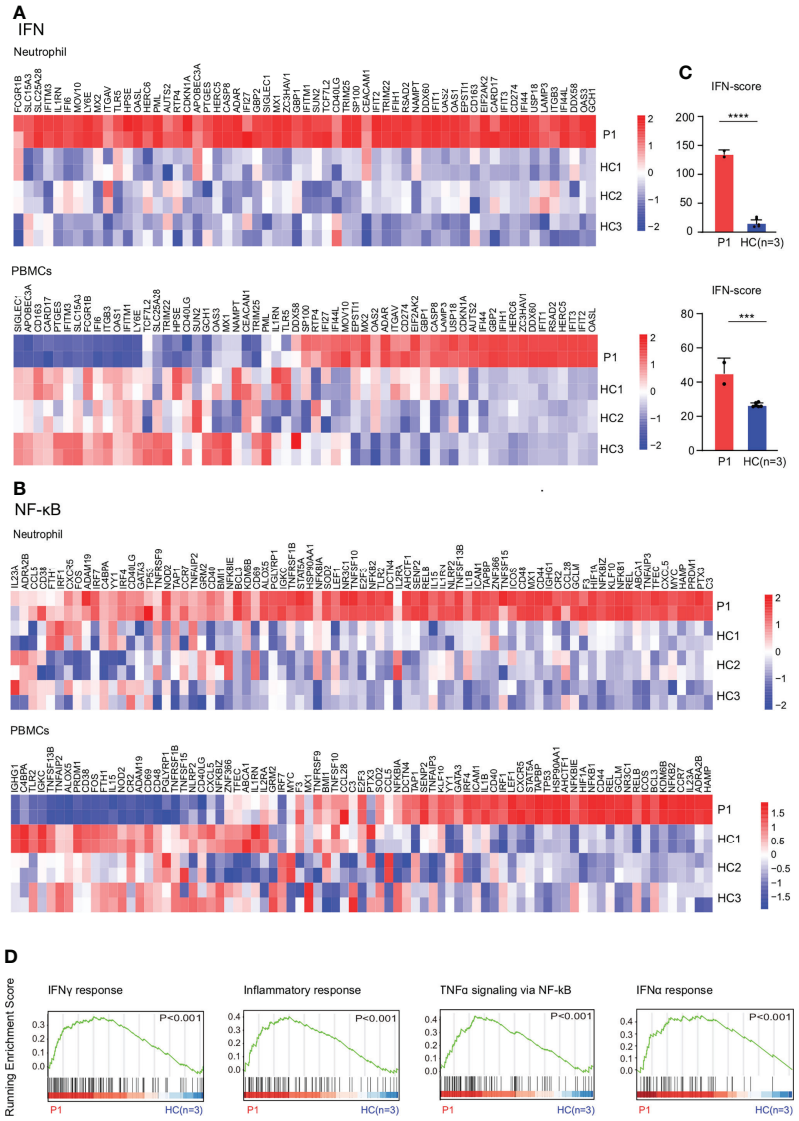

FIGURE 3
Expression patterns of genes involved in IFN and NF-κB by RNA sequencing. **(A)** Upregulation of IFN pathway in neutrophils and PBMCs of the patient1 compared with three healthy controls. **(B)** Upregulation of NF-κB pathway in neutrophils and PBMCs of the patient1 compared with three healthy controls. **(C)** IFN response gene scores (28 genes) in neutrophils and PBMCs of the patient1 and healthy controls, determined with RNA sequencing data. **(D)** GSEA plot of IFN-γ response, inflammatory response, tumor necrosis factor−α (TNF-α) signaling *via* NF-κB, and IFNα response in neutrophils from the patient1 and controls. ****p<0.0001. ***p<0.001.

(GSDMD). The N-terminal of GSDMD was released to form pores in cell membrane for cell contents release and cell rupture and leads to inflammation (18–20). We measured LDH release, a cytoplasmic enzyme released by dying cells that lose cell membrane integrity (21), and increased LDH protein could be detected in culture supernatant of patient neutrophils at the basal level (Figure 2E). The results indicated increased cell death in the neutrophils of the patient with PAMI promoted by pyrin inflammasome activation.

RNA sequencing results showed upregulation of NF-κB and IFN signaling pathways in the patient's neutrophils and PBMCs compared with healthy controls (Figures 3A, B). The results were confirmed by higher IFN scores in the patient with PAMI compared with healthy controls (Figure 3C). In addition, the inflammatory gene expression is more elevated in neutrophil than in PBMCs, and the IFN score is also higher in neutrophil than in PBMCs in the patient. Therefore, the inflammatory signature is higher in neutrophils compared with PBMCs in the patient with PAMI (Figures 3A–C). The Gene Set Enrichment Analysis (GSEA) plot showed higher IFN-γ pathway, inflammatory response, TNF-α signaling *via* NF-κB, and IFNα pathway in patient neutrophils than controls (Figure 3D). Consistent with NF-κB pathway activation, the patient showed elevated IL-6 level of 120.5 pg/ml (normal range, 1.7–16 pg/ml) in serum.

Discussion

The main hematologic manifestation in PAMI syndrome is neutropenia, but pancytopenia is also observed in a few cases in acute active phase just like our patient in this study (22, 23). In our patient, serum ferritin was high accompanied with hypertriglyceridemia and hypofibrinogenemia, so it fulfilled the criteria of MAS but misdiagnosed as septic shock at that time. MAS has been reported in the previous literature (24), and we speculate that the operation may be the stimulating factor of MAS in our patient, because the recurrence of MAS was not observed in the subsequent acute active phase. Bone marrow biopsy in this case showed myelofibrosis. Although myelofibrosis was not a common finding and the specific pathogenesis is not clear, it had also been reported in other patients with PAMI (25, 26).

Our study showed that the patient with PAMI exhibited strong inflammatory signals in neutrophils including over production of proinflammatory cytokines IL-1β and IL-18, hyperactivation of pyrin inflammasome, and excess cell death and upregulation of NF-κB and IFN signaling pathways, which resulted in severe inflammation and may contribute to develop neutropenia in patients with PAMI syndrome.

Patients with PAMI syndrome were rarer than PAPA syndrome, and some cases remain refractory to treatment, suggesting that the disease is more difficult to be controlled. hematopoietic stem cell transplant (HSCT) has been proved as effective treatment for patients with PAMI syndrome (24). According to previous reports, IL-1 inhibition did not resolve neutropenia in PAMI syndrome (1, 25), whereas it was effectively used for pyrin inflammasome–associated inflammation control in other autoinflammatory disorders (27, 28). Targeting IL-1 combination with IL-18 inhibition might be helpful for disorders with overproduction of both IL-1β and IL-18. Our study identified hyperactivation of inflammatory signals in both NF-κB and IFN pathways in the patient. In addition, the patient responded well to TNF inhibitor, etanercept, and cyclosporine combined with steroid and methotrexate, suggesting that targeting NF-κB signaling pathway could also help to suppress the inflammation in PAMI syndrome. Nevertheless, this study has certain limitations. First, immunosuppressive therapy implemented in the patient could influence the results. Second, more patients' samples are needed to provide a comprehensive analysis of the inflammatory signature of PAMI syndrome, including a comparison of inflammatory patterns of neutrophils with macrophages or monocytes of PAMI syndrome and with neutrophils of PAPA syndrome. The inflammatory signatures in neutrophils revealed in the patient with PAMI in our study provided insights for better understanding of the disease mechanisms.

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/s.

Ethics statement

The studies involving human participants were reviewed and approved by The Institutional Review Board and the Medical Ethics Committee of Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University (number 2021-K-349-02). The patients/participants provided their written informed consent to participate in this study.

Author contributions

QZ and XL designed the study and directed and supervised the research. WZ, ZY, TJ, and XF performed experiments and analyzed the data. WZ, YS, YL, JH, and XY enrolled the patients and collected and interpreted clinical information. WZ, XF, TJ, QZ, and XL wrote the manuscript with input from other authors. All authors contributed to the review and approval of the manuscript.

Funding

This work was supported by the National Natural Science Foundation of China (NSFC81370787, NSFC31771548, and NSFC 81971528), Zhejiang Provincial Natural Science Foundation of China (LR19H100001), Zhejiang Provincial Health Science and Technology Project (2022KY904), Suzhou Key Discipline of Pediatric Immunization (szxk 202106), and Suzhou Civil Biotechnology Key Technology Project (ss02067).

Acknowledgments

We thank the patient and healthy controls for support and help.

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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frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 30 September 2022
DOI 10.3389/fimmu.2022.1012868

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Autoimmune and Autoinflammatory Disorders : Autoimmune Disorders, a section of the journal Frontiers in Immunology

RECEIVED 06 August 2022
ACCEPTED 20 September 2022
PUBLISHED 30 September 2022

CITATION
Shuai Z, Zheng S, Wang K, Wang J, Leung PSC and Xu B (2022) Reestablish immune tolerance in rheumatoid arthritis.
Front. Immunol. 13:1012868.
doi: 10.3389/fimmu.2022.1012868

Reestablish immune tolerance in rheumatoid arthritis

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Rheumatoid arthritis (RA) is a chronic progressive autoimmune disease. Despite the wide use of conventional synthetic, targeted and biologic disease modifying anti-rheumatic drugs (DMARDs) to control its radiological progress, nearly all DMARDs are immunologically non-selective and do not address the underlying immunological mechanisms of RA. Patients with RA often need to take various DMARDs long-term or even lifelong and thus, face increased risks of infection, tumor and other adverse reactions. It is logical to modulate the immune disorders and restore immune balance in patients with RA by restoring immune tolerance. Indeed, approaches based on stem cell transplantation, tolerogenic dendritic cells (tolDCs), and antigen-based tolerogenic vaccination are under active investigation, and some have already transformed from wet bench research to clinical investigation during the last decade. Among them, clinical trials on stem cell therapy, especially mesenchymal stem cells (MSCs) transplantation are most investigated and followed by tolDCs in RA patients. On the other hand, despite active laboratory investigations on the use of RA-specific peptide-/protein-based tolerogenic vaccines for T cell, clinical studies on RA patients are much limited. Overall, the preliminary results of these clinical studies are promising and encouraging, demonstrating their safety and effectiveness in the rebalancing of T cell subsets; particular, the recovery of RA-specific Treg with increasing anti-inflammatory cytokines and reduced proinflammatory cytokines. Future studies should focus on the optimization of transplanted stem cells, the preparation of tolDCs, and tolerogenic vaccines with RA-specific protein or peptide, including their dosage, course, and route of administration with well-coordinated multi-center randomized clinical control researches. With the progress of experimental and clinical studies, generating and restoring RA-specific immune tolerance may bring revolutionary changes to the clinical management of RA in the near future.

KEYWORDS

rheumatoid arthritis, immune tolerance, stem cell transplantation, immune tolerogenic dendritic cells, immune tolerogenic vaccination, treatment

Introduction

Rheumatoid arthritis (RA) is an autoimmune chronic disease, primarily characterized by synovial inflammation (synovitis), which further leads to cartilage damage and bone erosion. Extra-articular damages caused by systemic inflammation are very common in RA. If untreated properly, chronic RA can lead to disability and extra-articular multiple systemic damages, some of which may be even life-threatening (1, 2). From the 1890s, when the first nonsteroidal anti-inflammatory drug (NSAID) Aspirin was chemically synthesized and used for RA treatment and glucocorticoids (GCs) was applicated in RA therapy in the 1940s, the goal of RA treatment was directed at symptomatic relief (3, 4). In the 1980s, methotrexate (MTX), the first disease modifying anti-rheumatic drug (DMARD), was approved for RA therapy; however, NSAIDs and GCs could not effectively prevent the radiologic progression of RA (5, 6). With an increasing understanding of RA pathogenesis, the treatment methods for RA and their effects have been much diversified and improved. So far, DMARDs have developed from conventional synthetic DMARDs (csDMARDs) to biological DMARDs (bDMARDs) and targeted synthetic DMARDs (tsDMARDs) with rapid effect and high efficiency (1, 7). Nevertheless, all existing DMARDs cannot overcome the adaptive immune disorders underlying RA. In order to maintain the disease stability and delay disease progress, RA patients have to endure various adverse reactions from DMARDs lifelong while taking a variety of DMARDs (8, 9). Therefore, it is crucial to explore novel treatment aiming to alleviate immune disorders and restore immune balance in RA patients. Here, we will briefly describe present clinical approaches in RA treatment and further discuss the current research progress on reestablishing immune tolerance in RA.

Conventional therapeutical approaches

Agents treating RA have been increasing and varying, ranging from NSAIDs and GC that mainly alleviate symptoms and inflammation reactions, to DMARDs that can effectively retard and even stop the destruction of joint structure (Figure 1). Their characteristic efficacy and various adverse reactions in RA treatment are outlined below.

Non-steroidal anti-inflammatory drugs and glucocorticoids

NSAIDs are the oldest anti-rheumatic agent that has been used for over 100 years, and are still widely used in the treatment of rheumatism due to their analgesic and anti-inflammatory effects (3). The principal mechanism shared by nearly all NSAIDs is to suppress inflammation in RA by inhibiting the activity of cyclooxygenase (COX) to produce prostaglandins, the common inflammatory mediators. However, it has been verified that all NSAIDs cannot affect disease progression (3). Compared with NSAIDs, GCs are more potent in reducing inflammation and arthritis symptoms by inhibiting the transcription of inflammatory genes, reducing production of cell adhesion molecules, and decreasing the key inflammatory mediators (10). Although it was regarded as a breakthrough in RA treatment when firstly used in the 1940s (4), long-term use of GCs may lead to serious multisystem metabolic side effects and increase the risk of infection (4). Therefore, GCs are often used in combination with DMARDs to relieve RA symptoms under the principle of lower dose and short treatment course (11).

Disease-modifying anti-rheumatic drugs

Existing DMARDs are mainly composed of synthetic DMARDs (sDMARDs) and biological DMARDs (bDMARDs). sDAMRDs can be divided into csDMARDs and tsDMARDs. All DMARDs are able to delay/modify disease progression and improve the prognosis of RA, just as the abbreviated name DMARDs implies (1, 7). Commonly used representative csDMARDs include methotrexate, leflunomide and hydroxychloroquine. They exert their immunosuppressive and regulatory effects through their respective non-target selective pathways (12, 13). bDMARDs approved for RA include four different modes of action: tumor necrosis factor α (TNF-α) inhibition, inhibitors of co-stimulation, interleukin-6 (IL-6) receptor inhibition, and the depletion of B cells (12, 14–16). While benefiting patients, DMARDs inevitably have toxic and side effects such as hair loss, stomatitis, nausea and hepatotoxic anaphylaxis, thrombocytopenia, ocular toxicity and even autoimmune diseases (9, 17–20). RA patients receiving prolonged DMARDs are susceptible to severe infections and malignancies because of the non-selective immunosuppression of DMARDs (21, 22). In addition, both tsDMARDs and bDMARDs are costly (23). Hence, many RA patients cannot adhere to or even have to terminate DMARDs treatment.

Logically, immunomodulatory therapy to restore tolerance is the ideal way to cure RA. Innovative immunotherapy approaches using stem cells, tolerogenic dendritic cells (tolDCs) and immune tolerant vaccines induced by special peptide have attracted attentions. Results from preliminary studies have been encouraging. Here, they are summarized below.

Immunotherapy approaches to reestablish immune tolerance in RA

The loss of immune tolerance to autoantigens is the culprit of autoimmune diseases including RA. Regulatory T cells (Tregs) play a pivotal role in maintaining self-tolerance while the

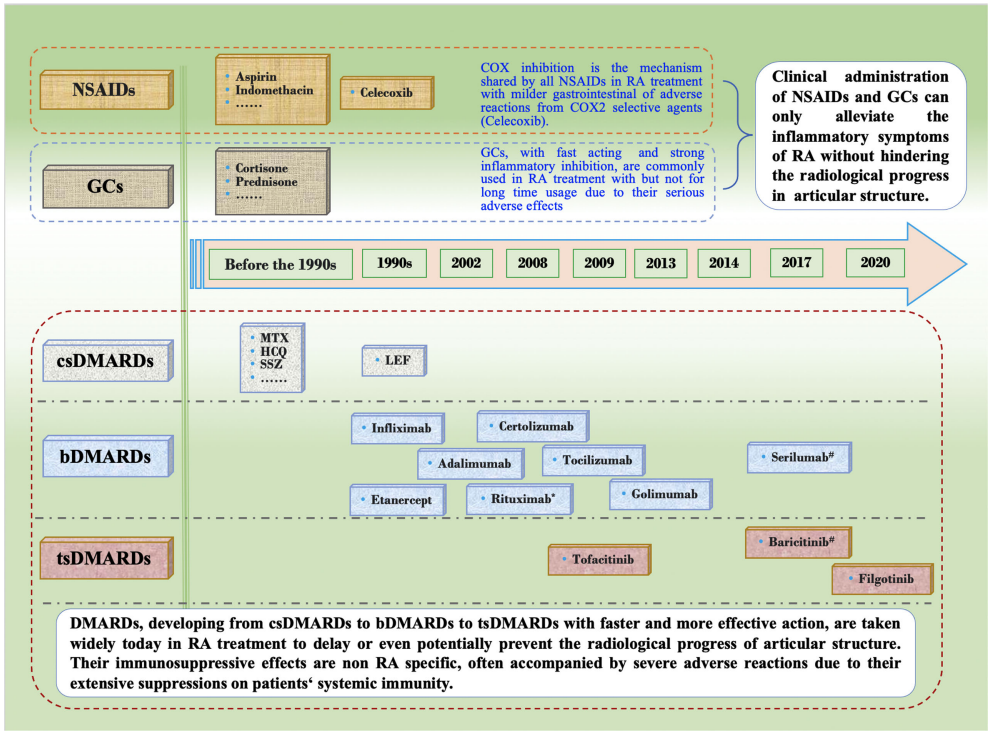

FIGURE 1
Development of Conventional Agents in the Treatment of Rheumatoid Arthritis. NSAIDs and GCs have stagnated for more than 20 years mainly due to their mere ability to alleviating RA inflammatory symptoms without interfering with the progress of articular erosion. Since DMARDs was proven to be able to slow down the erosive progress, they have been widely used in RA treatment as essential drugs, and have developed from csDMARDs to bDMARDs to tsDMARDs. Choice of csDMARDs, also known as slow acting antirheumatic drugs with non-targeted immunosuppressive effect, have remained the same for the last two deacdes. hbDMARDs, composed of recombinant monoclonal antibodies and receptors of proinflammatory cytokines, were rigorously developed in about the 2000s. tsDMARDs, targeting intracellular various signaling pathways, are becoming widely used in the recent 10 years due to its fast onset, high effect and more convenient oral medication. However, the immunosuppressive effects of all conventional medications above are not RA specific, and therefore cannot correct the autoimmune disorder to cure RA while they have the risk of serious adverse reactions after long-term use. Restoring the disordered autoimmune balance by reconstruction of RA specific immune tolerance is an ideal approach to cure RA. *, approved firstly by Food and Drug Administration (FDA) of USA for refractory RA treatment in 2006. #, approved by European Union but not FDA for RA treatment. NSAIDs, non-steroidal anti-inflammatory drugs; GCs, glucocorticoids; DMARDs, disease modifying anti-rheumatic drugs; csDMARDs, conventional synthetic DMARDs; bDMARDs, biological DMARDs; tsDMARDs, target synthetic DMARDs; COX, cyclooxygenase; RA, rheumatoid arthritis; MTX, methotrexate; HCQ, hydroxychloroquine; SSZ, sulfasalazine; LEF, leflunomide.

imbalance between Treg/Th17 and Th1/Th2 cells influence the development of RA (24, 25). In several pre-clinical models of autoimmune diseases, such as RA, experimental autoimmune encephalomyelitis (EAE), and anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (26), the protective role of antigen- specific Tregs was clearly documented. Thus, it is promising to specifically intervene chronic autoimmune disorders *via* restoring the balance of T cell subgroups by enhancing Tregs function (27). For instance, stem cell transplantation has been suggested to be a potential effective treatment for RA for reconstruction of the immune tolerance (24). In addition, $CD4^+Foxp3^+$Tregs and dendritic cells (DCs), are master regulators and major antigen presenting cells, respectively, and are thus key players in maintaining immune tolerance (28). There are numerous tolerogenic vaccine platforms which have been developed to deliver autoantigens to specific antigen presenting cell subtypes, including protein/peptide, nanoparticle, and DNA/RNA-based vaccines (29). Here, we summarize the recent development in mesenchymal stem cells (MSCs), tolDCs, and protein- or peptide-based tolerogenic vaccination as treatment for RA.

Stem cell transplantation therapy

Mesenchymal stem cells (MSCs) are pluripotent cells with differentiation potential, which can originate from bone marrow (BM), umbilical cord (UC), adipose tissue (AD), peripheral blood, dental pulp and other tissues. They are capable of self-renewal and can differentiate into chondrocytes, adipocytes,

osteoblasts and other cell types. In particular, they have marked immune regulatory effects and are regarded as a powerful tool for the restoration of immune tolerance in controlling autoimmune diseases (30).

Mechanism of MSCs treatment

MSCs mainly act through cell-cell contact mediated immune regulation and paracrine mediated immune regulation (31) (Figure 2). When MSCs are in the context of proinflammatory cytokines (IL-1β, TNF-α, IFN-γ), their immunosuppressive activity can be enhanced through secretory inhibitory factors, such as TGF-β, prostaglandin (PGE2), indoleamine-2,3-dioxygenase (IDO), or inhibition of effector cells through cell-cell contact and induce the formation of Tregs (32).

Cell to cell contact between MSC and T cells can affect T cell functions in various ways. When MSCs and $CD4^+$T cells are co-cultured, BM-MSCs can inhibit the proliferation of $CD4^+$T cells and increase the ratio of $Foxp3^+$Treg (33). Its ability to promote Treg differentiation depends on IFN-γ level (34). It can also inhibit the secretion of IL-2 and TNF-α by T cells, and restrain its transformation to Th1 cells (33). Using a collagen-induced arthritis (CIA) models in rats, Ma et al. showed that human UC-MSCs (HUCMSCs) therapy could down-regulate RAR-related orphan receptor gamma (RORγt) mRNA and its protein expression, reduce the ratio of Th17 cells, and induce T cell apoptosis. They might up-regulate Foxp3 mRNA and protein expression, and increase Treg cell ratio in the spleen. RORγt and Foxp3 are markers of Th17 and Treg cells, respectively. These findings indicated that the immunomodulation capacity of HUCMSCs could inhibit synovial hyperplasia in CIA rats, with delay in the progression disease, reducing foot swelling and arthritis index (35). Another study found that HUCMSCs had the ability in rats to inhibit the proliferation of spleen T cells and the serum expression of IL-17 and promote serum level of TGF-β (36, 37). Systemic infusion of human AD-MSCs could down-regulate Th1-driven autoimmunity and inflammatory reaction and induce the production of IL-10 in lymph nodes and joints to increase the ratio of $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs (38). A significant increase in the percentage of Tregs was observed one month after MSCs infusion. The increase of Tregs detected by Foxp3 mRNA was parallel to the increase of T-bet and GATA3 transcription factor mRNA levels and the increased levels of IL-10 and TGF-β (39).

BM-MSCs express high levels of toll-like receptor (TLR)-3 and TLR-4, which can determine the phenotypic differentiation of MSCs. Stimulated TLR-4 lead to the production of IL-6, IL-8, and TGF-β and resulted in the development of the pro-inflammatory phenotype of MSC1. Moreover, TLR-3 stimulation of immunosuppressive MSC2 cells can also increase IDO secretion (40). It was found that BM-MSC could

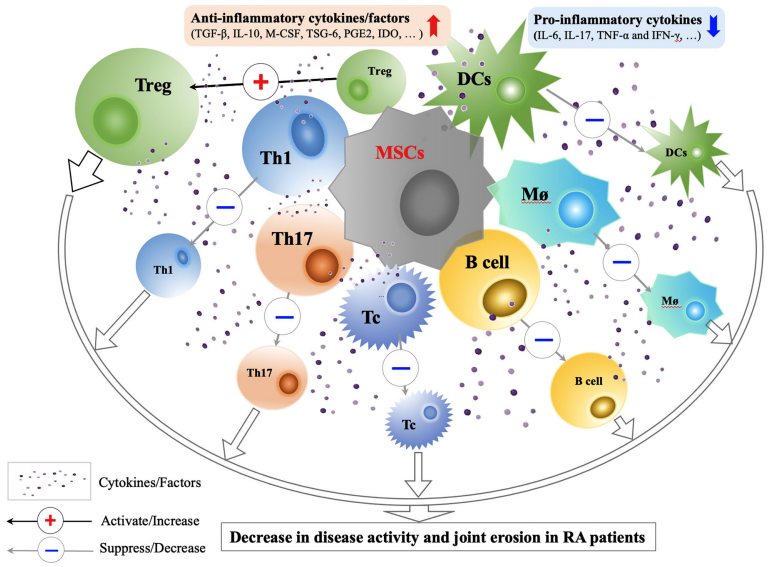

FIGURE 2
Immunological Mechanisms and Effects of MSCs Transplantation in RA Treatment. Through cell-cell contact and secretion of anti-inflammatory factors (TGF-β, IL-10, M-CSF, TSG-6, PGE2, IDO and etc.), MSCs can improve the functions of Tregs, suppress the activity of Th1, Th17, Tc, B cells, Mø and DCs, correct the immune imbalance between Treg and Th17, and restore the immune tolerance in RA. Animal and preliminary clinical studies on efficacy and safety of MSCs transplantation in RA treatment show that the use of MSCs treatment in RA patients are promising. CCP, cyclic citrullinated; CPR, C-reactive protein; DAS, disease activity score; DCs, dendritic cells; IDO, indoleamine-2,3-dioxygenase; INF, interferon; M-CSF, macrophage colony stimulating factor; MSCs, mesenchymal stem cells; Mø; macrophage; PGE2, prostaglandin E2; Tregs, regulatory T cells; Tc, cytotoxic T cell; TGF, transforming growth factor; TNF, tumor necrosis factor; TSG-6, TNF-stimulated gene-6 protein.

inhibit the function and differentiation of B cells and reduce the expression of chemokine receptors during co-culture, including CXCR4, CXCR5, and CCR7 on B cells, thereby inhibiting the chemotaxis of B cells. In the meantime, they could also inhibit the generation of dendritic cells from monocytes (33, 41). Indeed, MSCs therapy resulted in the decrease in levels and proliferation of B cells for at least one year following the MSC treatment in RA patients (42).

With respect to the findings on paracrine effect, the effects of MSCs on Th17/Treg balance have been attributed to various soluble molecules, including IDO, IL-10, PGE2, and nitric oxide (NO) (31, 43, 44). IL-6 and macrophage colony stimulating factor (M-CSF) are produced by MSCs and cause the inhibition of T cell, B lymphocytes, and dendritic cells. This ability of MSCs in releasing anti-inflammatory and anti-apoptotic molecules can effectively protect damaged tissues (38). BM-MSC could inhibit the pro-inflammatory cytokine cells and cytotoxic T cells by modulating anti-inflammatory gene expression, indicating its potential therapeutic effect on molecular level (45). BM-MSC could not only secrete IDO to inhibit T cell proliferation, but could also secret NO synthase to inhibit cell proliferation and to decease the toxicity and secretory function of T cells (33, 41) (Table 1). In addition, Ueyama et al. refuted that the levels of proinflammtory cytokines, such as IL-6, IL-17, TNF-α, and IFN-γ, were found to be decreased after the introduction of AD-MSCs in another experimental arthritis animal model. Their study found that HADMSC treatment could inhibit the function of activated inflammatory cells and macrophages, down-regulated inflammatory cytokines, and up-regulated TNF-stimulated gene-6 protein (TSG-6) and TGF-β1 (46).

Clinical trials of MSCs in RA treatment

In 2010, the first clinical pilot study of MSC in RA therapy was conducted in Korea. Ten patients were enrolled in this study, three of which were RA patient who did not respond to traditional treatment. In this study, isolated and expanded autologous AD-MSCs were used to treat the patients (more than 10^9 cells after 3 to 4 passages), with one patient receiving intra-articular injection. All patients were followed up for 13 months. The results showed that after treatment, there was significant improvement in the clinical status, and the condition of the patient with intra-articular injection greatly improved, from previously needing crutches for walking to being able to stand up and be off steroids. This study demonstrated that MSC therapy is safe and without severe adverse effect in patients with RA (47).

The first randomized multicenter double-blind placebo-controlled phase Ib/IIa clinical trial of allogeneic AD-MSCs for RA (NCT01663116; Table 2) was conducted in Spain in 2011. This study included 53 refractory RA patients with a long history of disease (more than 13 years) and who resistant to at least two biological agents with a DAS28-ESR>3.2. All patients maintained low-dose DMARD, NSAIDs, and/or steroid treatment, but without biologic treatment. The patients were monitored for 6 months. Based on the EULAR criteria, the study had a good response with low DAS28-ESR and C-reactive protein (CRP). According to the authors, the very refractory characteristics of RA patients in the study might have hindered the beneficial effect of MSC treatment. In this study, 19% of RA patients produced MSCs specific anti-human leukocyte antigen I (HLA-I) antibody with no obvious clinical consequences and without anti-HLA-II antibody. This was the first study on the immunogenicity of RA treatment based on allogeneic MSCs (49).

There were two large-scale UC-MSCs clinical trials on RA patients in China. One study (NCT01547091, Table 2) included 172 refractory patients, 64 of which had a follow-up period of 3 years. All patients maintained low-dose csDMARDs treatment. The study reported a significant remission of disease according to the ACR, DAS-28, ESR and the Health Assessment Questionnaire, with decreased levels of CRP, rheumatoid factor (RF), and anti-cyclic citrullinated peptide (anti-CCP) antibodies, as well as the levels of TNF-alpha and IL-6 in sera. The percentage of Tregs in peripheral blood was increased. This study, for the first time, demonstrated the long-term beneficial effect of MSC-based treatment in combination with low dose of

TABLE 1 Immunomodulatory Effects of MSCs on the T cell subsets.

T cell subsets	Specific regulatory changes	Cytokine levels	Effects of MSCs on T cells	Reference
Th1	T-bet↑	IL-2↓, TNF-α↓, IFN-γ↓	Inhibit T cells transformation to Th1 cells and down-regulate Th1-driven autoimmunity and inflammatory reaction. (The level of T-bet can be influenced by the conventional medications taken at the same time)	(33, 38, 39)
Th2	GATA3↑	IL-4↓, IL-10↑	Not clear	(38, 39)
Th17	RORγt↓	IL-17↓, TNF-α↓	Reduce the ratio of Th17 cells and induce T cells apoptosis.	(35–37)
Treg	Foxp3↑	IL-10↑, TGF-β↑	Promote Treg differentiation and increase the ratio of Tregs by cell to cell contact and by secreting IL-10 and TGF- β in the regulation of autoimmune tolerance.	(33, 38, 39)

IDO, indoleamine-2,3-dioxygenase; MSCs, mesenchymal stem cells; RORγt, RAR-related orphan receptor gamma; Tregs, regulatory T cells.

TABLE 2 Summary of Clinical Trials with MSCs in Treating Rheumatoid Arthritis.

Clinical trial identifier	Clinical phase	Status	Source	Registration year	Country	RA patients	MHC context; route of administration	Cells/kg of body weight; number of doses	Estimated/enrolled number of RA patients	Follow-up (Months)	Control group	Ref./ estimated completion date
NCT03333681	I	Completed	BM	2016	Iran	Refractory	Autologous; IV	1 to 2 $\times 10^6$; 1 dose	15	12	No	(48)
NCT01663116	I/II	Completed	AD	2011	Spain	Refractory	Autologous; IV	1, 2 or 4 $\times 10^6$; 3 doses, weekly	53	6	Yes	(49)
NCT02221258	I	Completed	UC	2014	Korea	Refractory	Allogeneic; IV	2.5, 5 or 10 $\times 10^7$ 1 dose	9	1	No	(50)
NCT01547091	I/II	Completed	UC	2013	China	Refractory	Allogeneic; IV	4×10^7/patient; 1 dose	172	36	Yes	(51)
NCT01851070	II	Completed	MPCs	2013	USA	Refractory	Allogeneic; IV	1 or 2 $\times 10^6$; 1 dose	48	3	Yes	(52)
ChiCTR-ONC-16008770	I	Completed	UC	2016	China	Refractory	Allogeneic; IV	1 $\times 10^6$; 1 dose	53	12	No	(53)
ChiCTR-INR-17012462	I/II	Completed	UC	2017	China	Refractory	Allogeneic; IV	1 $\times 10^6$; 1 dose	63	3	No	(54)
NCT03691909	I/II	Completed	AD	2018	USA	Stable treatment	Autologous; IV	Unknown	15	12	No	(55)
NCT01873625	I/II	Unknown	BM	2009	Iran	RA	Autologous; IA	Unknown	30	12	Yes	
NCT01985464	I/II	Unknown	UC	2000	Panama	DMARD-resistant	Autologous; IV	Unknown	20	12	No	June 2020
NCT02643823	I	Unknown	UC	2016	China	RA	Allogeneic; IV	2×10^7/patient; 4 doses, weekly	40	12	Yes	June 2017
NCT04170426	I/II	Not yet recruiting	AD	2022	USA	Refractory	Autologous; IV	2.0-2.86$\times 10^6$; 3 doses	54	12	No	December 2023
NCT04971980	I/II	Recruiting	UC	2021	China	Refractory	Allogeneic; IV	0.5,1.0,1.5$\times 10^6$; 1 dose	9	28 ± 3 days	No	April 2022
NCT03798028	N/A	Unknown	UC	2017	China	Anemia or pulmonary disease associated	Allogeneic; IV	1$\times 10^6$; 1 dose	250	6	Yes	June 2020
NCT03186417	I	Recruiting	MPCs	2017	USA	During onset	Allogeneic; IV	2, 4, 6$\times 10^6$; 1 dose	20	12	Yes	December 2020
NCT05003934	I	Recruiting	UC	2022	USA	RA	Allogeneic; IV	10×10^7; 1 dose	20	48	No	September 2025
NCT03828344	I	Recruiting	UC	2020	USA	Refractory	Allogeneic; IV	0.75 or 1.5$\times 10^6$; 1 dose	16	12	Yes	September 2020

(Continued)

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TABLE 2 Continued

Clinical trial identifier	Clinical phase	Status	Source	Registration year	Country	RA patients	MHC context; route of administration	Cells/kg of body weight; number of doses	Estimated/enrolled number of RA patients	Follow-up (Months)	Control group	Ref./ estimated completion date
NCT02348086	Observational	Unknown	AD	2015	USA	RA	Autologous; IV	Unknown	50	12	No	May 2019
NCT03618784	I/II	Recruiting	UC	2018	Korea	Refractory	Allogeneic; IV	10×10^7/patient; 3 doses	33	4	Yes	April 2021
NCT04170426	I/IIa	Active, not recruiting	AD	2022	USA	Refractory	Autologous; IV	2.0 or 2.86×10^6; 1 dose or 3 doses, every 3 days	54	12	Yes	December 2025

AD, adipose tissue; BM, bone marrow; DMARD, disease-modifying antirheumatic drug; IA, intra-articular; IV, Intravenous; MPCs, mesenchymal progenitor cells; MSCs, mesenchymal stem cells; UC, umbilical cord.

DMARDs for RA patients. No serious adverse effects were reported and only 4% of the patients showed mild adverse effects, such as flu-like symptoms. It would be desirable to conduct a multicenter clinical trial to further validate these enthusing results (51). Another study (ChiCTR-ONC-16008770, Table 2) included 53 refractory RA patients. The study was intended to find a marker for determining the effectiveness of MSC treatment. It found that high serum interferon was present in high-reactive RA patients before and 4 weeks after injection, but this change was not seen in non-reactive RA patients. The authors believed that high serum IFN-γ levels are associated with decreased DAS28 values in the responder RA population and claimed that serum IFN-γ levels could be used as biomarkers to predict the clinical benefits of patients (53). These data were consistent with preclinical studies that showed that BM-MSCs might play an immunosuppressive role when they encountered an inflammatory environment in the host. A later clinical trial (ChiCTR-INR-17012462, Table 2) also confirmed that BM-MSCs plus interferon greatly improved the clinical efficiency based on MSCs. ACR20 response rates were 53.3% and 93.3% in patients with MSCs monotherapy and with MSCs combined with IFN-γ treatment, respectively. All patients participating in the study were followed up and no unexpected safety problems were observed (54).

Besides the above clinical studies, other clinical trials conducted to date are summarized in Table 2. We noted that, firstly, current clinical trials mostly focused on phase I/II studies. The sources of MSCs included BM, AD, and UC (30%, 20% and 50%, respectively) (56). Only a small number of studies adopted allogeneic mesenchymal precursor cells (MPCs) and multipotent progenitor cells in the treatment of refractory RA (MPCs; NCT01851070, Table 2) (52). Currently, a clinical trial based on MPCs for the treatment of RA has been launched in the United States (NCT 03186417, Table 2). Most trials exhibited good safety results and a few with transient mild symptoms (51, 57, 58). Secondly, the scales of most clinical trials were small. In the early stage, most of the injections were performed intravenously, and only recently, intra-articular injections were conducted. However, improvement could not be significantly sustained beyond 12 months (59). We noted that doses and the use of multiple infusions of bone marrow MSCs seem to be not associated with their beneficial effect, and MSC could tolerate a wide range of doses (56). Thirdly, most of the subjects involved were refractory RA patients while patients in RA early stage and stable treatment period were also included. The general treatment outcome is that the early intervention could produce better effects than later intervention. In addition, there was a study launched to evaluate the improvement of anemia and interstitial pneumonia of RA (NCT03798028, Table 2) and the effectiveness for new onset RA (NCT03186417, Table 2). Fourthly, not only the clinical effect was evaluated by DAS28, CRP, ESR, RF, anti-CCP antibody, Health Assessment Questionnaire and scores (49, 50, 53, 55), but also patients'

immune balance was evaluated by the ratio of Treg/Th17 cells, serum levels of TNF- α, IL-6, IL-10, and even anti-HLA antibodies (39, 49), which might account for mechanisms of the effect and adverse reactions of MSCs in RA treatment. Lastly, the follow-up times were reported between 1 month to 4 years (Table 2). The overall response was that the curative effect was better at 6 months and the introduction of IFN-γ could enhance the effect of MSC. Interestingly, the addition of Chinese herbal medicine was found to improve the anti-inflammatory effect of MSCs (51).

Collectively, based on the current researches, MSCs treatment in RA is safe and promising. However, it still faces the challenges of short-time effectiveness and multiple injections. More multi-center clinical trials are needed to verify the effectiveness of MSCs in RA treatment in the future.

Tolerogenic dendritic cells therapy

A major objective of DCs-based therapies for RA are to induce and maintain immune tolerance and to restore immune homeostasis by tolDCs (60). To date, tolDCs are mainly derived from monocytes and bone marrow cells, which are obtained by *in vitro* culturing in the presence of GM-CSF, IL-4, by impairing the DC maturation in normal progression and by modulating their specific pro-inflammatory function with various agents loaded (61–63).

The principle behind the treatment with tolDC include directing DCs toward tolerogenic state *ex vivo* and *in vivo*, decrease of effector Th17 cells, reduction of proinflammatory cytokines, and increased number of Treg (64). *In vitro* generation of tolDCs from human monocytes (mo-tolDCs) indicated that tolDCs possess tolerogenic properties (65). Various agents, including dexamethasone, vitamin D3, rapamycin, minocycline, and ethyl pyruvate, were found to contribute to the increased number of human tolDCs *in vitro*, the variation in cytokines secretion, the co-stimulatory protein expressions, as well as the ability to inhibit T cell growth (65). Agents such as dexamethasone, ethyl pyruvate, acetylsalicylic acid, minocycline, and vitamin D3 were also often used to induce tolDCs in mice (66).

Animal trials of tolDCs in RA treatment

Advancement in generation of tolDCs were largely developed from animal trials. Collagen induced arthritis (CIA) animal model is a common experimental model for studying the efficacy of tolDCs in RA. It was found that heat-shock proteins (HSPs) loaded tolDC induce increased ratio of type 1 Treg (Treg1) in antigen-specific T cells in RA murine model (67). Additionally, HSP-specific Tregs effectively suppressed established arthritis. HSP peptides could be ideal antigens for tolDCs loading, not only for the treatment of RA, but also for tolDCs-based treatments of other auto-immune diseases. Thus, HSP-loaded tolDCs is worthy of further exploration in RA treatment in the future.

Vasoactive intestinal peptide loaded DCs (VIP-DCs) can contribute to the improvement of arthritis in experimental CIA mouse model (68). VIP-DCs with low expression of MHC-II, CD40, and the costimulatory molecules CD80 and CD86 produce lower levels of pro-inflammatory cytokines. The marked advantages of VIP-DCs on inhibiting inflammation and bone destruction is consistent with a previous clinical study that VIP-DC treatment could clinically inhibit the progression of RA (69).

Subcutaneous administration of microparticle "regulatory vaccine" (REGvac) induced tolDCs could ameliorate the joint arthritis of CIA mice (70). The REGvac contained dendritic cell chemoattractant, potent immunosuppressive molecules and the RA-relevant autoantigen. In REGvac treated mice, IL-6 in the joint tissue was significantly decreased and $CD25^{+}Foxp3^{+}$ Treg in the lymph nodes and the spleen increased markedly (70). More interestingly, the IL-10 level was increased while the IL-6 level was decreased. Collectively, this regulatory vaccine targeting tolDCs reversed the progression of joint erosion in CIA mouse and is potentially a promising approach for RA immunotherapy.

Another unique tolDCs approach was based on B cell activating factor (BAFF)-silenced DCs *via* RNA interference techniques (71). BAFF-silenced DCs distinctly suppressed arthritic progression and could re-establish the Th17/Tregs balance in CIA mice. *In vitro*, the regulatory effects of BAFF-silenced DCs on T cells were also verified.

In addition to immature DCs, semimature DCs have also been investigated in RA treatment. Semi-mature DCs could be induced by *Bacteroides vulgatus* and proinflammatory cytokines. These semi-mature DCs would lose their ability to generate proinflammatory cytokines, including TNF-α, IL-1β, and IL-6 (72). In the CIA animal model, DNA-induced-semi-mature DCs were demonstrated to contribute to the increased ratio of Tregs and were effective in preventing CIA (72). It has been reported that both allogeneic tolDCs (allo-tolDCs) and autologous tolDCs (auto-tolDCs) could suppress the expressions of pro-inflammatory molecules and improve the manifestations of arthritis in CIA rats and, therefore, displayed similar efficacy of tolerance between allo- and auto- tolDCs once tolDCs were successfully constructed (73).

Clinical trials of tolDCs in RA treatment

The first human phase I study of tolDCs in RA was conducted in 2009 on the safety and efficacy of autologous modified tolDCs exposed to citrullinated peptides (74). This study provided the rationale for advanced research on clinical efficacy of autoantigen immunomodulatory therapy in RA. Autologous tolDCs are often isolated from peripheral blood

monocytes of RA patients. DCs were cultured in the presence of NF-κB inhibitor and loaded with citrullinated self-peptides, namely Rheumavax (60). Administration of Rheumavax remarkably improved the ratio of Tregs and decreased the number effector T cells (Teff) and reduced the levels of IL-15, IL-29, chemokine CX3C motif ligand 1 (CX3CL1), and CXCL11 in RA patients (75). The decrease in disease activity score (DAS28) and circulating CRP were also evident in RA patients.

The AuToDeCRA trial is an unblinded, phase I trial, investigating the safety of tolDCs administrated by intra-articular injection (76). Before injecting different doses of tolDCs into the inflamed knee, tolDCs needed to be pulsed with synovial fluids containing self-antigens. This is the first trial to administer tolDCs *via* intra-articular route, in which tolDCs were cultured with dexamethasone/vitamin D3 and loaded with autologous synovial fluid. Dexamethasone/vitamin D3 was used to suppress the activity of NF-κB signaling. These tolDCs expressed a low level of co-stimulation proteins CD40, CD86 and CD83, together with decreased MHC-II expression. Significantly, obvious improvements in symptoms were observed in patients treated with the high dose tolDCs and there was no worsening knee flare, which indicated that intra-articula tolDC therapy was well-tolerated and effective. Furthermore, this study firstly revealed the safety of intra-articular injective tolDCs.

CreaVax-RA are semi-mature autologous DCs, which is heterogeneous nuclear ribonucleoprotein A2/B1, pulsed with recombinant protein-arginine deiminase type-4 (PAD4), citrullinated vimentin antigen, and citrullinated filaggrin. Interestingly, a decrease in numbers of IFN–γ producing T cells and autoantibody levels were observed when CreaVax-RA was administrated (60). This was the first clinical study that suggested that the utilization of semi-mature autologous DCs contributed to the control of autoantibody levels.

In fact, the efficacy of antigen-specific tolDC therapy relies not only on the different tolDCs but also on the different methods of administration in clinical studies. For tolDCs migration towards secondary lymph nodes in humans, intradermal injection of tolDCs should be better than injection by other routes (77). However, tolDCs migration by intradermal injection has not been used widely. Animal and clinical trials with tolDCs to treating RA hitherto are summarized in Table 3. Collectively, immunotherapies centered on the administration of tolDCs produce promising results as one of alternatives to immunomodulators for RA treatment, given their ability to specifically suppress auto-immune responses without inducing general immunosuppression.

Peptide-based tolerogenic vaccination therapy

Some antigens may have the capability of inducing either an immune response or immunologic tolerance under different exposure conditions and concomitant stimulators (78). Attempts to restore immune homeostasis have been executed with a variety of peptides, especially the disease-related antigenic peptides, and have shown positive effects in various experimental animal models of RA (28). Autoantigens including type II collagen (CII) and proteoglycan (PG) have been proposed as key basements to induce antigen-specific immune tolerance in RA models (78).

A recent research on the therapeutic effect of DerG peptide conjugate vaccines was verified in the human PG G1 domain-induced arthritis (GIA) mouse model of RA (79). The ligand epitope antigen presentation system (LEAPS) was developed by attaching an immune cell binding ligand peptide to a T cell epitope-containing peptide to promote immunogenicity and to determine the resultant response. DerG-PG70 and DerG-PG275Cit vaccines were LEAPS DerG conjugated with the epitope PG70 and the citrullinated form of another epitope (PG275Cit) from PG. Single or combination of subcutaneously administered DerG-PG275Cit and DerG-PG70 vaccines were shown to be protective in the GIA model. Furthermore, splenic T cells and $CD4^+$T cells from GIA mice treated with the vaccines preferentially produced anti-inflammatory (IL- 4 and IL-10) rather than pro-inflammatory (IFN-γ or IL-17) cytokine profile in culture. However, the DerG-PG70 (alone or with DerG-PG275Cit) vaccine, but not DerG-PG275Cit vaccine, could induce antibody responses, which indicated that the different peptide vaccines might elicit therapeutic immune responses *via* different immunomodulation. Hence it is critical to select and construct the relevant peptide conjugations as T cell vaccines.

In another study, the long-term protective effect of peptide 90578, a novel fructosylated peptide derived from the immunodominant T cell epitope of bovine CII (bCII) was studied using a bCII and human fibrinogen (FIA-CIA) immunized arthritis mice (80). Intravenous administration of peptide 90578 could lead to significantly beneficial effects on clinical outcome parameters and arthritis histology scores for 12 weeks, as well as improved survival time (80). Additionally, several studies have demonstrated that co-administration of self-antigen with an immunomodulator (e.g., calcitriol, aryl-hydrocarbon receptor ligands, or NF-kB inhibitors) could improve autoimmunity, whereas free antigen could not (26). For example, it has been demonstrated that low dose rapamycin could provide an immunosuppressive microenvironment for tolerance induction by producing anti-inflammatory cytokines and tolDCs. A preclinical study has explored the restoring immune of "tolerogenic polypeptide vaccine" (TPvax), which carried a multiepitope citrullinated peptide and rapamycin in CIA (81). This study demonstrated that TPvax led to increased ratios of secreted anti-inflammatory to pro-inflammatory cytokines and decreased antibody titers.

Protein-based tolerogenic vaccination therapy

Protein carriers such as monoclonal antibody, cytokines, cells, and pathogen derived immunosuppressive or adhesion

proteins have served as both tolerogenic adjuvants and targeting moieties, and have demonstrated efficacy in preclinical models of RA and other autoimmune diseases (82). B29 is a conserved epitope of heat shock protein (HSP) 70, a major ligandome to the MHC-II. It was found that B29-induced $CD4^+CD25^+Foxp3^+$ T cells could suppress arthritis in the proteoglycan-induced RA mice model both prophylactically and therapeutically, indicating these self-antigen-specific Treg cells could regulate immune disorder *in vivo* (83). HSP 65 is a ubiquitous protein overexpressed in inflamed tissues and capable of inducing immunoregulatory mechanisms. *L. lactis* has probiotic properties and is commonly and safely used in dairy products (84). A recent study has shown that oral co-administration of HSP 65 and *L. lactis* in CIA mouse model ameliorated clinical and histological signs of arthritis, reduced inflammatory cytokines (IFN-γ and IL-17), as well as increased $CD4^+Foxp3^+$Tregs and $CD4^+LAP^+$ T cells (85). This study suggested oral co-administration of HSP 65 and *L. lactis* might be a useful therapeutic approach to regulate inflammatory process of RA.

There are some recent studies on the mechanism of RA that may be potentially applied to therapeutic vaccines for RA Figure 3). The tonicity-responsive enhancer-binding protein (TonEBP) and the 14-3-3ζ protein have been shown the potential therapeutic role in RA models. The TonEBP, which is a Rel family protein involved in the pathogenesis of autoimmune disease and increased inflammation, is required for maturation and function of DCs. This protein is also known as a nuclear factor of activated T-cells 5 (NFAT5). It has been demonstrated that deletion of myeloid cell-specific TonEBP could reduce disease severity in a murine model of CIA and also inhibit maturation of DCs and differentiation of pathogenic Th1 and Th17 cells *in vivo* (86). The 14-3-3ζ protein is an adaptor that can regulate cellular signaling by binding to a wide range of proteins and act as an alarmin. It was recently reported that 14-3-3ζ could cause immune suppression in pristane-induced arthritis (PIA) and CIA models (87). 14-3-3ζ knockout (KO) rats exhibited elevated inflammatory cytokines, particularly for IL-1β and increased bone surface damage. Furthermore, 14-3-3ζ immunization during the pre-symptomatic phase could result in significant suppression of arthritis in both wild-type and 14-3-3ζ KO animals, especially for bone formation. More studies are urgently needed to clarify the underlying molecular mechanisms of inverse vaccination in advancing therapeutic vaccination in RA treatment.

Although previous studies on peptide- or protein- based vaccinations for RA have been encouraging, most of these studies were mainly based on RA animal models, while evidence from clinical studies is still limited. A phase I randomized clinical trial (RCT) was conducted in anti-citrullinated protein Ab (ACPA) positive patients to determine efficacy of three different doses (0.3ml, 1ml and 3 ml) of subcutaneously injected DEN-181, a liposome encapsulating 40mg/ml CII 259-273 peptide and 400ng/ml calcitriol (88). Overall, immunological tolerance was most prominent with the 0.3ml dose, which is also associated with decreased DAS28-CRP and increased naive CII-specific T cells. Further data from this RCT are necessary to determine its efficacy.

TABLE 3 Animal and Clinical Trials involving tolDCs in RA treatment.

Origin	Immature/ semimature	Target cell generation	Efficacy	Reference
PBMC	Immature	NF-κB inhibitor loaded with citrullinated peptides	Increase in Treg ratio; decrease in Teff and multiple cytokines; decrease in DAS28 and CRP;	(74)
PBMC	Immature	dexamethasone/vitamin D3; loaded with autologous synovial fluid	Improvements in symptoms and well tolerated	(76)
PBMC	Semi-immature	pulsed with PAD4, citrullinated filaggrin, and vimentin antigens	Decrease of numbers of IFNγ producing T cells and decreased autoantibody levels	(60)
PBMC	Immature	HSP	Increased ratio of Tr1	(67)
PBMC	Immature	Vasoactive intestinal peptide	Decreased levels of proinflammatory cytokines; inhibition of inflammation and bone destruction	(68)
PBMC	Immature	Microparticle regulatory vaccine	Increase inTreg cells and IL-10; decrease in IL-6	(70)
PBMC	Immature	BAFF-silenced DCs	Suppression of arthritic progression and re-establishment of the Th17/Treg balance	(71)
PBMC	Semi-immature	Bacteroides (*Bacteroides vulgatus*)	Reduction of proinflammatory cytokines	(72)
PBMC	Semi-immature	DNA	Increase of Tregs ratio and effective in preventing CIA	(72)

BAFF, B cell activating factor; CRP, C-reactive protein; CIA, Collagen-induced arthritis; DAS28, Disease Activity Score; DCs, dendritic cells; HSP, heat-shock proteins; NF-κB, nuclear transcription factor-κB; PAD4, protein-arginine deiminase type-4; PBMC, peripheral blood mononuclear cell; tolDCs, tolerogenic dendritic cells; Tr1, type 1 regulatory T-cells.

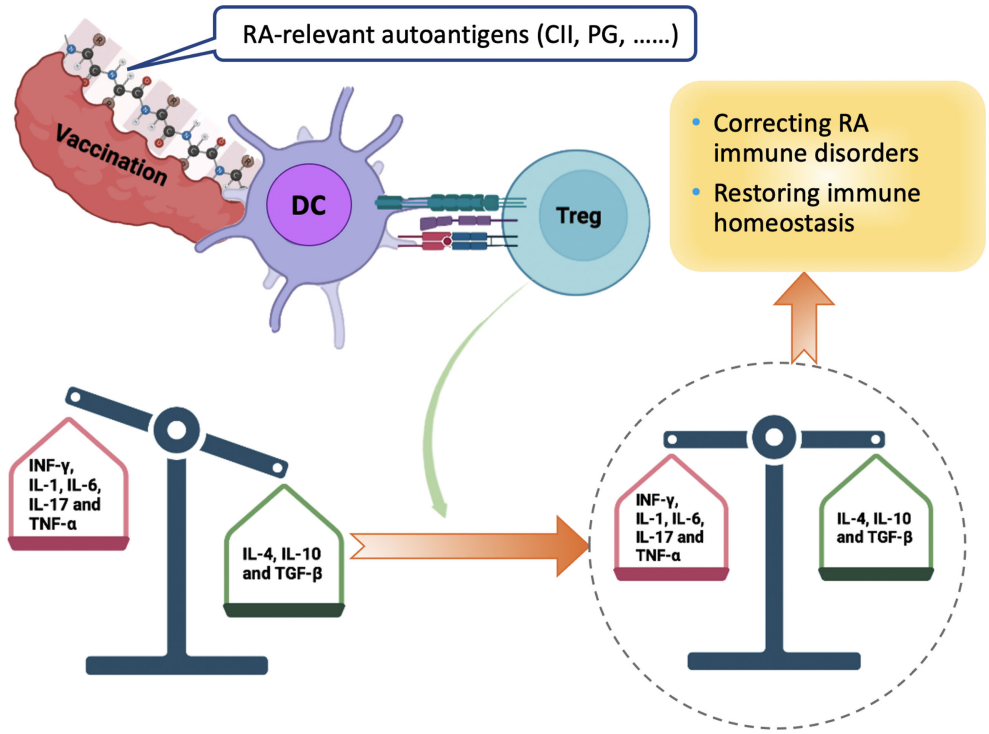

FIGURE 3
Mechanism of Peptide-based tolerogenic vaccination to Induce the Immune Homeostasis in RA Treatment. Vaccine containing autoantigens related with RA can be processed by DC and presented to Tregs in the context of TCR and cytokines. Activated Tregs function as the main regulator of the immunosuppressive process by increasing the ratio of anti-inflammatory cytokines (green box), restoring immune homeostasis and modulating the balance between pro-inflammatory (red box) and anti-inflammatory cytokines. CII, type II collagen; PG, proteoglycan; DC, dendritic cell; INF, interferon; Tregs, regulatory T cells; TGF, transforming growth factor; TNF, tumor necrosis factor.

Perspective

Restoring immune tolerance is an ideal but challenging goal in treating autoimmune diseases. In rheumatoid arthritis, experimental studies on the use of stem cell, tolDCs, and T-cell tolerogenic vaccines induced by peptide or protein specific for RA have yielded promising results and are gradually translated to clinical investigations. Current preliminary results showed these therapies are safe and effective. To further develop these therapeutic strategies, it is necessary to focus on how to effectively acquire RA specific stem cells, tolDCs, and T-cell vaccines with increased immunomodulatory capability in down regulating proinflammatory responses. Future work should also focus on the optimization on doses as well as duration and the methods administration, which are validated by large scale clinical trials. In summary, current data from studies on RA treatment by reestablishing immune tolerance are promising and encouraging. We believe that efforts devoted to developing treatment in this direction will transform our current treatment regimen on RA, greatly improve its prognosis and improve the quality of life in RA patients.

Author contributions

PL originated the topic and supervised the manuscript. ZS wrote the introduction, sections of conventional therapeutic approaches and tolerogenic dendritic cells therapy. SZ wrote the section of peptide-based tolerogenic vaccination therapy and protein-based tolerogenic vaccination therapy. KW wrote the section of stem cell transplantation therapy. JW wrote the perspective. BX wrote the abstract. BX, PL and JW jointly prepared the outline, coordinated the writing of the manuscript and revised the entire manuscript. All authors contributed to the article and approved the submitted version.

Funding

This study has been supported by the National Natural Science Foundation of China [81800906

07] and Anhui Provincial Major Projects of Science and Technology [202103a07020012].

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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10.3389/fimmu.2022.1012868

Glossary

AD	adipose tissue
anti-CCP	anti-cyclic citrullinated peptide
BAFF	B cell activating factor
BM	bone marrow
CCP	cyclic citrullinated peptide
CIA	collagen-induced arthritis
bCII	bovine type II collagen
CII	type II collagen
CRP	C-reactive protein
DAS	disease activity score
DCs	dendritic cells
bDMARDs	biological disease modifying anti-rheumatic drugs
csDMARDs	conventional synthetic disease modifying anti-rheumatic drugs
DMARDs	disease modifying anti-rheumatic drugs
EAE	experimental autoimmune encephalomyelitis
GCs	glucocorticoids
GIA	G1 domain-induced arthritis
HSPs	Heat shock proteins
IDO	indoleamine-2,3-dioxygenase
IFNs	interferons
IL-6	interleukin-6
JAK	Janus-kinase
KO	knockout
LEAPS	ligand epitope antigen presentation system
M-CSF	macrophage colony stimulating factor
MPCs	mesenchymal precursor cells
MSCs	mesenchymal stem cells
NO	nitric oxide
NSAIDs	non-steroidal anti-inflammatory drugs
PG	proteoglycan
PGE2	prostaglandin E2
RA	rheumatoid arthritis
RCT	randomized clinical trial
REGvac	regulatory vaccine
RF	rheumatoid factor
RORγt	RAR-related orphan receptor gamma
STAT	signal transduction and transcriptional activator
tDMARDs	targeted synthetic disease modifying anti-rheumatic drugs
TNF-α	tumor necrosis factor α
tolDCs	tolerogenic dendritic cells
Tregs	regulatory T cells
TSG-6	TNF-stimulated gene-6 protein
TonEBP	tonicity-responsive enhancer-binding protein
TPvax	tolerogenic polypeptide vaccine
UC	umbilical cord
VIP-DCs	vasoactive intestinal peptide loading DCs.

frontiers | Frontiers in Immunology

TYPE Mini Review
PUBLISHED 17 October 2022
DOI 10.3389/fimmu.2022.1003054

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Autoimmune and Autoinflammatory Disorders: Autoimmune Disorders, a section of the journal Frontiers in Immunology

RECEIVED 25 July 2022
ACCEPTED 20 September 2022
PUBLISHED 17 October 2022

CITATION
Vílchez-Oya F, Balastegui Martin H, García-Martínez E and Corominas H (2022) Not all autoantibodies are clinically relevant. Classic and novel autoantibodies in Sjögren's syndrome: A critical review. *Front. Immunol.* 13:1003054. doi: 10.3389/fimmu.2022.1003054

Not all autoantibodies are clinically relevant. Classic and novel autoantibodies in Sjögren's syndrome: A critical review

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Sjögren's syndrome (SjS) is a heterogeneous systemic disease. The abnormal responses to La/SSB and Ro/SSA of both B-cells and T-cells are implicated as well as others, in the destruction of the epithelium of the exocrine glands, whose tissue characteristically shows a peri-epithelial lymphocytic infiltration that can vary from sicca syndrome to systemic disease and lymphoma. Despite the appearance of new autoantibodies, anti-Ro/SSA is still the only autoantibody included in the American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) classification criteria and is used extensively as a traditional biomarker in clinical practice. The study and findings of new autoantibodies in SjS has risen in the previous decade, with a central role given to diagnosis and elucidating new aspects of SjS physiopathology, while raising the opportunity to establish clinical phenotypes with the goal of predicting long-term complications. In this paper, we critically review the classic and the novel autoantibodies in SjS, analyzing the methods employed for detection, the pathogenic role and the wide spectrum of clinical phenotypes.

KEYWORDS

autoimmunity, Sjögren's syndrome, anti-Ro/SSA antibodies, Sjögren, antinuclear antibodies

10.3389/fimmu.2022.1003054

Introduction

Sjögren's syndrome (SjS) is a systemic autoimmune disease which characteristically presents organ lymphocytic infiltration and has a specific predisposition for the exocrine glands. Its main consequence is the development of sicca symptoms, principally in the form of xerostomia and keratoconjunctivitis sicca (1). Nonetheless, a third of patients with long-standing SjS suffer from different systemic complications such as neurological, pulmonary or nephrological manifestations and nearly 5% of them end up developing lymphoma (2).

Multiple factors are involved in the pathogenesis of SjS and it is triggered in individuals with genetic predisposition by environmental factors. The core components of the disease process are autoimmunity and chronic inflammation secondary to the activation of an innate and adaptive immune response (1, 2).

It has been proven that the innate immune system plays a paramount role in early SjS through a type I interferon (IFN), cytokines such as IL-21 and the B-cell-activating factor (BAFF). The initial tissue and cell damage caused by different factors results in the production of IFN I and II in a first stage, inducing in turn the production of BAFF, which is responsible for the activation of autoreactive B-cells, among other processes. Thus, the TNF cytokine family connects innate immunity with the autoimmune activation of B-cells. Other cytokines, such as IL-12, are also central to its pathogenesis, by the activation of the type II IFN system through the innate immune system (natural killers' cells), as well as the adaptive one *via* type 1 T-helper cells. In addition, many other molecules are also important in the pathogenesis, such as IL-21, with a role in the regulation of B-cells and follicular cells, and the CXCR5-CXCL13 axis, with a key role in lymphocyte recruitment and possibly, in the formation of ectopic germinal centers (GCs) (2).

B-cells up-regulation is a crucial feature of SjS, reflected by the broad array of autoantibodies found in the serum of those patients and confirmed by the presence of ectopic GCs in the affected organs tissue (2–4). The development of ectopic GCs on the site of inflammation, often within the salivary glands, has been associated with a higher frequency of local production of anti-Ro/SSA and anti-La/SSB autoantibodies in SjS patients (5). Ectopic GCs are functional structures that offer all the suitable machinery necessary for the activation of autoreactive B-cells and the production of autoantibodies. A complex array of cytokines and immune cells are thought to be involved in the formation of these structures. BAFF cytokine, a key molecule in SjS pathogenesis, is produced by infiltrated immune cells in salivary glands and regulates B-cells activation, proliferation and, importantly, B-cells selection through a ligand competition mechanism. Contrary to what happens in the bone marrow, BAFF has an effect in B-cells tolerance at the periphery, with increased levels of circulating BAFF leading to a decreased competition and resulting in the escape of autoreactive B-cells (6). Consistent with this finding, increased levels of BAFF in serum of SjS patients is correlated with the presence of anti-Ro/SSA and anti-La/SSB autoantibodies (7).

Serum autoantibodies are present in most patients with SjS, and some show a strong association with specific clinical features, possibly contributing directly to the phenotype of individual patients, though SjS classification criteria include just anti-Ro/SSA and anti-La/SSB (8, 9).

Recently, the research seeking novel autoantibodies in SjS has increased, opening up a wide range of clinical phenotypes in an attempt to predict long-term complications (8).

In this review, we will analyze different SjS autoantibodies, including old and newly identified biomarkers and discuss their utility as diagnostic tools, the pathogenic role and their association with some clinical phenotypes.

Classic autoantibodies in Sjögren's syndrome

Anti-nuclear antibodies (ANA)

ANA are antibodies that target antigens in the nucleus of the cell. The gold standard method used for screening to detect ANA is the indirect immunofluorescence assay (IIFA) on HEp-2 cells, which is sensitive but not specific. The HEp-2 IIFA test provides further information than just whether or not autoantibodies are found, including antibody titration and the HEp-2 IIFA pattern (10). Higher antibody levels are associated with systemic autoimmune rheumatic diseases (SARD) and are more likely to identify a specific autoantigen during patient follow-up (10–12). It is of importance that ANAs are widespread in the healthy population and are not suitable for screening in connective tissue diseases, thus a positive result for a specific ANA in the absence of a clinical context or clinical findings is not equivalent to the diagnosis of a connective tissue disease.

To summarize the classification and descriptions of the specific patterns of HEp-2 IIFA, the international consensus on ANA patterns (ICAP) established each pattern and sub-pattern, which were identified with an anti-cell (AC) pattern code (AC-1 to AC-29) (10, 13). The HEp-2 IIFA antibody patterns, their specific antigens and their clinical relevance in SARD are summarized in Table 1.

ANA have been found to be positive in 59 to 85% of SjS patients in whom a higher prevalence of recurring parotidomegaly and a heightened frequency of extra-glandular symptoms are observed (14, 15).

The naming of the autoantigens is based on their biochemical structure or the associated disease, and the antigens in Sjögren's syndrome are named SS-A/B. The frequency of elevated ANA titres in SjS is 40-95%. In

TABLE 1 HEp-2 cell IIFA patterns and their correlated clinical relevance in Systemic Autoimmune Rheumatic Diseases (SARD) (10, 13).

Code	AC pattern	Specific antigens	Clinical relevance
		Nuclear patterns	
AC-1	Homogeneous	Nucleosome (dsDNA, ssDNA, histone)	SLE, chronic autoimmune hepatitis, juvenile idiopathic arthritis.
AC-2	Dense fine speckled	DSF70/LEDGF	Healthy individuals or patients without systemic autoimmune rheumatic diseases (SARD).
AC-3	Centromere	CENP-A/B	Limited cutaneous SSc.
AC-4	Fine speckled	SS-A/B, Mi-2, TIF1gamma, TIF1betta, Ku	SjS, SLE, subacute cutaneous lupus erythematosus, neonatal lupus erythematosus, congenital heart block, DM, SSc and SSc-AIM overlap syndrome.
AC-5	Large/coarse speckled	Sm, U1RNP, RNA polymerase III	SLE, SSc, MCTD, SSc-AIM overlap syndrome, UCTD.
AC-6	Multiple nuclear dots	Sp-100, PML proteins, MJ/NXP-2	PBC, AIM (DM) and other inflammatory conditions.
AC-7	Few nuclear dots	P80-coilin, SMN	Low positive predictive value for any disease.
AC-8	Homogeneous nuclear	PM/Scl-75, PM/Scl-100, Th/To. B23/nucleophosmin, nucleolin, No55/SC65	SSc, SSc-AIM overlap syndrome.
AC-9	Clumpy nucleolar	U3-RNP/fibrilarin	SSc.
AC-10	Punctate nucleolar	RNA polymerase I, NOR-90	SSc, Raynaud's phenomenon, SjS and cancer.
AC-11	Smooth nuclear envelope	Lamins A, B, C lamin-associated proteins	Autoimmune cytopenias, autoimmune liver diseases, linear scleroderma, APS and other SARDs.
AC-12	Punctate nuclear envelope	Nuclear pore complex proteins (gp210, p62, LBR, Tpr)	PBC, autoimmune liver diseases.
AC-13	PCNA-like	PCNA	SLE (debated).
AC-14	CENP-F like	CENP-F	Neoplastic conditions (breast, lung, colon, lymphoma, ovary, brain).
AC-29	TOPOI-like	DNA-topoisomerase/SCL70	SSc (particularly with diffuse cutaneous SSc and more aggressive forms).
		Cytoplasmatic patterns	
AC-15	Fibrillar linear	F-actin, non-muscle myosin	AIH type 1, chronic HVC infection, celiac disease. Rare in SARD.
AC-16	Fibrillar filamentous	Cytokeratin, vimentin, tropomyosin	Not typically found in SARD.
AC-17	Fibrillar segmental	Alpha-actinin, vinculin	SLE, AIH type I, chronic inflammatory demyelinating neuropathy.
AC-18	Discrete dots	GW (182)	Neurological symptoms in a variety of diseases.
AC-19	Dense fine speckled	PL-7, PL-12, ribosomal P proteins	SLE, anti-synthetase syndrome, interstitial lung disease, polyarthritis, Raynaud's phenomenon, mechanic's hands.
AC-20	Fine speckled	Jo-1	Anti-synthetase syndrome, interstitial lung disease, polyarthritis, mechanic's hands.
AC-21	Reticular/AMA	Mitochondrial structures: PDC-E2/M2, BCOADC-E2, E1alpha, E3BP/protein X	PBC, SSc, PBC-SSc overlap syndrome and PBC-SjS overlap syndrome.
AC-22	Polar/Goldi-like	Golgi structures: giantin/macrogolgin, golgin-95/GM130, golgin-160, golgin-97, golgin-245	Small number of patients with: SjS, SLE, RA, MCTD, GPA, adult onset Still's disease, viral infections.
AC-23	Rods and rings	IMPDH2	HCV patients treated with pegylated interferon-alpha/ribavirin combination therapy.

(Continued)

10.3389/fimmu.2022.1003054

TABLE 1 Continued

Code	AC pattern	Specific antigens	Clinical relevance
Nuclear patterns			
AC-24	Centrosome	Pericentrin, ninein, Cep250, Cep110	Low positive predictive value for any disease.Low percentage in SSc, SLE, Raynaud's phenomenon.
AC-25	Spindle fibers	HsEg5	Low positive predictive value for any disease.SjS and SLE (not specific).
AC-26	NuMA-like	NuMA	SjS, SLE, UCTD, limited SSc, RA.
AC-27	Intercellular bridge	CENP-E, CENP-F, TD60, MSA36, KIF-14, MKLP-1, MPP1/KIF20B.	Low positive predictive value for any disease.Very rare: SSc, SLE, Raynaud's phenomenon and malignancy.
AC-28	Mitotic chromosomal	DNA-topoisomerase/SCL70	SSc (difuse).

AIM, autoimmune myopathy; AMAs, antimitochondrial antibodies; APS, antiphospholipid syndrome; CENP, centromere-associated protein; DFS, dense fine speckled; DM, dermatomyositis; ENA, extractable nuclear antigens; HCV, hepatitis C virus; IIFA, indirect immunofluorescence assay; LAP, lamin-associated polypeptide; LBR, lamin B receptor; LEDGF, lens epithelial derived growth factor; NOR, nucleolus organiser region; NXP, nuclear matrix protein; PBC, primary biliary cholangitis; PCNA, proliferating cell nuclear antigen; PML, promyelocytic leukaemia; PM/Scl, polymyositis-scleroderma; RA, rheumatoid arthritis; RNApol, RNA polymerase; RNP, ribonucleoprotein; SARD, systemic autoimmune rheumatic diseases; SLE, systemic lupus erythematosus; SMN, survival of motor neuron; SSc, systemic sclerosis; SjS, Sjögren's syndrome; TIF, transcription intermediary factor; TRIM, tripartite motif; Tpr, translocated promoter region; UCTD, undifferentiated connective tissue disease; ssDNA, single stranded DNA; dsDNA, double stranded DNA; hUBF, human upstream binding factor; AIH, autoimmune hepatitis; Ago, argonaute protein; CLIP, class 11-associated invariant chain peptide; EEA, early endosome antigen; SRP, signal recognition protein; tRNA, transfer ribonucleic acid; Cep, centrosomal protein; DCA, dividing cell antigen; INCENP, inner centromere protein; KIF, kinesin family; MCA, mitotic chromosomal antigen; MKLP, mitotic kinesin-like protein; MPP, M-phase phosphoprotein; MSA, mitotic spindle apparatus; NMP, nuclear matrix protein; NuMA, nuclear mitotic apparatus; PCM, pericentriolar material; UCTD, undifferentiated connective tissue disease.

suspected SjS patients, one of the most characteristic patterns is AC-4 (nuclear fine speckled), in which case it would be advisable to have follow-up tests done in search of anti-Ro/SSA and anti-La/SSB antibodies (10). Figure 1 shows different ANA patterns of primary SjS patients.

Anti-Ro/SSA

Throughout many autoimmune diseases, anti-Ro/SSA antibodies appear to be the most prevalent specificity. Specially among patients with SjS and in the lupus spectrum (SLE, cutaneous lupus, congenital heart block and neonatal lupus). The Ro antigen consists of two proteins that weight 52 and 60 kDa. In humans, Ro 60 kDa is a ribonucleoprotein which forms small cytoplasmic complexes with non-coding RNAs (known as Y RNA). Among its functions, it mediates the quality control of misfolded non-coding RNAs and is involved in diverse cellular-stress responses such as survival after ultraviolet radiation damage (16). Ro 52-kDa (or TRIM21) is a E3 ubiquitin ligase implicated in the ubiquitination of many inflammatory related proteins like IRF transcription factors associated to IFN-I pathway. In addition, acts as cytosolic Fc receptor, being able to bind diverse isotype antibodies and playing a role in intracellular antibody-mediated immunity (17, 18). The human 60 kDa Ro protein is encoded by a 1.8 kb gene in chromosome 19 which possesses an RNA binding site, whereas the gene responsible for the 52 kDa Ro fraction is located in chromosome 11 and lacks any specific RNA binding domain (19, 20). Although RNA precipitation assay has showed the highest specificity and sensitivity to detect anti-Ro/SSA as well as anti-La/SSB, it can't be used in routine analysis, in spite of its usefulness as a reference and confirmation assay (19, 20). Therefore, counter-immunoelectrophoresis (CIE) is regarded as the most specific (100%) and sensitive (89%) assay in order to find anti-Ro/SSA antibodies, and better than enzyme-linked immunosorbent assays (ELISA) or the Immunoblotting (IB) assay (19, 21).

ELISA is frequently used in order to detect these antibodies, since they are straightforward to perform and the interpretation of the results is fast. However, ELISA is not more specific than other kind of analyses. Depending on the commercial kit, various types of antigens, such as recombinant, native proteins and synthetic peptide fragments are used for the detection of SSA/SSB antibodies. Nevertheless, their sensitivity and specificity may vary notably, from 39 to 77% and from 79 to 100% respectively, and most of the ELISAs detecting anti-Ro/SSA and anti-La/SSB antibodies are generally specific for a single target, in spite of a few assays being able to detect several antibodies (22). The Immunoblotting (IB) assay showed high specificity for anti-60 and anti-52 kDa (97 and 95% respectively), but lower overall sensitivity (17 and 36% respectively) (19, 20). Meanwhile, line immunoassay (LIA), an enzyme-linked

10.3389/fimmu.2022.1003054

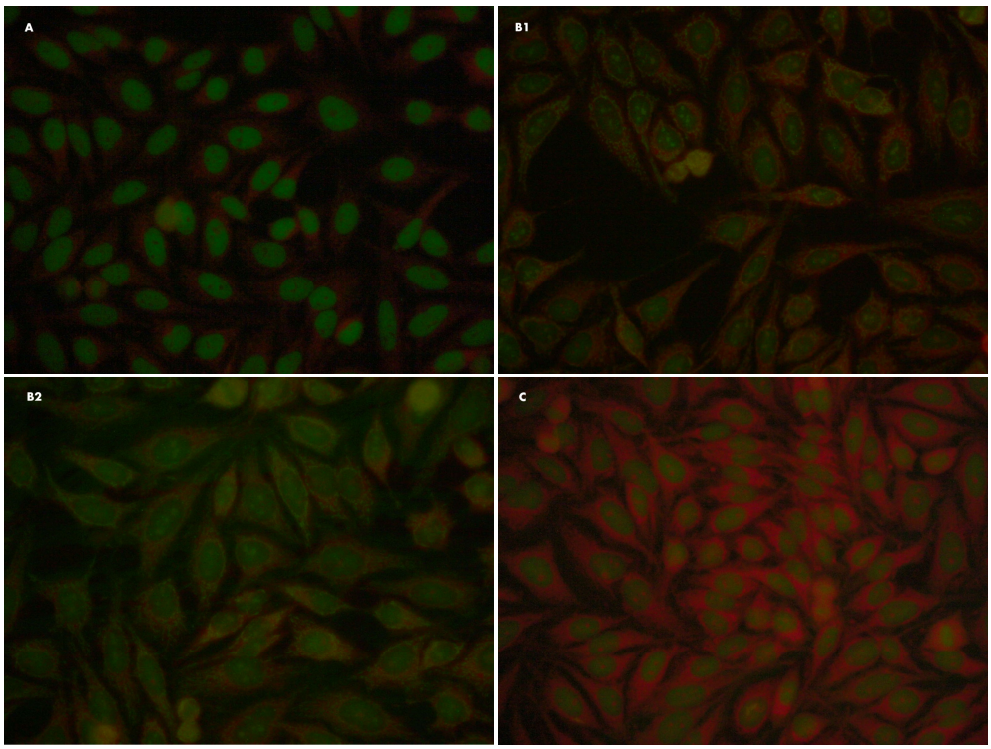

FIGURE 1
Different ANA patterns of primary SjS patients. **(A)** ANA IFI: Fine speckled (AC-4) 1/160. Negative specificity study for extractable nuclear antigens (ENAs). **(B1, B2)** ANA IFI: nuclear dots/nucleolar pattern (AC-7/10) 1/80; it also presents a minor reticular cytoplasmic pattern (AC-21) compatible with anti-mitochondrial antibodies. Organ-specific antibodies: specifity M2/nPCD. **(C)** Patient with anti-Ro/SSA antibody (Ro-52 kDa) and negative ANA-IFI on HEP2-cells.

immunosorbent assay, showed a much better sensitivity (91.4%) and specificity (87%) for detecting anti-Ro/SSA (19, 23).

In clinical practice, anti-Ro/SSA antibodies can be associated with a wide range of SARDs, such as subacute cutaneous lupus, neonatal lupus, systemic lupus erythematous (SLE), SjS, SjS/SLE overlap syndrome, myositis, rheumatoid arthritis (RA) and primary biliary cirrhosis (24–28). Anti-La/SSB and anti-52 Ro antibodies are both directly implicated in the pathogenesis of neonatal lupus. In this context, fetal complete heart block is due to tissue injury mediated by the expression of Ro (both 52 and 62 kDa fractions) and La antigens in the cardiac tissue, particularly located on the surface of myocardial cells', from the 18th to the 24th week. Although the main autoantibody involved in the neonatal pathogenesis is anti-Ro 52 (19, 29).

In SjS, anti-Ro/SSA and anti-La/SSB antibodies have been associated with diagnoses at a younger age, recurring parotidomegaly, more severe dysfunction of the exocrine glands, a more intense lymphocytic infiltration of the minor salivary glands and longer disease duration (30, 31). In addition, some authors suggest a higher prevalence of extra-glandular manifestations, for instance Raynaud's phenomenon, vasculitis, splenomegaly and lymphadenopathy (30, 32).

Anti-La/SSB

The La/SSB antigen is a phosphorylated protein which weighs 48 kDa. It can be found in the nucleus and the cytoplasm and binds to several RNA molecules (19). It participates in a broad spectrum of RNA metabolism processes as protecting nascent RNA polymerase III transcripts from exonuclease digestion by binding to their poly(U) termini, processing of 5' and 3' ends of pre-tRNA precursors and resolving misfolded RNA structures by acting as RNA chaperone (33). IgG anti-La/SSB antibodies are the major antibody class found in serum and it has been observed its strong association with anti-Ro/SSA antibodies. The latter can be found on their own in many patients' sera, whereas anti-La/SSB are generally found together with anti-Ro/SSA. The presence of anti-La/SSB without anti-Ro/SSA antibodies hasn't got any significant association with SjS phenotypic characteristics (34).

Anti-Ro/SSA and anti-La/SSB antibodies are commonly detected in patients suffering from cryoglobulinemia, hypergammaglobulinemia and in the presence of rheumatoid factor independently of autoimmune disease (19, 35).

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Autoantibodies in Sjögren's syndrome associated with overlap syndromes

Anti-mitochondrial antibodies (AMA)

AMAs are the serologic distinguishing finding of primary biliary cholangitis (PBC). AMAs are found in 95% of patients with PBC with a specificity of 98% for the disease (36).

There are four main autoantigens targeting antimitochondrial antibodies, referred to as an M2 subtype of mitochondrial autoantigens and which are identified as the E2 subunits of the pyruvate dehydrogenase complex, the ketoglutaric acid dehydrogenase complex, the branched chain 2-oxo-acid dehydrogenase complex and the dihydrolipoaminade dehydrogenase-binding protein (37). When IIFA is used, between 1.7 and 13% of SjS patients test positive for AMA. However, the positivity rate is higher (3 to 27%) if Western Blot or ELISA is used (38–40). Some SjS patients with a positive AMA test, besides developing more frequently hepatic manifestations, show also a higher prevalence of hypergammaglobulinaemia, peripheral neuropathy, Raynaud's phenomenon and higher titer in the erythrocyte sedimentation rate (ESR) (14, 38).

The similarity of the histological lesion in PBC and SjS is noteworthy. Both are characterized by lymphocytic infiltrates with a predominance of $CD4^+$ cells that initiate around the ductal epithelium (salivary or bile ducts). Hence, both entities may share some pathogenetic mechanisms within the context of some kind of autoimmune epithelitis, in spite of their different autoantibody profiles (38, 41).

Anti-centromere antibodies (ACA)

ACAs are a heterogeneous group of antibodies targeting different antigens clustered around the kinetochore plates (42). In systemic sclerosis (SSc), ACAs recognize three centromeric proteins (CENP): CENP-A (19 KDa), CENP-B (89 KDa) and CENP-C (140 KDa). Anti-CENP-B autoantibodies seem to have a greatest relevance for clinical practice among the various ACA, with a sensitivity of 20 to 30% for SSc (42). Among SARD, ACAs are also present in approximately 15% of patients with PBC and this association has been described with worsening outcomes compared in those seronegative patients (43).

In SjS, the prevalence of ACAs ranges from 3.7 to 27% when IIFA is used to detect them, but when ACAs are detected by other methods its prevalence varies between 20 and 27%, depending on which CENP is used as target antigen (44–46). CENP recognition patterns in SjS and SSc are dissimilar. Most SjS sera recognize CENP-C alone (70% of SjS versus 6% of SSc), whereas most SSc sera recognize CENP-B as well as CENP-C (83% of SSc versus 0% of SjS) (38, 46).

Regarding immunological and clinical features, some studies have shown that seropositive ACA patients with SjS have a higher disease onset mean age, a higher frequency of Raynaud's phenomenon, peripheral neuropathy and keratoconjunctivitis sicca compared to seronegative patients (38, 47, 48). Moreover, ACA positive SjS patients showed an inferior prevalence of anti-Ro/SSA and anti-La/SSB antibodies, a lower frequency of cytopenia, inferior rates of rheumatoid factor and hypergammaglobulinaemia (38, 49).

The singular features presented in ACA positive SjS patients suggests that they could be a SjS clinical subset with some overlapping traits of SjS and SSc (38). Interestingly, a study of SjS patients by capillaroscopy showed that those patients with ACA had a scleroderma-like pattern in 10.2%, showing dilated capillaries, low capillary density and pericapillary haemorrhages, raising the suspicion of an overlapping syndrome between SjS and SSc (50). The percentage of patients progressing to SSc span from 0 to 40% according to different cohorts (38, 49, 51), even though more accurate and prospective design studies are needed to determine it.

Anti-citrullinated peptide antibodies (ACPAs)

ACPAs are autoantibodies targeting proteins that have been citrullinated by a calcium-dependent enzyme family of peptidylarginine deiminases. Its specificity in RA is around 95% and it identifies a phenotype of patients affected by a more serious clinical profile and erosive disease (52). It is widely known that SjS may exist together with other autoimmune diseases, being autoimmune hypothyroidism the most frequent, followed by RA (53). In patients with SjS, the prevalence of ACPAs ranges between 3 and 9.9%, but no agreement exists regarding its clinical significance (54–58), although a small number of authors have described an association with non-erosive arthritis (54, 55, 57).

Some reports have described a low progression to RA in cohorts of patients with SjS during a long-term follow-up (59, 60). Nonetheless, it has been suggested that ACPAs positive SjS patients may have a higher risk of progression to RA (57, 61). Thus, ACPAs constitute a valuable tool for the accurate control of this subgroup of patients in order to ensure their immediate diagnosis and treatment (62).

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Novel autoantibodies in Sjögren's syndrome

As previously commented, classical autoantibodies have been used extensively in the setting of SjS diagnosis and currently, anti-Ro/SSA is widely accepted and considered useful for SjS classification criteria as a diagnostic tool (9).

In recent years, several studies have analyzed new autoantibodies, opening up a window of opportunities for the diagnostic approach, trying to discern the pathologic attributions of disease-associated autoantibodies and providing the opportunity to identify pre-clinical subjects before SjS onset (8).

Despite the fact that most of these antibodies are infrequent and, may even be shared with other connective tissue diseases, their study may help to elucidate some of the clinical features of patients with SjS and help to tackle potentially serious complications in those patients with suspected or confirmed SjS, as well as proving to be useful in order to reach a diagnosis when other autoantibodies turn out to be negative. Among the antibodies that we will discuss are anti-alphafodrin, anti-muscarinic type 3 receptor (M3R), anti-salivary gland protein 1 (SP1), anti-carbonic anhydrase, anti-parotid secretory protein (PSP), anti-interferon-inducible protein-16, anti-NA-14, anti-MDM2, anti-stathmin-4, anti-PUF60, anti-NR2, anti-TRIM38, anti-calponin-3, anti-saccharomyces cerevisiae (ASCA), anti-aquaporin (AQ), anti-ganglionic acetylcholine receptor (gAChR), anti-P-selectin, anti-moesin, anti-carbamylated, anti-alpha-enolase, anti-cofilin-1 and anti-Rho GDP-dissociation inhibitor 2 (RGI2) (63–92).

In addition, it is worth highlighting two ANA patterns mentioned in Table 1 and observed in patients with SjS that may have additional clinical value in the spectrum of clinical manifestations. These are the antibodies for nuclear transcription factor NOR 90/hUBF (anti-NOR 90) and anti-NuMA antibodies. Anti-NOR 90, are antinuclear antibodies that recognize the distal ends of the short arms of chromosomes 13, 14, 15, 21 and 22 (nuclear organizer region) on immunofluorescence analysis and 90-kd doublet proteins on immunoblot analysis (93). Fujii T et al. identified that anti-NOR 90 antibodies are rare, although they are associated with SjS and overlap syndromes (SjS-RA and SjS-SSc) in Japanese patients. Anti-NuMA antibodies (antinuclear mitotic apparatus), although rare, are mostly associated with SLE and SjS, and could be useful in order to reach a diagnosis when other autoantibodies are negative. Those patients' both clinical and biological profiles were milder, suggesting that these antibodies may imply a good prognosis marker in both syndromes. SjS patients with anti-NuMA antibodies presented fewer ocular sicca syndrome and dryness complications and anti-Ro/SSA and/or anti-La/SSB antibodies were less frequently present. Anti-NuMA positive patients received antimalarial agents less frequently than negative patients, with no difference regarding other treatments (94).

In Table 2, we summarize the novel autoantibodies found in SjS patients that may be associated with some clinical features.

Other relevant antibodies: Rheumatoid factors and cryoglobulins

Rheumatoid Factors (RF) and cryoglobulins, require special attention for their important clinical implications in SjS, partly due to their relationship with potentially serious disease complications.

Rheumatoid Factors (RF)

Rheumatoid factors (RF) are autoantibodies targeting the constant part (Fc) portion of other immunoglobulins. The most distinguished are IgM and IgA binding to the Fc portion of IgG (95).

RF levels are found in 36 to 74% of SjS patients with an increased prevalence during follow-up and have been unmistakably associated with the number of extra glandular features (14, 32). The wide spectrum of clinical manifestations in patients with RF includes a higher frequency of articular damage, Raynaud's phenomenon, parotidomegaly, cutaneous vasculitis, cytopenias, renal involvement and central nervous system manifestations (32, 96). In addition, RF levels are correlated to an active serological profile with higher presence of some antibodies, including ANA, anti-Ro/SSA, anti-La/SSB and cryoglobulins, as well as hypergammaglobulinaemia and hypocomplementemia (14, 32, 38). RF have been associated with diagnoses at a younger age and with lymphocytic infiltration in the biopsy of salivary glands. This last is associated with the lymphomagenesis process related to the role of monoclonal RF secreted by the B-cells through the chronic stimulation at the target organ (95).

Cryoglobulins

Cryoglobulinemia is characterized by the precipitation of circulating immunoglobulins at temperatures down 37°C. Serologically, it is classified into three subtypes according to the composition of the immunocomplex. Thus, it is differentiated as type I cryoglobulinemia (monoclonal immunoglobulins), type II (mixed cryoglobulinemia containing monoclonal and polyclonal immunoglobulins) and type III (mixed cryoglobulinemia containing only polyclonal immunoglobulins). Type II cryoglobulinemia is commonly associated with infectious diseases, with special mention to hepatotropic viruses, while type III cryoglobulinemia is most

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TABLE 2 Novel autoantibodies in Sjögren's syndrome.

Author	Number of patients	Autoantibodies	Technique	Prevalence	Sensitivity and specificity	Clinical features
Hu Q at al. (63)	Meta-analysis	**Anti-alpha fodrin**	Immunoblot and ELISA	38-42%	Sensitivity 39.3% Specificity 83%	Moderate accuracy for the diagnosis of SjS.Clinical manifestations were not evaluated.
Willeke P et al. (64)	62		ELISA	31-35%	Sensitivity 31-35%Specificity unknown	Shorter disease duration. Increased prevalence of recurrent parotid swelling with IgG isotype.
Mona M et al. (65)	156	**Anti-muscarinic type 3 receptor (M3R)**	On-Cell-Western assay	N/A	Sensitivity 75-98%Specificity 85%	Correlated with ocular dryness and glandular hypofunction and the haematological/biological domains of ESSDAI. Useful in SjS diagnosis, especially where clinical assessments are limited.
Deng C et al. (66)	956		ELISA	N/A	Sensitivity 4-98%Specificity 58-100%	Potential diagnostic biomarker for SjS.Clinical features were not evaluated.
Shen L et al. (67)	123	**Anti-salivary gland protein 1 (SP1)**	ELISA	19% isolated34% associated to other autoantibodies.	N/A	Associated with anti-Ro/SSA and anti-La/SSB. No distinct clinical manifestations were identified in patients expressing anti-SP1.
Xuan J et al. (68)	134		Western-blot	40%	N/A	Higher levels during earlier stages of the disease.
Karakus S et al. (69)	136		N/A	27 %	N/A	Dry eye. Correlated with having a Schirmer test ≤ 5 mm.
Karakus S et al. (69)	136	**Anti-carbonic anhydrase 6 (CA6) and anti-parotid secretory protein (PSP)**	N/A	27% (CA6)54% (PSP)	N/A	Anti-CA6 was associated with severe ocular surface staining (corneal and conjunctival). Anti-CA6 may indicate early stages of SjS.Anti-PSP was the only autoantibody that correlated with primary SjS.
Pertovaara M et al. (70)	74	**Carbonic anhydrase auto-antibodies (CA)**	ELISA	N/A	N/A	CA-II, CA-VI and CA XIII (associated with renal manifestations).CA-VII and CA-XIII (correlated to β_2 microglobulin)CA-I (oral dryness and associated with interstitial lung disease in other connective tissue diseases).
Alunno A et al. (71)	30	**Anti-Interferon-Inducible Protein-16**	ELISA	33%	N/A	Pathogenesis of glandular inflammation.
Baer AN et al. (72)	133		ELISA	29%	N/A	Severe disease: greater prevalence of abnormal Schirmer's test, ANA >1:320 and germinal center-like structures in the labial salivary gland lymphocytic infiltrates. Focus scores were significantly higher.
Alunno A et al. (73)	67		ELISA	34%	N/A	Pathogenesis of glandular inflammation.
Uomori K et al. (74)	72	**Anti-NA-14**	ELISA	11.1%	N/A	Elevation of IgA levels. Low prevalence of ANA positive patients. Disease duration tended to be shorter (although the difference did not reach statistical significance).
Liu Y et al. (75)	100	**Anti-MDM2**	ELISA	21%	N/A	Longer disease duration and more lymphocytes focal gathering in labial gland. Higher prevalence of anemia, thrombocytopenia and anti-Ro/SSA.
Duda S et al. (76)	72	**Anti-stathmin-4**	ELISA	33% (pSS with PNP) 7.8% (pSS without PNP)15% in sSS	N/A	Polyneuropathy.

(Continued)

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TABLE 2 Continued

Author	Number of patients	Autoantibodies	Technique	Prevalence	Sensitivity and specificity	Clinical features
Zhang YM et al. (77)	79	**Anti-PUF60**	ELISA and immunoblotting	10.1%	N/A	Overlap syndrome with myositis.
Fiorentino DF et al. (78)	84		ELISA	30%	Specificity 29%	May be more associated with Asian and African-American ethnicity, hypergammaglobulinemia, anti-Ro/SSA, anti-La/SSB and rheumatoid factor.
Lauvsnes MB et al. (79)	66	**Anti-NR2**	ELISA in serum and electrochemiluminescence in CSF	20%	N/A	Cognitive disturbances and mood disorders.
Lauvsnes MB et al. (80)	50		Electrochemiluminescence in CSF	12%	N/A	Loss of hippocampal gray matter.
Tjensvoll AB et al. (81)	71		Electrochemiluminescence in CSF	N/A	N/A	Cognitive impairment.
Wolska N et al. (82)	235	**Anti-TRIM38**	TNT Quick coupled transcription/translation system and immunoprecipitation assay	10.21%	N/A	Higher severity of disease: severe sialadenitis, higher van Bijsterved scores and lower Schirmer's test scores.
Birbaum J et al. (83)	209	**Anti-calponin-3**	ELISA	11%	N/A	Neuropathy.
Alunno A et al. (84)	104	**Anti-saccharomyces cerevisiae (ASCA)**	Immunodot test	4.8%	Very low sensitivity, 100% specificity	Patients displayed a triple combination of circulating anti-Ro60/SSA, anti-Ro/52/SSA and anti-La/SSB antibodies associated with low complement and cutaneous involvement.
Birnbaum J et al. (85)	109	**Anti-aquaporin (AQ)**	Fluorescence-activated cell sorting (FACS) assay	10%	N/A	Neuromyelitis optica spectrum disorder.
Alam J et al. (86)	112		Indirect immunofluorescence assay	76.8%	Sensitivity 73%Specificity 68%	Low resting salivary flow.
Tzartos JS et al. (87)	34		ELISA verified by radioimmunoassay, western blot and AQP-transfected cells.	38.2%	N/A	Severe xeropthalmia, suggesting a potential pathogenic role.
Mukaino et al. (88)	39	**Anti-ganglionic acetylcholine receptor (gAChR)**	LIPS assay	23.1%	N/A	Autonomic symptoms.
Hu YH et al. (89)	70	**Anti-P-selectin**	ELISA	40.6% (SjS patients with thrombocytopenia) and 7.8% (SjS patients without thrombocytopenia).	N/A	May lead to platelet destruction and endothelial injury. Possible role in the pathogenesis of thrombocytopenia.
Zhang Y et al. (90)	50	**Anti-moesin**	ELISA	42%	N/A	N/A
Bergum B et al. (91)	78	**Anti-carbamylated**	ELISA	27%	N/A	Increased focal lymphocytic infiltration, formation of ectopic GC-like structures in minor salivary glands and diminished salivary gland function.

(Continued)

TABLE 2 Continued

Author	Number of patients	Autoantibodies	Technique	Prevalence	Sensitivity and specificity	Clinical features
Cui L et al. (92)	70	**Anti-cofilin-1**	ELISA in saliva samples	N/A	Sensitivity 80%Specificity 90%	May predict progression to MALT lymphoma
Cui L. et al. (92)	70	**Anti-alpha-enolase**	ELISA in saliva samples	N/A	Sensitivity 90%Specificity 84%	May predict progression to MALT lymphoma
Cui L. et al. (92)	70	**Anti-Rho GDP-dissociation inhibitor 2 (RGI2)**	ELISA in saliva samples	N/A	Sensitivity 90%Specificity 80%	May predict progression to MALT lymphoma

ELISA, enzyme-linked immunosorbent assay; SjS, Sjögren's Syndrome; ESSDAI, EULAR Sjögren's syndrome disease activity index; ANA, antinuclear antibodies; anti-NA-14, nuclear autoantigen of 14 KDa; anti-MDM2, human homologue of mouse double minute 2; pSS, primary Sjögren 's Syndrome; sSS, secondary Sjögren's Syndrome; anti-PUF60, poly(U)-binding-splicing factor 60 KDa; anti-NR2, N-methyl-D-aspartic acid receptor 2; CSF, cerebrospinal fluid; anti-TRIM38, tripartite motif-containing protein 38; LIPS assay, luciferase immunoprecipitation system assay; MALT, mucosa-associated lymphoid tissue; N/A, not available.

commonly associated with autoimmune connective tissue diseases (95).

Cryoglobulins differentiate a subgroup of SjS patients with a worse prognosis, as they have been associated with an increased risk of lymphoproliferative disease (96). The wide spectrum of clinical manifestations in patients with cryoglobulinemia includes a higher frequency of Raynaud's phenomenon, parotidomegaly, vasculitis, cytopenias, renal involvement and peripheral neuropathy (32, 96). Cryoglobulinemia is also commonly associated with the presence of other immunological markers such as hypocomplementemia, RF and anti-Ro/SSA (32, 96).

Discussion

The pathogenesis of SjS is still elusive, although the role of abnormal autoreactive B-cells, which lead to the production of autoantibodies and the formation of immune complexes, seems to be fundamental in developing the syndrome. The presence of these autoantibodies is one of the hallmarks of SjS. Amid them, the most common are antibodies against Ro/SSA and La/SSB ribonucleoprotein complexes, which are included in the classification criteria of the American College of Rheumatology/ European League Against Rheumatism (ACR/EULAR). Both anti-Ro/SSA and anti-La/SSB positive SjS patients are diagnosed at a younger age, have a graver glandular dysfunction and a higher frequency of extraglandular features.

One of the diagnostic criteria for the majority of autoimmune diseases is the presence of autoantibodies, whose detection may be overlapping in the spectrum of other autoimmune diseases. As commented throughout this review, there are no specific autoantibodies for SjS. Anti-Ro/SSA and anti-La/SSB constitute a diagnostic marker for the disease, however, these autoantibodies are found in up to 30-40% and 10-15% of patients with SLE, respectively, but anti-Ro/SSA and anti-La/SSB autoantibodies are not the only ones that can be linked to SjS or SLE. To data, these autoantibodies do not correlate with the etiopathogenesis of the disease, although in pregnancy, it may increase the risk of developing neonatal lupus (97). The detection of ANAs has been considered one of the most sensitive techniques in the screening of autoimmune diseases, but may appear related to other conditions such as infectious diseases, malignancy or in healthy population. There are numerous shared autoantigens between SjS and SLE, and it seems that the differential pathogenic mechanism between them may be the inflammatory tropism at the glands in primary Sjögren's Syndrome, although the molecular explanation of that is still unknown (2). For all these reasons, despite that autoantibodies are important tools in the diagnosis of autoimmune diseases, they may be analyzed in a specific clinical context to evaluate their diagnostic value.

Antinuclear antibodies are serological biomarkers with a crucial role in the classification of systemic autoimmune rheumatic diseases. There is a continuous needed for harmonization of the methods for autoantibody determinations and in the research setting for the identification of novel autoantibodies, where several assay methods have become available in the last decades.

The HEp-2 indirect immunofluorescence assay (IIFA) is broadly used in order to detect ANA, and its outcome is included in both diagnostic and classification criteria when a systemic autoimmune disease is suspected. We agree with Damoiseaux J et al. that the HEp-2 IIFA test reveals a lot, considering the fluorescence pattern to be an important diagnostic tool which reveals clinically suitable information. It is important to reach a consensus in order to standardize the current diagnostic tools and achieve greater accuracy in our daily clinical practice, as well as aligning pattern descriptions across laboratories to homogenize the same criterion to help clinicians. We consider that the initiative by the International Consensus

on ANA Patterns (ICAP) eases to be more accurate and emerge as an important tool in the diagnostic work-up, promoting the understanding of HEp-2 IIFA staining pattern nomenclature, as well as optimizing usage in patient care by providing new guidelines. Despite this, ANA test results still need to answer some questions, methodologic consensus statements and semantic issues.

Previous SjS reviews have focused on traditional autoantibodies, and then on some of the novel autoantibodies described in the recent years. Thus, there are two main works that analyze the usefulness of the different antibodies related to Sjögren's syndrome. Firstly, Shen L et al. in 2017, discussed autoantibodies including the traditional ones, those identified initially from mouse models and those associated with other autoimmune diseases, examining their detection methods and their prevalence. Secondly, in 2019, Martín-Naresa E et al. mainly discussed their usefulness in order to identify a clinical subtype.

The advance in biological techniques for antigen determination have resulted in the knowledge of more auto-antigen specificities associated with autoimmune diseases, including SjS. Due to this heterogeneity in autoantibody responses and in clinical phenotypes, a broad-spectrum of diagnostic tools as antigen microarrays are increasingly demanded, targeting multiple specificities.

Conventional techniques used in the majority of diagnostic laboratories bring different non-specific initial screening methods based on the gold standard immunofluorescence assay of ANA autoantibodies or ELISAs, that use antigen mixtures. Although useful for its high sensitivity, these techniques would have to be evaluated for its efficacy in detecting patients with new autoantibody targets as positive. Some assays of ANA-ELISA have resolved this issue by adding a number of extra purified recombinant antigens, as is the case of some of the classical epitopes of Sjogren's syndrome (Ro/SSA and La/SSB) and also in other rheumatic autoimmune diseases (98). To further identify the specificity of auto-antibodies, the initial screening is usually followed by a number of assays based on different technologies (i.e., line immunoassays, immunoblots or multiplex color-coded bead assays as Luminex®) that allow the study of only a reduced number of auto-antibody specificities. With the appearance of autoantibody arrays assays, the ability to measure a large number of autoantibodies with a small sample volume on a single platform matrix, opened the possibility of reducing the time and cost of performing several tests that included only small subsets of the most classical antigens. Thus, with the increasingly knowledge of novel auto-antibody targets, antigen microarrays may become an established tool for routine diagnostic procedures in the future. Also, these new technologies provide the opportunity of defining new autoantibody profiles and explore the relevance of different isotypes, also identifying patient subsets that could correlate with different prognostic outcomes or be candidates to novel personalized treatments. However, currently there are a number of obstacles when standardizing these new tools in daily clinical practice: issues in the design and optimization of all targets in a single array, the lack of available normalized standards protocols and the lack of batch-to-batch reproducibility and inter-laboratory comparison (99). On the other hand, given the importance of inflammatory cytokines in the pathogenesis of SjS, techniques with a more functional approach based on the measurement of cell expression of different immune-related molecules such as IFN, and its correlation with the presence of certain auto-antibodies, are being studied in other rheumatic autoimmune diseases with interesting results related to their possible role in the autoimmunity response in those patients (100).

Our work aims to combine the information collected in recent years regarding classic and novel autoantibodies in SjS, with a special emphasis on the role of ANA and the HEp-2 IIFA staining pattern, trying to define the usefulness of "organ-specific" biomarkers in the creation of new patient phenotypes that may step up the diagnosis of SjS in the future. In this way, as new biomarkers are identified, more subtypes of patients may be established, with their own intrinsic features, helping other physicians in the clinical suspicion of SjS and additionally guiding the clinician in the face of possible potentially serious complications. In this sense, we also emphasize the role of anti-Ro/SSA antibodies, review the antibodies shared with other autoimmune diseases and analyze the novel antibodies that could provide valuable information for understanding the pathophysiology of the disease, predicting new clinical profiles in the future and probably helping in the development of new therapeutic targets. Likewise, understanding and validating this new spectrum of autoantibodies may make it possible to carry out a diagnostic approach in those patients with negative anti-Ro/SSA antibodies with specific clinical phenotypes that do not meet the American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) classification criteria.

To sum up, detecting serum antibodies is practical in order to determine both diagnosis and prognosis in autoimmune diseases. The identification of new autoantibodies in SjS opens up a window of opportunity to gain a better understanding of the SjS pathophysiology, to ascertain clinical phenotypes and to foresee long-term associated complications.

The characterization of autoantibodies and their target autoantigens in patients with primary SjS is helping to unravel more information regarding this common systemic autoimmune disease. Any positive serological result with clinically defined sicca symptoms or other extraglandular features, should raise red flags in the follow up of those patients. Some of the autoantibodies discussed in this review were identified in a research environment, but in the near future they may also be used as helpful tools in common clinical practice. Many of the autoantibodies mentioned in this review are found in subpopulations of SjS patients without any specificity and their real usefulness should be balanced and powered in later studies.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Acknowledgments

We are grateful to Dr. David Cañadas Bustos for his comments that greatly improved the manuscript. We also thank to the autoimmunity section of the clinical laboratory of Hospital Universitario Gregorio Marañón for their help in the preparation of the images used in this review.

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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frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 19 October 2022
DOI 10.3389/fimmu.2022.953726

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Autoimmune and Autoinflammatory Disorders, a section of the journal Frontiers in Immunology

RECEIVED 26 May 2022
ACCEPTED 24 August 2022
PUBLISHED 19 October 2022

CITATION
Ma H, Murphy C, Loscher CE and O'Kennedy R (2022) Autoantibodies - enemies, and/or potential allies? *Front. Immunol.* 13:953726. doi: 10.3389/fimmu.2022.953726

Autoantibodies - enemies, and/or potential allies?

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Autoantibodies are well known as potentially highly harmful antibodies which attack the host *via* binding to self-antigens, thus causing severe associated diseases and symptoms (e.g. autoimmune diseases). However, detection of autoantibodies to a range of disease-associated antigens has enabled their successful usage as important tools in disease diagnosis, prognosis and treatment. There are several advantages of using such autoantibodies. These include the capacity to measure their presence very early in disease development, their stability, which is often much better than their related antigen, and the capacity to use an array of such autoantibodies for enhanced diagnostics and to better predict prognosis. They may also possess capacity for utilization in therapy, *in vivo*. In this review both the positive and negative aspects of autoantibodies are critically assessed, including their role in autoimmune diseases, cancers and the global pandemic caused by COVID-19. Important issues related to their detection are also highlighted.

KEYWORDS

autoantibody, diagnosis, treatment, autoimmune disease, cancer, COVID - 19, biomarker

Introduction

It is well known that an antibody (Ab) produced by B cells helps the immune system to identify and neutralize non-self-antigens (e.g. antigens from bacteria, viruses, toxins and fungi etc.). However, sometimes the immune system fails to distinguish between self and non-self-antigens, leading to the generation of autoantibodies against self-antigens, or autoimmunity. This can cause autoimmune diseases, and, increasingly, autoimmunity has been found to be associated with a wide range of diseases, such as cancer, infectious disease (e.g. such as COVID-19), cardiovascular disease and neurodegenerative disease. However, autoantibodies are also found in healthy populations, albeit usually not in high levels and, for the most part, do not cause damage or attack the host.

Autoantibodies were first reported by Hargraves et al. (1) in lupus erythematosus (LE). LE cell factors, which could bind nuclear antigens, were eventually identified as autoantibodies. While it is widely reported that autoantibodies play a crucial role in the

pathogenesis of various autoimmune diseases, they may mediate both systemic inflammation and tissue injury (2). The exact reasons for autoantibody generation in certain diseases (e.g. cancer) have still not been fully elucidated. However, there are several suggested theories for autoantibody generation in cancer. They may include a) tolerance defects and inflammation, b) altered antigen expression, c) changes in exposure or presentation of antigen, and d) cellular death mechanisms (3). Some autoantibody production is due to a combination of genetic and environmental factors (e.g. exposure to viruses, certain toxins and hazardous chemicals). In systemic lupus erythematosus (SLE), autoantibody generation is triggered by genetic abnormalities and environmentally induced defects in immune cells, mutations in regulatory components which are involved in cellular apoptosis and ineffectual cellular debris clearance (4).

Approximately 5% of the general population suffers from one or more autoimmune diseases (5). About 50 million Americans may have some form of an autoimmune disease and among them, over 75% are women. Autoimmune diseases cause significant deaths in young to middle-aged women (6, 7). In some cancers and other diseases autoantibodies are generated and these can be used as biomarkers in diagnosis. It is also suggested that some cancers may be promoted by certain autoantibodies (8, 9). Increasingly reports suggest that in COVID-19, especially severe COVID-19, new autoantibodies are generated (10). Moreover, these new autoantibodies directly cause harm, including blood clotting, blood vessel inflammation, and tissue damage. Autoantibodies may also play a role in long COVID symptoms (11).

Autoantibodies may provide both harmful and potentially beneficial effects. For example, in malaria, autoantibodies appear to be involved in aspects of pathogenesis, which then lead to various symptoms, such as cerebral malaria, anemia, acute kidney injury and respiratory distress syndrome. However, autoantibodies which are produced during malarial infection may also assist in host protection (12). Autoantibodies can also make useful contributions in disease diagnosis, prognosis and treatment. The longer half-lives of autoantibodies (when compared with their sometimes less stable antigens) *in vivo* (e.g. in blood and other bodily fluids) often makes autoantibody detection easier and more effective compared to measurement of their related antigens. The overall stability of autoantibodies (with half-lives of up to several weeks in blood circulation and over years when blood samples are stored at -20° C and even longer at -80°C) may be much higher than that of their associated antigen (sometimes with a half-life of a few hours or a few days in the blood circulation). For instance, the *in vivo* half-lives of IL-1b and IL-18 are 20 min and 16 h, respectively (13), while the average half-life of IgG autoantibody in circulation is about 3 weeks (14). Moreover, many autoantibodies may be detected well in advance of clinical manifestations of the disease, which enables earlier diagnosis and, thus, benefits the selection and application of effective treatments (15). In addition, the immune response against self-antigens amplifies the signal, which makes the autoantibody detection easier and earlier than antigen detection. Finally, autoantibody-based immunoassays (e.g. enzyme-linked immunosorbent assay (ELISA) and lateral flow) are very easy to be translated to clinical diagnosis platforms (16).

Since autoantibodies can be used as promising biomarkers for diagnosis/prognosis of various diseases (e.g. autoimmune diseases, cancer and cardiovascular disease) this may aid potential treatment using a more targeted approach (17, 18). It was also noted that patients with some autoimmune diseases have a lower cancer risk, which suggests that certain autoantibodies may contribute to the protection of the individual (19).

Problems caused by autoantibodies

Role of autoantibodies in autoimmune diseases

In autoimmune diseases, the immune system 'mistakenly' recognizes self-cells/tissues/organs as non-self, which leads to autoantibody production. Such autoantibodies then bind/attack self-cells/tissues/organs causing damage, inflammation and/or organ dysfunction. However, some autoantibodies do not cause injury directly (e.g. Graves' disease is directly caused by thyroid-stimulating autoantibodies), but they are thought to be part of an overall complicated immune response that causes inflammation and damage. Various roles of autoantibodies in relation to the activation of autoimmune diseases are detailed below.

There are approximately 100 identified autoimmune diseases (20). Nine of the most common and/or harmful autoantibody-associated autoimmune diseases are Type 1 diabetes (T1D), rheumatoid arthritis (RA), multiple sclerosis (MS), systemic lupus erythematosus (SLE), Graves' disease (GD), psoriasis, inflammatory bowel disease (IBD), Sjögren's syndrome (SS) and celiac disease (CD).

It was reported that 9.5% of the world's population was affected by T1D, in which the immune system attacks insulin-producing cells in the pancreas (21). Insulin, produced by the pancreas plays a crucial role in blood sugar regulation. High blood sugar caused by T1D leads to damage of blood vessels and organs (e.g. heart, kidneys, eyes, and nerves). Many different harmful autoantibodies are generated including autoantibodies against glutamic acid decarboxylase (GAD), an enzyme which aids pancreatic function. Such anti-GAD autoantibodies promote T1D. They do this by directing the immune system to kill the insulin-producing pancreatic cells. Moreover, autoantibodies against islet cell cytoplasmic insulinoma-associated antigen-2, as well as insulin were usually found in T1D patients. These autoantibodies also are involved in T1D development (22).

In rheumatoid arthritis (RA) the immune system attacks the joints resulting in their damage and destruction and, eventually, disability occurs. Approximately 0.5-1% of adults worldwide suffer from RA (23). Autoantibodies are also important biomarkers for RA, and among these are rheumatoid factor (autoantibody against the fragment-crystallizable (Fc) region of IgG) and antibodies against post-translationally modified proteins involving citrullination (ACPA) and carbamylation (anti-CarP antibodies). Immune complexes in the joint may be formed by these autoantibodies, which cause swelling, redness, stiffness and warmth (24, 25).

Multiple sclerosis (MS) is a chronic autoimmune disease, where the protective coating (known as myelin sheath) that surrounds nerve cells in the central nervous system is damaged, and this is associated with characteristic inflammatory lesions and demyelination. Mobility limitations are a key feature, while other typical symptoms are weakness, numbness, balance dysfunction and trouble with walking. Over 2.8 million people are estimated to live with MS worldwide (26) and about 50% of these people will require the use of a walking aid within 15 to 25 years following diagnosis with MS (27). Kuerten et al. (28) demonstrated that B cells and autoantibodies play crucial roles in MS pathogenesis, also, broadly increased anti-myelin autoantibody levels were detected in the plasma of MS patients. Identification of specific pathogenic autoantibodies in MS and their target antigens remains a significant challenge.

Systemic lupus erythematosus (SLE) is well known to cause skin rash, however, it is a chronic autoimmune disease which affects many organs, including the joints, kidneys, brain, and heart. Other common symptoms of SLE are joint pain/swelling, fatigue, and fever.

It was reported that the prevalence of SLE in 2018 in the US was 0.073% (29). Autoantibody production in SLE is thought to be triggered by a complex interaction of genetics, the environment, and hormones, leading to harmful self-attack and inhibition of immune regulation (30). Leptin, an adipocytokine, plays a crucial role in the development and maintenance of proinflammatory immune responses and SLE was found to be promoted by autoantibodies increased by leptin (31).

Graves' disease (GD) is caused by thyroid-stimulating autoantibodies (TSAbs) that activate the thyrotropin receptor on the thyroid cell membrane, leading to the over-production of thyroid hormones. GD affects approximately 2-3% of the world's population (32). While thyroid hormones play a crucial role in control of metabolism, high levels hyper-stimulate the body's activities, resulting in nervousness, a fast heartbeat, heat intolerance, and weight loss. Bulging eyes, an associated symptom of GD, was found in circa 30% of GD patients (33).

Psoriasis is an autoimmune condition where T cells mistakenly attack the host's skin cells. It is a common skin disease affecting approximately 2–3% of the population globally (34). The occurrence of psoriasis in children varied from 0% in Taiwan to 2.1% in Italy, whilst in adults it ranged from 0.91% in the USA to 8.5% in Norway (35). In psoriasis, skin cells grow too quickly. This leads to build up of extra cells and this causes inflamed red patches. Over 30% of psoriasis patients also develop psoriatic arthritis, a form of inflammatory arthritis that can cause pain, swelling and sometimes damage to the joints. Four autoantigens involved in psoriasis have been reported. They are cathelicidin LL-37, melanocytic ADAMTSL5, lipid antigen PLA2G4D and keratin 17. Autoantibodies against LL-37 and ADAMTSL5 have been reported to play a potential role in pathogenesis of psoriatic arthritis (36).

Inflammatory bowel disease (IBD) is an immune-mediated inflammatory disease, which causes inflammation in the lining of the intestinal wall. There are two types of IBD, namely Crohn's disease and ulcerative colitis (UC) (37). Crohn's disease can produce inflammation in any part of the gastrointestinal tract, from the mouth to the anus, whereas ulcerative colitis affects only the lining of the large intestine (colon) and rectum. It is reported that autoantibodies may promote the pathological phenotype by activating M1 monocytes in the animal model where NOD/ScidIL2Rγnull mice were reconstituted with PBMC from ulcerative colitis donors (NSG-UC), and also in patients with ulcerative colitis (38). Antinuclear autoantibodies (ANA, the antibodies that attack contents in the cell nucleus) may represent a factor that enhances the propensity to the development of ulcerative colitis (39).

Sjögren's syndrome is an autoimmune disorder where the immune system attacks the glands providing lubrication to the eyes and mouth and leads to dry eyes and dry mouth. It also affects the joints and skin. About 0.2-4% of the world's population are affected (40). There are various autoantibodies associated with Sjögren's syndrome. These include anti-salivary protein 1 (SP1), anti-carbonic anhydrase II and IV, anti-parotid secretory protein (PSP), anti-Ro (SS-A) and anti-La (SS-B), rheumatoid factor, and ANA (41, 42).

The pathogenic role of these autoantibodies in the development of Sjögren's syndrome remains to be elucidated. However, Kim et al. (43) reported a pathologic role of primary Sjögren's syndrome autoantibodies associated with down-regulation of the major histocompatibility complex I (MHC I) molecule with muscarinic type 3 receptor (M3R) through internalization. It was also found that autoantibodies against Ro and La cause apoptosis in the A-253 cell line. Moreover, anti-carbonic anhydrase II autoantibodies have been detected in approximately 13 to 21% of Sjögren's syndrome patients and are believed to have a pathogenic role in renal tubular acidosis, a common extra-glandular manifestation of primary Sjögren's syndrome (44).

Celiac disease (CD), an autoimmune enteropathy, is triggered by dietary gluten in genetically susceptible individuals. The pooled global prevalence of CD was 1.4%,

based on serologic test results published from January 1991 through March 2016 (45). In CD, the immune system attacks the small intestine with gluten in it, which leads to inflammation, diarrhea and abdominal pain. While the autoantibodies in CD do not trigger the disease directly, they have pathogenic potential. It is reported that CD autoantibodies induced ataxia *in vivo*, and, moreover, induce epithelial proliferation and neuronal apoptosis *in vitro* (46). CD can be easily identified by the presence of autoantibodies against a self-antigen, tissue transglutaminase (tTG). The specificity of anti-tTG autoantibodies (IgA and IgM in IgA-deficient subjects) test can achieve 99% for CD patients (47).

Autoantibodies which promote the progression of cancer

Autoantibodies are produced in the early stage of cancer by the humoral immune response, which is activated by abnormal expression of tumor-associated antigens (TAAs) (48). The presence of autoantibodies is well established as early-stage biomarkers in cancer diagnosis. However, some autoantibodies are believed to contribute to cancer progression and resistance to cancer therapy, while some contribute to cancer suppression (19, 49, 50). Lin et al. (51) reported two autoantibodies (antibody 93 and 641) in breast cancer patients which play a role in cancer progression. Antibody 93 stimulated tumor growth, while antibody 641 inhibited tumor cell growth. The role of autoantibodies in promoting and inhibiting cancer is poorly defined. Andreu et al. (52) reported that autoantibodies promote chronic-inflammation-induced tumorigenesis. They found that through interaction with activating Fc gamma receptors, they may control several crucial functions of leukocytes in neoplastic tissue. It was also noted that stromal accumulation of autoantibodies in premalignant skin appears to promote neoplastic progression and subsequent carcinoma development.

It is reported that among patients with autoimmune diseases, that the risk of certain cancers is increased significantly. For instance, hematological, vulvar, thyroid, pancreatic, lung and hepatic cancers occur more frequently in SLE patients (53). Anti-double stranded DNA (anti-dsDNA) autoantibodies were found in about 30% of SLE patients but were less than 1% in healthy individuals. These anti-dsDNA autoantibodies, which attack and damage DNA, cause increased release of intracellular contents (e.g. DNA) from dying cells, which may then lead to further inflammation and autoantibody production, and, thus form a destructive cycle. Anti-dsDNA autoantibodies could increase the cancer risk directly among SLE patients through DNA damage or inflammation. (8, 9).

Some non-B cell-produced antibodies were found to influence tumor progression. For example, antibodies or 'antibody-like' molecules produced by malignant epithelial cells of various tumors (e.g. breast tumor, colon tumor, lung tumor and liver tumor) were able to aid the growth and survival of tumor cells *in vitro* and *in vivo* (54). This leads to the intriguing question as to whether or not these are autoantibodies and preliminary evidence suggests some are, as they target self-antigens (e.g. HEp2 cell antigens) (49, 55). However, the situation is still complex. Findings seem to indicate that antibodies expressed and secreted by various cancer cells (e.g. colorectal cancer, epithelial cancer, prostate cancer) enable cancer cell proliferation but suppress cancer cell apoptosis (56–58). Tumor-derived antibodies have been found to aid tumor development and progression in the following aspects i.e. tumor cell growth and proliferation, tumor cell migration, invasion, and metastasis; tumor immune escape and other biological functions (e.g. immunity regulation, promotion of drug resistance, involvement in cancer-associated diabetes, influence of tumor-associated thrombosis, mediation of CSC potential, regulation of cell morphogenesis, cell cycle process, fatty acid biosynthetic process, protein biosynthesis, and antimicrobial activity) (55). Xu et al. (57) found out that after IgG_1 knockdown, colony formation, survival, cell cycle progression, migration and invasion of LNCaP (lymph node carcinoma of the prostate) cells decreased significantly. Furthermore, reduction of proliferation [assessed *via* the proliferation marker, proliferating cell nuclear antigen (PCNA)], and increasing of numbers of apoptotic cells (detected using the apoptotic marker, caspase-3) were observed after IgG_1 silencing.

Autoantibodies could drive severe and long COVID-19

Several publications have suggested a link between autoantibody generation and severe/long COVID-19 (10, 59–62). It is reported that 52% of 172 people hospitalized with COVID-19 had autoantibodies against phospholipids, which contribute to the control of blood clotting, which is one of the severe COVID-19-associated symptoms (63). Therefore, scientists concluded that these anti-phospholipid autoantibodies are potentially pathogenic. It was demonstrated that autoantibodies neutralizing high concentrations of type I interferons (IFNs) were found in 9.5% of patients admitted to the ICU for COVID-19 pneumonia in a hospital in Barcelona (64). Troya et al. (65) also reported in a hospital in Madrid, anti-type I IFN autoantibodies were found in over 10% of COVID-19 patients at severe/critical COVID-19 stages. Moreover, Chauvineau-Grenier et al. (66) reported that the presence of anti-type I IFN autoantibodies was associated with higher risk of mortality, as these autoantibodies were found in 21% of patients who died from COVID-19 pneumonia in a hospital in France. Therefore, the presence of these anti-type I IFNs autoantibodies was recommended for testing as soon as possible after COVID-19 diagnosis, as they may indicate the possibility of life-

threatening issues. Inhibition of annexin A2 leads to systemic thrombosis, cell death, and non-cardiogenic pulmonary edema. Zuniga et al. (67) reported increased anti-annexin A2 autoantibodies among hospitalized COVID-19 patients and these autoantibody levels were associated with and may predict mortality levels. Anti-annexin A2 autoantibodies can be included in testing to predict severe COVID-19.

Cytokines are crucial in the immunopathology of infections caused by viruses, including COVID-19. They are well known to assist the immune and inflammation responses *via* controlling the growth and activity of blood cells and cells of the immune system. Increased levels of a wide range of cytokines [e.g., interferon (IFN)-α, IFN-ϵ, IL-1β, IL-2, IL-6, IL-7, IL-8, IL-10, IL-17, IL-22, macrophage colony-stimulating factor (M-CSF), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), 10 kD interferon-gamma-induced protein (IP-10), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein 1-α (MIP 1-α) and TNFα] as well as anti-cytokine autoantibodies have been identified in hospitalized/severe COVID-19 patients (68). Feng et al. (69) reported that more than 60% of hospitalized COVID-19 patients have one or more autoantibodies that recognize cytokines. Interestingly enough, these anti-cytokine autoantibodies are also highly prevalent (over 50%) in non-COVID-19 infections patients in ICU. Moreover, Chang et al. (10) stated that various autoantibodies (including anti-cytokine autoantibodies and autoantibodies against some intracellular antigens) found in COVID-19 patients are also associated with connective tissue diseases (e.g. systemic sclerosis, myositis, and overlap syndromes). It was found that autoantibodies against IFN-γ, GM-CSF, IL-6 and TH-17 contribute to or are closely related to infection susceptibility (70). Bastard et al. (71) reported that autoantibodies against type I IFNs neutralize their corresponding type I IFNs to block COVID-19 infection both *in vitro* and *in vivo*.

But one key question is, do pre-existing autoantibodies cause severe COVID-19 or does COVID-19 trigger the production of new autoantibodies which then cause severe COVID-19 symptoms, or both? Some scientists suggested that pre-existing autoantibodies against type I IFNs are predictive of critical COVID-19 pneumonia (64). However, more and more publications showed that new autoantibodies developed during and after COVID-19 (especially severe COVID-19) cause serious problems (10, 72). Wang et al. (73) found autoantibodies which attacked B cells, and some that attacked interferon in COVID-19 patients. They also suggested the possibility that COVID-19 triggers the body to generate new autoantibodies which attack self-tissues. Such autoantibodies were found against proteins in patients' blood vessels, hearts and brains. These new autoantibodies can do harm to individuals by causing blood clotting, blood vessel inflammation, tissue/organ/nerve damage, and attack the immune system, resulting in impaired ability to fight infection (74).

It is highly possible that both pre-existing and new autoantibodies generated during/after COVID-19 infection play a crucial role in severe COVID-19 individuals. Chang et al. (10) screened blood (serum and plasma) samples from 147 hospitalized COVID-19 patients, and then concluded that 52% of the patients with severe COVID-19 had at least one type of pre-existing autoantibody in their blood, while in healthy controls, only 15% had these autoantibodies. They also found that about 20% of hospitalized COVID-19 patients did not have any autoantibodies when they were first admitted but developed them during their illness. This evidence suggests that while pre-existing autoantibodies do correlate with severe COVID-19 it also leads to the development of new-onset IgG autoantibodies. These new autoantibodies, which break tolerance to self, were also found to correlate positively with the severity of COVID-19 (10).

Liu et al. (72) demonstrated that COVID-19 triggers the development of autoantibodies directly. These new autoantibodies were against both structural proteins similar to COVID-19, but also to proteins which are dissimilar to COVID-19 proteins. Moreover, they found sex-specific patterns of autoantibody reactivity, which last up to 6 months following associated symptomatology. Namely, males carry the risk of diverse autoimmune activation following symptomatic COVID-19, while females carry the risk for a distinct profile of autoimmune activation following asymptomatic COVID-19.

Many scientists believed that autoantibodies may play a role in long COVID symptoms, as these autoantibodies can remain for over 6 months, or much longer, after the original COVID-19 virus disappeared (11). Therefore, relevant autoantibody presence could be tested at an early stage following diagnosis of COVID-19, to predict which patients are at high risk or, for long COVID, with a view to more specific and effective treatment. (64).

Exploiting the benefit of autoantibodies

Autoantibody value in the detection and treatment of autoimmune and other disorders

Autoantibodies are widely used in the diagnosis and prognosis of autoimmune diseases (Table 1). For some autoimmune diseases, the diagnosis can be easily achieved through the observation of symptoms and detection of autoantibodies. For example, Graves' disease and Hashimoto's thyroiditis can be easily diagnosed and monitored by anti-thyroid autoantibody levels (95). Celiac disease can mainly be diagnosed and monitored by anti-tTG and DGP autoantibody tests (112). Moreover, some autoantibodies may be detected many years before the onset of autoimmune disease. It was

TABLE 1 Summary of autoantibody applications in various autoimmune disease.

Autoimmune disease type	Autoantibody targets for diagnosis and prognosis	Autoantibody targets for treatment
Type 1 diabetes (T1D)	Insulin; cytoplasmic proteins in beta cells, glutamic acid decarboxylase (GAD-65); protein tyrosine phosphatase (IA-2A); zinc transporter 8 (ZnT8); Pdx1 and Reg1A, cytokine CCL3, Rab GDP dissociation inhibitor beta (GDIb) (75)	CD3, CD20, CD2, interleukin (IL)-1β, IL-1R, IFNα, IFNγ, IL-12, IL-21, IL-17A, IL-25, CD4 and CD8α, CD127, IL-7Rα, IL-2, CD127, IL-7Rα (76)
Rheumatoid arthritis (RA)	Rheumatoid factor (RF), citrullinated antibodies (ACPA), carbamylated protein (anti-CarP), peptidyl arginine deiminase-4 (PAD-4), glucose-6-phosphate isomerase (anti-GPI), Type II collagen (CII), Heterogeneous nuclear ribonucleoprotein A2, RA33, malondialdehyde (MDA), malondialdehyde-acetaldehyde (MAA), CCP2 (25, 77)	TNFα, integrin alpha-9 (α9), IL-2, IL-10, IL-6R, CD20, CD80/86 (78, 79)
Multiple sclerosis (MS)	Potassium channel KIR4.1 (80), anti-α-D-Glcp-(1→4)-α-D-Glcp (GAGA4) IgM (81), myelin oligodendrocyte glycoprotein (MOG), myelin basic protein (MBP), KIR4.1, Neurofilaments-Heavy chain (NF-H), Chitinase-3-Like-1 precursor, miR-19a, miR-21, miR-22, miR-142-3p, miR-146a, miR-146b, miR-155, miR-210, and miR-326 (82)	Integrin α, α4β1-integrin, CD52, CD20 (83–85);
Systemic lupus erythematosus (SLE)	C1q, panel of Erythrocyte-bound C4d (E-C4d), B-cell-bound C4d (B-C4d), nuclear contents, and mutated citrullinated vimentin (MCV), panel of antibodies against dsDNA, nuclear contents, MCV, E-C4d, and B-C4d (86); Serum Complement 3 (C3), Complement 4 (C4), Nucleosome, Erythrocyte Sedimentation Rate (ESR), C-Reactive Protein (CRP), Sm, C1q, C1q (87); extractable nuclear antigens (anti-ENA), B lymphocyte stimulator (BLyS), TNF-like weak inducer of apoptosis (TWEAK) (88), myc-associated zinc finger protein (MAZ) (89), TRIM21 (90)	B-lymphocyte stimulator (BLyS) (91); Thyroid peroxidase (TPO), BLyS, CD 20, CD22, CD19, Cereblon Modulator (CC-220), CD40, IL-12/23, IL-10, IL-6, IFNα, Interferon-γ (IFNγ), IFNα Kinoid (IFNα-K) (92) CD38 (93) a proliferation-inducing ligand (APRIL) (94)
Graves' disease (GD)	Thyroglobulin (TG), thyroid peroxidase (TPO), and thyroid-stimulating hormone receptor (TSHR) (95)	CD20 (96), TSHR/IGF-IR receptor complex, IL-6, TNF-α (97)
Psoriasis	Cyclic Citrullinated Peptide, Rheumatoid factor (RF), nuclear contents (98)	TNF-α, (99) IL-12, -23, and -17 (100) CD6 (101), CD4 (102)
Inflammatory bowel disease (IBD)	Neutrophil cytoplasmic, Exocrine Pancreas, *Saccharomyces cerevisiae*, glycan, outer-membrane porin C, Cbir1, I2, *Mycobacterium avium* subspecies paratuberculosis, *Caenorhabditis elegans*, cocktail multiple antigenic peptide, tailless complex polypeptide (TCP), granulocyte macrophage colony-stimulating factor (103, 104)	TNF-α, IL-12, IL-23, α4 integrin, α4β7 integrin (105–107)
Sjögren's syndrome (SS)	Nuclear contents, Sjögren's syndrome type, rheumatoid factor, Panel of murine parotid tissue proteins, including parotid secretory protein, carbonic anhydrase 6, and salivary protein-1 (108), carbonic anhydrase 6 (CA6) (109)	CD-20 (110), IFN-α (111)
Celiac disease (CD)	Tissue Transglutaminase (tTG), Deamidated gliadin peptide, endomysium (112)	IL-15 (113)

reported that over 88% of SLE patients showed at least one SLE autoantibody-positive test (e.g. aANA, antiphospholipid, anti-Ro, anti-La, anti-Sm, anti-nuclear ribonucleoprotein and anti-double-stranded DNA autoantibodies) before the diagnosis of SLE (up to 9.4 years earlier; mean, 3.3 years) (114).

Nevertheless, for the autoimmune diseases which involve systemic autoantibodies against various organs or systems (e.g. rheumatoid arthritis, systemic lupus erythematosus, scleroderma, and dermatomyositis), the diagnosis is much harder. For instance, in order to diagnose systemic lupus erythematosus, in addition to symptom assessments, physical examination and X-rays, levels of various autoantibodies should be determined against a panel of targets, namely, anti-erythrocyte-bound C4d (E-C4d), anti-B-cell-bound C4d (B-C4d), ANA, and anti-mutated citrullinated vimentin (MCV); or, against a panel including anti-dsDNA, ANA, anti-MCV, anti-E-C4d, and anti-B-C4d (86).

Profiling the autoantibodies presented in serum is commonly used for the diagnosis of diseases, including various autoimmune diseases and cancers. Protein array technology enables the identification of novel panels of autoantibody biomarkers through the screening of the humoral immune response against thousands of proteins (115). For example, thousands of recombinant proteins may be expressed, purified, and then spotted on microarrays or chips to enable easy screening of test samples (e.g. serum) (116–118). Moreover, the potential autoantibodies can be screened and identified using tiny amounts of samples [e.g. autoantibodies to over 10,000 human antigens can be investigated in one experiment using only 50 μL serum sample by applying Engine protein arrays (Engine – a biomarker company in Germany)]. Antigens thus identified can then be used as potential novel probes for disease diagnosis, stage, progression, response to therapy, as well as treatment (119). For example, B-lymphocyte stimulator (BLyS)

has been found to increase significantly in SLE patients, and autoantibodies against BLyS have been successfully applied in SLE treatment (120). An anti-BLyS human monoclonal antibody, Belimumab, was approved by the US FDA, and is still proven to be safe and effective in SLE therapy (121).

Scientists have shown great interest in anti-idiotypic (anti-ID) use. Here the antibody against the binding site of the autoantibody (Ab1) is known as anti-ID antibody (Ab2). Autoantibodies offer a potential target for anti-ID antibody prevention/treatment for autoimmune disease. The antigen-binding regions of anti-ID antibodies (Ab2) that are specific for autoantibody (Ab1) can structurally mimic/resemble that of the target antigens. Thus, the Ab2 antigen-binding domain can potentially represent an exact mirror image of the initial targeted antigen of Ab1. Moreover, Ab2 may display a similar functional activity with the original antigen (122). Such Ab2s have been validated for potential application as a surrogate for the original antigen in vaccine studies (123).

There are several advantages of using anti-ID antibodies for immunotherapy. Firstly, anti-ID antibodies enable the inhibition of specific autoantibody responses while the rest of the immune system remains unaffected, thus, avoiding potential side effects. Secondly, anti-ID antibodies trigger a memory response (through T-helper memory cell generation) promoting longer-lasting immunity and preventing relapses. Finally, it is relatively safe to use anti-ID antibodies *in vivo*, since anti-ID antibodies naturally occurred in the body and the immune response caused by anti-ID antibodies should be similar with that caused by the original antigens, which are mimicked by anti-ID antibodies (124). For example, high levels of anti-dsDNA autoantibodies can be detected years before the onset of SLE, and these harmful autoantibody levels are associated with the severity of SLE. Anti-dsDNA autoantibodies are often correlated with continuing inflammation and kidney damage (125). Lee et al. (126) reported that high levels of anti-dsDNA antibody were successfully neutralized and decreased through the binding of anti-ID antibodies, which then lead to apoptosis of anti-dsDNA antibody-producing cells. Therefore, there is potential to employ anti-ID antibodies for prevention (e.g. vaccine) and treatment purposes.

Table 1 summarizes the targets for autoantibodies which have been used clinically or have high potential (as proved at research level) in diagnosis, prognosis and therapy of autoimmune diseases. It is very interesting to notice that for diagnosis and prognosis of different types of autoimmune disease, the autoantibody targets vary significantly for disease-specificity, while for treatment, one target can be used in several different types of autoimmune disease. For example, anti-cluster of Differentiation 20 (CD20) autoantibody showed therapeutic effect in Type 1 diabetes, rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus, Graves' disease and Sjögren's syndrome. Anti-tumor necrosis factor (TNF) α autoantibody is effective in the treatment of rheumatoid arthritis, Graves' disease, psoriasis and inflammatory bowel disease. Anti-IL-12 autoantibody can be useful for the therapy of Type 1 diabetes, systemic lupus erythematosus, psoriasis and inflammatory bowel disease. This indicates that a panel of autoantibodies may be able to provide effective treatment for various autoimmune diseases/conditions.

The role of autoantibody in cancer diagnosis, prognosis and tumor inhibition/treatment

Cancer requires early detection and effective treatment, while early diagnosis is usually essential for effective therapy. Tumor-associated autoantibodies are popular candidates for both early detection and treatment of cancer, as increasing numbers of autoantibodies against tumor specific antigens have been reported and their detection exploited for research and clinical analysis (127–129).

Tumor-associated autoantibodies are antibodies produced as an immune response against various tumor-associated autoantigens (i.e. over expressed antigens, mutated or post-translationally modified proteins). Various tumor-associated autoantibodies have been identified in virtually all types of cancers. However, there are some problems associated with autoantibody detection. This includes issues due to their very low levels which may be undetectable (130). Autoantibodies may start to be produced before disease symptoms are manifested but their detection may be difficult due to their low concentrations. Some autoantibodies may be very good potential biomarkers (sensitive and specific), but there is the possibility that their corresponding target antigens are still not identified, either because they are unknown or have not been linked to specific diseases (131). There may also be an issue for detection of autoantibodies that have very low antigen binding affinities (132).

Kijanka et al. (133) demonstrated that by screening high-density protein arrays, colorectal cancer-special antibody profiles (e.g. autoantibodies against p53, HMGB1, TRIM28, TCF3, LASS5 and ZNF346) can be identified for colorectal cancer diagnosis in symptomatic patients. Fitzgerald et al. (134) further described a novel ELISA assay which showed high predictive value for the presence of colorectal cancer, through the detection of IgM and IgG autoantibody immune responses in human serum. This novel blood-based test has potential for enhanced patient uptake, as such a blood-based test is generally more acceptable than a fecal-based test.

Some autoantibodies have potentially good diagnostic capacity, but some are also applicable for use in prognosis. It is reported that the serum autoantibodies against GAGE7, MAGEA1, PGP9.5, CAGE and p53 could be used for lung cancer diagnosis, while autoantibodies to PGP9.5 particularly correlate with poor prognosis for lung cancer patients (135).

Some autoantibodies were associated with good prognosis, while others indicated bad prognosis. Denkert et al. (136) found that high levels of autoantibodies against tumor-infiltrating lymphocytes (TILs) and p53 were associated with better prognosis in HER2-positive breast cancer patients, while autoantibodies against MSH2, EZR, PGK1, VCL and ANXA2 were associated with poorer pancreatic cancer patient outcomes (137). Autoantibodies against CDC25B also indicated poor prognosis in advanced esophageal squamous cell carcinoma (ESCC) patients (138). In some circumstances, an autoantibody can be used in both diagnosis and treatment (139) with panels of autoantibodies being more useful. For example, an autoantibody against human epidermal growth factor receptor 2 (HER2) can be used for diagnosis for HER2-positive breast cancer. Moreover, as HER2 promotes cancer cell growth, anti-HER2 autoantibodies may also provide potentially effective anti-cancer outcomes (140). For prostate cancer, an anti-prostate-specific antigen (PSA) autoantibody could serve as a diagnostic biomarker (since PSA is a FDA-approved prostate cancer diagnosis biomarker, although it has clearly established limitations, but is routinely used in clinics), and may mediate anti-cancer effects (141). The value of anti-PSA antibodies was shown by Sinha et al. (142) who successfully used anti-PSA IgG as a selective delivery agent for conjugated chemotherapeutic drugs to PSA-producing neoplastic prostate cells in nude mice, without causing cytotoxic effects on mouse organs.

Early diagnosis improves cancer outcomes significantly, but, is also challenging and demanding. Wang et al. (143) identified an anti-ALDH1B1 autoantibody which may have potential for early detection of colorectal cancer. Anti-TOPO48 autoantibody was reported to be a potentially useful biomarker for early diagnosis and prognosis of ESCC (144). Autoantibodies against aberrantly glycosylated MUC1 in early-stage breast cancer are believed to predict a better prognosis (145). Detection of a panel of autoantibodies against seven various targets (p53, GAGE7, PGP9.5, CAGE, MAGEA1, SOX2 and GBU4-5) was suggested to have significant clinical value for early diagnosis of lung cancer (146). However, autoantibody panels are generally proven to be more effective and accurate than the use of a single autoantibody in cancer diagnosis (147). O'Reilly et al. (148) reported that a panel of zinc finger proteins, including ZNF346, ZNF638, ZNF700 and ZNF768, are suitable for use as capture antigens in a blood-based autoantibody biomarker assay for colorectal cancer. Jiang et al. (48) successfully developed a panel of seven autoantibodies (reactive with: TP53, NPM1, FGFR2, PIK3CA, GNA11, HIST1H3B, and TSC1) for effective early detection of lung cancer, as well as providing novel targets for lung cancer immunotherapy.

In relation to the determination of autoantibodies, IgG, IgM and IgA have been demonstrated to have differential discriminatory abilities (134, 149). While protein targets are predominant, changes in glycation, citrullination or phosphorylation are also potentially significant. The linkage of autoantibody profiles to genomic findings and other sets of clinical data, with associated analysis using complex computational approaches, should be capable of providing greater insights and diagnostic capacity and risk analysis of diseases including cancer and autoimmunity (150).

Increasing numbers of studies have shown that some autoantibodies play a crucial role in cancer inhibition. Autoantibodies could inhibit tumors *via* surveillance mechanisms thereby controlling prolonged survival for various cancers, for instance, melanoma, esophago-gastric, breast gastric, colon, lung, pancreatic and tongue cancers (19). It is reported that the generation of autoantibodies specific to tumor antigens is derived from the migration, differentiation, and maturation of TIL-B (tumor infiltrating B-cells) in tumor-associated tertiary lymphoid structures. Thus tumor associated autoantibodies, which are believed to be indicative of more significant immunological reactivity, will induce functional anti-tumor humoral immunity and promote immune surveillance for cancer cells (151). Evidence suggests that certain pre-existing tumor associated autoantibodies (e.g. NY-ESO-1, XAGE1, and SIX2) are associated with clinical benefit in anti-PD-1 treatment for non-small-cell lung cancer (152–154). Karagiannis et al. (155) observed that impairment of autoantibody IgG1-mediated tumoricidal functions, generated poor clinical outcomes in melanoma.

The autoantibodies or autoantibody-derived antibodies involved in cancer therapy function in four ways. Firstly, autoantibodies induce tumor cell death directly, which includes blockade of growth factor receptor signaling, as well as ligand binding blockage that induces tumor cell apoptosis. Secondly, autoantibodies induce tumor cell death indirectly by engaging components from the host immune system, which include antibody-dependent cellular cytotoxicity, complement-dependent cytotoxicity, and antibody-dependent cellular phagocytosis (156). Thirdly, neutralization of harmful tumor-specific antigens and/or overexpressed tumor-associated antigens may occur. Finally, delivery of chemotherapy or radiotherapy specifically to cancer cells, but not to healthy cells and tissues, can be mediated with less side effects (157). There are now many potential autoantibodies for use in diagnosis and therapy of various cancers. For instance, there are around 100 autoantibodies with possible utility for lung cancer diagnosis and therapy (48, 146, 158, 159). Table 2 summarizes autoantibody targets which have been approved by the EU and US FDA for cancer diagnostics and therapeutics.

The advantages and principles of using the anti-ID antibodies in treatment were stated previously. Racotumomab (Vaxira) is the first approved (approved only in Cuba and Argentina) anti-ID antibody therapeutic vaccine. Racotumomab, which is well tolerated by patients, has successfully increased the overall survival rate of Non-Small Cell Lung Cancer patients in recurrent or advanced stages (161).

TABLE 2 Summary of EU and/or US FDA-approved autoantibody targets for cancer diagnosis and of autoantibodies/autoantibody-derived antibodies for cancer treatment .

Cancer Type	Autoantibody targets for diagnosis	Treatment autoantibodies or autoantibody-derived antibody drug conjugates (ADC) (Antibody name/target/type)
Breast cancer	HER2/neu, CA27-29, and CA15-3 (Mucin-1 [MUC1])	Margetuximab/HER2/Chimeric IgG; Atezolizumab/PD-L1/Humanized IgG1; Ado-trastuzumab emtansine/HER2/Humanized IgG ADC; Pertuzumab/HER2/Humanized IgG1; Trastuzumab emtansine/HER2/ Humanized IgG1; [fam]-trastuzumab deruxtecan/HER2/ Humanized IgG1 ADC; Margetuximab/HER2/Chimeric IgG1; Sacituzumab govitecan/TROP2/Humanized IgG1 ADC.
Lung cancer		Atezolizumab/PD-L1/Humanized IgG1; Bevacizumab/VEGF/Humanized IgG1; Necitumumab/EGFR/recombinant human IgG1; Nivolumab/PD-1/Human IgG4; Pembrolizumab/PD-1/Humanized IgG4.
Bladder cancer	Nuclear Mitotic Apparatus protein (NuMA, NMP22)	Atezolizumab/PD-L1/Humanized IgG1; Durvalumab/PD-L1/Human IgG1; Enfortumab/vedotin Nectin-4/human IgG1.
Colorectal cancer	carcinoembryonic antigen (CEA)	Bevacizumab/VEGF/Humanized IgG1; Cetuximab/EGFR/Chimeric IgG1; Edrecolomab/EpCAM/Murine IgG2a; Panitumumab/EGFR/Human IgG2; Nivolumab/PD-1/Human IgG4; Ramucirumab/VEGFR2/Human IgG1.
Renal / kidney cancer		Bevacizumab/VEGF/Humanized IgG1; Ipilimumab/CTLA-4/Human IgG1; Nivolumab/PD-1/Human IgG4.
Ovarian cancer	CA125 (MUC16), ROMA (HE4+CA-125), OVA1 (multiple proteins), HE4	Bevacizumab/VEGF/Humanized IgG1.
Multiple Myeloma		Belantamab mafodotin/BCMA/Humanized IgG1 ADC; Daratumumab/CD38/Human IgG1; Elotuzumab/SLAMF7/Humanized IgG1; Isatuximab/CD38/Chimeric IgG1.
Melanoma		Ipilimumab/CTLA-4/Human IgG1; Nivolumab/PD-1/Human IgG4; Pembrolizumab/PD-1/Humanized IgG4; Tebentafusp/gp100 CD3/Bispecific immunoconjugate (TCR-scFv).
Lymphoma		Loncastuximab tesirine/CD19/Humanized IgG1 ADC; Tafasitamab/CD19/Humanized IgG1 ; Mogamulizumab/(T cell) CCR4/ Humanized IgG1; Rituximab/(B cell) CD20/Chimeric IgG1; Brentuximab vedotin/CD30/Chimeric IgG1 ADC; Polatuzumab vedotin/CD79B/Humanized IgG1 ADC; Ibritumomab tiuxetan/CD20/Murine IgG1; Iodine (I131) tositumomab/CD20/Murine IgG2a; Pembrolizumab/PD-1/Humanized IgG4.
Leukemia		Moxetumomab pasudotox/CD22/Murine IgG1 dsFv-immunotoxin; Obinutuzumab/CD20/Humanized IgG1; Ofatumumab/CD20/ Human IgG1; Glycoengineered Blinatumomab/CD19, CD3/Murine bispecific tandem scFv; Alemtuzumab/CD52/Humanized IgG1; Inotuzumab ozogamicin/CD22/recombinant humanised IgG4 ADC; Gemtuzumab ozogamicin/CD33/Humanized IgG4 ADC.
Sarcoma		Olaratumab/PDGFRα/Human IgG1.
Gastric cancer		Ramucirumab/VEGFR2/Human IgG1.
Cervical cancer		Tisotumab vedotin/Tissue factor/Human IgG1 ADC; Pembrolizumab/PD-1/Humanized IgG4.
Pancreatic cancer	CA19-9	LYT-200/galectin-9/human IgG4.
Prostate cancer	PSA	Evaluating Panitumumab/(ABX-EGF) EGFr/human IgG2.
General cancer type	Carcino-embryonic antigen	

This table is derived from a combination of reports (156, 157, 160).

How could autoantibody utilization aid the treatment of COVID-19?

Certain autoantibodies (e.g. anti-type I IFNs autoantibody) could drive severe and long COVID-19. These 'harmful' autoantibodies should be determined at an early stage following diagnosis of COVID-19 infection to predict the severity and possible long-term effects of infection, thus, hopefully enabling more effective therapy. Anti-cytokine autoantibodies (e.g., antibodies against IFNα, IFNϵ, IL-6, IL-22, GM-CSF and TNFα) may also provide a potential target for COVID-19 treatment. Troya et al. (65) analyzed clinical data from COVID-19 patients receiving subcutaneous IFN-beta-1b treatment from March to May 2020, at the Infanta Leonor University Hospital in Madrid, Spain. However, no improved clinical outcomes were observed. It was suggested that IFN-beta

treatment was given too late, after two weeks of symptoms. Therefore, an earlier, ambulatory IFN-beta treatment appears to be required (162).

Cytokines play an important role in protection of the host against bacterial and viral (including COVID-19) infections. However, an over-activated immune response may cause an acute inflammatory reaction called a 'cytokine storm' (acute overproduction and uncontrolled release of pro-inflammatory cytokines), leading to multiple organ dysfunction. This is quite common (ca 21%) in COVID-19-infected pneumonia patients (163–165). Therefore, autoantibodies could be employed for COVID-19 treatment. The therapeutic functions of monoclonal neutralization antibodies against IL-6 and GM-CSF have been reported. Temesgen et al. (166) successfully used an anti-human GM-CSF monoclonal antibody for the treatment of patients with severe COVID-19 pneumonia, which proved to be safe and effective, with improved clinical outcomes, as well as a reduced cytokine storm. Moreover, an anti-IL-6 monoclonal antibody decreased IL-6 levels, which lead to the reduction of the inflammatory process in COVID-19 patients with severe respiratory disease. Therefore, there is high potential to use anti-IL-6 neutralization antibody for prevention of a cytokine storm and death caused by it (167). Similarly, anti-ID antibodies, which showed significant value for autoimmune disease treatment could be used in the same way.

Autoantibody-triggered autoimmune responses are often associated with severe and long COVID-19. Therefore, anti-ID antibodies of autoantibody targets may also have potential in COVID-19 treatment (122, 168, 169).

Conclusions and future trends

Autoantibodies have various roles and can be exploited as enemies, as well as friends, capable of doing harm and good. The levels and stability of autoantibodies can cause challenges (e.g. in autoimmune disorders and long COVID-19), but also enable potentially better and more reliable diagnosis.

Overall, in order to take full advantage of autoantibodies, and avoid/limit their negative aspects, more research is required. Luckily, more and more mature and advanced technologies will aid research on autoantibodies, for instance, protein arrays and use of anti-ID antibodies (119, 169).

The use of protein arrays for autoantibody detection offers advantages including high multiplexing capacity, availability of multiple detection systems, well-established quality control procedures, small sample volume requirements, high sensitivity, good dynamic ranges and rapid generation of results. Challenges include the need to detect autoantibodies at highly variable concentrations, issues with effective immobilization of proteins/antigens depending on their characteristics, epitope availability and stability and the need to identify appropriate sets of targets with the required sensitivity and specificity (170). The availability of artificial intelligence (AI) and other approaches for processing results from multiple analytical determinations from many patient cohorts and controls should also provide enhanced discrimination for diagnosis and follow-up. Linking autoantibody determination with genomics analysis should provide opportunities for precision health for greatly improved patient welfare, but the associated analysis may be complex (171).

Author contributions

HM and RO'K contributed to the conception and design of the work; HM drafted the work; CM, CL and RO'K revised it critically for important intellectual content and provided approval for publication of the content. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by Science Foundation Ireland, grant number 14/IA/2646.

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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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 25 October 2022
DOI 10.3389/fimmu.2022.1008146

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Autoimmune and Autoinflammatory Disorders: Autoinflammatory Disorders, a section of the journal Frontiers in Immunology

RECEIVED 31 July 2022
ACCEPTED 11 October 2022
PUBLISHED 25 October 2022

CITATION
Huang J, Zhang J, Wang F, Zhang B and Tang X (2022) Comprehensive analysis of cuproptosis-related genes in immune infiltration and diagnosis in ulcerative colitis. *Front. Immunol.* 13:1008146. doi: 10.3389/fimmu.2022.1008146

Comprehensive analysis of cuproptosis-related genes in immune infiltration and diagnosis in ulcerative colitis

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Objectives: Cuproptosis is a recently discovered form of programmed cell death; however, its role in ulcerative colitis (UC) remains a void.

Methods: Three gene expression profiles were acquired from the GEO database. Subsequently, the single sample gene set enrichment analysis (ssGSEA) was performed to identify the immune infiltration characteristics of UC. Correlation analysis between cuproptosis and immune infiltration was further conducted, and the cuproptosis-related genes were applied to construct a UC diagnostic model. Subsequently, analysis results of microarray data were experimentally validated by DSS-induced colitis in mice. Finally, therapeutic agents for the cuproptosis-related genes were screened owing to the gaping field of therapeutic agents on cuproptosis.

Results: Three gene expression profiles with 343 samples (290 UC and 53 healthy samples) were included. Immune infiltration revealed that UC patients had a higher level of DCs, B cells, $CD8^+$ T cells, iDCs, Macrophages, neutrophils, pDCs, T helper cells, Tfh, Th1 cells, Th2 cells, TIL and Treg than normal subjects. Moreover, almost all cuproptosis-related genes were significantly negatively associated with immune infiltration in UC patients. The risk prediction model based on cuproptosis-related genes showed an excellent discrimination for UC. Animal experiments revealed significant alterations in genes essential for cuproptosis between DSS-induced colitis mice and healthy controls, providing experimental validation for the analysis results of microarray data. Further analysis revealed that latamoxef, vitinoin, clomipramine, chlorzoxazone, glibenclamide, pyruvic acid, clindamycin, medrysone, caspan, and flavin adenine dinucleotide might be the target agents for cuproptosis-related genes.

Conclusions: In conclusion, cuproptosis was significantly associated with immune infiltration in UC, and the cuproptosis-related genes showed an excellent discrimination for UC.

KEYWORDS

Ulcerative colitis, cuproptosis, genes, immune infiltration, diagnosis

1 Introduction

Ulcerative colitis (UC) is a complex disease characterized by chronic inflammation of the colon (1). Worldwide, UC is estimated to affect 9-12/100,000 people annually, and the incidence is increasing year by year (2). The growing number of UC patients places a heavy economic burden on society, with direct and indirect costs associated with UC of $8.1 – $14.9 billion per year in the United States and €12.5 – 29.1 billion in Europe (3). The treatment goal in UC is the induction and maintenance of remission. Although therapeutic tools are expanding, the treatment of UC is highly challenging because of its incompletely understood pathogenesis (4). Therefore, an in-depth understanding of disease pathogenesis and identification of biomarkers of disease progression at the molecular level may provide new ideas for the early diagnosis of UC.

It is reported that various types of cell death are closely related to UC, and the regulation of cell death is an important strategy for its treatment (5). Recently, a novel copper-dependent cell death mediated by proteolipid acylation has been identified and termed "cuproptosis" (6). As a metal trace, copper is involved in a variety of physiological activities in living organisms and is essential for the maintenance of normal biological activities (7, 8). Copper deficiency impairs the function of copper-binding enzymes, and cell death can be induced by excess copper (9). Excess intracellular copper has been reported to bind directly to the lipid acylated components of the tricarboxylic acid cycle, leading to lipoylated protein aggregation and subsequent iron-sulfur cluster protein loss (6). This process leads to proteotoxic stress and ultimately to cuproptosis (Figure 1) (6). In addition, FDX1 and protein lipoylation are identified as the key regulators of copper ionophore induced cell death (6).

Accumulating evidence revealed that significant abnormalities in copper metabolism were present in UC patients and were strongly associated with its development (10–13). Furthermore, copper-containing metabolic structural domain 1 suppresses genes that promote inflammation and protects mice from colitis and colitis-associated cancers (14). Similarly, copper-mediated oxidation of mesalazine, a pro-oxidant interaction through a copper redox cycle mechanism, may exert anti-cancer effects in patients with ulcerative colitis (15). Moreover, cumulative evidence indicated that copper has also been involved in the regulation of the immune system (16–18). All these findings suggest an important role of copper in UC. However, as a newly identified form of regulated cell death, the role of cuproptosis in the pathogenesis, development, and immune system of UC remains a void, and its potential in being the therapeutic target for UC is far to be understood. Therefore, we hypothesized that cuproptosis was involved in UC and that cuproptosis-related genes may contribute to the early diagnosis and treatment of UC. The workflow of the present study is shown in Figure 2.

2 Materials and methods

2.1 Microarray data acquisition

Gene expression profiles were acquired from GEO database (www.ncbi.nlm.nih.gov/geo/) with the following criteria: (a) patients were diagnosed as UC; (b) data on colonic tissue from healthy controls and UC patients from the same GEO platform; (c) inclusion of datasets with at least 10 UC and healthy tissue samples; (d)) GEO platforms containing >5000 genes. Finally, three gene expression profiles (GSE87473, GSE92415, and GSE 75214) were included (Table 1). The batch effects among these datasets were eliminated by applying the combat algorithm in the "sva" R package (https://www.bioconductor.org/packages/release/bioc/html/sva.html). The "sva" package can remove batch effects in three ways: (1) identifying and estimating surrogate variables for unknown sources of variation in high-throughput experiments (19), (2) removing known batch effects directly using ComBat (20) and (3) removing batch effects with known control probes (21). Removal of batch effects and use of surrogate variables in differential expression analysis has been shown to reduce dependence, stabilize error rate estimates, and improve reproducibility.

Furthermore, cuproptosis-related genes (FDX1, LIPT1, LIAS, DLD, DBT, GCSH, DLST, DLAT, SLC31A1, PDHB, PDHA1, ATP7A, and ATP7B) were obtained from previous literature (16).

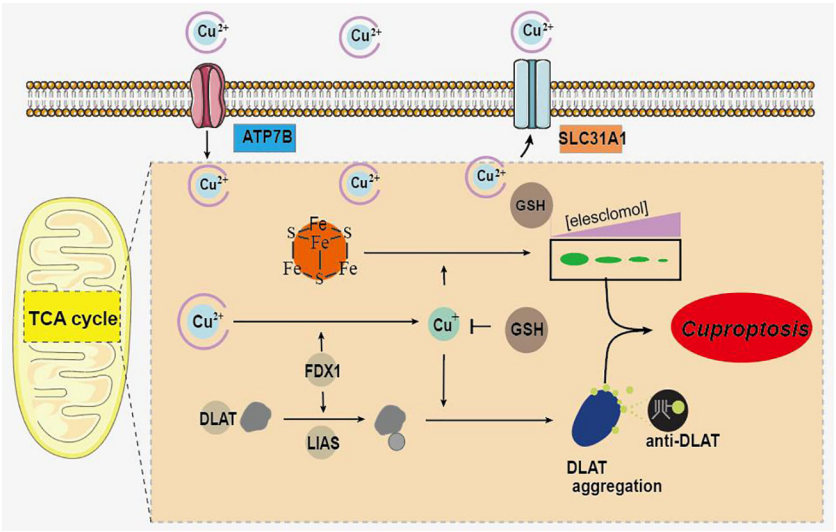

FIGURE 1
Schematic of mechanisms promoting cuproptosis.

2.2 Immune infiltration analysis

Single sample gene set enrichment analysis (ssGSEA) was performed to define the immune infiltration status between control and UC samples by calculating the normalized enrichment score. $P < 0.05$ was used to filter the samples. Heat map of immune infiltration in samples was produced by the "pheatmap" package (https://cran.r-project.org/web/packages/pheatmap/). Levels of immune cells and immune function between UC and control samples were visualized by the "vioplot" package (https://cran.r-project.org/web/packages/vioplot/).

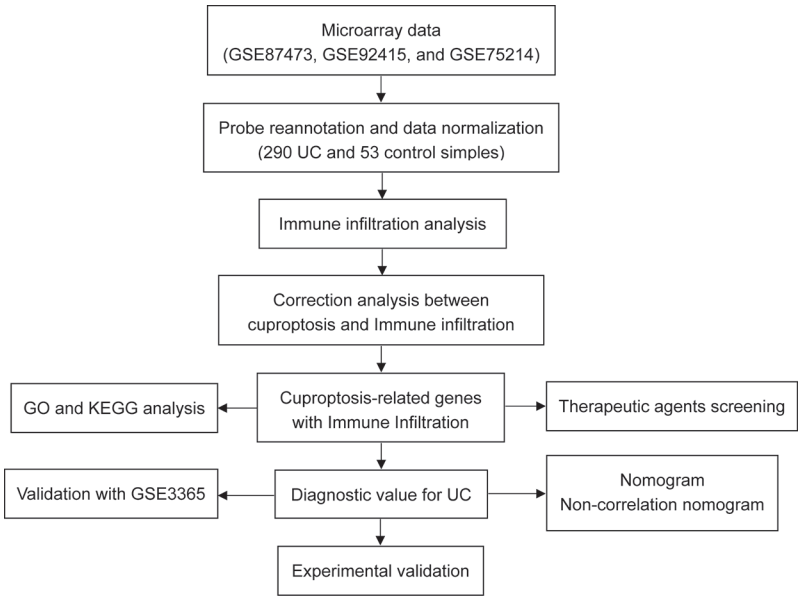

FIGURE 2
Flowchart of the present research.

TABLE 1 Details of microarray data.

Dataset	Platform	Tissue	UC	Normal	Reference (PMID)
GSE87473	GPL13158	Colon	106	21	29401083
GSE75214	GPL6244	Colon	97	11	28885228
GSE92415	GPL13158	Colon	87	21	23735746

2.3 Correlation analysis between immune infiltration and cuproptosis-related genes

The correlation between the cuproptosis-related genes and immune infiltration in UC was evaluated by the "psych" (https://cran.r-project.org/web/packages/psych/) and "ggcorrplot" (https://cran.r-project.org/web/packages/ggcorrplot/) packages. The "ggplot2" package (https://sourceforge.net/projects/ggplot2.mirror/) was performed to analyze results and $p < 0.05$ was considered significant.

2.4.The construction of risk prediction model

After the feature selection, the cuproptosis-related genes most strongly associated with immune infiltration were used to construct risk prediction model for UC with "rms" package (https://cran.r-project.org/web/packages/rms/). The prediction performance of the model was quantified by nomogram, non-correlated nomogram, and ROC curve. ROC-AUC ≥ 0.9 indicates outstanding discrimination. 0.8≤ ROC-AUC < 0.9 indicates excellent discrimination, 0.7 ≤ ROC-AUC < 0.8 indicates acceptable discrimination, and ROC = 0.5 indicates no discrimination (22). ROC analysis was performed with "ROCR" package (https://www.rdocumentation.org/packages/ROCR/).

2.5 Enrichment analysis for cuproptosis-related key genes

Enrichment analysis of GO and KEGG were performed for the cuproptosis-related genes in our model by the Enrichr (https://maayanlab.cloud/Enrichr/). Results of enrichment analysis were visualized with the "ggplot2" package (https://sourceforge.net/projects/ggplot2.mirror/).

2.6 Therapeutic agents screening for cuproptosis-related key genes

Therapeutic agents for the cuproptosis-related key genes were screened using Enrichr (https://maayanlab.cloud/Enrichr/). The threshold for enrichment analysis was set to p-value < 0.05.

2.7 Experimental validation

Twelve male C57BL/6J mice (18–22 g) were obtained from SPF Biotechnology Co., Ltd (Beijing, China). Before starting the experiment, animals were fed with free access to food and water for seven days to adapt to the environment. Animal experimental protocols were performed with the approval of the Animal Ethics Committee of Xi Yuan Hospital of China Academy of Chinese Medical Sciences (Approval NO. 2019XLC003-2).

Animals were randomly divided into control group and dextran sulfate sodium (DSS) group. To induce acute experimental colitis, mice in the DSS group were given 3.0% (w/v) DSS (Cat. No. 160110, MP Biomedicals) in the drinking water ad libitum for 7 days. Control group received regular diet and drinking water throughout the experimental period. Body weight, stool consistency, and rectal bleeding of all animals were recorded daily. At the end of the experiment, mice were sacrificed, the colonic tissues were quickly excised and measured for length, and then stored in the refrigerator at -80° C until use (Figure 8A).

2.7.1 Histological analysis

Colon tissue was fixed in 4% paraformaldehyde and then processed into paraffin-embedded tissue blocks to produce 5 μm-thick sections for hematoxylin and eosin (H&E) staining, in which a blinded colitis activity score, according to previous criteria (23), was given.

2.7.2 Immunohistochemistry

Immunohistochemistry (IHC) was performed with paraffin-embedded sections. Slices were incubated with ZO-1 (Cat. No. AF5145, Affinity Biosciences, 1:100), occludin (Cat. No. 27260-

1-AP, Proteintech, 1:200), claudin-1 (Cat. No. 28674-1-AP, Proteintech, 1:200), FDX1 (Cat. No. BS-11426R, Bioss, 1:400), LIAS (Cat. No. 11577-1-AP, Proteintech, 1:200) and DLAT (catalog no. 13426-1-AP, Proteintech, 1:300) antibody overnight at 4°C. Later, the slides used for IHC staining were incubated with the secondary antibody (Cat. No. GB23303, Servicebio, 1:200). The DAB chromogen was used for incubation, and then hematoxylin was used for counterstaining. Finally, images were acquired under a light microscope (Olympus BX41, Shanghai, China). Staining intensity was analyzed using Image Pro Plus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA).

2.7.3 Quantitative real-time PCR

Total RNA was extracted from colon tissue and reverse transcribed as cDNA template. Next, quantitative real-time PCR (qRT-PCR) was carried out with the CFX96 real-time PCR detection system (Bio-Rad, USA). The expression levels of target genes were analyzed with $2^{-\Delta\Delta CT}$, and the results were presented with GAPDH as an internal control. Primer sequences in this study are listed in Table 2.

2.8 Statistical analysis

Categorical variables are presented as percentages, while continuous variables are presented as the mean ± standard deviation. For bioinformatics analysis, the R software (Version 4.1.2, https://www.r-project.org/) was used for all data analysis in the present study. For experimental validation, student's t-test was applied for comparisons between two groups with Prism GraphPad software (Version 7.04, https://www.graphpad.com/scientific-software/prism/). A value of $p < 0.05$ indicates statistically significant difference.

3 Results

3.1 Immune infiltration analysis for UC

The normalized enrichment score of immune infiltrates is presented in the heat map (Figure 3). The results of differential analysis of immune cell infiltration revealed that UC patients had a higher level of aDCs, B cells, $CD8^+$ T cells, DCs, iDCs, macrophages, neutrophils, pDCs, T helper cells, Tfh, Th1 cells, Th2 cells, TIL and Treg than healthy subjects (Figure 4A). Moreover, a significant higher level of all immune function subtypes was observed in UC patients (Figure 4B).

3.2 Correlation analysis between immune infiltration and cuproptosis-related genes

Results of Pearson correlation analysis revealed that all cuproptosis-related genes were significantly negatively associated with almost all immune cell subtypes and immune function subtypes (except mast cells). PDHB, PDHA1, LIAS, FDX1, DLD, DLAT, DBT, and ATP7B were negatively associated with more than 24 immune cell subpopulations and immune function subtypes (Figure 5).

3.3 Construction of risk prediction model

Inspired by previous results, PDHB, PDHA1, LIAS, FDX1, DLD, DLAT, DBT, and ATP7B were used to construct a risk prediction model for UC. Coefficients of the cuproptosis-related genes in this model are presented in Table 3. The clinical information and genetic characteristics of UC patients were integrated to develop nomogram. Results showed that the constructed model with these predicted diagnostic genes had diagnostic efficacy for UC (Figure 6A). Based on the calibration curve predicted by the uncorrelated nomogram, the performance of the column line plot was close to the ideal model, suggesting that the predictive value of the model is reliable (Figure 6B). Similarly, ROC-AUC of the risk score was 0.889, that is an indicative of excellent model discrimination (Figure 6C). Furthermore, the microarray data of GSE3365 68 clinical simples (26 UC patients and 42 healthy controls) were used to verify the robustness of the model. Results revealed that ROC-AUC of the risk score was 0.857, indicating excellent model discrimination (Figure 6D).

TABLE 2 The primer sequences were used in this study.

Genes	Forward primer	Reverse primer
FDX1	ACAGACAGGAACCTGGAAGACC	GAGACAATCTGTATGGGGTGGTT
LIAS	CGTTAAGACCGCAAGAAATCC	CCACATCATCTCGATCCACC
DLAT	TCACAGACATCCCCATCAGCA	TTAAGTTCCTTCCGTACCAACAG
GAPDH	CCTCGTCCCGTAGACAAAATG	TGAGGTCAATGAAGGGGTCGT

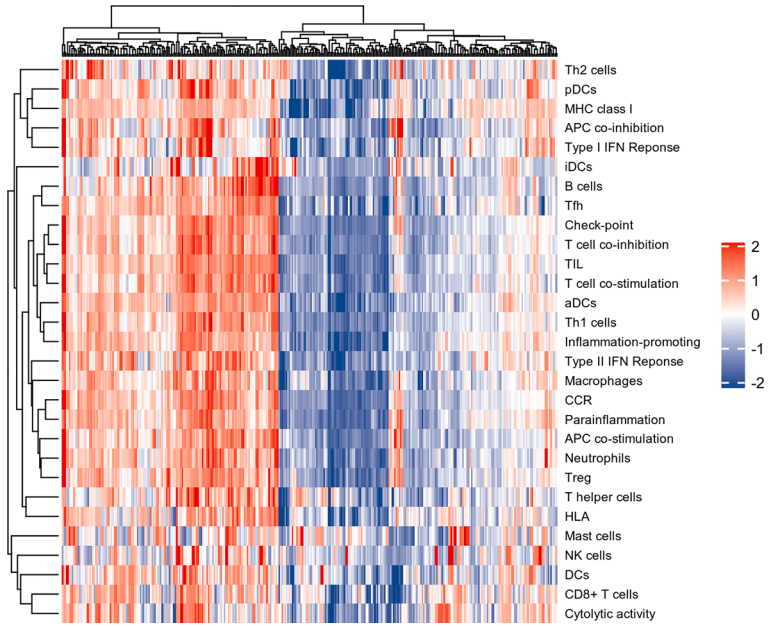

FIGURE 3
The heat map of Immune infiltration. Each row represents one sample, either normal or UC, each column represents a type of immune cell or immune function.

3.4 Enrichment analysis for cuproptosis-related key genes

Further, enrichment analysis for cuproptosis-related key genes in this model was performed. GO analysis revealed that these genes were significantly enriched in many metabolic pathways, such as: acetyl-CoA, pyruvate, monocarboxylic acid, branched-chain amino acid, cellular amino acid, copper ion transport and serine amino acid family (Figure 7). For KEGG analysis, genes were significantly enriched in citrate cycle (TCA cycle), HIF-1 signaling pathway,

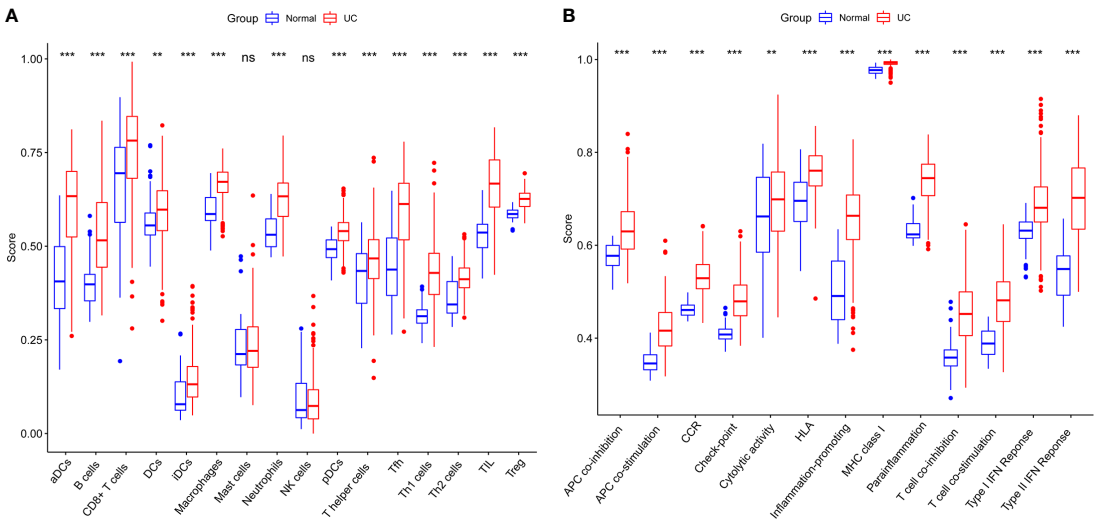

FIGURE 4
(A) Comparison of 16 immune cell subtypes between patients with UC and controls. **(B)** Comparison of 13 immune function subtypes between patients with UC and controls. *p < 0.05 compared to the normal, **p < 0.01 compared to the normal, ***p < 0.001 compared to the normal, ^{ns}p > 0.05 compared to the normal.

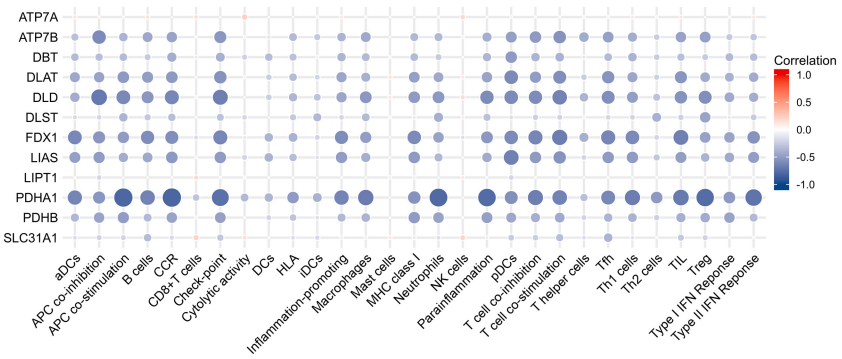

FIGURE 5
Correlation analysis between immune infiltration and cuproptosis-related genes.

pyruvate metabolism, glycolysis/gluconeogenesis propanoate metabolism, valine, glucagon signaling pathway, leucine and isoleucine degradation, diabetic cardiomyopathy, glyoxylate and dicarboxylate metabolism, and central carbon metabolism in cancer (Figure 7).

3.5 Therapeutic agents screening for cuproptosis-related key genes

Therapeutic agents for the cuproptosis-related genes were screened using Enrichr. And the predicted results suggested that latamoxef, vitinoin, clomipramine, chlorzoxazone, glibenclamide, pyruvic acid, clindamycin, medrysone, caspan, and flavin adenine dinucleotide might be the target agents for cuproptosis-related genes (Table 4).

3.6 Altered expression of cuproptosis-related genes in DSS-induced colitis

Compared to control group, the body weight and colon length of mice in DSS group were significantly decreased, while DAI score increased significantly (Figures 8B–E). In addition, disappearance of crypt glands, mucosal damage and inflammatory cell infiltration were observed in the colonic tissue of mice with colitis, while in the control group the colonic mucosa was intact, and the crypt structure was clear (Figures 8F, G). The IHC analysis results revealed that positive expression of ZO-1, claudin-1, and occludin were observed in the control group, however, they were significantly inhibited in the DSS group (Figures 8H, I). Taken together, these results indicate the successful establishment of an acute experimental colitis model.

To further explore the role of cuproptosis in UC, genes that are essential for cuproptosis (6) were evaluated. The IHC analysis results revealed that positive expression of FDX1, LIAS and DLAT were observed in the control group, however, they were significantly inhibited in the DSS group (Figures 9A, B). Furthermore, the results of qRT-PCR (Figure 9C) revealed that the mRNA levels of FDX1, LIAS and DLAT were significantly altered in the DSS group compared to control group. Taken together, these results indicate that the

TABLE 3 Coefficients of the cuproptosis-related genes in modle.

Genes	Estimate	Std. Error	z value	Pr (>|z|)
ATP7B	-0.08443	0.45697	-0.185	0.853423
DBT	-0.60574	0.41556	-1.458	0.144944
DLAT	-0.96232	0.50883	-1.891	0.058593
DLDH	0.06694	0.53848	0.124	0.901073
FDX1	-2.34911	0.64954	-3.617	0.000298
LIAS	1.13356	0.51425	2.204	0.027505
PDHA1	-2.34070	0.61410	-3.812	0.000138
PDHB	-0.99644	0.46688	-2.134	0.032823

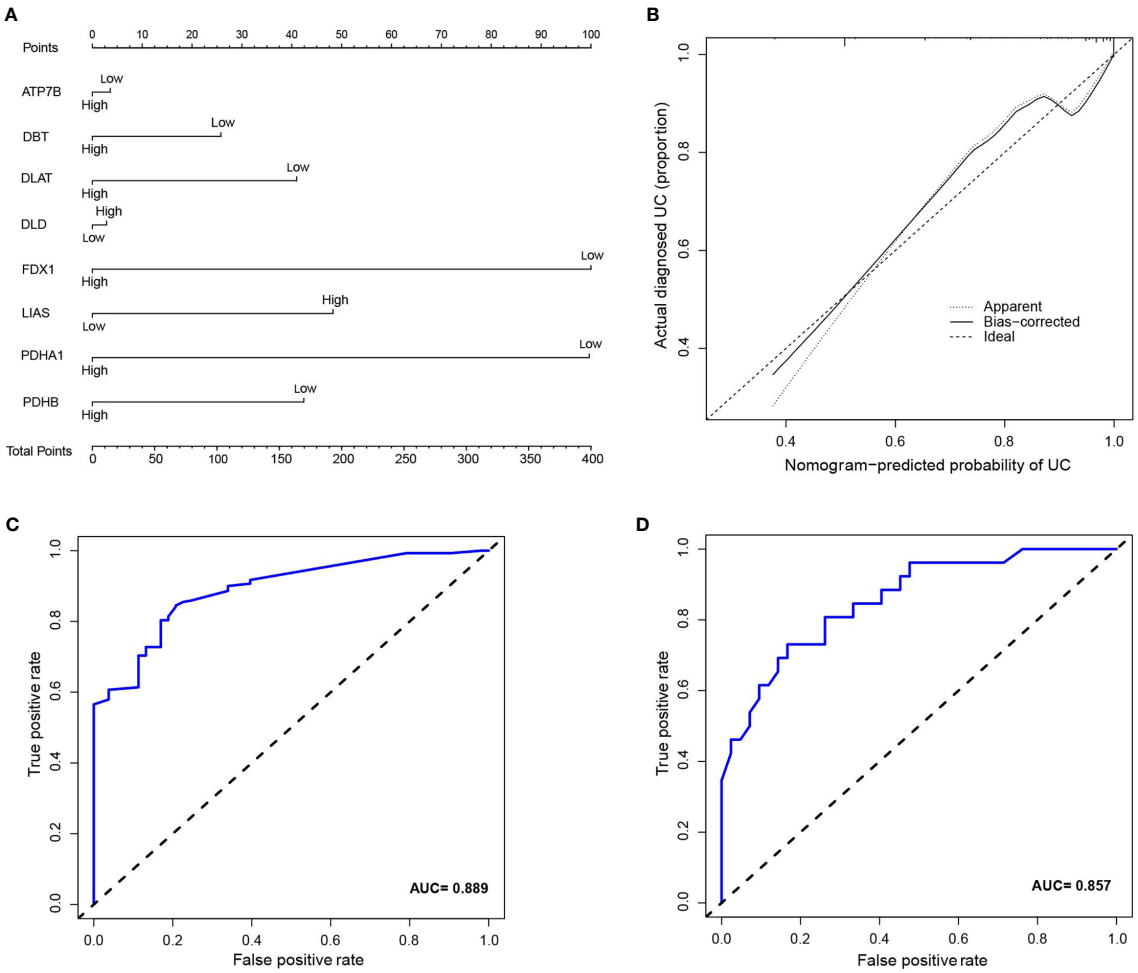

FIGURE 6
(A) nomogram of diagnostic marker genes. **(B)** Calibration curve of non-correlation nomogram prediction in the cohort. **(C)** ROC curve for the diagnostic efficacy of diagnostic model. **(D)** ROC curves analysis of test set in GSE3365.

cuproptosis-related genes presented well discrimination for UC, which validate the analysis results of microarray data.

4 Discussion

Copper metabolism plays an important role in gastrointestinal disorders. Excessive storage of copper may cause intestinal cell damage and even lead to pathological diseases. Cuproptosis is a novel kind of regulation of cell death that differs from other forms of regulation of cell death such as necroptosis, pyroptosis, and ferroptosis (Table 5) (6). In contrast to healthy individuals, UC patients showed copper accumulation (10–12). Regulation of cell death plays an important role in many pathological and physiological processes, including inflammatory bowel disease (24). To date, what role cuproptosis plays in UC has not been explored.

UC is a chronic inflammatory bowel disease characterized by a dysregulated mucosal immune response. GSEA is a method to analyze gene sets in groups and can identify differential gene expression profiles among different phenotypes and groups. Whereas ssGSEA is an extension of GSEA to calculate separate enrichment scores for each pairing of a sample and gene set (25). In this way, ssGSEA converts the gene expression profiles of individual samples into gene set enrichment profiles. By defining immune cell and immune function-related gene sets, the enrichment score of the gene set can represent the immune cell and immune function characteristics of each sample (25). This transformation enables researchers to characterize the immune infiltrate profile of individual samples rather than by flow cytometry or immunohistochemistry (26). Developed and validated with microarray data, CIBERSORT is a method for characterizing the cellular composition of complex tissues from their gene expression profiles (27). CIBERSORT requires Gaussian distribution data,

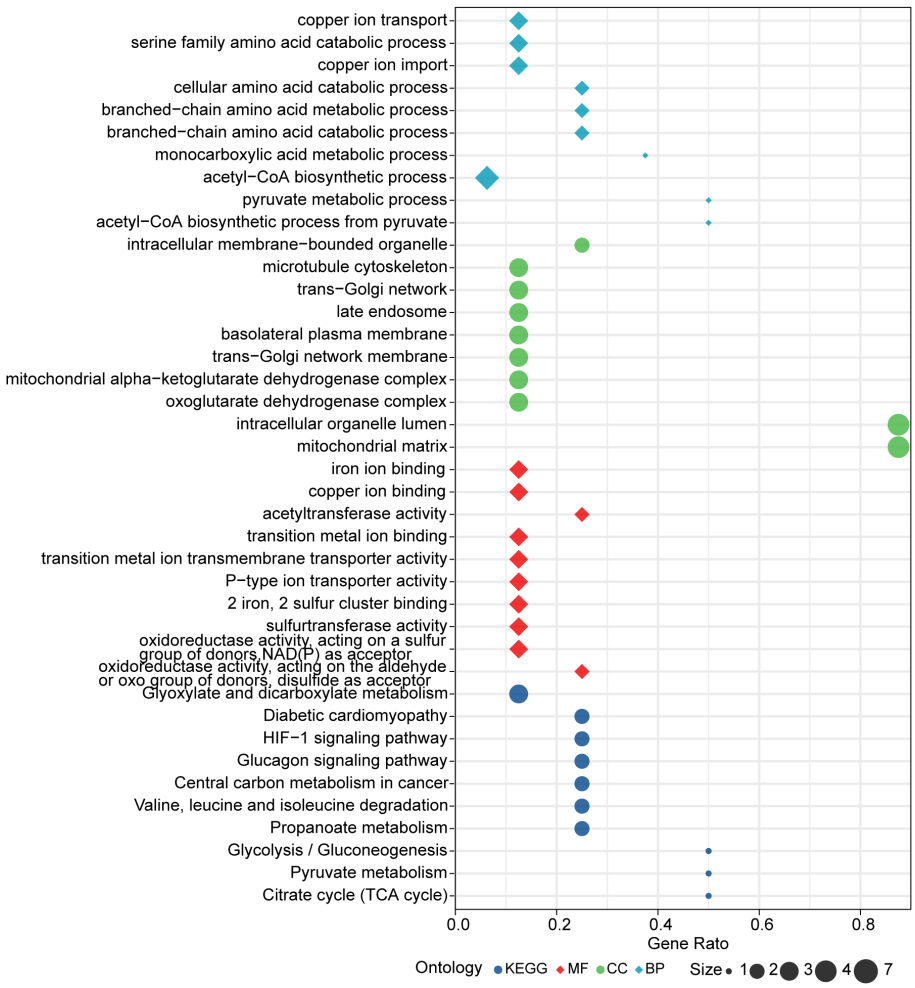

FIGURE 7
Results of GO and KEGG enrichment analysis.

whereas unnormalized RNA-seq counts are negatively binomially distributed (27). Hence, RNA sequencing data must be converted to "microarray-like" data when analyzed before it can be used for subsequent analysis (28). Interestingly, ssGSEA does not require secondary transformation of the data when analyzing RNA sequencing data (26). In addition, ssGSEA is based on the actual analysis of immune cells and immune function-related genes and is not limited by immune cells and immune function types (25), whereas CIBERSORT can only evaluate a fixed set of 22 cell types (27). Therefore, the ssGSEA method was used to quantify the enrichment scores of immune cells and immune functions for each specimen in the present study.

We hypothesized that cuproptosis was involved in UC and that cuproptosis-related genes may contribute to the early diagnosis and treatment of UC. In the present study, microarray data was applied to reveal the potential significance of cuproptosis in the UC disease process. First, the immune infiltration characteristics of UC were identified, and the results revealed a significant difference in immune infiltration between the colonic tissue of UC patients and the normal group. This finding implies that there may be excessive survival and proliferation of multiple immune cells in UC patients, further mediating inflammation and barrier disruption in the intestine. Second, the association between cuproptosis-related genes and UC pathological states was explored, and the results suggested that cuproptosis-related genes were closely associated with immune infiltration in UC. We next identified key cuproptosis-related genes based on their degree of association with UC immune infiltration and used them to construct a risk prediction model for UC, which was found to have excellent discrimination. These results suggest that cuproptosis may be involved in UC, and the cuproptosis-related genes may serve as diagnostic biomarkers for UC. Furthermore, after successfully inducing experimental colitis in mice, genes that are essential for

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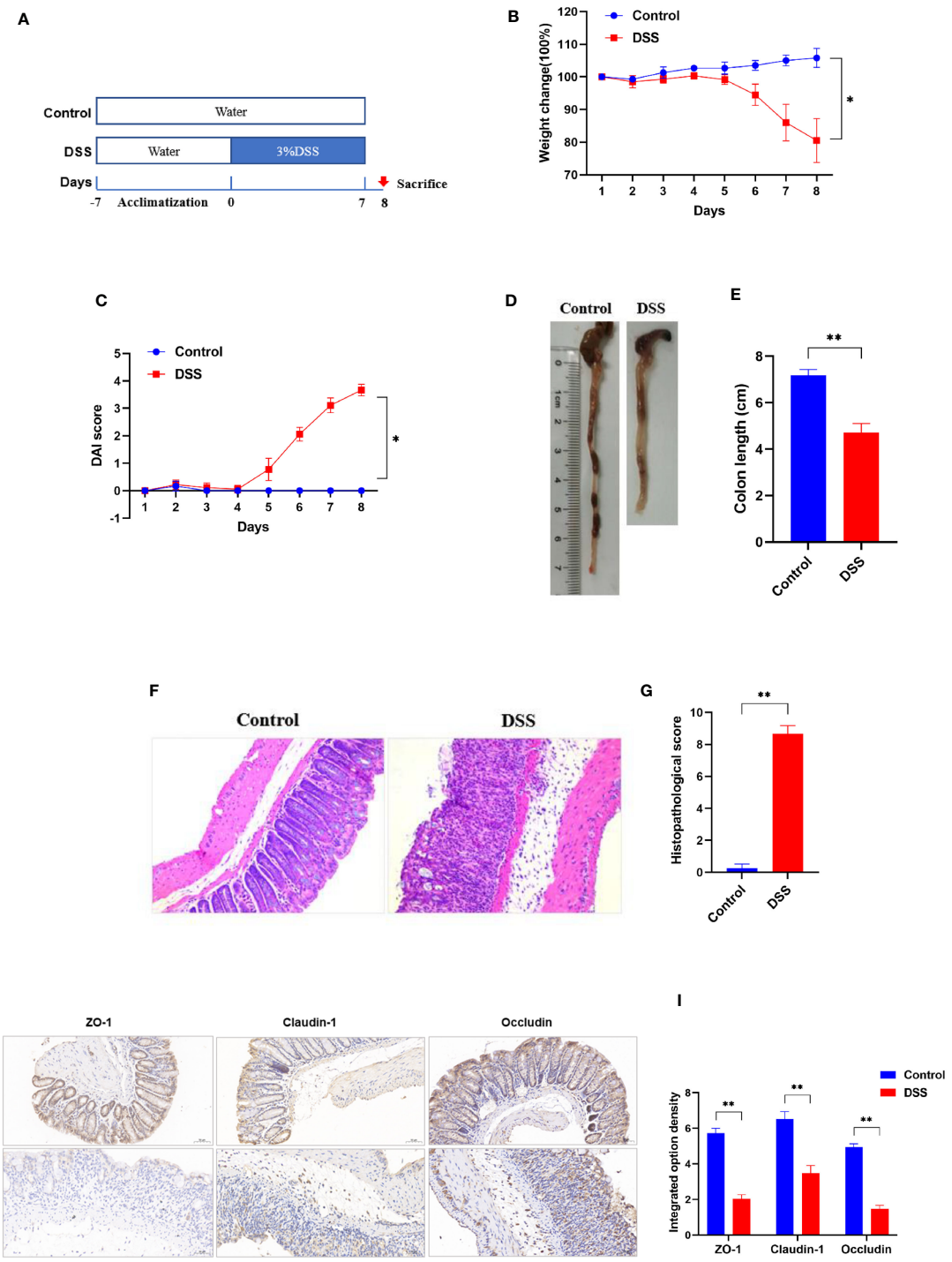

FIGURE 8
Successful establishment of DSS-induced colitis *in vivo*. **(A)** Schematic overview of DSS-induced colitis mice. **(B)** Daily changes in body weight and **(C)** DAI score in different groups (n = 6). **(D)** Representative colon pictures of mice from different groups and the comparison of colon length (n = 6). **(E)** Statistics of colon length. **(F)** Representative histopathological images of mice colon sections and histopathological scores (200× magnification, n = 6). **(G)** The histopathological score. **(H)** Representative images of immunohistochemical staining of ZO-1, claudin-1, and occludin in colonic tissues (200× magnification, n = 3). **(I)** The integrated option density was used in order to quantify ZO-1, claudin-1, and occludin proteins. *p < 0.05 compared to the control, **p < 0.005 compared to the control.

10.3389/fimmu.2022.1008146

TABLE 4 Therapeutic agents for cuproptosis-related genes.

Term	*p*-value	Combined Score	Genes
latamoxef	1.39E-04	173.328	PDHA1, FDX10, DBT, DLAT, LIAS
Vitinoin	1.42E-04	219.439	PDHA1, DBT, PDHB, DLD
clomipramine	7.92E-04	446.560	DBT, LIAS
chlorzoxazone	9.66E-04	101.323	PDHA1, FDX1, DLAT, DLD
glibenclamide	0.001	95.586	PDHA1, FDX1, DLAT, DLD
Pyruvic acid	0.002	270.716	LIAS, DLD
clindamycin	0.002	68.639	PDHA1, FDX1, DLAT, LIAS
medrysone	0.003	90.444	FDX1, DLAT, LIAS
Caspan	0.003	82.339	ATP7B, DBT, DLAT
flavin adenine dinucleotide	0.004	1549.437	DLD

cuproptosis were evaluated using qRT-PCR and IHC, which revealed that the levels of FDX1, LIAS and DLAT in colonic tissues were significantly altered in the DSS group compared to control group, validating the analysis results of microarray data. These results all indicate that cuproptosis may be involved in UC and that its related genes presented well discrimination for UC.

The tricarboxylic acid cycle provides carbon for biosynthesis and a reducing agent for ATP production, which is essential for oxidative metabolism (29). Cuproptosis depends on copper binding to components of the tricarboxylic acid cycle and subsequent disruption of normal respiratory function of mitochondria (6). Thus, the tricarboxylic acid cycle is crucial for the regulation of cuproptosis. A markedly different profile of tricarboxylic acid cycle-related molecules has been found in colonic lesion tissue and serum of UC patients compared to healthy volunteers (30). D-2-hydroxyglutaric acid (D2HG) is a

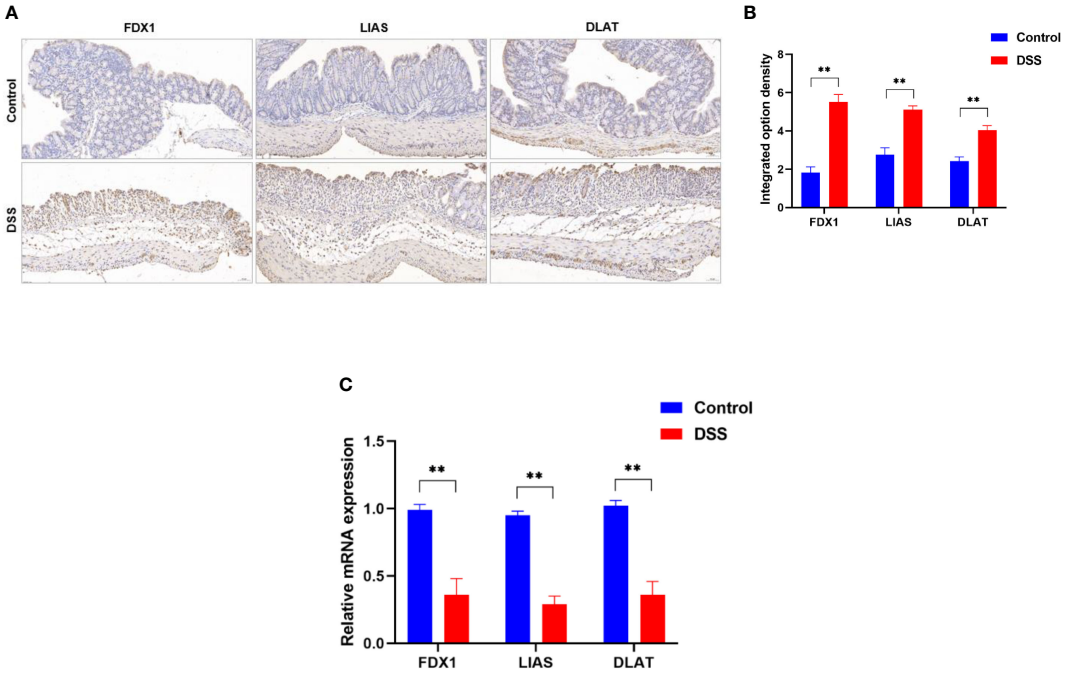

FIGURE 9
Altered expression of cuproptosis-related genes in DSS-induced colitis. **(A)** representative images of immunohistochemical staining of FDX1, LIAS, and DLAT in colonic tissues (200× magnification, n = 3). **(B)** The integrated option density was used in order to quantify FDX1, LIAS, and DLAT proteins. **(C)** FDX1, LIAS, and DLAT expression levels in colon tissues were detected by qRT-PCR (n = 6). **p < 0.005 compared to the control.

TABLE 5 Hallmarks of major types of regulation of cell death.

Type	Trigger factors	Morphological features	Representative inhibitors
Cuproptosis	Intracellular excess copper	Abnormal morphology of mitochondria	Tetrathiomolybdate
Alkaliptosis	Intracellular alkalinization	Necrosis-like morphology	N-acetyl alanine acid
Oxeiptosis	Accumulation of reactive oxygen species	Apoptosis-like morphology	N-Acetyl-L-cysteine
Autophagy-dependent cell death	Molecular machinery of autophagy	Autophagic vacuolization	Chloroquine
Ferroptosis	Intracellular iron accumulation	Smaller mitochondria; reduced mitochondria crista; elevated mitochondrial membrane densities	Ferrostatin-1
Parthanatos	Oxidative stress	Chromatin condensation; large DNA fragmentation	Iniparib
Entotic cell death	Activation of adhesion proteins and actomyosin	Cell-in-cell structure	C3-toxin
Immunogenic cell death	A relatively restricted set of stimuli	Not appliable	KIRA6
Necroptosis	Extracellular stimulation	Cell swelling; rupture of plasma membrane	Necrostatin-1
Netotic cell death	Formation of neutrophil extracellular traps	Plasma membrane rupture; nuclear membrane collapse; chromatin fibre release	Lactoferrin
Pyroptosis	Activation of inflammasome	Rupture of plasma membrane; bubbling	Ac-YVAD-cmk
Lysosome-dependent cell death	Release of lysosomal hydrolytic enzymes	Lysosome and plasma membrane rupture	CA-074Me
Apoptosis	Activation of caspases	Apoptotic body formation	Emricasan

product of the tricarboxylic acid cycle, which a positive correlation was observed between the level of D2HG in the urine of colitis-associated colon cancer mice and the number of subsequent polyps and severity of dysplasia (31). Furthermore, intravenous administration of D2HG resulted in delayed recovery from colitis and severe tumorigenesis in mice (31). Therefore, excess copper might disrupt the tricarboxylic acid cycle and subsequent mitochondrial respiration causing death of intestinal epithelial cells, thereby disrupting the intestinal mucosal barrier, and ultimately inducing UC.

PDHB, PDHA1, LIAS, FDX1, DLD, DLAT, DBT, and ATP7B were included in the diagnostic model. The further bioinformatic analysis of these cuproptosis-related genes revealed the enrichment of some cell death, metabolic and immune response-related pathways. Pathways, such as tricarboxylic acid cycle, Pyruvate metabolism, Valine, leucine and isoleucine degradation, and HIF-1 have been identified to be involved in UC (32–34). Hence, cuproptosis-related genes may regulate the pathological process of UC by mediating these classical pathways associated with cell death, metabolism, and immune response.

As a novel mode of copper-induced cell death, therapeutic agents targeting cuproptosis are a gaping field. Therefore, therapeutic agents for the cuproptosis-related genes were screened in the present study. The predicted results suggested that latamoxef, vitinoin, clomipramine, chlorzoxazone, glibenclamide, pyruvic acid, clindamycin, medrysone, caspan, and flavin adenine dinucleotide might be the target agents for cuproptosis-related genes. These therapeutic agents, such as latamoxef and clindamycin belong to antibiotics family. It is well known that microbial dysregulation has been increasingly appreciated in the pathogenesis of inflammatory bowel diseases (35, 36), and antibiotics used to modulate gut microbes and fecal microbiota transplantation have been considered to treat UC (37). However, it remains to be verified whether antibiotics or fecal microbiota transplantation can target cuproptosis-related genes.

To our knowledge, this is the first study to explore the role of cuproptosis in UC. Although encouraging results were found, limitations should be acknowledged. First, the clinical data for this study were obtained from public databases, and the clinical information of the samples was incomplete, such as the clinicopathological characteristics of the GSE series were not available. Second, the data used in this study was constructed on RNA sequences. Although animal experiments validated the results of bioinformatics analysis, their reproducibility and broad applicability still need to be validated with clinical samples in the future, as we were unable to obtain enough clinical samples of UC within a tight timeframe. Third, results of IHC suggested that the protein expression level of cuproptosis-related genes was significantly increased in DSS-induced colitis mice, while the mRNA expression level of cuproptosis-related genes was significantly decreased in DSS-induced colitis mice, suggesting a complex regulatory mechanism of cuproptosis in

UC. In this study, we could not explore the regulatory mechanism of copper death in UC within a tight timeframe, but further studies in the future are highly warranted. Fourth, almost all cuproptosis-related genes were significantly correlated with immune infiltration in UC, and these genes showed an excellent discrimination of UC, suggesting that cuproptosis may be a potential therapeutic target in UC and the predictive value of cuproptosis-related genes in the early diagnosis of UC. Admittedly, these findings provide new clues to investigate the role of cuproptosis in UC, however, more studies are still needed to confirm our findings.

5 Conclusion

In conclusion, our study revealed that cuproptosis was significantly associated with immune infiltration in UC, and cuproptosis-related genes showed an excellent discrimination for UC. Therefore, cuproptosis may be a therapeutic target for UC, and the model based on cuproptosis-related genes facilitates the early diagnosis of UC.

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 animals were reviewed and approved by the Animal Ethics Committee of Xi Yuan Hospital of China Academy of Chinese Medical Sciences (Approval NO. 2019XLC003-2).

Author contributions

JH and JZ contributed equally to this paper. JH drafted the manuscript. JZ, FW, and BZ helped with implementation of this work. XT contributed to the methodology, review, and editing of the manuscript. All authors read and approved the final manuscript.

Funding

This work was supported by the National Natural Science Foundation of China (No. 81830118), China Academy of Chinese Medical Sciences Innovation Fund (No. CI 2021A01012), China Academy of Chinese Medical Sciences Excellent Young Talent Cultivation Fund (No. ZZ 15-YQ-002), and Administration of Traditional Chinese Medicine Digestive Refractory Disease Inheritance and Innovation Team Project (No. ZYYCXTD-C-C202010).

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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frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 27 October 2022
DOI 10.3389/fimmu.2022.966766

OPEN ACCESS

EDITED BY
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SPECIALTY SECTION
This article was submitted to Autoimmune and Autoinflammatory Disorders,
a section of the journal
Frontiers in Immunology

RECEIVED 11 June 2022
ACCEPTED 20 September 2022
PUBLISHED 27 October 2022

CITATION
Jiao L and Guo S (2022) Anti-IL-6 therapies in central nervous system inflammatory demyelinating diseases.
Front. Immunol. 13:966766.
doi: 10.3389/fimmu.2022.966766

Anti-IL-6 therapies in central nervous system inflammatory demyelinating diseases

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Current treatments for central nervous system (CNS) inflammatory demyelinating diseases (IDDs) include corticosteroids, plasma exchange, intravenous immunoglobulin, and immunosuppressant drugs. However, some patients do not respond well to traditional therapies. In recent years, novel drugs, such as monoclonal antibodies, targeting the complement component C5, CD19 on B cells, and the interleukin-6 (IL-6) receptor, have been used for the treatment of patients with refractory CNS IDDs. Among these, tocilizumab and satralizumab, humanized monoclonal antibodies against the IL-6 receptor, have shown beneficial effects in the treatment of this group of diseases. In this review, we summarize current research progress and prospects relating to anti-IL-6 therapies in CNS IDDs.

KEYWORDS

IL-6, central nervous system inflammatory demyelinating diseases, tocilizumab, satralizumab, monoclonal antibodies

Introduction

Central nervous system (CNS) inflammatory demyelinating diseases (IDDs) comprise a heterogeneous group of disorders that mainly includes clinically isolated syndrome, multiple sclerosis (MS), neuromyelitis spectrum disorders (NMOSDs), myelin-oligodendrocyte glycoprotein antibody-associated disease (MOGAD), and acute disseminated encephalomyelitis. These disorders are characterized by myelin loss and axonal damage associated with inflammatory lesions (1–5).

Current treatments for CNS IDDs are primarily aimed at relieving acute attacks and preventing relapse. High-dose corticosteroids, plasma exchange, and intravenous immunoglobulin are frequently employed for the treatment of acute attacks in CNS IDDs (6–14), whereas immunosuppressant drugs, such as oral corticosteroids, azathioprine (AZA), and mycophenolate mofetil, are commonly used for relapse prevention in NMOSDs and MOGAD (15–19). Disease-modifying drugs, such as IFN-β, dimethyl fumarate, and glatiramer acetate, are recommended as mainstream

treatments for MS (20, 21). However, some patients do not respond well to these traditional therapies. Over recent years, novel drugs, such as monoclonal antibodies targeting the complement C5 protein, CD19 on B cells, and the interleukin-6 (IL-6) receptor have been used for the treatment of patients with refractory CNS IDDs (22–24).

IL-6 is a cytokine that plays a key role in host defenses, and dysregulated IL-6 signaling is implicated in various autoimmune and inflammatory diseases (25–27). IL-6 signals are transmitted *via* two routes, namely, the classical and trans-signaling pathways. In the classical pathway, IL-6 binds to the membrane-bound IL-6 receptor, forming a complex that recruits glycoprotein 130. In the trans-signaling pathway, IL-6 binds to the soluble form of the IL-6 receptor, and then to membrane-anchored glycoprotein 130 (28, 29). During the adaptive immune response, IL-6 exerts its effects *via* stimulating B-lymphocyte differentiation, promoting antibody production, modulating blood–brain barrier permeability, and enhancing T-lymphocyte activation (30–32). Studies demonstrate that serum and cerebrospinal fluid (CSF) IL-6 levels are significantly increased in patients with NMOSD, whereas IL-6 inhibition is shown to mitigate disease progression (33–38). Several studies have also identified a positive association between serum IL-6 receptor levels and the risk for MS (39–41). Accordingly, IL-6 receptor blockade may represent a novel therapeutic approach for the prevention of relapse in CNS IDDs. Tocilizumab and satralizumab, humanized monoclonal antibodies against the IL-6 receptor, have recently been shown to elicit beneficial clinical effects in the treatment of CNS IDDs (42–46). In this review, we summarize current studies regarding the effects of tocilizumab and satralizumab in the treatment of these disorders.

Tocilizumab

Tocilizumab in NMOSD

In 2013, Araki et al. reported the case of a 36-year-old woman who experienced an improvement in her disability burden and neuropathic pain 6 months after tocilizumab therapy (44). The same group described another case series involving seven patients with NMOSD who received monthly injections of tocilizumab (8 mg/kg) for 12 months. The authors reported a significant reduction in the annualized relapse rate (ARR), the Expanded Disability Status Scale (EDSS) score, neuropathic pain, and general fatigue among the patients. The anti-AQP4 antibody titers were also decreased 6 and 12 months after tocilizumab treatment (45).

In a German retrospective observational study in which the patients were followed up for a mean of 30.9 months, eight patients who received tocilizumab displayed a marked decrease in ARR and EDSS scores. Their AQP4 antibody titers and pain levels were also significantly reduced during tocilizumab treatment (46). Subsequently, data from a clinical study confirmed the long-term efficacy and safety of tocilizumab. Nineteen patients were given tocilizumab monthly, and the number of relapses decreased in all cases. Among 15 patients who received tocilizumab for more than 1 year, the EDSS score, ARR, neuropathic pain, and general fatigue were all significantly improved (47). Similar results were reported for 12 NMOSD patients who received at least 6 months of subcutaneous tocilizumab (48). In the same study, both median and annualized relapse rates were significantly decreased (from 2 to 0). The efficacy of subcutaneous tocilizumab appears to render it an alternative to infusion for patients with NMOSD. In Spain, an observational, retrospective study analyzed the effectiveness and safety of tocilizumab in five NMOSD patients who failed to respond to other immunosuppressant drugs. The authors reported that the mean ARR was reduced by 88.9% during the first year of treatment (from 1.8 ± 1.3 to 0.2 ± 0.4, $P<.05$) (49).

The TANGO trial (NCT03350633) was a randomized, open-label phase II trial that included 118 patients who were followed up for 90 weeks at six hospitals in China (50). The patients were randomly given tocilizumab (8 mg/kg every 4 weeks) or AZA (2–3 mg/kg per day). The median time to first relapse was longer in the patients treated with tocilizumab than in those administered AZA (78.9 weeks *vs*. 56.7 weeks; $P=.0026$). In the per-protocol analysis, 89% of the patients treated with tocilizumab were relapse-free after 60 weeks of treatment compared with 56% for AZA treatment ($P<.0001$).

A meta-analysis of five clinical trials that included 89 patients reported that the ARR was significantly reduced in patients treated with tocilizumab. Moreover, a significant correlation was found between the proportion of relapse-free patients and tocilizumab treatment (51). Another meta-analysis involving 775 patients from seven randomized controlled trials found that patients treated with tocilizumab or satralizumab exhibited significantly lower EDSS scores compared with patients treated with other monoclonal antibodies (52). Meanwhile, in a meta-analysis comprising a total of 202 patients with NMOSD from nine studies, Kharel et al. found that 76% of the patients treated with tocilizumab were relapse-free and the ARR was significant reduced (mean difference: −2.6) at follow-up (ranging from 12 to 31.8 months) (53).

Recently, Du et al. undertook a retrospective study on the effects of tocilizumab in 19 NMOSD patients with moderate-to-severe myelitis. The authors found that disease disability scores were significantly improved in patients treated with tocilizumab when compared with those in patients treated with steroids at 3 months. In addition, compared with steroids, tocilizumab treatment led to a significantly lower ARR and risk of relapse (54). A longitudinal study investigated retinal damage in 50 patients with NMOSD who received disease-modifying drugs

and reported that, compared with AZA, tocilizumab and rituximab could delay macular ganglion cell complex thinning in the eyes of patients without a history of optic neuritis (55).

Tocilizumab In MOGAD

In 2019, Novi et al. reported that a 20-year-old patient with MOGAD experienced clinical deterioration despite receiving rituximab treatment, following which he received tocilizumab infusion every 4 weeks. At the 24-month follow-up, the patient was relapse-free, and a spinal MRI showed a reduction in cervical and thoracic lesions (56). Subsequently, Hayward-Koennecke et al. described the case of a 59-year-old man with recurrent optic neuritis who had received high-dose steroids, plasmapheresis, rituximab, natalizumab, and cyclophosphamide due to disease deterioration. Following further relapse, the patient tested positive for anti-MOG antibodies. Tocilizumab was initiated for 12 months and then tapered to an application every 6 weeks. No further relapses occurred (57).

Masuccio et al. reported a patient with MOGAD who experienced severe acute respiratory syndrome coronavirus (SARS-CoV-2) infection during tocilizumab therapy. Given the high risk of relapse, the patient continued taking tocilizumab, and the symptoms stabilized. The patient also retained walking ability (58).

A retrospective study analyzed seven patients with inflammatory CNS disorder (four with NMOSD and positive for anti-AQP4 antibodies and three with MOGAD) who were treated with tocilizumab. The median follow-up period was 23 months (4–50 months). All the patients were relapse-free throughout tocilizumab treatment (59). Similarly, a single-center report involving 10 patients with relapsing MOGAD who received intravenous or subcutaneous tocilizumab found that none of the patients had clinical or radiographic recurrence over an average treatment duration of 28.6 months (60).

Recently, a retrospective study described 57 patients, including 14 with MOGAD, 36 with AQP4-IgG-seropositive NMOSD, and seven with AQP4-IgG-negative NMOSD who switched to tocilizumab from other immunotherapies. The authors reported that 60% of all the patients were relapse-free (79% for patients with MOGAD) during tocilizumab treatment. For MOGAD, the median ARR decreased from 1.75 to 0, and the inflammatory activity on MRI also decreased significantly under tocilizumab treatment. Eleven of the patients with MOGAD who received tocilizumab for more than 12 months had reduced ARR, and 73% of these were relapse-free (61).

Tocilizumab in MS

To date, relatively few studies have focused on tocilizumab therapy in MS, and the efficacy of tocilizumab as a treatment for this disease remains unclear. In 2014, Sato et al. reported the case of a 53-year-old Japanese woman with MS and rheumatoid arthritis (RA) who received tocilizumab and was relapse-free for more than 5 years (62). Moreover, in 2020, the neurological condition of a Japanese boy who was diagnosed with MS with a tumefactive lesion in the cervical spine deteriorated when his oral prednisolone dose was tapered off. After tocilizumab treatment, the prednisolone dose was reduced without symptom exacerbation, and the EDSS score effectively improved from 8.5 to 5.0 points (63).

Satralizumab

Satralizumab in NMOSD

Satralizumab is another IL-6 receptor-targeting monoclonal antibodies. It has better pharmacokinetic properties and a longer half-life than tocilizumab, resulting from modifications in the constant and variable regions of the antibody (64–66).

SAkuraStar (NCT02028884) and SAkuraSky (NCT02073279) are two randomized, double-blind, placebo-controlled phase III studies that compared the efficacy and safety of satralizumab as, respectively, an add-on treatment to other immunosuppressants and as monotherapy in patients with NMOSD (67, 68). In the two trials, 120 mg subcutaneous satralizumab or placebo were administered at weeks 0, 2, and 4 and then every 4 weeks thereafter. The primary endpoint was time to the first protocol-defined relapse. Secondary endpoints were changes in the Functional Assessment of Chronic Illness Therapy–Fatigue score and visual analog scale pain score.

In the SAkuraStar trial, the rate of relapse (8/41, 20%) was lower in the satralizumab group than in the group given the placebo (18/42, 43%). Additionally, 89% and 78% of patients treated with satralizumab were still relapse-free after 48 and 96 weeks, respectively. Among 55 AQP4-IgG-seropositive patients, the relapse rate was 11% in the satralizumab group *versus* 43% in the placebo group. No significant difference between satralizumab and placebo was observed in the AQP4-IgG seronegative subgroup (67). In the SAkuraSky trial, satralizumab elicited a 55% reduction in the relapse risk compared with the placebo. At 48 and 96 weeks, 76% and 72% of patients were relapse-free in the satralizumab group compared with 62% and 51%, respectively, in the placebo group. For the AQP4-IgG-seropositive subgroup, relapse occurred in 22% of patients who received satralizumab *versus* 57% for the placebo. No significant difference was observed between satralizumab and placebo in the AQP4-IgG-negative subgroup (68). Similarly, no significant differences in neuropathic pain or fatigue were detected in either study.

Based on the positive results of these two phase III clinical trials, on June 1, 2020, Health Canada approved satralizumab for use in the treatment of AQP4-IgG-seropositive NMOSD in

adults and children aged ≥2 years. Subsequently, satralizumab was also approved in Japan on June 29, 2020, and in Switzerland on July 13, 2020 (69).

Recently, a Japanese study reported a patient with AQP4-IgG-seropositive NMOSD whose painful tonic seizures disappeared after 6 months of satralizumab treatment (70).

Adverse events

Overall, anti-IL-6 therapy is well-tolerated by patients with NMOSD. Infections, anemia, leukocytopenia, and hypercholesterolemia are the main adverse events associated with tocilizumab treatment in NMOSD (44); however, most are mild, and serious adverse events are rarely reported. Although a meta-analysis found that two patients with NMOSD died during tocilizumab therapy, neither death was related to tocilizumab treatment (50). Most adverse events related to satralizumab therapy are mild to moderate. The most commonly reported adverse events are upper respiratory tract infections, urinary tract infections, nasopharyngitis, and headaches. No deaths occurred during satralizumab treatment (67, 68, 71, 72).

Perspectives and challenges

Are IL-6 receptor antagonists also suitable as treatments for MOGAD and MS, in addition to NMOSD?

Growing evidence supports that IL-6 plays a key role in the pathophysiology of NMOSD. *In vitro*, dysregulated IL-6 activity leads to reduced blood–brain barrier function, increased leukocyte migration, enhanced chemokine production, and activation of AQP4 antibody secretion (32, 63, 73). *In vivo*, serum and CSF levels of IL-6 were found to be elevated in relapsing patients with NMOSD (33, 36, 66). Several studies report the efficacy and safety of IL-6 receptor inhibitor therapy in the treatment of NMOSD (Figure 1, Table 1).

The pathogenic effect of IL-6 signaling in MS may be exerted through the induction of IL-17-producing T cells. The effect of lL-6 inhibition in MS treatment is unclear. Evidence from mouse models of MS (experimental autoimmune encephalomyelitis) indicates that Th17 cells play an important role in MS progression (74, 75). In the mouse, IL-6 mainly induce the differentiation of naive CD4-positive T cells into Th17 cells, whereas anti-IL-6 therapy effectively suppresses the onset of experimental autoimmune encephalomyelitis *via* the inhibition of the development of autoantigen-specific Th17 cells. In humans, the data are limited to a few studies. One patient with RA and MS received tocilizumab for more than 5 years without exacerbation of MS (62), whereas another RA patient developed MS during tocilizumab treatment (76). Although this result indicates that IL-6 inhibition might trigger inflammatory demyelination in the CNS, elevated levels of the soluble IL-6 receptor, an indirect marker for reduced IL-6 signaling, were found to be significantly correlated with a reduced risk of MS. This strongly suggests that IL-6 receptor inhibitor therapy may be suitable for use in MS treatment (77). Additionally, there are two recent case reports of patients with MS who have been successfully treated with tocilizumab (62, 63) (Figure 1, Table 1). Accordingly, the role of IL-6 in MS pathogenesis and the efficacy of IL-6 receptor inhibitors in MS treatment merit further evaluation.

Biopsy and autopsy data demonstrate complement and immunoglobulin deposition in demyelinating lesions of

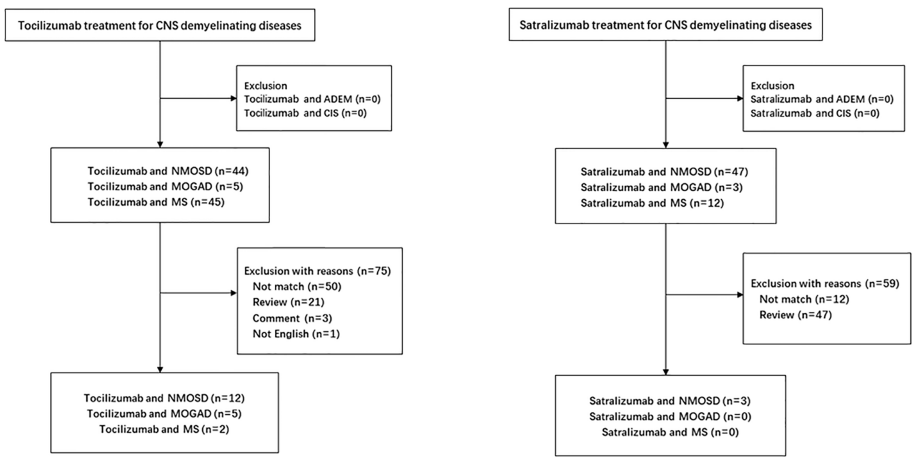

FIGURE 1
Flowchart enrolled in the study.

TABLE 1 Summary of the main clinical trials on tocilizumab and satralizumab in central nervous system inflammatory demyelinating diseases.

Reference	Year	Disease	Patients (*n*)	Treatment	Duration	Study design and outcomes
Araki et al.	2013	AQP4 Ab-positive NMOSD	1	Intravenous TCZ at 8 mg/kg was given monthly	6 months	Case report Serum anti-AQP4 autoantibody levels were rapidly reduced Neuropathic pain and disability scores gradually improved TCZ was well tolerated
Araki et al.	2014	AQP4 Ab-positive NMOSD	7	Intravenous TCZ at 8 mg/kg was given monthly	12 months	Prospective study The ARR decreased from 2.9 ± 1.1 to 0.4 ± 0.8 after 12 months (P<0.005) The EDSS score, neuropathic pain, and general fatigue also exhibited a marked declined TCZ was well tolerated
Ringelstein et al.	2015	AQP4 Ab-positive NMOSD	8	Intravenous TCZ at 6 or 8 mg/kg was given monthly	30.9 months	Retrospective study The median ARR decreased significantly (from 4.0 to 0.4; P=0.008) as did the median EDSS score (from 7.3 to 5.5; P=0.03) Active magnetic resonance imaging lesions were seen in 6 of 8 patients at tocilizumab initiation and in 1 of 8 patients at the last magnetic resonance imaging The AQP4 Ab titers and pain levels dropped significantly during TCZ treatment TCZ was tolerated
Araki	2019	NMOSD	19	Intravenous TCZ at 8 mg/kg was given monthly	Maximum 6 years and 8 months	Retrospective study The number of relapses decreased in all cases Among 15 patients who received TCZ for more than twelve months: The ARR decreased markedly from 2.2 ± 1.1 to 0.3 ± 0.7 (P<0.001); the EDSS improved significantly from 4.5 ± 1.8 to 3.8 ± 1.4 (P<0.05); neuropathic pain and general fatigue also improved significantly (P<0.001) TCZ was well tolerated
Lotan et al.	2019	NMOSD 7 AQP4 Ab-positive 2 MOG Ab-positive 3 seronegative	12	Subcutaneous TCZ at a dose of 162 mg every 1–2 weeks	31.8 months	Retrospective study The median relapse rate was significantly reduced from 2 to 0 (P=0.04) The ARR decreased from a median of 2 to 0 (P=0.0015) One TCZ-treated patient died
Zhang et al.	2020	NMOSD 103 AQP4 Ab-positive	118	Intravenous TCZ at 8 mg/kg was given monthly Oral AZA reached the daily target dose (2–3 mg/kg)	90 weeks	Open-label, randomized phase 2 trial The median time to the first relapse was longer in the TCZ group than in the AZA group (P=0·0026) A total of 89% of patients treated with TCZ were relapse-free compared with 56% for treatment with AZA at 60 weeks (P<0.0001) Adverse events occurred in 61% of TCZ-treated patients and 83% of AZA-treated patients Two patients died but the deaths were not treatment-related
Xie et al.	2020	NMOSD	89	Intravenous TCZ at 6 mg/kg or 8 mg/kg monthly Subcutaneous TCZ at a dose of 162 mg every 1–2 weeks		Meta-analysis The ARR ratio was significantly lower in the TCZ therapy group (P<0.001) A significant correlation was observed between the proportion of patients with relapse-free NMOSD and TCZ therapy (OR=67.78; P<0.001) TCZ was well tolerated
Xue et al.	2020	NMOSD	775	485 patients were treated with monoclonal antibodies 290 were given a placebo		Meta-analysis Satralizumab and tocilizumab treatment reduced the EDSS score relative to treatment with other monoclonal antibodies (P=0.02) TCZ was well tolerated
Zeng et al.	2021	AQP4 Ab-positive NMOSD	50	Intravenous TCZ at 8 mg/kg was given monthly Oral AZA reached the daily target dose (2–3 mg/kg)	48 weeks	Retrospective study Compared with AZA, TCZ (P=0.012) and RTX (P=0.015) delayed macular ganglion cell complex thinning in the eyes of patients

(Continued)

TABLE 1 Continued

Reference	Year	Disease	Patients (*n*)	Treatment	Duration	Study design and outcomes
				RTX dose according to CD19+ B-cell counts		without a history of optic neuritis TCZ was well tolerated
Du et al.	2021	NMOSD	19	Intravenous TCZ at 8 mg/kg within 2 weeks of attack onset, and then at 4-week intervals Prednisone 10–15 mg/day for maintenance	12 months	Retrospective study Compared with steroids, patients treated with TCZ displayed significant improvements in the EDSS score, HAI, mRS, ADL, and EQ-5D-3L at 3 months (P<0.05) Compared with steroids, TCZ significantly lowered the risk of relapse (P=0.017) as well as the ARR (P=0.013). TCZ was well tolerated
Kharel et al.	2021	NMOSD	202	Intravenous TCZ at 6 mg/kg or 8 mg/kg monthly Subcutaneous TCZ at a dose of 162 mg every 1–2 weeks		Meta-analysis 76.95% of patients treated with tocilizumab were relapse-free at follow-up (P<0.001) The ARR was significantly reduced (P<0.001) TCZ was well tolerated
Carreón Guarnizo et al.	2022	NMOSD	5	Intravenous TCZ at 8 mg/kg every 4 weeks.	Mean duration was 2.3 ± 1 years	Retrospective study The mean ARR was reduced by 88.9% during the first year of treatment (from 1.8 ± 1.3 to 0.2 ± 0.4, P<0.05) TCZ was well tolerated
Novi et al.	2019	MOGAD	1	Intravenous TCZ at 8 mg/kg was given monthly	24 months	Case report No relapses occurred and spinal MRI showed a reduction in cervical and thoracic lesions at a 24-month follow-up TCZ was well tolerated
Hayward-Koennecke et al.	2019	MOGAD	1	Intravenous TCZ at 8 mg/kg was given monthly	12 months	Case report No further relapses occurred, MRI remained stable throughout, and no new lesions were detected TCZ was well tolerated
Masuccio et al.	2020	MOGAD	1	Intravenous TCZ at 8 mg/kg was given monthly	9 months	Case report After TCZ treatment, the clinical situation of the patient did not deteriorate (EDSS score: 6.5) The patient retained the ability to walk TCZ was well tolerated
Elsbernd et al.	2021	MOGAD	10	Intravenous TCZ at 8 mg/kg per month or a subcutaneous dose of 162 mg per week	28.6 months	Retrospective study None of the patients had clinical or radiographic relapses over an average treatment duration of 28.6 months TCZ was well tolerated
Ringelstein et al.	2021	14 MOGAD 36 AQP4 Ab-positive NMOSD 7 seronegative NMOSD	57	Intravenous dose of 8.0 mg/kg (median; range 6–12 mg/kg) every 31.6 days	23.8 months	Case-series 60% of all patients were relapse-free (79% for those with MOGAD) The median ARR decreased from 1.75 to 0 (P=0.0011) and inflammatory activity on MRI decreased in patients with MOGAD who received TCZ (P=0.04) 11 of the patients with MOGAD who received tocilizumab for more than 12 months had reduced ARR, and 73% of these were relapse-free TCZ was well tolerated
Sato et al.	2014	MS	1	Intravenous TCZ at 8 mg/kg was given monthly	5 years	Case report Complete remission was achieved at the second administration of TCZ. No recurrence of MS for more than 5 years TCZ was well tolerated
Hoshino et al.	2020	MS	1	Intravenous TCZ at 8 mg/kg was given monthly	2 years	Case report One year and 6 months after TCZ therapy, the oral PSL dose was tapered from 30 to 10 mg/day and the EDSS score improved from 8.5 to 5.0 Five years from the disease onset, brain MRI showed regression of the known lesions TCZ was well tolerated
Yamamura et al.	2019	NMOSD	83	Subcutaneous satralizumab 120 mg or matching placebo at weeks	216 weeks	Randomized, double-blind, placebo-controlled phase III study Relapse occurred in 20% of patients receiving satralizumab and in 43% of patients receiving a placebo

(Continued)

TABLE 1 Continued

Reference	Year	Disease	Patients (*n*)	Treatment	Duration	Study design and outcomes
				0, 2, and 4 and then once every 4 weeks		Among 55 AQP4-IgG-seropositive patients, the relapse rates were 11% in the satralizumab group and 43% in the placebo group No significant differences in neuropathic pain or fatigue were observed between the satralizumab and placebo groups Satralizumab was well tolerated
Traboulsee et al.	2020	NMOSD	95	Subcutaneous satralizumab 120 mg at weeks 0, 2, and 4 and then once every 4 weeks	216 weeks	Randomized, double-blind, placebo-controlled phase III study Relapses occurred in 30% of patients receiving satralizumab and in 50% of patients receiving a placebo For the AQP4-IgG-seropositive subgroup, relapse occurred in 22% of patients receiving satralizumab *versus* 57% for the placebo No significant differences in neuropathic pain or fatigue were observed between the satralizumab and placebo groups Satralizumab was well tolerated
Uzawa et al.	2022	NMOSD	1	Subcutaneous satralizumab 120 mg at weeks 0, 2, and 4 and then once every 4 weeks	12 months	Case report ARR was reduced from 2.3 to 0 times/year Painful tonic seizures disappeared after 6 months Satralizumab was well tolerated

CNS IDDs, central nervous system inflammatory demyelinating diseases; TCZ, tocilizumab; AQP4, Aquaporin 4; NMOSD, neuromyelitis spectrum disorders; MOGAD, myelin oligodendrocyte glycoprotein antibody-associated disease; MS, multiple sclerosis; ARR, annualized relapse rate; EDSS, Expanded Disability Status Scale score; HAI, Hauser ambulation index; mRS, modified Rankin score; ADL, activities of daily living; EQ-5D-3L, EuroQol five-dimension three-level questionnaire; AZA, azathioprine; PSL, prednisolone.

patients with MOGAD, indicative of a significant humoral immune component similar to that seen in AQP4 antibody-positive NMOSD (5, 15, 78). Although NMOSD and MOGAD have different underlying pathogenic mechanisms, they are both characterized by elevated levels of IL-6 in serum and CSF, especially during relapses (57, 79–83). The similarity in CSF cytokine profiles provides promising therapeutic options for NMOSD and MOGAD using IL-6 inhibitors. To date, relatively few reports have assessed the efficacy of tocilizumab treatment on patients with MOGAD (48, 53–57, 82, 83), (Figure 1, Table 1), and further studies are needed to confirm the efficacy and safety of anti-IL-6 receptor therapy in the treatment of this disease.

Intravenous or subcutaneous tocilizumab

Lotan et al. (48) reports 12 NMOSD patients who received at least 6 months of subcutaneous tocilizumab. The ARR decreased from a median of 2 (5.75–1.29) before subcutaneous tocilizumab to 0 (1.0–0) (P=.0015) after treatment. The efficacy of subcutaneous tocilizumab in NMOSD was similar to that of the intravenous formulation.

One case series reports seven patients (four with NMOSD and three with MOGAD), all of whom first received tocilizumab by intravenous injection; subsequently, three patients switched to the subcutaneous form of administration. None of these patients relapsed during tocilizumab treatment (59). Another case series describes 10 patients with relapsing MOGAD treated with tocilizumab, including six by intravenous injection, two by subcutaneous injection, and two who changed from intravenous to subcutaneous injection. All the patients were relapse-free during 28.6 months of follow-up (60), indicating that intravenous and subcutaneous tocilizumab have similar clinical efficacy. Similar results were reported by Lotan et al. (48).

The use of subcutaneous injection should be encouraged given its advantage of at-home administration. Prospective studies of subcutaneous tocilizumab treatment for CNS IDDs are warranted.

The usage of IL-6 receptor antagonists in pregnancy

Hoeltzenbein et al. undertook an analysis of a global safety database containing data for 399 women exposed to tocilizumab shortly before or during pregnancy, 288 of whom reported pregnancy outcomes. Of these 288 pregnancies, 60.6% resulted in live births, 21.7% in spontaneous abortions, 17.2% in elective terminations, and one in stillbirth. The rate of deformity was 4.5%. Although the rate of preterm birth increased (31.2%) compared with the general population, no substantial increase in the risk for deformity was observed (84). In a retrospective analysis from Japan, the authors analyzed pregnancy outcomes in patients with rheumatic disease exposed to tocilizumab. No increased risk of spontaneous abortion or congenital malformation was found in 61 pregnancies (85).

Data regarding the safety of tocilizumab during pregnancy and breastfeeding among patients with RA are limited, and these patients are advised to stop tocilizumab treatment 3 months before conception (86). In NMOSD, experts advise that tocilizumab can be used by pregnant women with severe

disease, whereas breastfeeding may be considered, but only under close monitoring (87). The effects of satralizumab treatment on pregnancy outcomes are unknown.

Analysis of the cost effectiveness of IL-6 receptor antagonists

A cost-utility analysis of tocilizumab in the treatment of patients with RA in Japan showed that quality-adjusted life years (QALYs) and lifetime costs of tocilizumab were approximately 1.3- and 1.5-fold higher, respectively, compared with those for methotrexate. The incremental cost per QALY for tocilizumab treatment was reported to be USD 49,359, which was below the assumed cost-effectiveness threshold of USD 50,000 per QALY (88). These findings indicated that tocilizumab may be cost-effective in the treatment of RA.

A cost-effectiveness analysis of tocilizumab in patients with RA from the United Kingdom, Greece, and Italy showed similar results; namely, that tocilizumab, used either as a first-line biologic monotherapy or an addition to the standard treatment strategy, can be a cost-effective option for the treatment of patients with RA who have not adequately responded to conventional drugs (89–91).

No economic evaluation of tocilizumab and satralizumab therapy in the treatment of CNS IDDs has been undertaken to date. An assessment of the cost-effectiveness of IL-6 receptor inhibitors is warranted in the near future.

Finally, the cost of satralizumab in the United States is USD 219,231 for the first year, subsequently decreasing to USD 190,000 per year (92). The high costs of these new drugs limit their usage in low-income countries, and finding ways of providing these drugs at more reasonable prices constitutes a major challenge for the governments of these countries.

Author contributions

LJ conceived and wrote the manuscript, SG read and approved the submitted version.

Funding

This work was supported by the National Natural Science Foundation of China (Grant no. 82072079).

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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frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 22 December 2022
DOI 10.3389/fimmu.2022.1051082

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Autoimmune and Autoinflammatory Disorders : Autoimmune Disorders, a section of the journal Frontiers in Immunology

RECEIVED 22 September 2022
ACCEPTED 09 December 2022
PUBLISHED 22 December 2022

CITATION
Wu D, Luo Y, Li T, Zhao X, Lv T, Fang G, Ou P, Li H, Luo X, Huang A and Pang Y (2022) Systemic complications of rheumatoid arthritis: Focus on pathogenesis and treatment. *Front. Immunol.* 13:1051082. doi: 10.3389/fimmu.2022.1051082

Systemic complications of rheumatoid arthritis: Focus on pathogenesis and treatment

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As a systemic autoimmune disease, rheumatoid arthritis (RA) usually causes damage not only to joints, but also to other tissues and organs including the heart, kidneys, lungs, digestive system, eyes, skin, and nervous system. Excessive complications are closely related to the prognosis of RA patients and even lead to increased mortality. This article summarizes the serious complications of RA, focusing on its incidence, pathogenesis, clinical features, and treatment methods, aiming to provide a reference for clinicians to better manage the complications of RA.

KEYWORDS

rheumatoid arthritis, complications, incidence, treatment, prospects

1 Introduction

Rheumatoid arthritis (RA) is defined as a systemic autoimmune disease associated with a chronic inflammatory process, which gradually leads to joint destruction, deformity, disability, and even death (1). It is a widely distributed disease worldwide, with a prevalence of approximately 0.5% to 2% and a higher prevalence in women, smokers, and those with a family history of it (2). At present, the etiology of RA has not been fully elucidated, but what attracts attention is the immune processes that occur in the joint synovium and synovial fluid (3, 4), during which synovial macrophages release cytokines, such as tumor necrosis factor α (TNF-α), interleukin-1 (IL-1) and interleukin-6 (IL-6), which co-stimulate the activity of osteoclasts with inflammation and fibroblast-like synoviocytes (FLS), thus leading to the progress of bone erosion (5). In addition, activated FLS can produce matrix metalloproteinase (MMP) that leads to cartilage degeneration (6). Nuclear factor-kappa-light-chain-enhancer of activated B cells (NF-κB) is involved in the pathogenesis of chronic inflammatory diseases, and FLS stimulates the NF-κB signaling pathway, allowing T cells to bind to proteins on the surface of osteoclasts, which also leads to further development of bone erosion as it increases

osteoclast activity (7). FLS can migrate from one joint to another, resulting in symmetrical joint destruction which is typical in RA (8). In addition, the presence of autoantibodies in the serum of RA patients is a mark of disease, with rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA) being the most prominent. These autoantibodies are found in 50-80% of RA patients (9), newly-detected antibodies such as anti-carbamylated protein antibodies and anti-acetylated protein antibodies were also identified in them (10). Antibody production leads to inflammation; citrullination leads to an immune response which indicates the formation of ACPA (11); ACPA may play an important role in the prolonged inflammatory process and its presence directly links bone erosion and pain in RA patients (12). The pathogenesis of rheumatoid arthritis mentioned above is shown in Figure 1.

As a systemic disease, RA usually causes damage to other tissues and organs besides joints, including the heart, kidneys, lungs, digestive system, eyes, skin, and nervous system (13, 14). The results of the study show that about 40% of RA patients suffer from complications, and the incidence of serious complications is 8.3%, among which cardiovascular disease, interstitial lung disease, osteoporosis, and metabolic syndrome are more common (15). The existence of complications seriously reduces the quality of life of RA patients and even leads to increased RA mortality (16). Complications of RA are usually closely related to prognosis and require early diagnosis and active intervention, and the main treatment goals include reducing disease activity and controlling extra-articular damage of RA (17). At present, the treatment methods for RA complications are relatively limited. In this article, we mainly summarize the manifestations of severe extra-articular damage in RA (as shown in Figure 2), and discuss its pathogenesis, incidence, clinical features, and treatment methods, hoping to provide some reference for clinical practice.

2 Cardiovascular disease in RA

2.1 Pathogenesis of RA-CVD

It is well known that RA patients may be disabled, but the main cause of their death is cardiovascular disease (CVD) (18). Many studies have shown that the incidence of CVD in RA patients is 30%-60%, mainly involving pericarditis, myocarditis and heart failure, and coronary artery disease (19). Epidemiological investigations suggest that synovial tissue and circulating immune cells in RA release pro-inflammatory cytokines such as TNF-α and IL-6, which directly lead to systemic inflammation and the occurrence of CVD (20, 21). Overactive immune cells, such as T lymphocytes and B lymphocytes, may affect the cardiovascular system through multiple mechanisms (22, 23). Autoantibodies in RA affect the cascade of all structures of the cardiovascular system, from the myocardium to the heart valves, conduction system, and vasculature (24). There is more severe disease activity in ACPA-

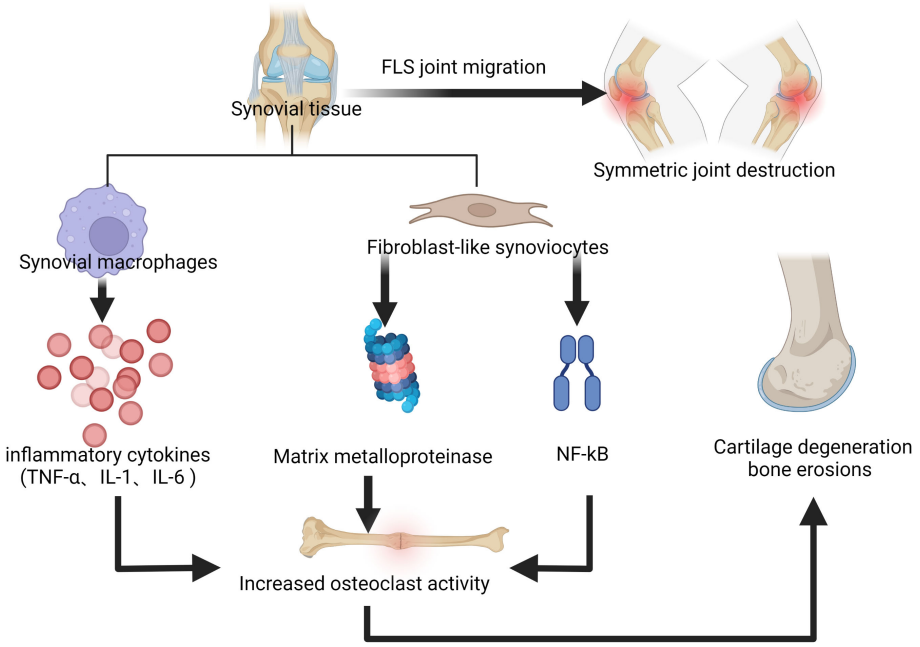

FIGURE 1
The immune processes that occur in the joint synovium and synovial fluid, leading to the progress of bone erosion and cartilage degeneration.

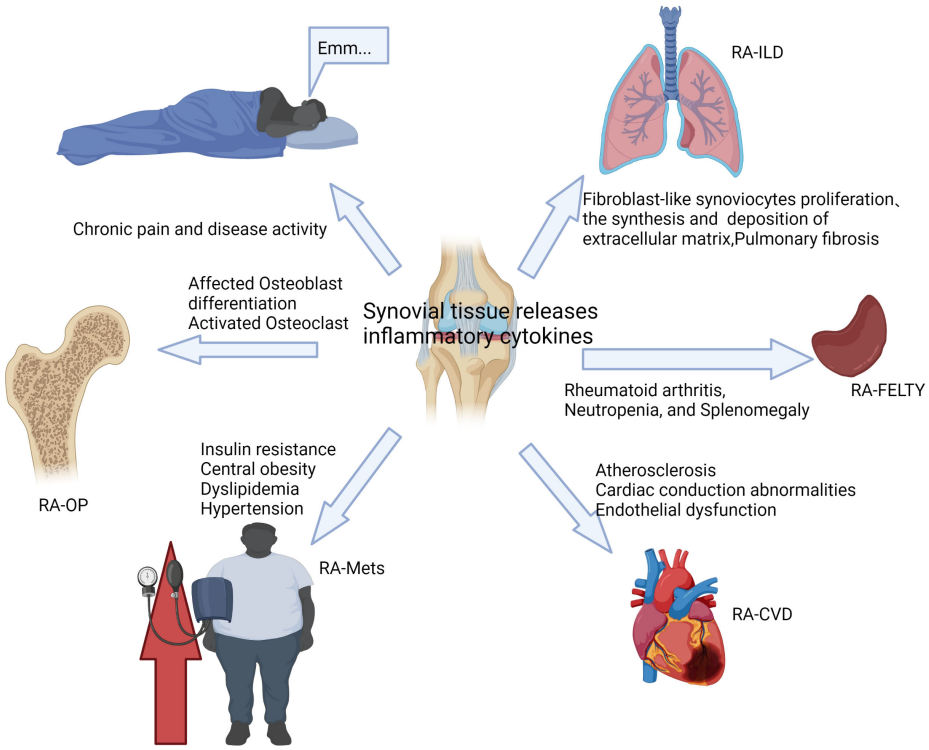

FIGURE 2
The complications of RA are usually closely related to disease activity and inflammation levels.

positive patients, which further leads to atherosclerosis and increases CVD mortality (25). In addition, ACPA is also seen in non-RA patients with cardiovascular disease and has adverse outcomes (26). Imaging methods are essential for the detection and assessment of CVD risk in RA, and carotid ultrasound, aortic pulse wave velocity or arterial enhancement index and ankle-brachial index, echocardiography, and cardiac magnetic resonance can be used to assess the CVD risk of patients with RA in clinical practice (27). Early detection and diagnosis of CVD in RA patients are critical for prognosis and management.

2.1.1 Pericarditis

Pericarditis is one of the common cardiac manifestations of RA. Many patients with early RA can be complicated with pericarditis or develop pericarditis before RA (28). Pericarditis is inflammation and fluid accumulation in the pericardium, and about 15% of RA patients will show corresponding symptoms. However, electrocardiography shows that about 20%-50% of patients have pericardial involvement, clinically manifested as chest pain or dyspnea (29). Therefore, strict physical examination and antibody screening are needed to detect whether RA is complicated by pericarditis as soon as possible. Early diagnosis and effective treatment of pericarditis will significantly improve the prognosis of RA patients.

2.1.2 Myocarditis

Myocarditis is the result of persistent inflammation in the myocardium and is histologically characterized by cellular infiltration composed of lymphocytes, histiocytes, and macrophages, which may form nodular granulomatous lesions (30). The degree of myocardial dysfunction is associated with disease activity of RA because key inflammatory cytokines in RA, such as TNFα, IL-1, and IL-6, may induce myocardial and vascular dysfunction and promote remodeling and fibrosis of the left ventricular (31).

2.1.3 Arrhythmia

Arrhythmia is another common cardiac complication in RA patients, which may be secondary to conduction abnormalities. Its causes include ischemia, rheumatoid nodules, and amyloidosis (32). Recent researches indicate that symptoms and increased sympathetic nerve activity can lead to abnormal heart rhythms, and Holter monitoring can capture latent arrhythmias with higher accuracy (33).

2.1.4 Coronary artery disease

The main etiology of coronary artery disease in RA may be related to atherosclerosis accelerated systemic inflammatory response and abnormal lipids and endothelial dysfunction (34,

35). The chronic inflammation and reactive oxygen species (ROS) response of RA is the core of the pathogenesis of atherosclerosis (36). ROS is a group of small active substances that play a key role in the regulation of biological cellular processes. The balance between ROS and antioxidants is critical for maintaining cellular homeostasis, thus an imbalance between oxidants and antioxidant mechanisms can lead to oxidative stress states (37). Excessive ROS may lead to vascular damage, the result of a complex cascade including oxidative modification of lipoproteins, endothelial activation, and accelerated atherosclerosis by leukocyte migration and differentiation (38). Pro-inflammatory cytokines and chemokines, as well as IL-1 and intercellular and vascular cell adhesion molecules (39), are highly expressed in atherosclerotic lesions, promote leukocyte recruitment, impair vasodilation, and induce oxidation stress and promote coagulation (40).

2.1.5 Heart failure

Heart failure is the main cause of death in RA patients, and the prevalence of heart failure in RA patients is also twice as high as that in the general population, with a higher incidence in women than men in general (41). Studies have found that RA patients are more likely to develop heart failure due to diastolic dysfunction, which may be related to systemic inflammation (42). Elevated levels of c-reactive protein (CRP) and erythrocyte sedimentation rate (ESR), RF, ACPA and inflammatory cytokines may contribute to the progression of heart failure in RA (43).

2.2 Treatment of RA-CVD

An increasing number of evidence supports that long-term use of NSAIDs has the potential of triggering cardiovascular risks despite reductions in disease activity and some adverse CVD outcomes with conventional RA drugs (44). NSAIDs anti-inflammatory drugs exert therapeutic effects by inhibiting cyclooxygenase isoforms. The drugs inhibit prostacyclin production, leading to vasoconstriction, increased blood pressure, rupture of atherosclerotic plaques, and thrombosis, thus are thought to be the main contributor to CVD in RA (45). Non-steroidal anti-inflammatory drugs (NSAIDs), such as rofecoxib, have been the fundamental treatment for patients with osteoarthritis and other types of pain, but as controlled trials and other meta-analyses indicated an increased risk of cardiovascular problems in RA patients, rofecoxib has withdrawn from the market (46). Glucocorticoids are usually used to treat RA, mainly for short-term control of disease activity. However, glucocorticoids can aggravate hypertension or cause abnormal blood lipid levels and glucose tolerance, insulin resistance, and obesity, and promote the occurrence and development of CVD (47). Studies have shown that the use of statins in RA patients can reduce the degree of arteriosclerosis and carotid plaque formation (48). RA patients treated with at least one disease-modifying Anti-Rheumatic Drugs (DMARDs) and statins at the same time have seen reduced RA-CVD mortality of 21% (49), and disease activity was significantly decreased in those RA patients whose methotrexate (MTX) and corticosteroid regimens are added with statins, and this may indicate a clear positive effect of statins in the control of RA (50).

MTX is the "gold standard" for RA treatment because it has important immunosuppressive and anti-inflammatory effects and inhibits dihydrofolate reductase (51). Many studies have demonstrated the benefits of MTX. Approximately 25-40% of patients receiving MTX alone have seen significant improvement because MTX can play a role in improving microvascular endothelial function by reducing the degree of RA disease activity, reducing the risk of CVD in RA patients, and reducing their mortality (52). In addition, methotrexate appears to have cardioprotective properties on lipids and endothelium, in contrast to patients receiving adalimumab (53). Similarly, Hydroxychloroquine (HCQ) was found to have a protective effect on the vascular endothelium of RA patients (54), and it causes a lower cardiovascular risk in RA patients (55, 56). HCQ treatment can reduce low-density lipoprotein and Triglyceride serum values, and plays an anti-platelet aggregation role, thus it is considered to be cardioprotective (57).Tumor necrosis factor inhibitor (TNFi) therapy in RA reduces CVD risk *via* inhibition of endothelial dysfunction and slows the progression of atherosclerosis by reducing the expression of pro-inflammatory cytokines and endothelial adhesion molecules (58). In a controlled study, TNFi preparations improved myocardial inflammation and myocardial perfusion in patients with RA-CVD compared with standard disease-modifying antirheumatic drugs (59).

Recently, metabolic modulation therapy has become a research hotspot. Sirtuin 1 (SIRT1) is a sirtuin involved in a wide range of transcriptional and metabolic regulation, which may affect cell proliferation and inflammatory responses and inhibit the activation of NF-κB-dependent inflammation (60). Some SIRT1 activators, such as resveratrol, a polyphenol found in wine, have been extensively studied as SIRT activators and they exhibit potent antioxidant, anti-inflammatory and anti-cancer properties (61). Resveratrol can inhibit NF-κβ-dependent inflammatory response and its effect on RA patients is under evaluation (62). Notably, serum biochemical markers such as CRP, ESR, MMP-3, and IL-6 were also significantly reduced in resveratrol-treated patients (63). In addition, metformin and its analog phenformin are hypoglycemic drugs used in diabetic patients; although the exact mechanism of action remains unclear, their effect on AMPK (Adenosine 5'-monophosphate-activated protein kinase) can be conducive to the beneficial secondary effects of these drugs such as cutting inflammatory markers, improving lipid metabolism, and reducing experimental autoimmune arthritis based on the importance of

AMPK on T cells in RA (64). In particular, metformin, as an activator of AMPK, can inhibit the migration of FLS, inhibit the expression of pro-inflammatory cytokines, and downregulate the level of inflammation in RA and its comorbidities (65).

Some pathways involve extracellular targets. Mavrilimumab is a monoclonal antibody against granulocyte–macrophage colony-stimulating factor (GM-CSF), and GM-CSF is expressed at high levels in synovial fluid and plasma as well as synovial tissue cells of RA patients. Phase I and II trials of mavrilimumab in the treatment of RA showed satisfactory safety and efficacy (66). GM-CSF emphasizes the impact of "inflammatory" pathways on arteriosclerosis and endothelial dysfunction. Based on this connection, it is expected that more potential therapeutic targets will be developed to better manage cardiovascular problems in RA patients. Recent clinical studies on RA-CVD are shown in Table 1.

3 Lung disease in RA

3.1 Pathogenesis of lung disease in RA

3.1.1 Interstitial lung disease

ILD is a serious pulmonary complication of RA, resulting in a 10-20% mortality in RA. Pulmonary involvement is common in RA patients, among which the occurrence of pulmonary complications is approximately 60-80% (67, 68). Clinical manifestations include interstitial lung disease, small airway disease, rheumatoid nodules, pleural effusion, pulmonary vasculitis, pulmonary fibrosis, etc (69). Although RA can involve many parts of the respiratory system, such as the airway or pleura, parenchymal lung involvement is associated with the highest morbidity and mortality (70). One diagnostic study showed that approximately 50% of RA patients had interstitial lung disease, of which only 10% had clinically significant symptoms such as cough and progressive exertional dyspnea (71), and that is because cytokine, chemotactic factor, and growth factor-mediated RA inflammatory process can promote FLS proliferation, increase the synthesis and deposition of extracellular matrix, and lead to pulmonary fibrosis (72, 73). The most common patterns of RA-ILD are usual interstitial pneumonia (UIP) and nonspecific interstitial pneumonia (NSIP) (74). There is no universal treatment guideline for RA-ILD, thus accurate screening and diagnosis of the characteristics of ILD development in RA patients is critical for future research and treatment of RA patients (75). Histological biopsy, pulmonary function tests, and high-resolution computed tomography (HRCT) are valuable tools for the diagnosis and evaluation of RA-ILD (76), and HRCT can accurately capture UIP cellular and traction bronchiectasis as well as reticular abnormalities and the "ground glass opacity" in NSIP (77).

TABLE 1 Recent clinical studies of RA-CVD.

Clinical therapeutic drug	Possible mechanism
Statins (49)	Reduce the degree of arteriosclerosis and carotid plaque formation
Methotrexate (51)	Inhibits dihydrofolate reductase, Reducing the degree of RA disease activity
Hydroxy Chloroquine (54)	Have a protective effect on the vascular endothelium, Anti-platelet aggregation
Resveratrol (63)	Exhibit potent antioxidant, Anti-inflammatory, Downregulate the level of inflammation in RA
Metformin, Phenformin (64)	Affects AMPK activity,Downregulate the level of inflammation, Improving lipid metabolism
Mavrilimumab (66)	Decreased leukocyte activation, Modulates immune and inflammatory processes

3.1.2 Pleurisy and pleural effusion

Pleurisy and pleural effusion are the most common pleural manifestations observed in RA patients, with only 3-5% of patients presenting with clinical symptoms such as cough, dyspnea, chest pain, and fever, which means the majority of RA patients with the pleural disease are with no clinical manifestations (78). In terms of pathogenesis, studies have suggested that IgG, IgE, and other antibodies contribute to the formation of immune complexes to destroy the capillary endothelium and increase the capillary permeability of the pleural cavity (79). Ultrasound-guided thoracentesis can be an important test in RA patients with pleural effusion.

3.1.3 Airway involvement (bronchiolitis, bronchiectasis, and cricoarytenoid arthritis)

The prevalence of airway disease is high in RA as it affects 39% to 60% of RA patients and may involve any part of the airway, including large and distal small airways. The most common manifestations are bronchitis, bronchiectasis, and cricoarytenoid arthritis (80). Pulmonary function tests and HRCT can help diagnose airway-related diseases. Chronic inflammatory infection is the main cause of bronchiectasis in RA patients, and bronchiolitis is characterized by damage to the airway epithelium, which leads to airflow obstruction (81). Because the midline of the vocal folds is adducted, cricoarytenoid arthritis manifests as hoarseness, sore throat, dyspnea, and stridor, which are primarily due to thickening of the synovial membrane of the cricoarytenoid joint and persistent cartilage erosion (82).

3.2 Treatment of lung disease in RA

Treatment options for RA-ILD are complicated by the possible pulmonary toxicity of many DMARDs, but their ability to improve lung function and stabilize pulmonary symptoms has been

demonstrated (83, 84). Therefore, joint and pulmonary involvement should be assessed independently for therapeutic purposes (85). The Spanish Society of Rheumatology recommends the use of abatacept and rituximab in patients with RA-ILD (86). A retrospective study showed that the use of abatacept, a costimulatory antagonist of T lymphocytes, improved ILD in approximately 88% of cases and reduced their risk of infection (87). In addition, abatacept significantly reduced lung density and fibrotic histology scores and improved ILD (88). Finally, data from a retrospective multicenter study conducted in Italy in 2020 showed that 86.1% and 91.7% of patients with RA-ILD treated with abatacept for at least 6 months had stable or increased forced vital capacity and carbon monoxide diffusing capacity, respectively, while 81.4% of patients had stable or improved chest HRCT (89). Rituximab is considered safe for the treatment of RA-ILD as evidenced by observational studies (90, 91).In addition, a large observational study of patients with RA-ILD showed that the pulmonary function of most ILD patients remained stable or improved after treatment with RTX during long-term follow-up (92). The British College of Rheumatology suggests that doctors be cautious in prescribing TNFi to patients with RA-ILD and recommends RTX for the treatment of refractory ILD (93).

Interstitial lung disease is characterized by alveolar inflammation and interstitial fibrosis, thus anti-fibrotic therapies, such as nintedanib and pirfenidone, have become the spotlight, and in fact, nintedanib and pirfenidone have been proven to slow the disease progression in patients with idiopathic pulmonary fibrosis (94, 95). In addition, tocilizumab as monotherapy can stabilize or even improve ILD (96), and as an IL-6 receptor antagonist, tocilizumab can achieve anti-fibrotic effects by blocking IL-6R, which means this treatment delivers potential benefits in RA-ILD-associated pulmonary fibrosis (97). Although there are still many challenges in practical clinical application, the efficacy and safety of anti-fibrotic agents in RA-ILD patients are still under continuous research (98, 99) for better control over RA-ILD.

In addition, non-drug conservative treatment methods, such as pulmonary rehabilitation and supplemental oxygen, can be used for aged or frail patients or those with multiple comorbidities (100). The role of pulmonary physical rehabilitation in RA-ILD is unclear, but it has beneficial effects on improving dyspnea, functional exercise capacity, and quality of life in idiopathic ILD (101). However, dyspnea and poor joint mobility in patients with ILD limit their pulmonary rehabilitation, thus patients with RA-ILD should take pulmonary rehabilitation in the early course of the disease (102). In addition, supplemental oxygen can be used as primary palliative therapy to improve the quality of life of patients with severe lung disease and reduce respiratory symptoms during daily activities (103). At the same time, smoking is a major risk factor for the progression of RA-ILD, and smoking cessation is important for RA-ILD patients (104).

Lung transplantation may be an option for end-stage RA-ILD, while only a few studies have evaluated post-transplant outcomes in patients with RA-ILD. A recent study reveals that patients with ILD of connective tissue disease (including RA) had similar rates of acute or chronic rejection after lung transplantation compared with patients with idiopathic pulmonary fibrosis, and there was no significant difference in survival (105). Lung transplantation may be an option for younger patients with advanced refractory disease but is not appropriate for patients at risk of advanced age, multiple comorbidities, immobility, and other severe extra-articular damage. Recent clinical studies on pulmonary complications in RA are shown in Table 2.

4 Metabolic syndrome in RA

4.1 Pathogenesis of RA-Mets

The main features of Mets in RA patients are related to inflammation-induced RA disease activity and mainly include insulin resistance (IR), central obesity, dyslipidemia, and hypertension; these manifestations (106). The prevalence of Mets in RA patients varies widely worldwide, ranging from 14.32% to 37.83% according to different criteria (107). In addition, Mets are strongly associated with accelerated atherosclerosis development and increased CVD risk, and are considered to be characteristic pathogenesis of CVD (108). Studies have shown that IR is a fundamental feature of Mets in RA, and is directly related to the levels of IL-6, TNF-α, CRP, and ESR (109). RA-induced IR leads to increased systemic inflammatory responses and directly affects endothelial dysfunction (110). In addition, the continuous increase of macrophages in obese adipose tissue has emerged as a key link to metabolic inflammation (111). Recent studies reveal the heterogeneity of adipose tissue macrophages and their interactions with adipocytes, endothelial cells, and other immune cells in the adipose tissue microenvironment (112). Adipose tissue is a multifunctional organ that, in addition to its central role in storing lipids, secrets a variety of hormones. These various product, collectively referred to as “adipocytokines” or “adipokines”, are responsible for the immune response and mediators of inflammation (113). RA is associated with IR, dyslipidemia, and changes in the adipokines profile (114). In RA, adipocytes and their surrounding macrophages induce innate and adaptive immune cells to release proinflammatory cytokines that cause cartilage

TABLE 2 Recent clinical studies of pulmonary complications in RA.

Clinical therapeutic drug	Possible mechanism
Abatacept (87)	Interferes with T cell activation, Reduces pulmonary fibrosis
Rituximab (91)	Improved pulmonary function
Nintedanib,pirfenidone (105)	Reduces pulmonary fibrosis
Tocilizumab (96)	Blocking IL-6R, Anti-fibrotic

degradation and osteoblast dysregulation, thus leading to arthritic disease and Mets (115).

4.2 Treatment of RA-Mets

In RA patients, TNF-α is an important mediator of IR; therefore, biological therapies that block proinflammatory cytokines, such as TNF-α antagonists, can reduce CRP levels in RA patients, as well as modulate lipid metabolism and improve IR (116). The majority of patients receiving anti-TNF-α biologic therapy (eg, infliximab) were observed to have significant reductions in serum insulin levels as well as insulin and glucose indices, indicating an improvement in IR (117).

Other non-TNF-α treatments,Such as Abatacept, a novel biologic already approved for the treatment of patients with RA, interferes with T cell activation and prompts the polarization of adipose tissue macrophages from pro-inflammatory M1 to anti-inflammatory M2 phenotype, thereby reducing adipose tissue inflammation to improve insulin sensitivity (118). Based on the close relationship between IR and the levels of inflammatory factors such as IL-6, a study on the IL-6 blocker tocilizumab found that intravenous administration of tocilizumab had a rapid positive effect on IR and insulin sensitivity in RA patients. These findings suggest that IL-6 blocker has a potential beneficial effect on mechanisms associated with Mets and CVD development in RA patients (119).

The Janus kinase and signal transducer and activator of the transcription pathway (JAK-STAT) has an important pathogenic role in the development of low-grade chronic inflammatory responses leading to obesity and type II diabetes (120). Tofacitinib, the first small-molecule oral selective JAK inhibitor approved for the treatment of RA patients in 2018, can reduce IR when used alone as proved by research, which brings the therapeutic potential to the JAK-STAT pathway (121, 122).

Lowering LDL cholesterol with statins is a commonly used treatment in patients with metabolic diseases, and there is evidence that statins have a direct anti-inflammatory effect because they reduce CRP levels (123) to improve RA-Mets. Recent clinical studies on RA-Mets are shown in Table 3.

TABLE 3 Recent clinical studies of RA-Mets.

Clinical therapeutic drug	Possible mechanism
Infliximab (117)	Blocking TNF-α, Modulate lipid metabolism
Abatacept (118)	Interferes with T cell activation, Downregulate the level of inflammation
Tocilizumab (119)	Blocking IL-6R, Improve insulin sensitivity
Tofacitinib (121)	Decreased insulin sensitivity
Statins (123)	Modulate lipid metabolism, Reduces CRP levels

5 Osteoporosis in RA

5.1 Pathogenesis of RA-OP

Osteoporosis is a common systemic skeletal disease characterized by low bone mass and degeneration of bone tissue microarchitecture that lead to bone fragility and fracture susceptibility (124). A fragility fracture is defined as a spontaneous fracture caused by minimal or no identifiable trauma and is a hallmark of OP (125). Bone erosion and systemic bone loss are typical features of RA. Systemic bone loss leads to the occurrence of OP, which is one of the main complications of RA (126). The incidence rate can reach 30% of RA patients, or even higher (127). Bone fragility in RA is caused by a combination of systemic inflammation, autoantibodies circulation, and the secretion of pro-inflammatory cytokines. Inflammatory cytokines such as TNF-α, IL-6, IL-1, and immune cell-derived cytokines undermine osteoblastogenesis while promoting osteoclastogenesis (128, 129). ACPA is a determinant of bone loss (130) as it has a direct and independent effect on osteoclasts (131). The effect may be mediated by IL-8-dependent osteoclast activation, so the bone loss is more likely to occur around joints of ACPA-positive RA patients. These factors all have a deleterious effect on bone (132).

5.2 Treatment of RA-OP

Teriparatide, a parathyroid hormone analog, can act as an anabolic drug by reducing osteoblast apoptosis and stimulating osteoblasts to increase bone formation with subcutaneous administration (133). The study showed that teriparatide resulted in a significantly greater increase in bone mineral density levels and a significant reduction in spinal fractures, compared with the active comparator and the anti-resorptive drug alendronate, and that was confirmed in clinical practice (134). Another study showed a significant reduction in spinal fractures in RA patients treated with teriparatide (135). Furthermore, in cases of high fracture risk, calcium and vitamin D should be supplemented with anti-osteoporotic therapy (136).

The receptor activator of NF-κB ligand (RANKL) is a key molecule in osteoclast differentiation and activation and is a potential therapeutic target for osteolytic diseases (137). Denosumab is a RANKL-specific human monoclonal antibody currently used to treat osteoporosis, osteosarcoma, multiple myeloma, and bone metastases (138). RANKL is expressed at moderate and high levels in the inflammatory state of RA patients, while denosumab can prevent the receptor activator of RANKL from binding to RANK on osteoclasts, thereby inhibiting bone resorption (139). In a phase II randomized controlled trial, the result of the combined use of methotrexate and denosumab in the treatment of RA was a significant increase in bone mineral density at the lumbar spine and hip of RA patients (140), suggesting that the combination of methotrexate and denosumab can prevent the development of bone erosions in RA (141).

TNFi is the first biological agent for RA treatment and is a key drug for inhibiting inflammation (142). Inflammatory cytokines induce osteoclast maturation and inhibit osteoblast activation to perturb bone homeostasis, thus, anti-TNF therapy can improve bone homeostasis in RA patients (143, 144). Infliximab has beneficial effects on bone metabolism in RA patients, studies on the effect of TNFi on bone loss have demonstrated that the use of infliximab can improve bone loss in RA patients (145). Another observational study indicated a lower incidence of vertebral fractures in RA patients treated with TNFi, suggesting that TNFi plays a bone-protective role in RA patients (146).

Janus kinases are a family of protein tyrosine kinases JAK1, JAK2, JAK3, and TYK2, which act on signal transducers and activators of transcription, and JAK inhibitors are approved for the treatment of RA (147). Tofacitinib, a JAK inhibitor, can regulate RANKL overexpression in the synovium by inhibiting the secretion of IL-17 and IL-6 to reduce the damage to joints caused by RA inflammation as proved by research (148). It is also proved that baricitinib can improve bone loss in RA by stimulating osteoblast function (149). The above results demonstrate that JAK inhibitors are effective therapeutics to increase osteoblast function and bone formation. Recent clinical studies on RA-OP are shown in Table 4.

6 Felty syndrome in RA

6.1 Pathogenesis of Felty syndrome in RA

Felty syndrome is a rare and severe extra-articular manifestation of RA, with an incidence of approximately 1% of RA patients. Typical manifestations are unexplained RA-complicated neutropenia and splenomegaly (150), and due to long-term granulocyte deficiency, patients are more prone to opportunistic infections, which results in increased mortality (151). Felty syndrome is common in RA patients with a disease history of more than 10 years while it is not uncommon that patients with short onset and atypical clinical symptoms are not diagnosed or misdiagnosed (152). The cause of peripheral blood cytopenia in Felty syndrome is not fully understood, and neutropenia is the most common symptom, which may be related to the presence of granulocyte-specific antinuclear factors (GS-ANF). It has been reported that the positive rate of GS-ANF in patients with Felty syndrome is as high as 75%, while that in RA patients is only 25% to 30% (153). At the same time, the presence of IgG-like granulocyte antibodies in the peripheral blood of patients with Felty syndrome can further destroy granulocytes and reduce their ability to phagocytose immune complexes, while T cell activation can inhibit granulocyte production (154). In addition, splenomegaly can cause thrombocytopenia, and the mechanism may be related to factors such as decreased platelet production, spleen retention, peripheral platelet depletion, and peripheral immune-mediated platelet destruction (155).

TABLE 4 Recent clinical studies of RA-OP.

Clinical therapeutic drug	Possible mechanism
Teriparatide (133)	Reducing osteoblast apoptosis, Stimulating osteoblasts to increase bone formation
Denosumab (139)	Affecting osteoclast differentiation, Inhibiting bone resorption
Infliximab (145)	Improve bone loss
Tofacitinib (148)	Inhibiting the secretion of IL-17 and IL-6, Regulate RANKL overexpression
Baricitinib (149)	Stimulating osteoblast function

6.2 Treatment of Felty syndrome in RA

Treatment of Felty syndrome is supportive and is aimed at controlling underlying RA while improving neutropenia to prevent life-threatening infections (156). However, due to the lack of evidence-based medicine, most drugs are empirical (157). Granulocyte colony-stimulating factor ameliorates neutropenia by inducing the production of neutrophils and has good efficacy and tolerance by patients (158). It has been reported that a patient with a 38-year history of RA and Felty syndrome had a significant increase in absolute neutrophil counts after treatment with abatacept (159). Both MTX and leflunomide can improve joint and vascular inflammation in patients with Felty syndrome (160). Currently, the most widely used drug is rituximab, an anti-CD20 monoclonal antibody that acts against mature B cells and has been approved for the treatment of complex RA. In addition, rituximab has also been reported to successfully treat refractory neutropenia in Felty syndrome (161). Another report of Felty syndrome told that the patient's clinical symptoms has been resolved after tocilizumab treatment, and his spleen had returned to normal size, the absolute neutrophil count had stabilized, and joint erosions had not continued to worsen (162). These case reports suggest new options for the treatment of Felty syndrome. Recent clinical studies on RA-Felty are shown in Table 5.

7 Sleep disorders in RA

7.1 Pathogenesis of sleep disorders in RA

Sleep disorder is closely related to the development of chronic disease. In the long course of RA, chronic pain and disease activity may be the main factors related to sleep disorders in RA (163, 164).

TABLE 5 Recent clinical studies of RA-Felty.

Clinical therapeutic drug	Possible mechanism
Abatacept (158)	Induce the formation of neutrophils
Methotrexate,Leflunomide (160)	Reducing disease activity
Rituximab (161)	Against mature B cells
Tocilizumab (162)	Reducing disease activity

Sleep disorder is multifactorial thus the degree of disease activity increases the risk of depression and anxiety in RA patients, while depression can affect the quality of life and treatment compliance of RA patients. The above factors, which are underestimated or even ignored, all contribute to sleep disorders caused by disease activities and emotional problems (165). In fact, the incidence of sleep disorder in RA patients is as high as 50% (166), and poor sleep quality severely undermines the physical function of patients. Therefore, it is necessary to pay attention to the treatment of sleep disorders in RA patients because of their crucial impact on patients' quality of life.

7.2 Treatment of sleep disorders in RA

Studies have shown that anti-TNF and other biologics can improve the sleep quality of RA patients. Abatacept significantly improves sleep disorders in RA patients as the MOS-Sleep Scale demonstrated its validity, reliability, and sensitivity to changes (167). Infliximab improves sleep quality and relieves vigilance disorders in RA patients, possibly a result of central effects by suppressing TNF-α circulation (168). In addition, adalimumab was proven to be beneficial in improving sleep disorder in RA patients for it reduces disease activity while improving sleep problems in RA patients (169). Another study has shown that the IL-6 antagonist tocilizumab improved sleep quality in RA patients, yet patients' disease activity was not significantly reduced, which deserves further study as it seems to indicate a potential role of IL-6 in sleep regulation (170). Recent clinical studies on sleep disorders in patients with RA are shown in Table 6.

8 Conclusion

RA complications are a major scientific issue worthy of attention. However, the current international research on the pathological mechanism of RA complications remains unclear, and safe and effective clinical drugs and methods are limited. Given that much of the extra-articular damage in RA is related to disease activity and disease severity, control of disease activity in RA should be the optimal treatment, and earlier and more aggressive management of RA can reduce the impact of complications on prognosis. Although there exist some guidelines on the management of RA-related complications, the range of recommendations including ILD and CVD is still limited. In this review, we discuss the pathogenesis, morbidity, and updated management guidelines of serious complications such as cardiovascular problems and pulmonary involvement in patients with RA. We hope that the recommendations reviewed in this article can provide clinicians with a better reference to treatment options for RA complications.

TABLE 6 Recent clinical studies of Sleep Disorders in RA.

Clinical therapeutic drug	Possible mechanism
Abatacept (167)	Reducing disease activity
Infliximab (168)	Inhibition of circulating TNF-α levels
Adalimumab (169)	Reducing disease activity
Tocilizumab (170)	Regulation of IL-6 levels

Author contributions

DW, YL designed the study together, equal contribution, Listed as co-first author. AH, YP as co-corresponding author, TLi, XZ, TLv, PO, HL, XL were all involved in the revision of the manuscript, GF, AH, YP made final critical revisions. All authors contributed to the article and approved the submitted version.

Funding

This research was funded by National Natural Science Foundation of China project Study of the Mechanism for Regulating the Th17/Treg Balance along Gut-immune-joint Axis in Treating Rheumatoid Arthritis by the Component Compatibility of the Zhuang Medicine Longzuan Tongbi Formula (Number 81973976), the Guipai Chinese Medicine Inheritance and Innovation Team of Guangxi University of Chinese Medicine – Clinical Medicine Research and Application Innovation Team of Zhuang Medicine Poison Disease (2022A003), the Guipai Xinglin Young Talents of Guangxi University of Chinese Medicine (2022C035), and the Qihuang Scholar Cultivation Program of Guangxi (YP).

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.2022.1051082/full#supplementary-material

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