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ORIGINAL ARTICLE

Identification of lipopolysaccharide-binding protein as a novel citrullinated autoantigen in rheumatoid arthritis

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Abstract

Background: A specific feature of the autoimmune response in rheumatoid arthritis (RA) is the presence of anti-citrullinated protein antibodies (ACPAs) in patient sera. These antibodies can appear several years before disease onset and are involved in the development of RA.

Objective: We performed proteomic analysis by mass spectroscopy to identify novel citrullinated antigens and autoantibodies in RA patients.

Methods: Polypeptides isolated from the sera of RA patients were identified by Orbitrap high-precision mass spectrometry and then citrulline-containing proteins were selected. The levels of ACPAs against these newly identified citrullinated autoantigens in sera of 100 RA patients and 50 healthy controls were determined by enzyme-linked immunosorbent assays.

Results: A total of 135 proteins were identified in RA patients and the protein profile included 11 citrulline-containing antigens. Three of the 11 citrullinated proteins had been reported in previous studies. ACPAs against the novel citrullinated epitopes from these proteins were increased in sera from the RA patients compared with those from healthy controls. Autoantibodies against one of the citrullinated antigens, lipopolysaccharide-binding protein (LBP), was significantly increased in RA patients and associated with disease activities. The titer of anti-citrullinated LBP antibodies (anti-cLBP) was closely related to the infection incidence in RA patients.

Conclusion: Serum protein analysis by high-precision proteomic technology is a feasible method to identify novel citrullinated epitopes in RA patients. Anti-cLBP antibodies are associated with disease severity and infection in RA patients.

KEYWORDS

ACPA, lipopolysaccharide-binding protein, rheumatoid arthritis

Highlights

- Serum protein analysis by high-precision proteomic technology is a feasible method to identify novel citrullinated epitopes in RA patients.
- Anti-cLBP antibodies are associated with disease severity and infection in RA patients.

1 | INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory joint disease that affects 0.5%–1.0% of the global population and 0.28% of people in China.[1–3] It is characterized by systemic inflammation that damages the cartilage and bone, which leads to restricted movement and disability. Several factors have been proposed to play a role in the pathogenesis of RA, which include infection, and genetic and hormonal factors.[4,5] Retrospective analyses have shown that anti-citrullinated protein antibodies (ACPAs) begin to accumulate in RA patient serum several years

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before clinical onset of symptoms[6,7], and that the detection of ACPAs is the most specific diagnostic test for RA.[8] Importantly, high ACPA titers correlate with a more severe disease course. As such, a positive anti-cyclic citrullinated peptide test, which detects ACPAs, is now a part of the clinical criteria to diagnose RA.[9]

Serum proteins contain various antigens that may be associated with underlying diseases. Citrullinated polypeptides incorporated into serum proteins are promising candidates for diagnostic biomarker screening of RA. At present, proteomics by mass spectroscopy has been applied to explore novel biomarkers in various diseases.[10] Van Beers et al.[11] applied this approach to RA synovial fluid and identified citrullinated apolipoprotein E as a novel autoantigen in RA patients, which suggested that apolipoprotein E might play an important role in the pathogenesis of RA.

In the case of Gram-negative bacteria, the principal stimulator of the innate immune system is lipopolysaccharide (LPS), a component of the bacterial outer membrane. LPS is bound by LPS-binding protein (LBP) and transferred to the CD14 LPS receptor on the macrophage surface or to high-density lipoprotein particles. Jack et al.[12] found that LBP is essential for rapid induction of the inflammatory response by small amounts of LPS or Gram-negative bacteria.

In this study, we applied high-resolution mass spectrometry (MS) by nano-liquid chromatography (LC) combined with a Orbitrap Q Exactive mass spectrometer, to identify novel citrullinated autoantigens in sera of RA patients. Then, we analyzed the clinical correlation of the newly identified ACPAs in RA patients.

2 | MATERIALS AND METHODS

2.1 | Patients and samples

Serum samples were obtained from 100 RA patients (mean age: 58.99 ± 11.58 years, 73 females and 27 males) and 50 healthy volunteers (mean age: 56.57 ± 10.61 years, 37 females and 13 males). The 28-joint count disease activity score (DAS28) was employed (5.11 ± 1.64). The characteristics of RA patients are listed in Table 1. The serum was collected from inpatient or outpatient clinics of the Department of Rheumatology and Immunology, Peking University People's Hospital. The diagnosis of RA was made in accordance with the 2010 revised American College of Rheumatology/European League Against Rheumatism criteria.[13] The healthy volunteers were free from any symptoms of infections. The study was approved by the Ethics Committee of Peking University People's Hospital in accordance with the Declaration of Helsinki. All patients had signed informed consent for participation in the study.

2.2 | Clinical and laboratory data of RA patients

Clinical and laboratory information of RA patients as collected from the medical record database at Peking University People's Hospital, which included age, sex, disease duration, number of swollen joints, and number of tender joints, organ involvement, treatments, history of smoking, infection, and metabolic disorders. Erythrocyte sedimentation rate (ESR) was evaluated by the Westergren method. Serum levels of immunoglobulins (IgG, IgM, and IgA), complement proteins (C3 and C4), C-reaction protein (CRP), and rheumatoid factor (RF)-IgM were examined by immunonephelometry. Antikeratin antibodies (AKAs), antiperinuclear factor (APF), and RF-IgG were detected by indirect immunofluorescence assays. Anti-citrullinated peptide (anti-CCP) antibodies and glucose phosphate isomerase were measured by enzyme-linked immunosorbent assays (ELISAs). The DAS28 was evaluated as described previously.[14]

2.3 | Proteomic analysis by LC-MS/MS

Serum samples were collected from 10 RA patients and pooled together. Low abundance proteins in serum samples were enriched by a ProteoMiner™ enrichment kit (Bio-Rad), separated by SDS-polyacrylamide gel electrophoresis, and stained with a Coomassie blue staining solution. Each lane was cut into 10 pieces, followed by reduction with 25 mM dithiothreitol and alkylation with 55 mM iodoacetamide, and then digested with sequencing-grade modified trypsin (Promega) at 37°C overnight. Peptides were extracted with 50% acetonitrile and 0.5% formic acid, and dried in a SpeedVac. The peptides were dissolved in a 200 mM tetraethylammonium bromide solution and labeled with tandem mass tags (TMT) reagent. Then, 5% hydroxylamine was used to quench the reaction after 1 h of incubation. The labeled peptides were mixed together and desalted using an in-house C18 stage tip. Peptides were analyzed by LC-MS/MS using a nano-LC combined with an LTQ Orbitrap Velos mass spectrometer. The LTQ Orbitrap Velos mass spectrometer was operated in the data-dependent acquisition mode using Xcalibur 2.1.2 software. In addition, there was a single full-scan mass

TABLE 1 Clinical characteristics of the 100 patients with RA

Index	RA ($n = 100$)
Male/female	27/73
Age (year)	58.99 ± 11.58
Duration (year)	12.34 ± 8.32
Swollen joint count ($M \pm SD$)	7.40 ± 8.01
Tender joints ($M \pm SD$)	9.45 ± 9.53
ESR (mm/h)	51.52 ± 34.22
CRP (mg/L)	36.90 ± 44.62
DAS28 scores	5.11 ± 1.64
RF (IU/ml)	435.79 ± 642.65
IgG (g/L)	13.62 ± 4.21
IgA (g/L)	3.18 ± 1.34
IgM (g/L)	1.26 ± 0.84

Abbreviations: CRP, C-reaction protein; DAS28, 28-joint disease activity score; ESR, erythrocyte sedimentation rate; RF, rheumatoid factor.

TABLE 2 Citrullinated sites detected in serum proteins from patients with rheumatoid arthritis

Protein name	Citrulline sites	IPI
Apolipoprotein B-100 precursor	NWE-Cit-QVSHAKEKL	IPI00022229.2
	KL-Cit-LEPLKHVAGNLK	
	NIQ-Cit-ANLFNKLVTELRGLS	
Apolipoprotein A-I	DSG-Cit-DYVSQFEGS	IPI00021841.1
Apolipoprotein C-III	QQA-Cit-GWVTDGFSS	IPI00021857.1
Apolipoprotein D	ENG-Cit-CIQANYSLME	IPI00006662.1
Complement C3	CNYLHLSVL-Cit-TEL	IPI00783987.2
	CLQ-Cit-SYTVAIAGYAL	
Complement C1q subcomponent subunit C	CSLI-Cit-FNAVLT	IPI00022394.2
Complement C1q subcomponent subunit B precursor	CNNYEP-Cit-SGKF	IPI00296165.7
58 kDa proteinkeratin, type II cytoskeletal 6B	CNFL-Cit-ALYDA	IPI00969456.1
Lipopolysaccharide-binding protein	CALQSELL-Cit-ITLP	IPI01009486.1
Angiotensinogen	CLVAQG-Cit-ADSQ	IPI00032220.3
Amyloid A-4 protein precursor	CQNSN-Cit-YLYAR	IPI00006146.4

spectrum in the Orbitrap (400–1800 m/z, 6000 resolution) with an automatic gain control target value of 2e6 followed by 10 MS/MS scans.

The raw data were searched against the ipi.HUMAN.v3.82 database using Protein Discoverer 1.2.0. The search parameters were as follows: two missed cleavages of full trypsin digestion were allowed; carbamidomethylation (C) and TMT sixplex (K and N-terminal) were set as the fixed modifications; oxidation (M) and citrullination (R) were set as the variable modification; precursor ion mass tolerances were set at 20 ppm for all MS acquired in the Orbitrap mass analyzer; the fragment ion mass tolerance was set at 0.5 Da for all MS2 spectra acquired. Relative protein quantification was performed on the reporter ion intensities per peptide using Proteome Discoverer software in accordance with the manufacturer's instructions.

2.4 | Enzyme-linked immunosorbent assays

Fourteen citrullinated polypeptides were synthesized by CHINESE PEPTIDE. Serum concentrations of antibodies against the 14 citrulline-containing peptides were determined by ELISA assays. Briefly, 96-well polysorp plates (NUNC) were coated with recombinant citrullinated polypeptides at 10 μg/ml in 0.05 M carbonate buffer at 4°C overnight. The wells were then washed with phosphate-buffered saline (PBS) that contained 0.05% Tween-20 (PBS-T) three times and blocked with 3% bovine serum albumin (BSA)-PBS for 2 h at 37°C. Serum samples were diluted at 1:100 with PBS-T that contained 1% BSA and were added to the 96-well plate. Wells filled with PBS-T that contained 1% BSA without human serum samples were set up to examine nonspecific background. After incubation for 30 min at 37°C, all wells were washed three times with PBS-T. Then, 100 μl of goat anti-human IgG conjugated to peroxidase and diluted at 1:16,000 with 1% BSA in PBS-T was added to each well, followed by incubation for 20 min at 37°C. After washing with PBS-T three times, the bound antibodies were detected with *o*-phenylenediamine as the substrate. The reaction was stopped by adding 100 μl of 2 M sulfuric acid to each well. OD was determined at 450 nm by a Bio-Rad plate reader. The OD values of ACPAs were transformed to arbitrary units (AUs) calculated as shown below:

$$\mathrm{AU} = [\mathrm{OD}_{\mathrm{peptide}} - \mathrm{OD}_{\mathrm{nonspecific\ background}}]_{\mathrm{test\ serum}} / [\mathrm{OD}_{\mathrm{peptide}} - \mathrm{OD}_{\mathrm{nonspecific\ background}}]_{\mathrm{positive\ control\ serum}} \times 100.$$

The AU value was considered positive if it was greater than the mean + 2 SD of the healthy group.

2.5 | Data analysis

Data analyses were performed using SPSS 19.0 for Windows. The distribution of numerical data was evaluated by the Shapiro–Wilk test. Numerical data with a normal distribution are expressed as the mean ± SD and differences between two groups were analyzed by the independent *t*-test. Numerical data with a skewed distribution are expressed as the median (P25 and P75) and differences between two groups were analyzed by the Mann–Whitney test. Ranked data are expressed as a percentage and differences between two groups were analyzed by the χ^2 test. Spearman's rank correlation coefficient was applied to determine correlations. A difference between groups was considered to be significant at $p < 0.05$.

3 | RESULTS

3.1 | Identification of citrullinated autoantigens from serum proteins in RA patients by high-resolution MS

The protein profile identified by proteomic analysis of RA patients consisted of 135 proteins. These proteins are involved in the immune response (complement C3), blood pressure (angiotensinogen), and lipid transportation (apolipoprotein B-100 precursor). We found that 11 proteins had citrullinated modifications (Table 2). Eight of these 11 proteins were novel antigens in RA, which have not been reported in previous studies, whereas Apolipoprotein A–I, complement C3, and angiotensinogen have been identified in RA synovial fluids.

3.2 | Autoreactivity of citrullinated antigens in patients with RA

Fourteen peptides, each containing a citrulline, were synthesized on the basis of the identified citrullination sites in the 14 proteins. The reactivity of RA sera with these newly identified citrullinated peptides was analyzed. The results showed that the positive rates of all peptides in RA patients was 16%–28% (Table 3).

We found the highest positive rate (28/28.00%) in the detection of anti-citrullinated LBP antibodies (anti-cLBP). The prevalence of anti-cLBP was 20.59% (7/34) and 19.05% (4/21) in RF-negative and anti-CCP-negative RA patients, respectively. Three out of 18 (16.67%) patients with high anti-cLBP were found among the 18 patients with both anti-CCP and RF negative (Figure 1A). These results revealed that the anti-cLBP antibody might be a valuable biomarker to distinguish seronegative RA patients from patients with arthralgia caused by other autoimmune diseases.

3.3 | Anti-cLBP antibody is associated with disease activity of RA

As shown in Figure 1B, the anti-cLBP antibody was marginally correlated to DAS28 ($r = 0.194$, $p = 0.059$). We also showed that RA patients with high serum levels of anti-cLBP antibodies had increased joint pain and incidence of swelling (53.57% vs. 44.44%, 67.86% vs. 48.61%; Table 4) and RA patients with high CRP had higher levels of anti-cLBP (78.40 ± 56.29 vs. 55.86 ± 40.90, $p = 0.049$, Table 5), which indicated that anti-cLBP antibodies were positively associated with the inflammation and disease activity of RA.

Further analysis suggested that anti-cLBP antibodies were positively correlated to the levels of anti-CCP, IgG, and IgA ($p < 0.05$, Figure 2B–D). We found that RA patients with high serum levels of anti-cLBP antibodies had higher anti-CCP, RF, APF positivity, AKA positivity, and hidden rheumatoid factor IgG antibody (HRF-IgG) ($p < 0.05$, Table 4). In addition, patients positive for RF, AKA, APF, anti-CCP, or HRF-IgG had higher levels of anti-cLBP antibodies ($p < 0.05$, Table 5), which suggested that anti-cLBP antibodies were positively associated with autoantibody production.

TABLE 3 Prevalence of anti-citrullinated protein antibodies in RA patients and controls

Antibodies		RA (100)	HC (50)
Anti-Cit-Apo B-100p-I antibodies	AU value	89.06 ± 54.65	55.88 ± 39.07
	Positive rate	16 (16.00%)	3 (6.00%)
Anti-Cit-Apo B-100p-II antibodies	AU value	52.98 ± 64.86	26.61 ± 27.79
	Positive rate	24 (24.00%)	3 (6.00%)
Anti-Cit-Apo B-100p-III antibodies	AU value	51.77 ± 53.66	24.03 ± 17.82
	Positive rate	26 (26.00%)	2 (4.00%)
Anti-Cit-Apo A-I antibodies	AU value	63.21 ± 59.87	30.44 ± 18.19
	Positive rate	21 (21.00%)	3 (6.00%)
Anti-Cit-Apo C-III antibodies	AU value	49.18 ± 42.71	27.47 ± 23.8
	Positive rate	24 (24.00%)	4 (8.00%)
Anti-Cit-Apo D antibodies	AU value	53.97 ± 50.41	22.31 ± 18.31
	Positive rate	19 (19.00%)	3 (6.00%)
Anti-Cit-C3-I antibodies	AU value	52.38 ± 39.18	30.68 ± 17.16
	Positive rate	26 (26.00%)	3 (6.00%)
Anti-Cit-C3-II antibodies	AU value	75.84 ± 52.84	50.17 ± 20.94
	Positive rate	25 (25.00%)	1 (2.00%)
Anti-Cit-C1qsC antibodies	AU value	115.91 ± 69.87	78.89 ± 29.8
	Positive rate	22 (22.00%)	2 (4.00%)
Anti-Cit-C1qsBp antibodies	AU value	150.36 ± 100.87	85.46 ± 39.55
	Positive rate	27 (27.00%)	3 (6.00%)
Anti-Cit-cytoskeletal 6B antibodies	AU value	68.63 ± 59.75	42.33 ± 21.17
	Positive rate	26 (26.00%)	1 (2.00%)
Anti-Cit-Amy-A4p antibodies	AU value	106 ± 67.76	71.2 ± 34.12
	Positive rate	26 (26.00%)	0 (0%)
Anti-Cit-Angiotensinogen antibodies	AU value	113.98 ± 87.33	78.09 ± 37.13
	Positive rate	19 (19.00%)	3 (6.00%)
Anti-cLBP antibodies	AU value	76.86 ± 53.1	47.31 ± 30.04
	Positive rate	28 (28.00%)	2 (4.00%)

Abbreviations: AU, arbitrary unit; HC, healthy control; RA, rheumatoid arthritis.

3.4 | Anti-cLBP antibody increases in RA patients with infection

As shown in Table 4, RA patients with high anti-cLBP antibody levels had an increased infection incidence compared with patients with normal anti-cLBP levels [11/28 (39.29%) versus 10/72 (13.89%), $p = 0.005$]. LBP is required to combat Gram-negative bacterial infection.[15,16] Twenty RA patients with infection had respiratory tract infections. Two of the patients were infected with *Pseudomonas aeruginosa* and the levels of anti-cLBP were

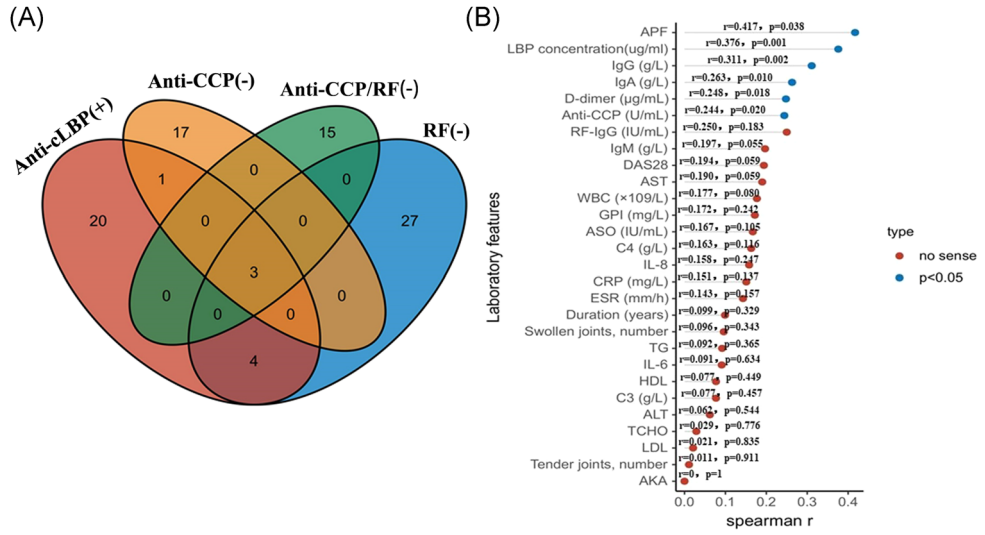

FIGURE 1 Prevalence of anti-cLBP antibodies in rheumatoid arthritis (RA) patients and controls. Prevalence of anti-cLBP antibodies in RA patients and controls. Correlation of serum anti-cLBP antibodies with clinical features of RA patients. $p < 0.05$ was considered statistically significant. AKA, antikeratin antibodies; ALT, glutamic-pyruvic transaminase; APF, anti-perinuclear factor; ASO, anti-*Streptococcus* hemolysin O; AST, aspartate aminotransferase; GPI, glucose-6-phosphate isomerase; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TCHO, total cholesterol; TG, triglyceride; WBC, white blood cell

high. We also found that RA patients with infection had higher levels of anti-cLBP antibodies (98.43 ± 76.59 vs. 65.19 ± 43.56, $p = 0.011$, Table 5). In addition, RA patients with various infections and high anti-cLBP had higher RF, HRF-IgG, ESR, and CRP levels (Figure 3A).

To determine whether the association of anti-cLBP antibodies with infection was specific, we examined the association between the incidence of infection in RA patients and the positivity of other RA-associated autoantibodies that included anti-CCP, AKA, APF, HRF, and RF As a result, no significant association was found, which showed that anti-cLBP was the only indicator of infection (Figure 3B).

4 | DISCUSSION

RA is a systemic autoimmune disease characterized by chronic and erosive polyarthritis caused by abnormal growth of synovial tissue, which leads to irreversible joint disability. The finding of ACPAs is a milestone in the history of RA serolog.[15] In this study, we applied high-resolution MS to screen serum proteins that incorporated citrulline modifications from RA patients. A total of 11 citrulline-containing proteins were identified in the serum of RA patients, which included LBP and Apo A-I (Table 2). Eight of the proteins were novel antigens in RA, which have not been reported previously. Apolipoprotein A-I and its citrulline epitope have been reported in RA synovial fluid.[11] Citrulline-containing complement C3 and angiotensinogen have also been identified in RA patients in previous studies, but the specific citrullinated sites identified in this study have not been reported. These proteins were involved in physiological processes that included the immune response, blood pressure, and lipid transportation. Two (Apo B-100p and C3) of the identified polypeptides appeared to have citrulline on at least one position (Table 2). In addition, it is noteworthy that this set could not include all citrulline-containing polypeptides in RA patients, not only because the MS data only partially covered the polypeptide sequences but also because of the heterogeneity of citrullination patterns in different RA patients.

In addition to the identification of a series of citrulline-containing proteins, we further found that the levels of anti-cLBP were significantly increased in RA patients and even in a portion of seronegative patients. We also confirmed that the anti-cLBP antibody was associated with RA disease activity. These results confirmed the feasibility and reliability of protein analysis of novel citrulline-containing antigens in RA patients.

LBP is a class I acute-phase protein that mediates innate immune responses after recognizing LPS from Gram-negative bacterium.[16,17] LBP forms a high-affinity complex with LPS and delivers LPS was to the cell through CD14 or TLR4-MD2 and triggers a cascade of cytokines and proinflammatory factors.[18] A previous study has suggested that serum LBP in sepsis patients is significantly elevated almost seven times higher than normal levels.[18] LBP not only had diagnostic value for sepsis, urinary tract infection, and periodontitis, but is also related to the prognosis and mortality of some types of infections.[19–21] In our study, we found a correlation between the anti-cLBP antibody and infections. A previous study showed that the serum LBP level is elevated in RA patients and the incidence of infection was higher in LBP-positive RA patients than in LBP-negative cases, but there was no significant difference.[18] LBP promotes infection immunity, contributes to eliminating pathogens, and controls further occurrence of infection. However, an anti-cLBP antibody may interfere with the normal physiological functions of LBP. Therefore, RA patients with elevated anti-cLBP antibodies are prone to infection. Thus, it is necessary to elucidate the pathological roles of anti-cLBP antibodies in RA.

TABLE 4 Clinical and laboratory characteristics of RA patients with elevated or normal serum levels of anti-cLBP antibodies

Clinical and laboratory features	Anti-cLBP antibodies					
	Positive (28)		Negative (72)			
	N	%	*N*	%	$t/u/\chi^2$	*p*
Female	21 (28)	75.00	52 (72)	72.22	0.079	0.779
Tender joints ≥ 6	15 (28)	53.57	32 (72)	44.44	0.672	0.412
Swollen joints ≥ 4	19 (28)	67.86	35 (72)	48.61	3.006	0.083
Somke	6 (28)	21.43	15 (72)	20.83	0.004	0.948
Interstitial lung disease	17 (27)	62.96	37 (72)	51.39	1.061	0.303
Infection	11 (28)	39.29	10 (72)	13.89	**7.838**	**0.005**
Cardiac involvements	1 (27)	3.70	1 (72)	1.39	0.532	0.466
Renal involvements	0 (27)	0	2 (71)	2.82	-	1.000
Skin	3 (27)	11.11	8 (71)	11.27	0.000	1.000
Haematological involvements	11 (27)	40.74	26 (71)	36.62	0.141	0.707
RF (IU/ml)	542.3 ± 543.82		335.49 ± 683.30		**1.358**	**0.035**
ESR (mm/h)	58.16 ± 37.17		49.96 ± 33.55		0.939	0.350
CRP (mg/L)	48.79 ± 46.22		34.26 ± 44.11		1.253	0.213
AKA	18 (26)	69.23	26 (70)	37.14	**7.863**	**0.005**
APF	2 (26)	76.92	34 (70)	48.57	**6.192**	**0.013**
Anti-CCP	166.41 ± 77.50		120.25 ± 83.17		**2.038**	**0.035**
HRF-IgG	12 (24)	50.00	16 (70)	22.86	**4.422**	**0.035**
GPI	12 (22)	60.00	30 (67)	44.77	0.634	0.426
ERY	0 (27)	0	7 (68)	10.29	1.682	0.195
PRO	0 (27)	0	2 (68)	2.94	-	1.000
IgG	14.62 ± 4.62		13.29 ± 4.11		1.126	0.263
IgA	3.37 ± 1.39		3.13 ± 1.34		0.674	0.502
IgM	1.46 ± 0.86		1.21 ± 0.83		1.134	0.260
C3 (g/L)	1.14 ± 0.27		1.09 ± 0.23		0.750	0.455
C4 (g/L)	0.25 ± 0.07		0.22 ± 0.07		1.386	0.169
ASO	61.93 ± 116.07		64.16 ± 91.98		0.087	0.930
D-dimer	2713.63 ± 2546.53		1745.59 ± 3050.81		1.181	0.241
WBC	7.31 ± 2.23		7.99 ± 15.26		0.197	0.845
TG	1.39 ± 0.75		1.22 ± 0.55		1.090	0.279
TCHO	4.31 ± 1.22		4.20 ± 0.84		0.452	0.7652
LDL	2.66 ± 0.85		2.56 ± 0.77		0.501	0.618
HDL	0.99 ± 0.34		1.06 ± 0.28		0.883	0.379
ALT	18.00 ± 11.63		15.34 ± 9.72		0.988	0.325
AST	23.35 ± 15.20		18.22 ± 7.17		1.988	0.052
DAS28 > 5.1	15 (28)	53.57	30 (72)	41.67	1.154	0.283

Notes: For normally distributed data, results are expressed as the mean ± SD. Differences between groups were analyzed by the *t*-test. Data not distributed normally are expressed as the median (range) and differences were analyzed by the Mann–Whitney *U*-test. Classified data are presented as *n* (%) and were analyzed by the χ^2 test. $p < 0.05$ was considered statistically significant. The bold entries emphasized the parameter reached statistical significance.

Abbreviations: AKA, antikeratin antibody; ALT, alanine aminotransferase; Anti-CCP, anti-citrullinated peptide; APF, antiperinuclear factor; ASO, anti-*Streptococcus* hemolysin O; AST, aspartate aminotransferase; CRP, C-reaction protein; DAS28, 28-joint count disease activity score; ERY, urine erythrocyte; ESR, erythrocyte sedimentation rate; GPI, glucose phosphate isomerase; HDL, high-density lipoprotein; HRF-IgG, hidden rheumatoid factor IgG antibody; LDL, low-density lipoprotein; PRO, urine protein; RA, rheumatoid arthritis; RF, rheumatoid factor; TCHO, total cholesterol; TG, triglyceride; WBC, white blood cell.

TABLE 5 Serum levels of anti-cLBP antibodies in the presence or absence of clinical or laboratory characteristics of RA patients

Clinical and laboratory features	Anti-cLBP levels (AU)			
	Presence	Absence	*t*/*u*	*p*
Female	72.24 ± 52.56 (*n* = 73)	60.01 ± 41.58 (*n* = 27)	1.363	0.176
Tender joints ≥ 6	72.72 ± 59.08 (*n* = 47)	70.99 ± 46.95 (*n* = 53)	0.162	0.872
Swollen joints ≥ 4	77.94 ± 57.29 (*n* = 54)	64.12 ± 46.73 (*n* = 46)	1.298	0.198
Somke	63.28 ± 49.218 (*n* = 21)	74.14 ± 54.15 (*n* = 79)	0.832	0.408
Interstitial lung disease	76.93 ± 57.57 (*n* = 54)	64.99 ± 47.37 (*n* = 45)	1.112	0.269
Infection	98.53 ± 76.59 (*n* = 21)	65.19 ± 43.56 (*n* = 79)	**2.583**	**0.011**
Cardiac involvements	80.57 ± 54.09 (*n* = 2)	71.32 ± 53.50 (*n* = 97)	0.242	0.809
Renal involvements	24.24 ± 17.99 (*n* = 2)	73.06 ± 53.30 (*n* = 96)	1.288	0.201
Skin involvements	82.63 ± 73.93 (*n* = 11)	70.73 ± 50.44 (*n* = 87)	0.697	0.488
Hematological involvements	78.39 ± 63.73 (*n* = 37)	68.22 ± 45.88 (*n* = 61)	0.916	0.362
RF > 20 (IU/ml)	78.42 ± 55.48 (*n* = 66)	59.13 ± 46.29 (*n* = 34)	**2.036**	**0.042**
ESR > 20 (mm/h)	72.38 ± 51.96 (*n* = 77)	70.12 ± 57.94 (*n* = 23)	0.178	0.859
CRP > 8 (mg/L)	78.40 ± 56.29 (*n* = 71)	55.86 ± 40.90 (*n* = 29)	**1.989**	**0.049**
AKA	86.68 ± 63.66 (*n* = 48)	55.98 ± 36.09 (*n* = 48)	**2.906**	**0.005**
APF	83.20 ± 61.61 (*n* = 54)	56.06 ± 36.83 (*n* = 42)	**2.523**	**0.013**
Anti-CCP	76.73 ± 56.90 (*n* = 76)	49.71 ± 30.12 (*n* = 21)	**2.091**	**0.039**
HRF-IgG	82.23 ± 60.53 (*n* = 31)	62.15 ± 45.24 (*n* = 63)	**2.090**	**0.042**
GPI	66.68 ± 42.68 (*n* = 46)	67.43 ± 54.70 (*n* = 43)	0.072	0.943
ERY	36.96 ± 18.53 (*n* = 7)	74.80 ± 55.27 (*n* = 88)	1.796	0.076
PRO	37.83 ± 37.21 (*n* = 2)	72.75 ± 54.50 (*n* = 93)	0.899	0.371
IgG ≥ 16 (g/L)	84.41 ± 53.42 (*n* = 20)	69.17 ± 54.24 (*n* = 75)	1.120	0.266
IgA ≥ 4.5 (g/L)	83.22 ± 61.28 (*n* = 16)	70.18 ± 52.74 (*n* = 79)	0.877	0.383
IgM ≥ 3.04 (g/L)	82.01 ± 68.70 (*n* = 7)	71.61 ± 53.24 (*n* = 88)	0.487	0.627
C3 < 0.78 (g/L)	63.18 ± 71.71 (*n* = 9)	73.34 ± 52.42 (*n* = 86)	0.533	0.595
C4 < 0.16 (g/L)	64.69 ± 52.36 (*n* = 16)	73.94 ± 54.69 (*n* = 79)	0.621	0.536
ASO ≥ 116 (IU/mL)	91.22 ± 63.73 (*n* = 7)	71.08 ± 52.70 (*n* = 91)	0.960	0.339
D-dimer ≥ 243 (ng/mL)	77.73 ± 58.13 (*n* = 75)	50.85 ± 26.85 (*n* = 15)	1.748	0.084
WBC ≤ 3.5 ($\times 10^9$/L)	64.42 ± 17.11 (*n* = 2)	71.65 ± 53.76 (*n* = 97)	0.189	0.850
TG ≥ 1.7 (mmol/L)	80.72 ± 59.36 (*n* = 16)	69.73 ± 52.20 (*n* = 83)	0.335	0.754
TCHO ≥ 6.20 (mmol/L)	116.43 ± 100.71 (*n* = 3)	70.10 ± 51.44 (*n* = 96	1.493	0.139
LDL ≥ 4.1 (mmol/L)	81.01 ± 48.58 (*n* = 3)	71.21 ± 53.60 (*n* = 96)	0.312	0.755
HDL ≤ 1.03 (mmol/L)	72.46 ± 50.79 (*n* = 50)	70.53 ± 56.16 (*n* = 49)	0.179	0.858
ALT ≥ 40 (U/L)	105.05 ± 76.35 (*n* = 5)	69.72 ± 51.71 (*n* = 94)	1.454	0.149
AST ≥ 35 (U/L)	67.41 ± 34.74 (*n* = 5)	71.72 ± 54.18 (*n* = 94)	0.178	0.861
DAS28 > 5.1	73.60 ± 54.91 (*n* = 51)	70.05 ± 51.65 (*n* = 49)	0.333	0.740

Notes: For normally distributed data, results are expressed as the mean ± SD. $p < 0.05$ was considered statistically significant. The bold entries emphasized the parameter reached statistical significance.

Abbreviations: AKA, antikeratin antibody; ALT, alanine aminotransferase; Anti-CCP, anti-citrullinated peptide; APF, antiperinuclear factor; ASO, anti-*Streptococcus* hemolysin O; AST, aspartate aminotransferase; AU, arbitrary unit; CRP, C-reaction protein; DAS28, 28-joint count disease activity score; ERY, urine erythrocyte; ESR, erythrocyte sedimentation rate; GPI, glucose phosphate isomerase; HDL, high-density lipoprotein; HRF-IgG, hidden rheumatoid factor IgG antibody; LDL, low-density lipoprotein; PRO, urine protein; RA, rheumatoid arthritis; RF, rheumatoid factor; TCHO, total cholesterol; TG, triglyceride; WBC, white blood cell.

There were several limitations in this study. First, the number of RA patients recruited from our clinical center was limited, which might hinder demonstration of the clinical correlation of anti-cLBP antibodies because of the lack of statistical power. It will be important to recruit more RA patients from multiple clinical centers in our future studies. Another shortcoming of this study is that we did not reveal the exact pathogenesis of anti-cLBP in RA,

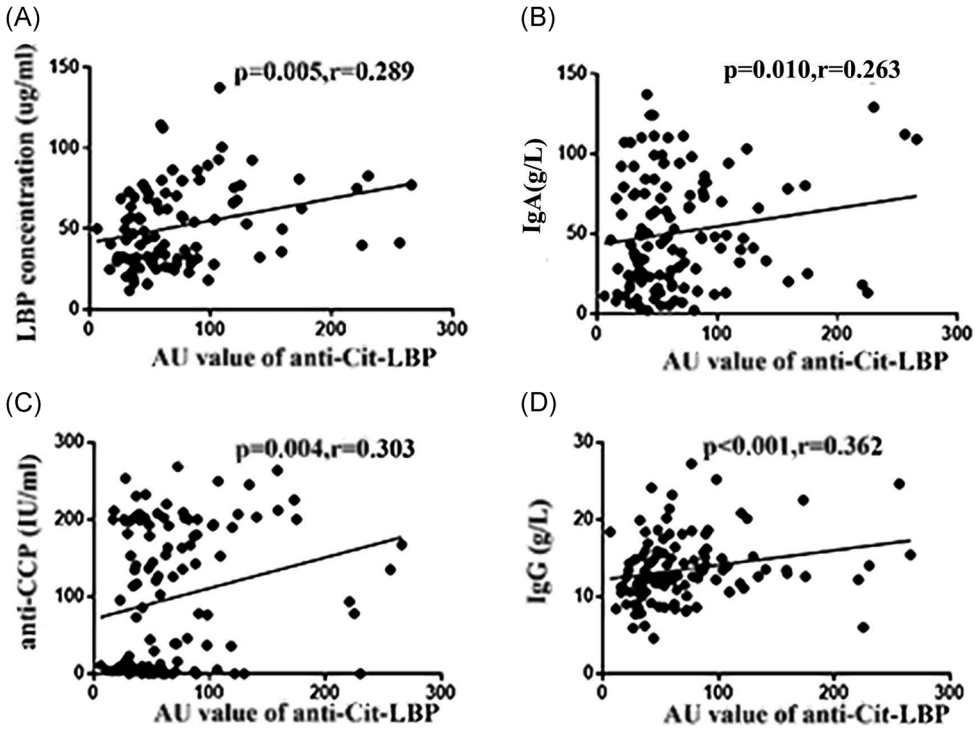

FIGURE 2 Correlation between anti-cLBP antibodies and clinical parameters. (A–D) Anti-cLBP antibodies positively correlated with the lipopolysaccharide-binding protein (LBP) concentration, IgA, anti-citrullinated peptide (anti-CCP), and IgG

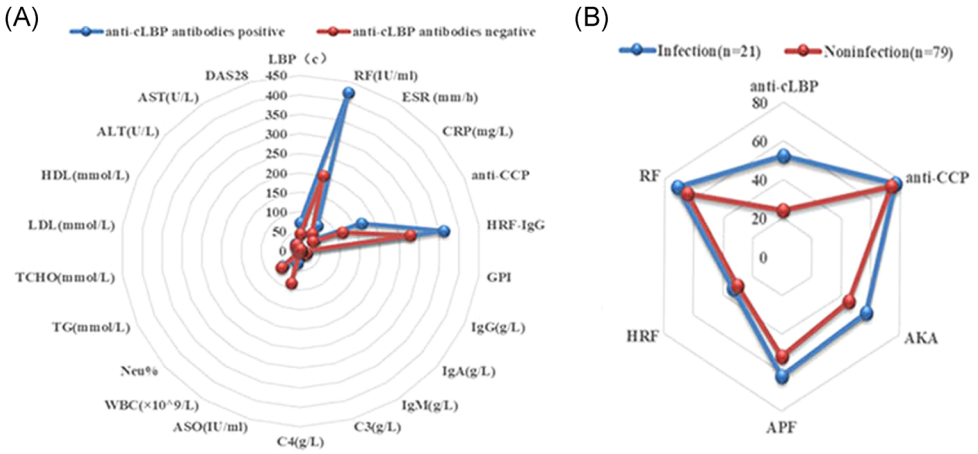

FIGURE 3 Correlation between anti-cLBP antibodies and infection. Comparison of clinical and laboratorial characteristics of positive and negative anti-cLBP antibodies in rheumatoid arthritis (RA) patients with infection. Correlation between anti-cLBP antibodies and other RA autoantibodies and infection

especially how it affects immune responses during infection, which should be revealed in future investigations.

In conclusion, this study developed a protein analysis method for RA-associated autoantigens by high-resolution MS, which identified 11 citrulline-containing proteins in the serum of RA patients. Eight of the proteins are novel antigens in RA, which have not been reported previously. Two (Apo B-100p and C3) of 11 citrulline-containing proteins appeared to have citrulline on at least one position. The identified epitopes of citrulline-containing proteins in RA will be helpful to develop assays for ACPA profiling. Such profiles may benefit ACPA-positive patients with RA in terms of disease responsiveness to some kinds of treatments. The biological functions of many proteins are affected by citrullination and it will be important to investigate to which extent this contributes to pannus formation as well as cartilage and bone damage. We also found that the anti-cLBP antibody was elevated in RA and correlated to disease activity and infection, which implies that anti-cLBP is involved in the infections of RA patients.

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CONFLICT OF INTERESTS

Professor Ru Li, Zhanguo Li are a member of Rheumatology & Autoimmunity editorial board and is not involved in the peer review process of this article.

ETHICS STATEMENT

This study was reviewed and approved by the Peking University People's Hospital Ethics Committee.

AUTHOR CONTRIBUTIONS

Xiaolin Sun, Zhanguo Li, and Haiteng Deng initiated the study and wrote, reviewed, and edited the manuscript. Xiaozhen Zhao and Yulin Chen obtained and analyzed the data, and wrote, edited, and reviewed the manuscript. Wen Wen and Yongjing Cheng obtained data and wrote, edited, and reviewed the manuscript. Yuhui Li, Rulin Jia, Ru Li, and Xu Liu contributed to the study design, and reviewed and edited the manuscript. All authors gave final approval of the manuscript to be published.

DATA AVAILABILITY STATEMENT

The data sets for this study are available from the corresponding authors upon reasonable request.

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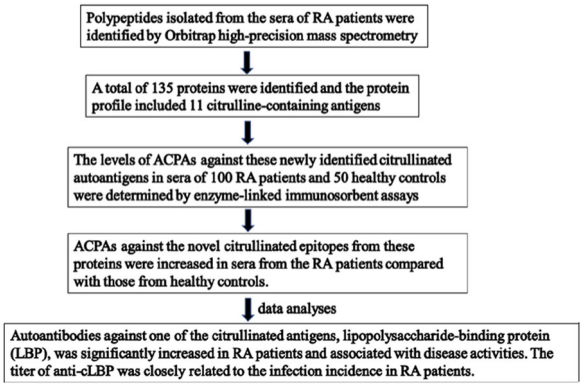
Graphical Abstract

This graphical abstract will be a part of HTML, Online and Print versions.



We performed proteomic analysis by mass spectroscopy to identify novel citrullinated antigens and autoantibodies in RA patients. Graphical Abstract image see the attachment.