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Genetic and environmental factors in rheumatic diseases

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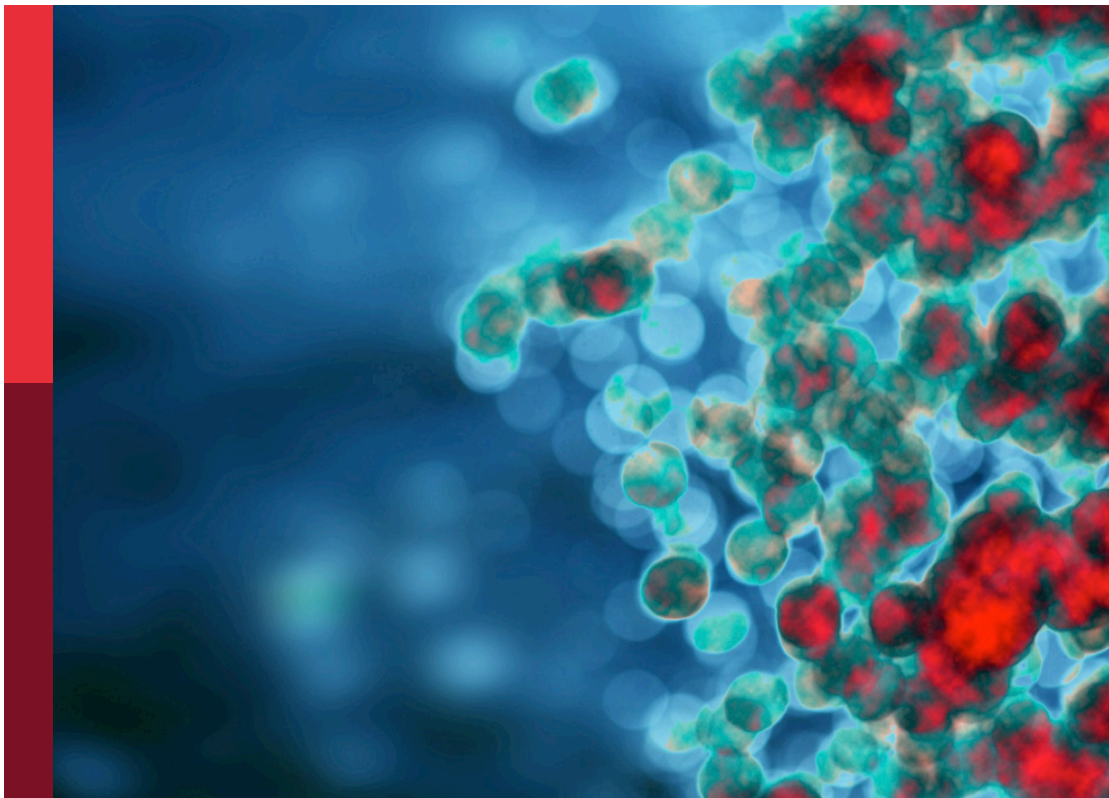

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Genetic and environmental factors in rheumatic diseases

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Association Between Sleep Traits and Rheumatoid Arthritis: A Mendelian Randomization Study

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Currently, the causal association between sleep disorders and rheumatoid arthritis (RA) has been poorly understood. In this two-sample Mendelian randomization (TSMR) study, we tried to explore whether sleep disorders are causally associated with RA. Seven sleep-related traits were chosen from the published Genome-Wide Association Study (GWAS): short sleep duration, frequent insomnia, any insomnia, sleep duration, getting up, morningness (early-to-bed/up habit), and snoring, 27, 53, 57, 57, 70, 274, and 42 individual single-nucleotide polymorphisms (SNPs) ($P < 5 \times 10^{-8}$) were obtained as instrumental variables (IVs) for these sleep-related traits. Outcome variables were obtained from a public GWAS study that included 14,361 cases and 43,923 European Ancestry controls. The causal relationship between sleep disturbances and RA risk were evaluated by a two-sample Mendelian randomization (MR) analysis using inverse variance weighted (IVW), MR-Egger regression, weighted median, and weight mode methods. MR-Egger Regression and Mendelian randomization pleiotropy residual sum and outlier (MR-PRESSO) were used to test for horizontal pleomorphism and outliers. There was no evidence of a link between RA and frequent insomnia (IVW, odds ratio (OR): 0.99; 95% interval (CI): 0.84–1.16; $P = 0.858$), any insomnia (IVW, OR: 1.09; 95% CI: 0.85–1.42; $P = 0.489$), sleep duration (IVW, OR: 0.65, 95% CI: 0.38–1.10, $P = 0.269$), getting up (IVW, OR: 0.56, 95% CI: 0.13–2.46, $P = 0.442$), morningness (IVW, OR: 2.59; 95% CI: 0.73–9.16; $P = 0.142$), or snoring (IVW, OR: 0.95; 95% CI: 0.68–1.33; $P = 0.757$). Short sleep duration (6h) had a causal effect on RA, as supported by IVW and weighted median (OR: 1.47, 95% CI: 1.12–1.94, $P = 0.006$; OR: 1.43, 95%CI:1.01–2.05, $P = 0.047$). Sensitivity analysis showed that the results were stable. Our findings imply that short sleep duration is causally linked to an increased risk of RA. Therefore, sleep length should be considered in disease models, and physicians should advise people to avoid short sleep duration practices to lower the risk of RA.

Keywords: autoimmune disease, rheumatoid arthritis, Mendelian randomization, sleep, causality

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INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory immune system disease that affects joint synovial tissue, tendon sheaths, and bursa, with symptoms of joint pain, stiffness, joint swelling, deformity, functional disability, and sleep disturbances (1). The global prevalence of RA is estimated to be around 1%, with a lower prevalence in some countries. The incidence rises with age, with women over 65 having the highest rate. RA Patients have a higher burden of mortality

and morbidity, as well as a lower life quality and a higher disability rate (2). Many patients were given disease-modifying antirheumatic drugs (DMARDs) do not achieve effective treatment or develop resistance (3, 4). The actual pathophysiological mechanisms of RA are unknown, though studies have suggested that Interactions between genes and the environment, immune dysfunctions, and interstitial tissue disorders may all play a role (3). As a result, it is critical that more efforts be made to investigate the etiology of RA in order to promote treatment strategies with minimal or no side effects.

Sleep disturbances are a common but unrecognized feature of rheumatism (5). Sleep has a significant and broad impact on human immunity, is critical to human health, and is increasingly recognized as a significant lifestyle choice, with studies reporting that decreased sleep duration is associated with an increased risk of cerebrovascular disease and death in the general public (6). An animal model study conducted in New Zealand suggests that sleep deprivation causes immune disorders that may include not only a reduced response to pathogens, but also destroys immune self-tolerance which promotes the emergence of autoimmune diseases (7). According to studies, 50–75% of RA patients suffer from sleep disturbances (8), poor sleep quality and decreased total sleep time are common complaints in RA patients (9, 10), insomnia affects more than half of all patients (11).

Sleep disorders have been linked to RA in cross-sectional studies, but the findings are conflicting (12–14), it is unknown whether sleep problems cause or are independent risk factors for RA. According to data from a baseline assessment of cohort studies of sleep disorders in RA revealed that 87.4% of people had short sleep duration. As disease activity worsens, physical health conditions include pain, fatigue, and dysfunction are impacted, and sleep disorders become more common and future studies will need to look into a causal relationship between poor sleep quality and disease activity (15–17). After controlling for variables, the baseline data from the UK Biobank cohort reveal that rheumatoid factor level (RF) status was still linked with sleep duration, implying that sleep length may influence RF (18). These results show that sleep disturbances may contribute to the development of rheumatoid arthritis. However, whether sleep disturbances are causally associated with RA has rarely been investigated, and conducting clinical trials to answer this question is very difficult.

Mendelian randomization study is an epidemiological study design and data analysis method based on Mendelian's law of independent distribution to verify the etiology hypothesis. It has been widely utilized to evaluate environmental risk variables and illness etiology (19). MR adopts germline genetic variants as proxies. Because environmental factors and disease processes have no effect on genetic variants, MR can reduce the influence of other factors, enhance the exposure-outcome relationship, and remove reverse causality (20). It is thought to be a reliable method for circumventing the limits of observational studies by employing genetic variation as an instrumental variable and large-scale data from GWASs (21). In this study, we aimed to conduct a two-sample MR study to estimate the causal relationship between sleep traits and RA.

MATERIALS AND METHODS

Data Sources

Genetic information is used as an IV in Mendelian randomization studies (19). We carried out a two-sample MR study using data based on publicly available GWAS. Preceding published SNPS associated with each sleep phenotype were chosen as IVs (**Supplementary Table 1**).

Short Sleep

Park et al. (22) used genetic instruments (genetic variants associated with short sleep behavior as instrumental variables) to conduct a MR analysis on 321260 White British individuals and defined short sleep as <6 h. To investigate sleep duration, a standardized touchscreen questionnaire was used: "How much sleep do you get every 24 hours?" (Naps should be included). Sleep time was collected on an hourly basis, and was classified as short (<6 h), medium (6–8 h), and long (≥9 h) groups. Data from the UK Biobank prospective cohort were studied, patients aged 40–69 years, contain 25,605 self-reported short duration sleep (6 h per 24 h), 404,550 reported intermediate sleep (6–8 h), and 35,659 reported long sleep (9 h). MR analysis was performed on 321,260 White British people in the clinical study. As a result, 27 SNPs were associated with short sleep duration and 8 SNPs with long sleep duration. We did not perform MR analysis on long sleep because there were insufficient SNPs in the long sleep data.

Insomnia Symptoms

Participants of European descent in the UK Biobank ($n = 453,379$) self-reported insomnia symptoms by answering the question "Do you have difficulty falling asleep at night or wake up in the middle of the night". In this sample, 29% of individuals self-reported frequent insomnia symptoms (usually), 57 loci for self-reported insomnia symptoms were discovered (23). The study performed two parallel GWAS: (1) Frequent insomnia symptoms: participants whose responses occurred frequently were defined as the case group, while those whose responses never or rarely occurred were defined as the control group ($n = 129,270$ patients) and 108,357 controls); (2) Any symptoms of insomnia: participants whose responses were sometimes or usually frequent were defined as cases, while those whose responses never or rarely appeared were defined as controls ($n = 345,022$ and 108,357 controls), resulting in 57 SNPs associated with frequent insomnia and any symptoms of insomnia.

Sleep Duration

Jansen et al. (24) obtained a sample size of 13,31,010 individuals by combining data from the UK Biobank (UKB) version 2 ($n = 386,533$) and 23 and Me, a privately held personal genomics and biotechnology company ($n = 944,477$). Jansen et al. assessed sleep duration by asking, "About how many hours sleep do you get in every 24 hours?". Only integer values could be used in the answer (round hours). The average sleep duration per 24 h was 7.10 h. The sleep duration GWAS analysis discovered 3,886 GWS SNPs ($P < 5 \times 10^{-8}$) and 53 SNPS were found to be related to sleep duration which were mapped to 49 independent genomic loci. Sleep duration was analyzed as a continuous outcome.

Ease of Getting up in the Morning

Jansen et al. (24) assessed the ease of getting up in the morning in a GWAS study of 1,331,010 people by answering, "In general, how easy do you find getting up in the morning?" There were four possible responses: "not at all easy," "not very easy," "fairly easy," and "very easy." The ease of getting out of bed in the morning was examined as a continuous outcome, divided into four categories. The ease of getting up GWAS analysis revealed 7248 GWS SNPs ($P < 5 \times 10^{-8}$), finally, 70 SNPs associated with ease of getting up were discovered, which were mapped to 62 independent genomic loci.

Morningness

In the GWAS study involving 1,331,010 individuals by Jansen et al. (24), the following responses were given to the question "Do you consider yourself to be?" "Definitely a 'morning' person," "More a 'morning' than an 'evening' person," "More an 'evening' than a 'morning' person," "Definitely an 'evening' person," and "Do not know," and was analyzed on a continuous scale. The morningness GWAS analysis discovered 16,805 GWS SNPs ($P < 5 \times 10^{-8}$), and 274 SNPs associated with morningness were discovered and mapped to 207 independent genomic loci.

Snoring

The snoring GWAS analysis discovered 3416 GWS SNPs ($P < 5 \times 10^{-8}$), represented by 42 independent lead SNPs that were mapped to 36 distinct genomic loci. Snoring was evaluated by asking the question, "Does your partner, a close relative, or a friend complain about your snoring?" Participants could respond with a "yes" or a "no." Finally, 42 snoring-related SNPs were discovered (24).

RA

The GWAS information about RA were acquired from a public GWAS website (https://gwas.mrcieu.ac.uk/), ID: ieu—a—832, the study from the European people involved in 14,361 cases and 43,923 controls and 87,47,963 SNPs. Okada Y et al. published the results of a GWAS meta-analysis of RA related loci in this database in 2014 (25).

SNP Selection

We used the following steps to select the IVs to ensure that genetic instruments were associated with sleep. First, as IVs, SNPs that were significantly associated with sleep were chosen. A set of SNPS that fell below the genome-wide statistical significance threshold ($P < 5 \times 10^{-8}$) was used. Second, for meaningful SNPs, the minor allele frequency (MAF) threshold was set at 0.01. Thirdly, in this study, the clumping process ($R^2 < 0.001$, clumping distance = 10,000 kb) was used to evaluate the linkage disequilibrium (LD) between the contained SNPs. Fourth, the effect of SNPs on exposure should correspond to the same allele as the effect on outcome. Palindromic SNPs are removed from instrumental variables. Fifth, if no exposure-related SNPs were found in the results, proxy SNPs that were significantly associated with meaningful variables ($R^2 > 0.8$) were chosen.

The Assumptions of MR

Two sample MR must meet three assumptions: that genetic instrumental variables are associated with the exposure (relevance assumption), that F statistics are usually employed to evaluate the intensity of the correlation between instrumental variables and exposures. The equation for the F statistic is $F = R^2(n\text{-}k\text{-}1)/k(1\text{-}R^2)$. R^2 denotes the exposure variance of the chosen SNPs interpretation, n is the sample size, and K represents the number of instrumental variables included. If F is <10, there is a weak relationship between the IVs and exposure (26); that they are unrelated to confounding factors (independence assumption); and that they influence the outcome solely through their effects on the exposure of interest (exclusion restriction assumption), implying that there is no horizontal pleiotropic effect between IV and outcomes (27).

MR Estimates

Four different methods were used to assess the causal effects of RA outcomes: Weighted median, MR-Egger regression, IVW, weighted mode (28). When each genetic variant meets the assumptions of an instrumental variable, IVW methods provide consistent estimates of the causal effect of exposure on outcomes (29). Egger's method and weighted median methods provide consistent causal estimates of multiple genetic variants from pooled data under weaker assumptions (28). Even when up to 50% of the information comes from the genetic variation of invalid IV, weighted median estimates still yield consistent estimates of causal effects. The weighted median estimate maintains a more accurate estimate than the MR-Egger analysis (30). The MR-Egger regression methods and MR-PRESSO analysis were adopted to identify and adjust for pleiotropy. The MR-Egger regression analysis, which is robust to invalid instruments, tests and explain the presence of unbalanced pleiotropy by mergering summary data estimates of causal effects from multiple individual variants (31). MR-Egger employs the weighted linear regression of the gene-outcome coefficients on the gene-exposure coefficients. Estimates of causal effects are expressed by the slope of linear regression, and estimates of the mean horizontal pleiotropic effects of genetic variation are shown by intercept (32). Heterogeneity between estimates for each SNP was evaluated using Cochran's Q statistic (33). Sensitivity analysis was performed by eave-one-out Sensitivity test to assess the stability of effect size and identify single SNPS that had a disproportionate impact on association (34). All statistical analyses were performed using R version 4.0.1 with the Two Sample MR 0.5.6 package. The statistical significance was double-tailed, $P < 0.05$.

RESULTS

Supplementary Table 1 shows the data sources. The detailed information of the IVs is provided in **Supplementary Tables S2–S8**. The details of the SNPS included in MR analysis are shown in **Supplementary Table S9**. The visualization results of MR analysis are shown in **Supplementary Figures S1–S6**.

Short Sleep Duration and RA

Initially a total of 27 genome-wide significant SNPs were identified as IVs from the Mendelian Randomization Study performed by Park et al. (22). However, due to the LD with other SNPs, two SNPs (rs75539574 and rs142180737) were removed. SNPs that not associated with RA were removed, the effect alleles were aligned, and all SNPs with palindromic structures were removed, finally, 22 SNPS were included in the next two-sample MR analysis, explaining 0.29% of the variance of short sleep (<6 h). We also collected SNP effect allele, other allele, EAF, beta, se and *P*-value. The results of IVW analyses and weighted median demonstrated that short sleep (<6 h) were positively correlated with the risk of RA (OR: 1.47,95%CI: 1.12–1.94, $P = 0.005$; OR: 1.43, 95%CI: 1.01–2.05, $P = 0.047$) (**Table 1**) (**Figure 1C**). The MR-Egger regression method failed to identify the horizontal pleomorphism between IV and outcomes (**Table 1**) (**Figure 1B**). However, the MR-PRESSO method found horizontal pleiotropy ($P = 0.031$), and rs60882754 was identified as outlier. Results did not change after removing abnormal SNPs (IVW, $\beta = 0.39$, 95% CI, 0.11–0.66, $P = 0.006$). No heterogeneity was detected ($Q = 23.65$, $P = 0.258$) (**Figure 1D**). The results of the leave-one-out sensitivity test showed that the results of the MR analysis were robust (**Figure 1A**). The detailed information of the IV was shown in **Supplementary Table S2**. The *F* statistics of the SNPs was >10, indicating that there was no weak instrumental variables bias (**Table 1**).

Frequent Insomnia and RA

A total of 57 SNPs were associated with frequent insomnia in the study by Lane et al. (23). 43 SNPs remained after removal of LD. After removing SNPs with independence from RA, aligning effect allele, and removing all SNPs with palindromic, the remaining 40 SNPs were finally included in the next two-sample MR analysis, explaining 0.68% of the variance of frequent insomnia. The results of IVW analyses demonstrated no association between frequent insomnia and RA (OR, 0.99; 95% CI, 0.84–1.16; $P = 0.858$) (**Table 1**) (**Supplementary Figure S1C**). Horizontal pleiotropy between instrumental variables and outcomes was not identified by the MR-Egger regression (**Table 1**) (**Supplementary Figure S1B**) method and the MR-PRESSO method ($P = 0.583$; $P = 0.457$) (**Table 1**), and no heterogeneity was detected ($Q = 28.67$, $P = 0.570$) (**Table 1**) (**Supplementary Figure S1D**). The detailed information of the IV was shown in **Supplementary Table S3**. There was no weak instrumental variables bias ($F = 51.25$).

Any Insomnia and RA

In the study of Lane et al. (23) a total of 57 SNPs were related to sleep difficulty, and 43 SNPs remained after removing the LD. After removing SNPs independent of RA, and aligning the effect allele, 40 SNPs remained, and 33 SNPs were included in the next two-sample MR analysis, explaining 0.29% of the variance of any insomnia. The results of IVW analyses did not find any insomnia to be associated with RA (OR, 1.09; 95% CI, 0.85–1.42; $P = 0.489$) (**Table 1**) (**Supplementary Figure S2C**). The MR-Egger regression method did not identify horizontal pleiotropy between IV and outcomes (**Table 1**) (**Supplementary Figure S2B**). The heterogeneity test showed the existence of heterogeneity ($Q = 46.52$, $P = 0.036$) (**Table 1**) (**Supplementary Figure S2D**). The MR-PRESSO method detected the outlier rs10156602. After removing the outlier, MR analysis showed no significant change (IVW, $\beta = 0.09$, 95% CI, −0.18–0.35, $P = 0.515$) the heterogeneity still existed ($P = 0.028$) (**Table 1**). The detailed information of the IV was shown in **Supplementary Table S4**. There was no weak instrumental variables bias ($F = 39.93$).

Sleep Duration and RA

We selected 53 SNPs in the GWAS of Jansen et al. (24) as IVs. 10 SNPs were deleted due to LD (rs1392817, rs11682175, rs2863244, rs11883686, rs34388845, rs1633063, rs6979198, rs7778250, rs1668331, rs62061734). SNPs that independent of RA were removed, and the effect allele was aligned and all SNPs with palindromic structures were removed. The last 30 SNPs were included in the next two-sample MR analysis, explaining 0.08% of the variance of sleep duration. MR analysis showed that sleep duration was not associated with RA (OR: 0.65, 95% CI: 0.38–1.10, $P = 0.269$) (**Table 1**) (**Supplementary Figure S3C**). The MR-Egger regression method did not identify horizontal pleiotropy between instrumental variables and outcomes ($P = 0.354$) (**Table 1**) (**Supplementary Figure S3B**). The MR-PRESSO method found horizontal pleiotropy ($P = 0.027$), and rs12215241 was identified as outlier, and rs12215241 was identified as outlier. However, the results did not change when the abnormal SNP values were removed (IVW, $\beta = -0.3$, 95% CI, −0.77–0.17, $P = 0.215$) (**Table 1**). Heterogeneity was detected between SNPs ($Q = 42.53$, $P = 0.039$) (**Table 1**) (**Supplementary Figure S3D**). The heterogeneity disappeared after removing outliers ($P = 0.233$). **Supplementary Table S5** displays detailed IV information. The SNPs had F-statistics >10, indicating that there was no weak instrumental variable bias (**Table 1**).

Getting up and RA

We selected 70 SNPs from the GWAS of Jansen et al. (24), 17 SNPs were removed due to LD (rs148173313, rs6691053, rs75650221, rs4652514, rs1402121, rs10180284, rs13393656, rs35333999, rs4483990, rs11520042, rs17464772, rs1017168, rs61963491, rs1949072, rs7222039, rs3746601, rs4634827). After removing SNPs with independence from RA, performing effect allele alignment and removing all SNPs with palindromic, 43 SNPs were included in the next two-sample MR analysis, explaining 0.09% of the variance of getting up. MR analysis showed that there was no causal relationship between getting up and RA (IVW, OR: 0.56, 95% CI: 0.13–2.46, $P = 0.442$) (**Table 1**) (**Supplementary Figure S4C**). The MR-Egger regression method did not identify horizontal pleiotropy between instrumental variables and outcomes ($P = 0.278$) (**Table 1**) (**Supplementary Figure S4B**) MR-PRESSO test found 3 outliers (rs2193749, rs553108, rs61773374), MR analysis was performed after removing outliers, and the results did not change significantly (IVW, $\beta = -0.23$, 95% CI: −0.80–0.35, $P = 0.440$) (**Table 1**). The detailed information of the IV was shown in **Supplementary Table S6**. No heterogeneity was found after removing outliers ($P = 0.18$) (**Table 1**). There was no weak instrumental variables bias ($F = 28.72$) **Table 1**.

TABLE 1 | MR results of causal links between sleep traits and RA risk.

Exposure	N SNP	Methods	β (95% CI)	OR (95%CI)	SE	*P* value	Horizontal pleiotropy							Heterogeneity			
							MR-Egger regression				MR-PRESSO			Cochran's *Q*	*P* value	*P* value*	*F*
							Egger intercept	SE	*P* value	Global test *P* value	Outliers	β* (95% CI)	*P** value				
Short sleep	22	MR Egger	−0.04 (−1.28–1.21)	0.96 (0.28–3.36)	0.64	0.954	0.01	0.02	0.503	0.031	rs60882754	−0.04 (−1.28–1.21)	0.954	23.65	0.258	0.258	42.65
		Weighted median	0.36 (0.01–0.72)	1.43 (1.01–2.05)	0.18	0.047						0.36 (−0.00–0.72)	0.068				
		Inverse variance weighted	0.39 (0.11–0.66)	1.47 (1.12–1.94)	0.14	0.006						0.39 (0.11–0.66)	0.006				
		Weighted mode	0.40 (−0.16–0.96)	1.50 (0.86–2.62)	0.29	0.172						0.40 (−0.14–0.14)	0.179				
Frequent insomnia	32	MR Egger	0.12 (−0.38–0.62)	1.13 (0.68–1.85)	0.25	0.645	0.01	0.01	0.583	0.457				28.67	0.535		51.25
		Weighted median	−0.14 (−0.37 0.10)	0.87 (0.69–1.10)	0.12	0.249											
		Inverse variance weighted	−0.01 (−0.17–0.35)	0.99 (0.84–1.16)	0.08	0.858											
		Weighted mode	−0.12 (−0.43 0.18)	0.88 (0.65–1.20)	0.15	0.430											
Any insomnia	33	MR Egger	0.01 (−0.73–0.76)	1.01 (0.48–2.14)	0.38	0.974	0.00	0.01	0.829	0.041	rs10156602	0.01 (−0.74–0.77)	0.972	46.52	0.036	0.028	39.93
		Weighted median	0.08 (−0.24–0.40)	1.08 (0.78 1.50)	0.16	0.625						0.06 (−0.27–0.39)	0.715				
		Inverse variance weighted	0.09 (−0.17–0.35)	1.09 (0.85–1.42)	0.13	0.489						0.09 (−0.18–0.35)	0.515				
		Weighted mode	0.01 (−0.39–0.41)	1.01 (0.67–1.51)	0.21	0.964						−0.03 (−0.42–0.36)	0.862				
Sleep duration	30	MR Egger	−1.02 (−2.36–0.32)	0.36 (0.09–1.37)	0.68	0.684	0.01	0.01	0.354	0.027	rs12215241	−0.87 (−2.06–0.32)	0.163	42.53	0.039	0.233	36.32
		Weighted median	−0.5 (−1.17–0.18)	0.61 (0.31–1.19)	0.34	0.344						−0.5 (−1.18–0.19)	0.157				

(Continued)

TABLE 1 | Continued

Exposure	N SNP	Methods	β (95% CI)	OR (95%CI)	SE	*P* value	Horizontal pleiotropy							Heterogeneity			
							MR-Egger regression				MR-PRESSO						
							Egger intercept	SE	*P* value	Global test *P* value	Outliers	β* (95% CI)	*P** *value*	Cochran's *Q*	*P* value	*P** *value*	*F*
		Inverse variance weighted	−0.43 (−0.96–0.10)	0.65 (0.38–1.10)	0.27	0.269						−0.3 (−0.77 0.17)	0.215				
		Weighted mode	−0.66 (−1.54–0.22)	0.52 (0.21–1.24)	0.45	0.448						−0.67 (−1.49 0.16)	0.124				
Getting up	43	MR Egger	1.46 (−2.47–5.38)	4.29 (0.08–216.94)	2.00	0.471	0.03	0.03	0.278	<5e-04	rs2193749, rs553108, rs61773374	0.16 (−1.37 1.69)	0.838	368.25	0.000	0.165	28.72
		Weighted median	0.22 (−0.62–1.05)	1.24 (0.54–2.85)	0.43	0.612						−0.29 (−1.11–0.52)	0.477				
		Inverse variance weighted	−0.58 (−2.06–0.90)	0.56 (0.13–2.46)	0.76	0.442						−0.23 (−0.80–0.35)	0.440				
		Weighted mode	−0.09 (−1.19–1.00)	0.91 (0.30–2.72)	0.56	0.867						−0.30 (−1.30–0.70)	0.566				
Morningness	109	MR Egger	0.95 (−0.31 2.21)	2.59 (0.73–9.16)	0.64	0.142	0.02	0.01	0.099	<0.0003	rs486416, rs6716898, rs9295795	0.68 (−0.07–1.43)	0.078	422.25	0.000	0.008	47.92
		Weighted median	0.30 (−0.10–0.70)	1.36 (0.91–2.02)	0.20	0.136						0.30 (−0.09–0.69)	0.126				
		Inverse variance weighted	−0.05 (−0.51 0.41)	0.95 (0.60–1.51)	0.23	0.837						0.12 (−0.15–0.40)	0.385				
		Weighted mode	−0.24 (−0.84–0.36)	0.78 (0.43–1.43)	0.31	0.427						−0.27 (−0.92–0.37)	0.405				
Snoring	22	MR Egger	1.24 (−0.41–2.88)	3.44 (0.67–17.77)	0.838	0.156	0.04	0.03	0.132	0.109				28.33	0.102		49.26
		Weighted median	0.27 (−0.11–0.66)	1.32 (0.90–1.93)	0.196	0.163											
		Inverse variance weighted	−0.05 (−0.39–0.28)	0.95 (0.68–1.33)	0.171	0.757											
		Weighted mode	0.3 (−0.30–0.89)	1.35 (0.74–2.434)	0.303	0.336											

**The result of recalculation after removing outliers.*

MR, Mendelian Randomization; MR-PRESSO, MR-Pleiotropy Residual Sum and Outlier method; OR, odds ratio; CI, confidence interval; IVW, inverse-variance weighted.

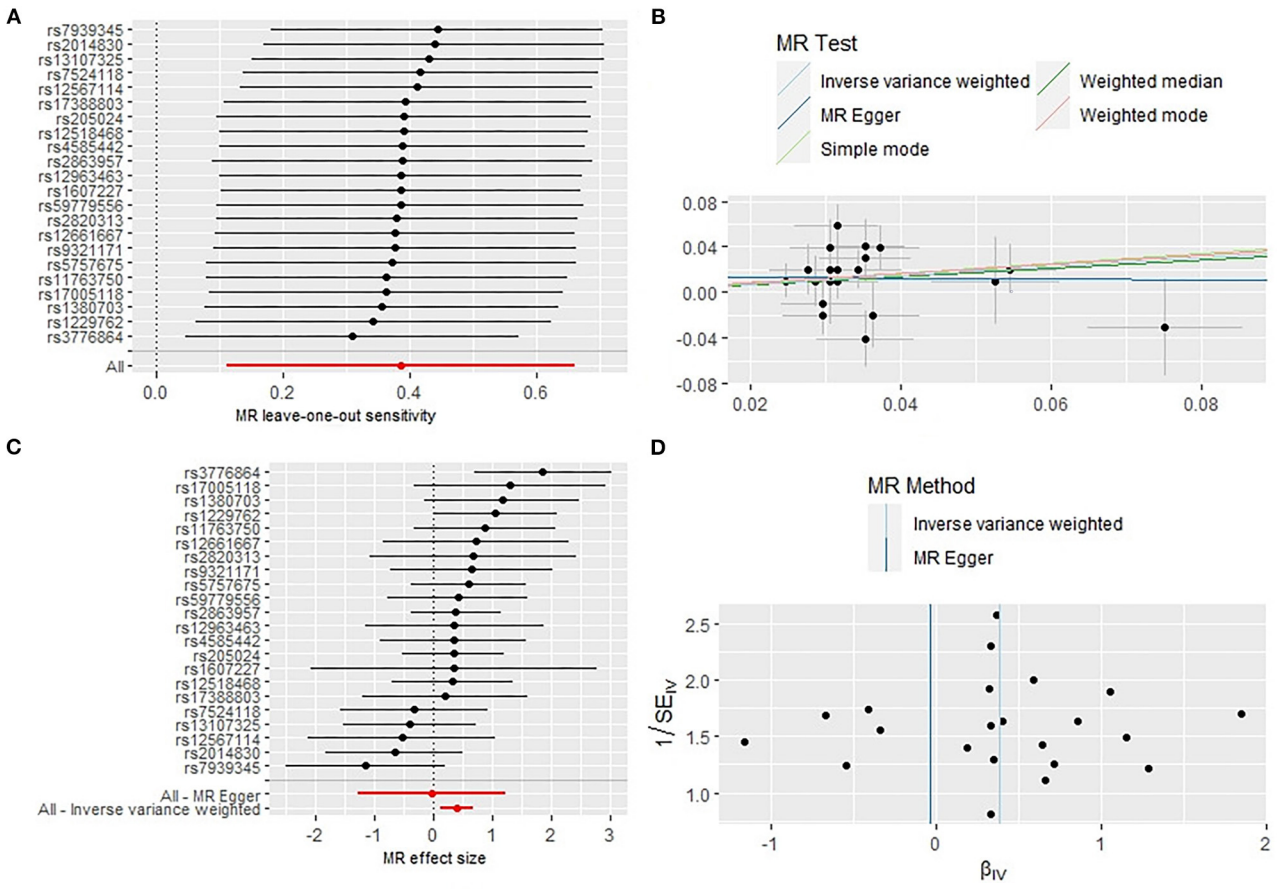

FIGURE 1 | Sensitivity analysis **(A)**, scatter plot **(B)**, forest plot **(C)**, and funnel plot **(D)** of the causal effect of short sleep duration on RA risk.

Morningness and RA

In the study of Jansen et al. (24), 274 SNPs were associated with morning phenotype, and 131 SNPs were removed due to LD, after removing the SNPs independent of RA, 131 SNPs remained after aligning the alleles. Finally, the remaining 109 SNPs were included in the MR analysis, explaining 0.39% of the variance of the morningness type, The results of IVW analyses indicated no association between the morningness type and RA (OR: 2.59; 95% CI: 0.73–9.16; $P = 0.142$) (**Table 1**) (**Supplementary Figure S5C**). The MR-Egger regression method did not identify horizontal pleiotropy between IV and outcomes ($P = 0.099$) (**Table 1**) (**Supplementary Figure S5B**). The MR-PRESSO method detected outliers rs486416, rs6716898, and rs9295795. Heterogeneity test was performed to find heterogeneity among SNPs (**Table 1**) (**Supplementary Figure S5D**). After removing the outliers, the results did not change, and the heterogeneity still existed ($P = 0.008$) (**Table 1**). The specific information of the IV was displayed in **Supplementary** Table S7. There was no weak instrumental variables bias ($F = 47.92$).

Snoring and RA

There were 42 SNPs related to snoring in the study of Jansen et al. (24), 11 SNPs were removed due to LD (rs35915391, rs10190879, rs745558, rs2762049, rs2408111, rs7217107, rs4792897, rs1563304, rs2924251, rs60, rs2924251, rs60, rs2924251, rs64). After SNPs that were independent of RA were removed, 28 SNPs remained after allele alignment and removal of the SNPS with palindromic structure. The last 22 SNPs were included in the MR analysis, explaining 0.08% of the variance in snoring. The results of IVW analyses did not find association between snoring and RA (OR: 0.95; 95% CI: 0.68–1.33; $P = 0.757$) (**Table 1**) (**Supplementary Figure S6C**). The MR-Egger regression (**Table 1**) (**Supplementary Figure S6B**) and MR-PRESSO method did not identify horizontal pleiotropy between instrumental variables and outcomes ($P = 0.132$; $P = 0.109$) (**Table 1**). There was no heterogeneity in the results ($P = 0.102$) (**Supplementary Figure S6D**). The detailed information of the IV was shown in **Supplementary Table S8**. There was no weak instrumental variables bias ($F = 49.26$).

DISCUSSION

In this TSMR study, we investigated the effects of seven sleep traits on RA, including short sleep, frequent insomnia, any insomnia, sleep duration, getting up, morningness, and snoring. Short sleep duration (<6 h) was linked to an increased risk of RA. However, because fewer IVs reached the genome-wide statistical significance threshold, the results and accuracy of sleep may have been affected.

RA is a chronic inflammatory autoimmune disease, the pathogenesis of which has not been fully clarified. It may be associated with inflammation, synovial cells and cartilage cells, autoantibodies, genetics, immune responses. Immune disturbances can have an effect on sleep quality, and sleep disturbances can also have an impact on immune function. Sleep is especially important for triggering an effective adaptive immune response, which results in long-term immune memory (35). T_{reg} inhibitory activity was reduced in healthy sleep-deprived subjects. T_{regs} play a crucial role in suppressing inappropriate immune responses and preserving self-tolerance. The disruption of self-tolerance is nuclear to the pathogenesis of most autoimmune diseases, establishing a linkage between sleep disorders and autoimmune diseases (36). Sleep disturbances cause an increase in several pro-inflammatory cytokines, and IL-17 remains elevated despite recovering within 7 days of sleep deprivation; IL-17 plays a key role in coordinating inflammation; and IL-17 is linked to the onset of RA (37). Sleep disorders can impair immune defenses and cause inflammatory changes throughout the body, triggering the onset of RA.

However, our findings revealed that frequent insomnia, any insomnia, sleep duration, getting up, morningness, and snoring has nothing to do with an increased risk of RA. According to data from a large, prospective study in Norway, after controlling for confounding factors, there is evidence that insomnia increases the risk of rheumatoid arthritis (38). A longitudinal study found that pain in RA patients predicted sleep problems 2 years later, but that sleep problems had no effect on subsequent pain (39). Several cross-sectional studies have also linked sleep problems to pain (2), depression (40), disease activity, and fatigue (41). These findings suggest that sleep disturbance may be an adverse consequence of RA, which may be related to joint pain, activity limitation, and medication in active disease, which cannot fully explain that sleep disturbance is the cause of RA. Since the immune system appears to play a significant function in regular sleep, variation in inflammatory load can influence sleep quality, and cytokines such as interleukin (IL)-1β, IL-6, or tumor necrosis factor (TNF) have been found to alter animals and play a key role in human sleep quality (42), Elevated levels of IL-6 are associated with sleep disorders and fatigue symptoms in inflammatory diseases such as RA, and insufficient sleep time and quality can also stimulate the elevation of IL-6 levels and promote the progression of RA(43), these inflammatory factors are closely related to the pathogenesis of RA(44). This suggests that sleep disorders do not directly influence the incidence of RA, but they may influence the incidence of RA via inflammatory mediators.

In recent years, more and more observational evidence has shown that short sleep duration is a risk factor for the progress of RA. A cohort study found that short sleepers (≤6 h/day) were 1.23 times more possible to develop RA than those who slept 7–8 h (95% CI 1.101–1.51), after adjusting for confounding factors (17). An observational study showed that RA patients do have sleep disorders, especially short sleep duration. Poor sleep quality and short sleep duration may be the inducement or risk factor of RA (45). However, most of these previous studies were cross-sectional, retrospective or prospective cohort studies and, due to their observational nature, could not overcome the influence of unmeasured confounding factors on the results. MR methods were adopted to research the causal effect of genetically predicted short sleep duration on RA risk, which may control for unmeasured confounders and reverse causality. The complex interactions between the central nervous system, endocrine system, and immune system play an important role in normal sleep, and sleep problems in RA patients may arise at various levels of these systems. Although sleep disturbances are more common in RA patients, studies have shown that sleep disturbances do not change with improvement in disease activity (46). The Swedish "RA Epidemiological Survey" from 1998 to 2018 collected sleep data from 1 to 12 years after diagnosis and did not find any major sleep problems. The existing sleep problems were mainly related to pain and functional decline (47). This may explain to a certain extent that short sleep time may be the cause of RA, and there is a one-way causal relationship between short sleep time and RA. Our findings also suggest a causal effect between short sleep duration and RA, suggesting that shorter sleep duration may increase the risk of RA.

Our study has the following advantages: First, as far as we know, this is the first study to investigate the genetic link between sleep traits and RA risk, and while some previous cross-sectional studies have controlled for confounding factors, there may still be undetected biases. Second, because of the large number of published genetic associations used to screen for suitable genetic instrumental variables, MR analysis is a time and cost-effective method for assessing and screening potential causal relationships. Based on large sample sizes, we were capable to investigate the effect of short sleep, sleep duration, frequent insomnia, any insomnia, morningness, getting up, and snoring on RA using a two-sample MR design (14,361 cases and 43,923 controls). This broadens the scope of our findings. Third, in the MR analysis, genes are used as IV, and the possibility of these genes being related to environmental confounders is very small. Genotypes are randomly distributed during pregnancy, prior to exposure, and the association between genotypes and disease is not affected by reverse causality. Exposure-related genotypes are typically linked from birth to adulthood, avoiding attenuation due to causal inference error (regression dilution bias).

Our study has several limitations that should be considered. First, because not all genes that determine all traits are isolated independently, Mendel's second law does not apply to all genetic variants (randomly) and genes as IVs cannot avoid bias due to weak instrumental variable, population

stratification, and developmental compensation. MR genetic instruments are chosen from hypothesis-free genome-wide association studies, which may lack a thorough understanding of the mechanisms underlying associations between genetic variants and diseases (48), this could have an impact on the results. Second, our study is limited to the European population. As a result, it is necessary to conduct additional studies to determine whether our study can be extended to other populations. Third, all sleep characteristics were described subjectively by the patient. As a result, misclassification is unavoidable. Fourth, even if we took steps to identify and eliminate outlier variants, we cannot rule out the possibility that horizontal pleiotropy influenced our results. Finally, in our MR analysis, each method has advantages and disadvantages. However, we used four methods based on different assumptions, which may result in inconsistent results, thereby clouding the study's conclusions.

CONCLUSION

Our results reveal the causal relationship between gene-predicted sleep traits and RA, and we only identify the causal relationship between short sleep and RA, which is somewhat inconsistent with many published observational studies. To verify our results more accurately, updated MR analyses are necessary when more advanced methods yield better accuracy or GWAS aggregate data, and more RA patient information becomes available.

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.

AUTHOR CONTRIBUTIONS

G-CW designed this study. R-CG and NS analyzed data, and wrote the draft of the manuscript. R-CG, NS, C-ZJ, M-YZ, B-HL, and MW discussed and reviewed the manuscript critically. G-CW and R-CG revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2022.940161/full#supplementary-material

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Risk of primary Sjogren's Syndrome following human papillomavirus infections: a nationwide population-based cohort study

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Introduction: Viral infection is an exogeneous factor for primary Sjogren's syndrome (pSS). This study investigated the association between human papillomavirus (HPV) infections and pSS through a nationwide population based cohort study.

Methods: Patients with HPV infections between January, 1999 and December, 2013 were included. The incidence of new-onset pSS in patients with HPV infections and non-HPV controls were derived. The multiple Cox regression model derived the risk of pSS in patients with HPV infections. Subgroup analysis and sensitivity analysis were performed to validate the association.

Results: During a follow-up period of 12 years, the adjusted hazard ratio (aHR) of pSS in patients with HPV infections was significantly higher than that in non-HPV controls (aHR=1.64, 95% CI=1.47-1.83, P<0.001). The risk of pSS increased with age and the risk increased by 2.64-fold (95% CI= 2.37-2.93) for those older than 45 years. The significant association between HPV infections and the risk of pSS persisted in the sensitivity analysis restricted in HPV infections that lasted over 12 months (aHR=1.63, 95%CI=1.45-1.83, P<0.0001). Subgroup analyses revealed that both male (aHR=1.83, 95%CI=1.47-2.28, P<0.0001) and female (aHR=1.58, 95%CI=1.40-1.79, P<0.0001) patients with HPV infections and HPV-infected patients aged between 16 and 45 years (aHR=1.60, 95%CI=1.34-1.91, P<0.0001) and over 45 years (aHR=1.67, 95%CI=1.46-1.91, P<0.0001) were associated with a significantly greater risk of pSS.

Conclusion: Patients with HPV infections presented with a significantly higher risk of pSS, regardless of age and sex.

KEYWORDS

Human papillomavirus, infection, autoimmunity, primary Sjogren's Syndrome, cohort study

Introduction

Primary Sjogren's syndrome (pSS) is a chronic autoimmune disorder manifested as lymphocytic infiltration, which causes inflammation of the exocrine glands. The destructed glands result in dryness of the eyes and mouth, pain of the joints and fatigue (1). 30 to 40% of pSS patients have other systematic complications involving lungs, kidneys, gastrointestinal (GI) tract, and nervous system. Epidemiological data has revealed that pSS prevalence is approximately 1% of general population and female predominance (16:1) (2). Genetic inheritance and environmental factors are previously reported to enhance pSS pathogenesis; however, pSS etiology remains unclear, for which viral infection has been as well considered an external factor for it induces chronic inflammation (3, 4). Viral particles translated by viral gene would act as autoantigens, which attract auto-antibodies to combine and trigger proliferation of B cells to cause autoimmune disease. Infected cells may also activate cytotoxic T cells in response through the major histocompatibility (MHC) class I (MHC-1) antigen presentation pathway. Various virus including human T-lymphocytic virus type-1 and type-5, Epstein-Barr virus, Coxsackie virus play an important role in activating auto-inflammation. Several other infection agents including human herpes virus 6, human immunodeficiency virus, and hepatitis B have also been reported to trigger pSS (3, 5). The potential associated mechanisms between viral infections and the immune-pathogenesis of pSS require further research.

Human papillomaviruses (HPVs) are transmitted through sexual intercourse or direct contact with macerated skin and they exclusively invade the epithelium and mucous membranes (6). The HPV prevalence in Taiwan is about 10-20% (7) and predominately in women, which corresponds to the prevalence of pSS in female sex. HPV 16 and 18 are the most prevalent types in female population in Taiwan and other Asian countries (8, 9). There are two prophylactic HPV vaccines available in Taiwan currently. The bivalent vaccine protects against HPV type 16 and 18 and young women before sexual debut are recommended to be vaccinated. The quadrivalent vaccine targets HPV type 6, 11, 16, and 18 (10), and both genders are recommended to be vaccinated (11, 12). Taiwan government supports HPV vaccination, but less than 4% of female population has been vaccinated (12). More than 80% of Taiwanese females are still susceptible to HPV infection due to lack of health education.

A recent study indicates that female patients with rheumatic diseases have higher risk for HPV infections, which might be due to disarranged immune system or immunosuppressive effect of treatments (8). However, currently there is no evidence that supports a reverse correlation of the incidence of pSS in HPV-infected patients, despite an autoimmune phenotype observed in HPV patients (13). As such, the present study aims to determine the incidence of pSS in patients with HPV infections.

Methods

Data source

We used the Longitudinal Health Insurance Database 2000 (LHID2000) as our data source, which is a subset of the National Health Insurance Research Database (NHIRD) (14, 15). The LHID2000 contains information on one million randomly selected patients' sex, date of birth, clinical interventions, hospitalization, medications, dosages, and diagnoses in accordance to the requirements of the International Classification of Disease–Ninth Revision, Clinical Modification, from 2000 to 2013. Demographic dates, records of inpatient or outpatient visits, catastrophic illness, items of medical expenditure, and other clinical materials were available in this database and extracted for research purpose of this study. With a large sample size, the database has been largely used in observational studies (16). The research protocol of the present study was approved and supervised by the Institutional Review Board (IRB) of Chung Shan Medical University Affiliated Hospital.

Study design

To clarify the association between HPVs and the risk of pSS, we designed a retrospective cohort study. The exposure group and the controlled group were followed up from the index date

to the date of pSS onset, study subjects withdrawal, or study completion. The index date was defined as the date of HPV diagnosis. The observational period was set between January, 1999 and December, 2013. We also selected several comorbidities to investigate their effects on the incidence of pSS. The HPV group included patients with new-onset HPV infections between 2002 and 2012; while the non-HPV group included patients who did not have HPV infections during the study period. Propensity score matching of 1:4 by sex, year of birth and the index date was used to select non-HPV controls. For both cases and controls, individuals of any missing demographic data, patients who developed pSS before index date, aged below 16 in 2002, or had HPV infections before 2002 and new-onset HPV infections after 2012, were excluded.

Study population

After excluded 362 patients with pSS diagnoses prior to HPV infections, 6,817 patients with missing demographic data, 185,298 patients younger than 16 years in 2002, 8,664 patients with previous HPV diagnoses before 2002, and 3,491 patients with HPV diagnoses after 2012, a total of 47,302 patients with first HPV diagnoses (ICD-9 code: 078.11, 079.4, 078.1, 078.10–078.12, 078.19, 759.05, 795.09, 795.15, 795.19, 796.75 and 796.79, along with records of positive polymerase chain reaction test results) between 2002 and 2012 were enrolled in the HPV group (Figure 1).

748,066 patients without HPV diagnoses between 2002 and 2012 were enrolled as the comparison group and matched to patients in the HPV group by sex, year of birth and the index date at a ratio of 1:4. As a result, there were 47,300 patients in the HPV exposure group and 189,200 matched-controls in the control group at baseline (Figure 1).

Variables and measurements

New-onset pSS was set as the primary endpoint of this study and the diagnoses were based on the American College of Rheumatology (ACR)-the European League Against Rheumatism (EULAR) classification criteria (17). To ensure the consistency of the diagnoses, the diagnostic records of pSS have to be confirmed by at least 3 outpatient records within 2 years or at least 1 hospital admission history between 2002 and 2012. Predictor variables included comorbidities such as rheumatoid arthritis, pneumonia, bronchitis, dental caries, chronic liver disease, cholelithiasis, interstitial nephritis, calculus of kidney, urinary tract infection, arthralgia, chronic obstructive lung disease (COPD), as identified within 2 years preceding the index date.

Statistical analysis

Propensity score matching (18–25) was used to homogenize the baseline characteristics of the two groups (26–35). To reveal the potential risk factors contributing to the pathogenesis of pSS during follow-up, we performed multiple Cox regression on selected comorbidities to estimate the adjusted hazard ratios (aHRs) of pSS and calculated the corresponding 95% confidence intervals (CIs) to ensure the precision and accuracy of the

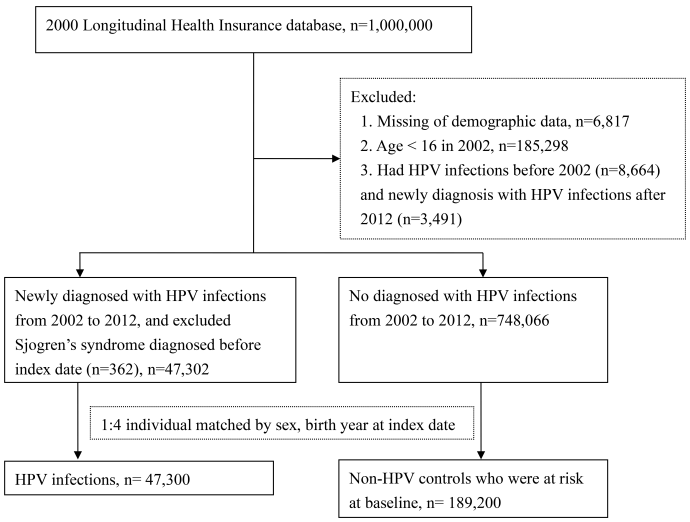

FIGURE 1
Flowchart for study design.

analysis. We recognized statistically significant results with a two-tailed p value <0.05. All data were analyzed using SAS software (version 9.4; SAS Institute, Inc., Cary, NC, USA).

Results

Baseline characteristics

In total, 47,300 HPV-infected patients and 189,200 non-HPV individuals were enrolled at the baseline. The mean age at the time of diagnosis of HPV was 42.59 ± 16.43 years, and 59.24% of HPV patients were between the age of 16 and 45. The female to male ratio was slightly greater than 1 (50.03% vs. 46.97%). After propensity score matching, baseline demographics and comorbidities were balanced (Table 1).

Incidence of pSS among patients with HPV infections

A total of 493 HPV patients and 1,081 controls were diagnosed with pSS, which corresponded to incidence rates of 13.61 and 7.53 per 100,000 person-months in the total follow-up period of 3,622,659 and 14,359,439 person-months for the HPV group and control group (Table 2). The pSS incidence rate in the HPV group was 1.81(95% CI=1.63-2.01) times higher than in the control group (Table 2). After almost 12 years of observation, the pSS cumulative incidence in the HPV group was significantly higher than in the control group (log-rank p<0.0001, Figure 2).

Risk for pSS in patients with HPV infections

Subsequent risk of pSS was estimated by using Cox proportional hazard regression (Table 3). Compared with the control group, patients with HPV exposure has a 1.64-fold aHR of pSS after adjusting for age, sex, and selected comorbidities (95% CI = 1.47–1.83) (Table 3). The risk of pSS increased with age by 2.64-fold (95% CI= 2.37-2.93) for over 45 years group (Table 3). Females has 2.78 times higher pSS risk than males (95% CI= 2.5-3.125) (Table 3). Patients with RA, COPD, pneumonia, dental caries, chronic liver disease, calculus of kidney, urinary tract infection, or arthralgia have increased the risk for pSS with aHRs of 3.67 (95% CI=2.86-4.70), 1.56 (95% CI=1.31-1.86), 1.29 (95% CI=1.00-1.67), 1.43 (95% CI=1.30-1.58), 1.32 (95% CI=1.14-1.53), 1.37 (95% CI=1.05-1.77), 1.29 (95%CI=1.14-1.46), and 1.31 (95% CI=1.14-1.49), respectively (Table 3).

Sensitivity analysis

After excluded individuals with at least 12 months of loss of follow-up, the aHR of pSS in HPV patients was 1.63 (95%

TABLE 1 Characteristics in study groups at baseline.

	HPV infections n = 47,300	Non-HPV controls n = 189,200	p value
Age at baseline, Mean ± SD	42.59 ± 16.43	42.59 ± 16.43	1.0000
16-45	28,021 (59.24%)	112,084 (59.24%)	
≧45	19,279 (40.76%)	77,116 (40.76%)	
Sex			1.0000
Female	25,083 (53.03%)	100,332 (53.03%)	
Male	22,217 (46.97%)	88,868 (46.97%)	
Comorbidities			
Rheumatoid arthritis	404 (0.85%)	1,179 (0.62%)	<.0001
Pneumonia	1,468 (3.10%)	5,022 (2.65%)	<.0001
Bronchitis	1,914 (4.05%)	5,478 (2.90%)	<.0001
Dental caries	23,425 (49.52%)	69,654 (36.82%)	<.0001
Chronic liver disease	5,100 (10.78%)	14,246 (7.53%)	<.0001
Cholelithiasis	905 (1.91%)	2,667 (1.41%)	<.0001
Interstitial nephritis	958 (2.03%)	2,786 (1.47%)	<.0001
Calculus of kidney	1,419 (3.00%)	4,143 (2.19%)	<.0001
Urinary tract infection	6,924 (14.64%)	21,431 (11.33%)	<.0001
Arthralgia	5,545 (11.72%)	18,085 (9.56%)	<.0001
COPD	4,418 (9.34%)	13,588 (7.18%)	<.0001

SD, standard deviation; COPD, chronic obstructive pulmonary disease; HPV, human papillomavirus.

TABLE 2 Incidence of primary Sjogren's syndrome.

	HPV infections n = 47,300	Non-HPV controls n = 189,200
Follow up person-months	3,622,659	14,359,439
Event of Sjogren's syndrome	493	1,081
Incidence rate* (95% C.I.)	13.61 (12.46-14.86)	7.53 (7.09-7.99)
IRR†	1.81 (1.63-2.01)	Reference

*per 10^5 person-months.
†IRR, Incidence rate ratio.

CI=1.45-1.83) (Table 4). The subgroup analysis was conducted to determine which HPV subgroup was most susceptible to pSS. The risk of developing pSS in male HPV patients was higher than female patients (male HR=1.83, 95% CI=1.47-2.28 vs. female HR=1.58, 95% CI=1.40-1.79) (Table 4). The risks of having pSS were similar in all age groups. Patients between the age of 16 and 45 has HR of 1.60 (95% CI=1.34-1.91) and older than 45 years has HR of 1.67 (95% CI=1.46-1.97) (Table 4).

Discussion

The etiology of pSS is still inconclusive. Currently, the genetic background, the environmental stimulation on epigenetics, and the use of immunotherapeutic agents (36–40) are proposed to trigger pSS. However, the evidence for the underlying mechanism is still insufficient. Out of all the external factors, viral infection is suspected to be the main risk. Several studies have suggested the possible role infections

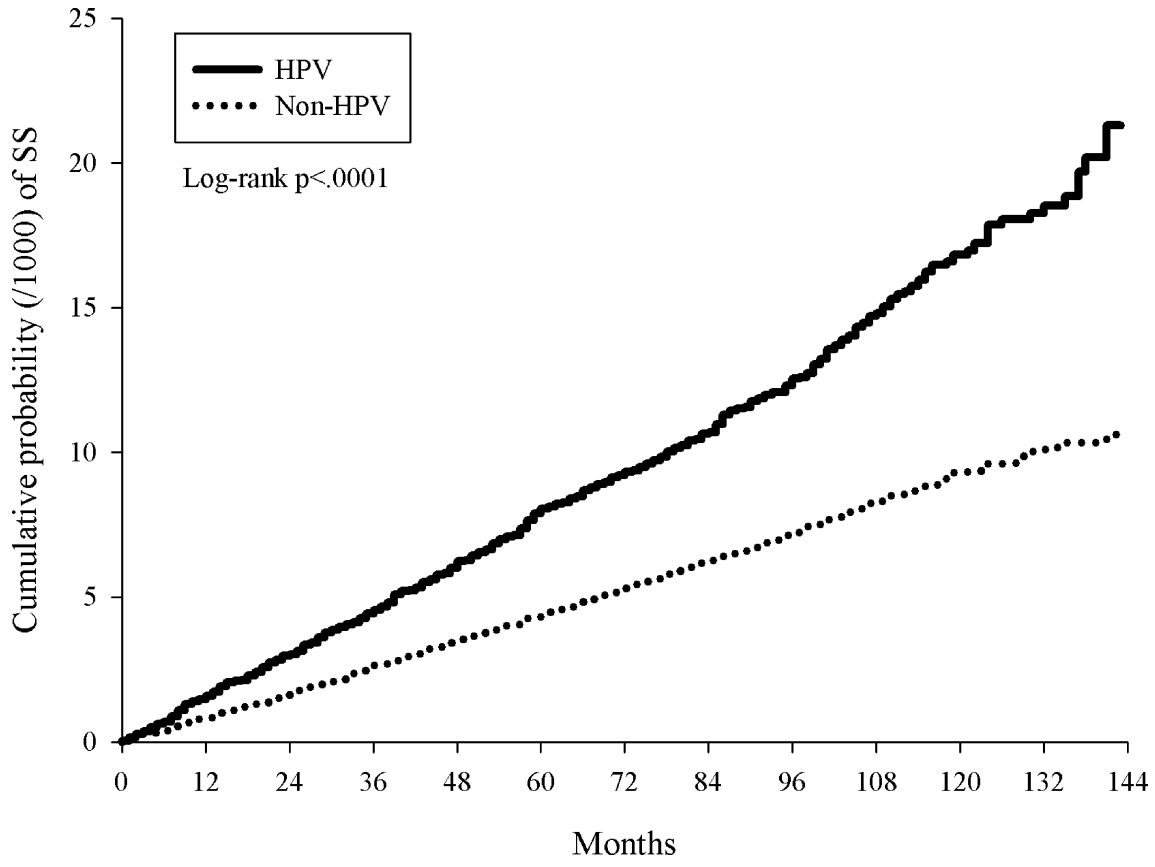

FIGURE 2
Kaplan-Meier curves of cumulative proportion of primary Sjogren's syndrome in patients with HPV infections and non-HPV controls.

TABLE 3 Risk factors for primary Sjogren's syndrome in multiple Cox regression.

	aHR	95% C.I.	p value
HPV infections (reference: non - HPV controls)	1.64	1.47 - 1.83	<.0001
Age at baseline (reference: 16 - 45)			
≧45	2.64	2.37 - 2.93	<.0001
Sex (reference: females)			
Male	0.36	0.32 - 0.40	<.0001
Comorbidities (reference: no status of the comorbidities)			
Rheumatoid arthritis	3.67	2.86 - 4.70	<.0001
Pneumonia	1.29	1.00 - 1.67	0.0512
Bronchitis	0.91	0.70 - 1.19	0.4779
Dental caries	1.43	1.30 - 1.58	<.0001
Chronic liver disease	1.32	1.14 - 1.53	0.0002
Cholelithiasis	1.02	0.75 - 1.40	0.8814
Interstitial nephritis	1.07	0.79 - 1.45	0.6535
Calculus of kidney	1.37	1.05 - 1.77	0.0188
Urinary tract infection	1.29	1.14 - 1.46	<.0001
Arthralgia	1.31	1.14 - 1.49	0.0001
COPD	1.56	1.31 - 1.86	<.0001

aHR, adjusted hazard ratio; C.I., confidence interval; COPD, chronic obstructive pulmonary disease; HPV, human papillomavirus.

play in the development of pSS. To date, no studies have clarified the association between HPV infections and pSS. This paper demonstrates epidemiological evidence in favor of a correlation by using nationwide, population-based data in Taiwan. In this study, patients with HPV exposure were associated with increased pSS incidence. RA, COPD, pneumonia, dental caries, calculus of kidney, urinary tract infection, and chronic liver disease also increased pSS incidence.

During the infected stage, the HPV deoxyribonucleic acid (DNA) in the infected cells may activate T cells or B cells through MHC pathways (41, 42). Majewski and Jablonska reported HPV could serve as super antigens to activate polyclonal T cells, which could trigger autoimmune phenomenon of psoriasis (13). The specific sequence of a certain type of HPV gene that codes for viral protein, which acts as autoantigens, need further research to identify. A previous epidemiological study in Taiwan found the association between HPV infection and psoriasis onset. In this study, pSS patients were prominent for the risk of psoriasis through activation of T cells, as similar to the reported associations between bacterial (43–47) or viral (48) infections in other autoimmune diseases.

In Taiwan, pSS is one of the catastrophic illness specified by the National Health Insurance program. Attending physicians must submit related clinical information including pSS patient histories, laboratory, and pathological data to the National

TABLE 4 Sensitivity analysis for the risk of primary Sjogren's syndrome following HPV infections.

	HR	95% C.I.	p value
Restriction			
Excluded the individuals cannot be tracked for at least 12 months (239 patients with HPV infections and 1,609 non - HPV controls were excluded)	1.63	1.45 - 1.83	<.0001
Subgroup analysis stratified by sex			
Female	1.58	1.40 - 1.79	<.0001
Male	1.83	1.47 - 2.28	<.0001
p for interaction			0.1499
Subgroup analysis stratified by age at baseline			
Aged16 - 45 years old	1.60	1.34 - 1.91	<.0001
Aged ≧45 years old	1.67	1.46 - 1.91	<.0001
p for interaction			0.8251

HR, hazard ratio; C.I., confidence interval; HPV, human papillomavirus.

Health Insurance Administration (NHIA) to apply for a catastrophic illness certificate (CIC). A committee under the NHIA would review all applications in according to the criteria of the American-European Consensus Group (AECG) for pSS (49). Patients must meet either 4 out of 6 AECG criteria including No.4 (Histopathology) or No.6 (Autoantibodies), or 3 out of 4 objective criteria including No.3, No.4, No.5, No.6. These features recognized by AECG could reflect the immune-pathogenesis of patients' adaptive immune systems.

There are limitations in this study. Firstly, the diagnoses of HPV infections may not be fully accurate. About 75% of females with history of sexual intercourse were susceptible to HPV infections, but the infection symptoms were not always clinically identifiable in patients with HPV infections, which could cause an underestimated rate of HPV-infected patients in this survey. Secondly, the diagnosis of HPV infection in Taiwan mainly depended on pap smear methods and viral DNA types analysis. Smear tests were convenient, but their like-hood ratios (LRs) of HPV infections were as low as 50%. Southern blot analysis, PCR, and other molecular level laboratory analysis were usually performed to increase the true positive rates and to identify specific type of HPV DNA (41). Furthermore, most physicians would not detect HPV antigens in pSS patients' lesions through biopsy. Therefore, the imperfect decision-making process could result in information bias in random. Thirdly, data on specific clinical subsets of pSS triggered by HPV infections that included data on Schirmer's test, history of HPV vaccinations and medications including steroids or other drugs for pSS, and subsequent mortality of participants, were not included in this study, which may be investigated in future studies to support findings of the present study.

Our study employed a large, nationwide sample with high external validity to neutralize deviation from selection bias. In the future, more clinical data are required to reflect a more accurate disease course of HPV infections. Genetic or cytological analyses of lesions that specify HPV genotypes and phenotypes of the infections and biomarkers with high LR values are key prognosis factors of HPV infections, which may solidify our knowledge on the relationship between HPV infections and the risk of pSS. On the other hand, this would help with future disease screenings, patient educations (50), and early treatments to be implemented.

Conclusion

In this population-based cohort study, patients with HPV infections presented with significantly higher risk of new-onset pSS. RA, COPD, pneumonia, dental caries, chronic liver disease, calculus of kidney, urinary tract infection, and arthralgia were also independent risk factors for pSS.

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 authors.

Author contributions

H-HC and KM contribute to conception and design of the study. KM and W-JC organized the database and performed the statistical analysis. H-HC wrote the first draft of the manuscript. KM and CD wrote sections of the manuscript. All authors contributed to manuscript revision, read 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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Identification of SLAMF1 as an immune-related key gene associated with rheumatoid arthritis and verified in mice collagen-induced arthritis model

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Background: Rheumatoid arthritis (RA) is the most common inflammatory arthropathy. Immune dysregulation was implicated in the pathogenesis of RA. Thus, the aim of the research was to determine the immune related biomarkers in RA.

Methods: We downloaded the gene expression data of RA in GSE89408 and GSE45291 from Gene Expression Omnibus public database (GEO). Differentially expressed genes (DEGs) were identified between RA and control groups. Infiltrating immune cells related genes were obtained by ssGSEA and weighted gene co-expression network analysis (WGCNA). We performed functional enrichment analysis of differentially expressed immunity-related genes (DEIRGs) by "clusterProfiler" R package, key genes screening by protein-protein interaction (PPI) network of DEIRGs. And mice collagen-induced arthritis (CIA) model was employed to verify these key genes.

Results: A total of 1,885 up-regulated and 1,899 down-regulated DEGs were identified in RA samples. The ssGSEA analysis showed that the infiltration of 25 cells was significantly different. 603 immune related genes were obtained by WGCNA, and 270 DEIRGs were obtained by taking the intersection of DEGs and immune related genes. Enrichment analyses indicated that DEIRGs were associated with immunity related biological processes. 4 candidate biomarkers (*CCR7, KLRK1, TIGIT* and *SLAMF1*) were identified from the PPI network of DEIRGs and literature research.In mice CIA model, the immunohistochemical stain showed *SLAMF1* has a significantly high expression in diseased joints. And flow cytometry analysis shows the expression of *SLAMF1* on CIA mice-derived CTL cells, Th, NK cells, NKT cells,

classical dendritic cell (cDCs) and monocytes/macrophages was also significantly higher than corresponding immune cells from HC mice.

Conclusion: Our study identified *SMLAF1* as a key biomarker in the development and progression of RA, which might provide new insight for exploring the pathogenesis of RA.

KEYWORDS

SLAMF1, rheumatoid arthritis, collagen-induced arthritis, bioinformatics, animal model, key gene

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by inflammatory changes in the synovial tissue of joints. The estimated global RA prevalence was 0.46%, accounting for 36 million people worldwide (1, 2). RA primarily during the productive years of adulthood, between the ages of 20 and 40 years, and it is more prevalent among women in developed countries (3). Eventually, RA causes irreversible damage to the joint, and it is an enormous burden on patients and society. The complications associated with RA, like respiratory problems and heart diseases and, can predispose to increased mortality (4).

Serological autoantibodies are crucial indicators in the diagnosis and prognosis of RA, the anti-immunoglobulin G (IgG) antibodies, including rheumatoid factor (RF) and anti-cyclic citrullinated peptide antibody (anti-CCP), have been used as preferred RA diagnostic criteria for decades (5). Whereas, when serum RF is negative in early onset of RA and can also be found in other autoimmune and inflammatory diseases, And anti-CCP is a highly specific but moderate sensitivity indicator for RA (6–8). Therefore, a multi-dimensional diagnosis system including more novel and effective auxiliary biomarkers is urgently needed for more accurate prediction and treatment of RA.

Studies have reported that some biomarkers, including cytokines, microRNA, and autoantibodies, may effectively diagnose early RA (9–12). Also, some works proved microbiota and metabolites maybe potential ways for RA prognosis and diagnosis (13–15). Moreover, with the clinical applications of flow cytometry, some membrane proteins have been considered to have potential as a diagnostic marker such as CD146, CD64 and CD154 (16–18).However, most biomarkers are not validated in prospective cohorts, or their clinical relevance is unclear.

RA has been recognized as a chronic immune-mediated disease resulting from combined genetic and environmental factors. Further, the malfunction of multiple immune cell types and signaling networks elicit a maladaptive tissue repair process, leading to tissue damage predominantly in the joints. RA is closely associated with various immune cells either residing in the synovium or circulating in peripheral blood, and each cell type contributes differently to the disease pathogenesis (19–21). RA runs through a relatively prolonged immune disorder stage, and the molecular characterization of this stage has the potential to identify upstream molecular targets. So, the immune system could be re-engineered to halt the disease process prior to irreversible tissue damage.

Recently, transcriptomic and analyses have been widely used to identify novel biomarkers in the diagnosis and treatment of RA. With high-throughput sequencing and bioinformatics analyses, it is possible to explore potential vital genes and signal pathways closely related to disease development. In this study, we initially selected some new hub genes in RA by downloading and re-analyzing datasets GSE45291 and GSE89408. Then we employed collagen-induced arthritis (CIA) mice as a RA model and verified signaling lymphocytic activation molecule family 1 (*SLAMF1, CD150*) as an immune-related key gene in RA using Flow Cytometry, immunohistochemistry, and real-time PCR. This work may shed light on the mechanisms of RA development at the transcriptome level and provide a new target for diagnosing, preventing, and treating RA.

Materials and methods

Data source

Two gene expression profiles of RA, GSE89408 and GSE45291, were identified after searching GEO. The GSE89408 data file including 152 RA samples and 28 normal samples was used as a training set. And GSE45291 including 493 RA samples and 20 normal samples was used as an external validation set.

Identification of DEGs

R software was used to process data using the "limma-voom" package (PMID: 34616422). The log transformation was applied to the original data. statistical methods p-value and false discovery rate (FDR) were initially used and results with adj.p value < 0.05 and |log2FC| > 1.5 were regarded as significant. The analysis results were presented by volcano plot and heatmap.

Functional and pathway enrichment analysis

The "clusterProfiler" R package (PMID: 22455463) was utilized to understand the functional and pathway enrichment information on the gene of interest. Gene Ontology (GO) analysis is a commonly used approach for annotating genes and gene products with functions including biological pathways (BP), cellular components (CC) and molecular function (MF). Kyoto Encyclopedia of Genes and Genomes (KEGG) is a database resource for understanding gene functions and utilities of the biological system. GO terms or KEGG pathways with adj.p value < 0.05 were considered statistically significant.

Analysis of immune cell characteristics

The composition of 28 immune cell (activated B cell, activated CD4 T cell, activated CD8 T cell, activated dendritic cell, $CD56^{bright}$ natural killer cell, $CD56^{dim}$ natural killer cell, central memory CD4 T cell, central memory CD8 T cell, effector memory CD4 T cell, effector memory CD8 T cell, eosinophil, gamma delta T cell, immature B cell, immature dendritic cell, macrophage, mast cell, MDSC, memory B cell, monocyte, natural killer cell, natural killer T cell, neutrophil, plasmacytoid dendritic cell, regulatory T cell, T follicular helper cell, Type 1 T helper cell, Type 17 T helper cell and Type 2 T helper cell) types in GSE89408 cohort was determined by the ssGSEA (Single-sample gene set enrichment analysis) method. ssGSEA was performed by "GSVA" R package (PMID: 23323831). The difference in the immune cell infiltration between the RA and normal groups was carried out using the Wilcoxon rank-sum test (p value < 0.05).

Weighted gene co-expression network construction and analysis

The WGCNA was used to determine the relationship between co-expression gene modules and differentially infiltrating immune cells in the R package "WGCNA" (PMID: 19114008). The WGCNA network and the co-expressed gene modules were established and detected using the soft threshold power of $\beta = 12$. The genes in the core module were selected for further analyses.

Construction of PPI network and identification of hub genes

The protein-protein interaction (PPI) of DEIRGs was obtained using the Search Tool for the Retrieval of Interacting Genes database (STRING, PMID: 12519996) and visualized by Cytoscape software (PMID: 14597658). The Core module in the PPI network was extracted by Molecular Complex Detection (MCODE, PMID: 32684073) plug-in. The parameters were set as: degree cutoff = 2, K-Core = 2, and Node Score Cutoff = 0.2. The genes in the core module were regarded as the potential hub genes.

Validation of SLAMF1 by ROC curve

Receiver operating characteristic (ROC) was used to evaluate the ability of SLAMF1 to discriminate between RA and control samples. And the expression and discriminating ability of SLAMF1 were also tested in the external validation set. "PRROC" R package was used to calculate the precision and recall, and draw the PR curve of SLAMF1.

Induction of collagen-induced arthritis (CIA) in mice

For CIA induction, Female DBA/1 mice were injected subcutaneously at the base of the tail with 100 μg of Chick type II collagen (Condrex, Seattle, WA, USA) emulsified in Freund's complete adjuvant (CFA) (Chondrex, Seattle, WA, USA), followed 14 days later by a booster injection of the same Chick type II collagen (100 μg) emulsified in Freund's incomplete adjuvant (Chondrex, Seattle, WA, USA), and mice injected with CFA/IFA only as a negative control. Which *via* the same route and following the protocol described by David et al (22). To assess the severity of arthritis, clinical symptoms were evaluated by means of a five-point scale: grade 0 = no swelling; grade 1 = paw with detectable swelling in a single digit; grade 2 = paw with swelling in more than one digit; grade 3 = paw with swelling of all digits and instep; and grade 4 = severe swelling of the paw and ankle.

Flow cytometry

Single-cell suspensions were prepared from the peripheral blood of mice using standard procedures. Following red blood cell lysis, Fc receptors were blocked with anti-CD16/32 Ab

(2.4G2), and cells were incubated with antibody in 0.5% FBS in PBS for 30 min, and then washed in FACS buffer before analysis. Data were collected on a FACSCanto™ II (BD Biosciences San Jose, CA, USA) instrument and analyzed using FlowJo software (Tree Star, Ashland, OR, USA). The Antibodies used for staining are as follows. FITC-conjugated anti-mouse CD4 (GK1.5), NKp46 (29A1.4), Siglec-F (S17007L), F4/80 (BM8), PE-conjugated anti-mouse T cell receptor γ/δ (GL3), CXCR5 (L138D7), α-GalCer : CD1d complex (L363), Gr-1 (RB6-8C5), 317 (927), PE/Cy7-conjugated anti-mouse CD8a (53-6.7), CD25 (3C7), B220 (RA3-6B2), CD11b (M1/70), CD11c (N418), APC-conjugated anti-mouse Slamf1 (W19132B) were purchased from BioLegend (San Diego, CA, USA).

RNA extraction and real-time PCR analysis

RNA of peripheral blood mononuclear cells was extracted by EZ-10 Total RNA Mini-Preps Kit (Sangon Biotech, Shanghai, China) and subjected to reverse transcription using HiFiScript cDNA Synthesis Kit (CoWin Biosciences, Beijing, China). The messenger RNA levels were assessed by quantitative real-time PCR using UltraSYBR Mixture (CoWin Biosciences, Beijing, China). β-actin was used as the internal control. The primers used in the study were as follows: *β-actin*, 5′-AGA GGG AAA TCG TGC GTG AC-3′ (sense) and 5′-CAA TAG TGA TGA CCT GGC CGT-3′ (antisense); *CCR7*, 5′-TGT ACG AGT CGG TGT GCT TC -3′ (sense) and 5′-GGT AGG TAT CCG TCA TGG TCT TG-3′ (antisense); *KLRK1*, 5′-ACT CAG AGA TGA GCA AAT GCC-3′ (sense) and 5′-CAG GTT GAC TGG TAG TTA GTG C-3′ (antisense); *TIGIT*, 5′- CCA CAG CAG GCA CGA TAG ATA -3′ (sense) and 5′-CCA CCA CGA TGA CTG CTG T-3′ (antisense); *Slamf1*, 5′-CAG AAA TCA GGG CCT CAA GAG-3′ (sense) and 5′- CAT GCC ACC CCA GGT CAA C -3′(antisense).

Statistical analyses

Data are presented as mean ± SD. The Wilcoxon rank-sum test was used for all statistical analyses between two conditions. A P value < 0.05 was considered statistically significant (*P < 0.05; **P < 0.01; ***P < 0.001).

Results

Identification DEGs and the immune infiltration in RA

First, GSE89408 was analyzed using the "limma-voom" package to identify DEGs. After standardization analysis, 3,784 DEGs were identified, including 1,885 up-regulated DEGs and 1,899 down-regulated DEGs (Figures 1A, B) in RA samples compared to normal samples.

Further, We analyzed whether RA and healthy control (HC) had different immune cell infiltration profiles using ssGSEA. Figure 1C shows the distribution of 28 infiltrating immune cells in the RA and normal samples. The Wilcoxon rank-sum test result showed that there were significant differences in the content of the 25 immune cells between RA and normal groups (Figure 1D), specifically, activated dendritic cell, monocyte, central memory CD4 T cell, MDSC, activated CD4 T cell, effector memory CD8 T cell, regulatory T cell, type 1 T helper cell, CD56dim natural killer cell, eosinophil, CD56bright natural killer cell, natural killer cell, natural killer T cell, activated CD8 T cell, gamma delta T cell, macrophage, T follicular helper cell, central memory CD8 T cell, mast cell, immature dendritic cell, type 2 T helper cell, immature B cell, effector memory CD4 T cell, activated B cell and plasmacytoid dendritic cell.

WGCNA and identification of the key module associated with immune infiltration in RA

We performed WGCNA to screen genes associated with differentially infiltrating immune cells. Supplementary Figure 1A shows that the GSM2371137 sample was an outlier removed from the subsequent WGCNA. The clustering of the remaining samples is shown in Supplementary Figure 1B. β value 12 was chosen as the appropriate soft-thresholding value (Supplementary Figure 1C). The WGCNA detected 19 co-expression modules, and the cluster dendrogram was established (Figure 2A). Then, their correlations with differentially infiltrating immune cells were analyzed. The yellow module was mainly associated with immune cells such as lymphocytes, including activated B cells, activated CD4 T cells, and activated CD8 T cells (|Cor| > 0.8, p-value < 0.05, Figure 2B). We defined the yellow module as an immune-related module, and a total of 603 genes in this module were extracted for subsequent studies.

Enrichment and identification analysis of hub genes

A total of 270 DEIRGs were obtained by intersecting 603 module genes with 3,784 DEGs (Figure 3A). The most enriched KEGG pathways were "cytokine-cytokine receptor interaction," "T cell receptor signaling pathway," "cell adhesion molecules", "natural killer cell-mediated cytotoxicity", "chemokine signaling pathway", "primary immunodeficiency" (Figure 3B). According to GO enrichment analysis, the most significantly enriched terms were "lymphocyte differentiation," "T cell activation,"

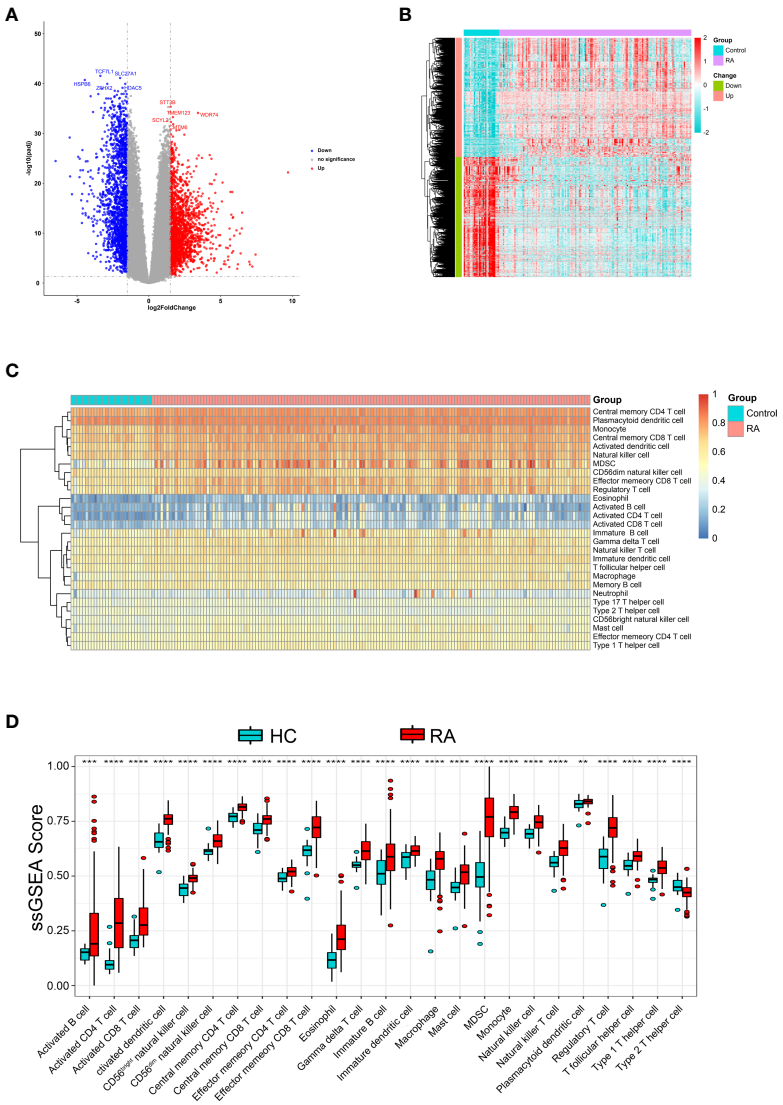

FIGURE 1
Identification DEGs and the immune infiltration in RA. **(A)** The volcano plot and **(B)** heatmap to show significant DEGs between RA and HC samples **(C)** Heatmap of the immune infiltration profiles of RA and HC samples analyzed by ssGSEA score-based method. **(D)** Comparison of immune cell infiltration between RA and HC samples. **p < 0.01, ***p < 0.001 and ****p < 0.0001.

"immunological synapse", "T cell receptor complex", "immune receptor activity" "cytokine receptor activity" (Figure 3C).

The PPI was analyzed with 270 DEIRGs using the STRING database (Figure 3D). The interactive relationships between the key genes in the whole network were determined using the Cytoscape plugin MCODE. There were 6 clusters: 45 nodes and 744 edges in cluster 1 (Figure 3E), 17 nodes and 36 edges in cluster 2, 4 nodes and 6 edges in cluster 3, 4 nodes and 5 edges in cluster 4, 3 nodes and 3 edges in cluster 5, and 3 nodes and 3 edges in cluster 6 (Supplementary Figure 2). Then, 27 hub TOP genes degree score≥30 in cluster 1 was used to identify hub genes.

We conducted literature research to find novel immune-related RA diagnostic biomarkers. We eliminated the genes encoding classical immune cell markers and genes with a clear function in or associated with RA. We identified 4 genes as candidate RA biomarkers for further validation, including *CCR7, KLRK1, TIGIT*, and *SLAMF1* (Supplementary Table 1). Figure 3F shows the expression levels of the above genes in the RA samples, and they were significantly higher than those in the control group.

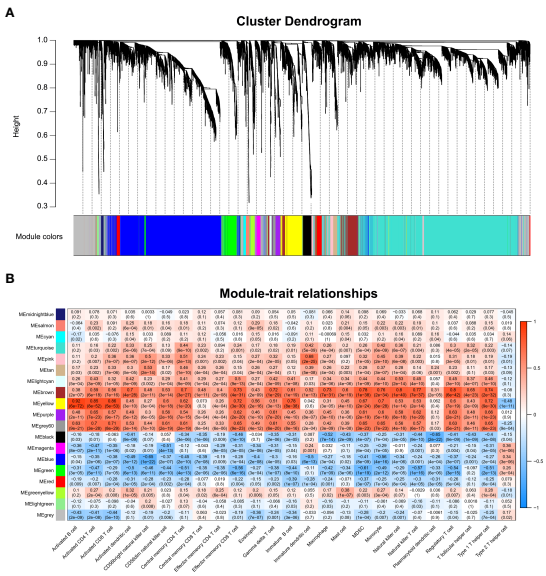

FIGURE 2
WGCNA and identification of the key module associated with immune infiltration in RA. **(A)** Clustering dendrograms for the 3,784 DEGs with dissimilarity based on the topological overlap together with the assigned module colors; 19 co-expression modules were constructed with various colors. **(B)** Heatmap of the correlation between module eigengene and differentially infiltrated immune cells. Each row represents a color-coded module eigengene, each column represents a type of infiltrated immune cells. The number in each cell means the correlation coefficient and p-value.

Validation of candidate biomarkers in mice model

To verify the role of candidate genes in RA, we employed classical collagen-induced arthritis (CIA) model in DBA/1 mice using subcutaneous injection of chick type II collagen. We observed a perfect joint pathological damage in this animal model (Figure 4A). To dynamically detect the expression of candidate genes in the pathological process, we isolated PBMCs from Healthy Control mice (HC), Freund's Complete Adjuvant injected mice (CFA Control) and CIA mice every week. We detected the mRNA expression level of *CCR7, KLRK1, TIGIT*, and *SLAMF1* and found that *SLAMF1* has the most consistent trend of significance with the pathological progression of CIA (Figure 4B). Further, the immunohistochemical stain of joint tissue showed *SLAMF1* has a significantly high expression in diseased joints (Figure 4C), suggesting that *SLAMF1* might be involved in the immune response caused by multiple immune cell infiltration.

Since *SLAMF1* is a typical membrane protein and is also known as CD150, we used flow cytometry to investigate the role of *SLAMF1* in the RA-related immune responses and detected the expression of *SLAMF1* on the surface of immune cells of CIA and HC mice using ssGSEA as mentioned above. To simplify the detection, we grouped 23 immune cells into 11 categories according to surface markers and gate strategy (Supplementary Table 2). We excluded plasmacytoid dendritic cell (pDCs) and mast cells from grouping as they cannot be detected in PBMCs. The results showed significantly elevated expressions of *SLAMF1* in the Cytotoxic T Cell (CTL), T helper cells (Th), Natural killer cells (NK), Natural killer T cells (NKT), classical dendritic cell (cDCs), Monocytes/Macrophages (Mφ) (Figure 5), But not in Tfh cells, Treg cells, B cells, Eosinophils, and MDSC (Data not shown).

Evaluation and verification of the diagnostic effect of *SLAMF1* on RA

We used the ROC curves to assess *SLAMF1* diagnostic efficiency and determine whether it has an excellent diagnostic value in RA patients. As showed in Figures 6A, B, the AUCROC and AUCPR of Slamf1 were 0.899 and 0.981, also which indicated that *SLAMF1* has a particular reference value in diagnosing RA patients.

We further verified the expressions and diagnostic values of *SLAMF1* using the GSE45291 as a validation dataset. We found that the expression trends (Figure 6C) and the AUCs

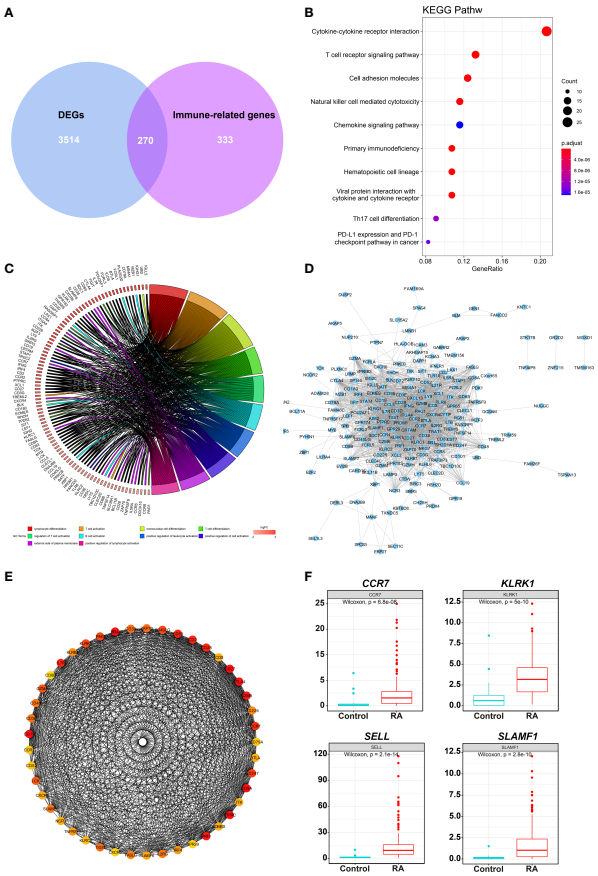

FIGURE 3
DEIRGs screening and enrichment analysis. **(A)** Venn diagram of 270 overlapped candidate genes shared by DEGs and Immune-related genes (MEyellow module). KEGG **(B)** and GO **(C)** enrichment analysis showed the most significantly enriched pathways. **(D)** Protein-protein interaction (PPI) analysis by STRING database for 270 DEIRGs. **(E)** PPI diagram of all the genes in cluster 1. **(F)** Expression levels of 4 potential biomarkers between RA and HC samples.

(Figures 6A, B) were consistent with those in the training set GSE89408.

Discussion

Rheumatoid arthritis (RA), characterized by joint inflammation leading to progressive tissue damage and joint disability, is among the most frequent chronic inflammatory diseases (23). In the last decade, from a microscopic to a macroscopic level, we have seen unprecedented significant insight into the cellular function and molecular mechanisms involved with RA. However, the pathogenesis of RA remains incompletely understood, and researching the key genes underlying RA development remains a priority. Our previous studies found that Thymocyte-expressed positive selection-associated 1 (*Tespa1*) plays a critical role in the pathogenesis of human rheumatoid arthritis and mice collagen-induced arthritis, thus defining *Tespa1* as a key gene in the pathogenesis of RA (24, 25). With the development of high-throughput sequencing and bioinformatic analytical techniques, DNA microarray has become an important method to obtain gene expression data of multiple diseases on a large scale and efficiently. Through integrated analysis of multiple sequencing databases, several studies have identified key genes and biomarkers that contribute to the occurrence and progression of RA (26–29). However, most results obtained in these studies were not physiologically validated. Therefore, further studies are required to screen and verify biomarkers involved in the pathogenesis of RA more precisely and reliably.

The present study aimed to identify and verify novel biomarkers highly expressed in RA compared to normal controls and reveal their potential mechanisms. We extracted

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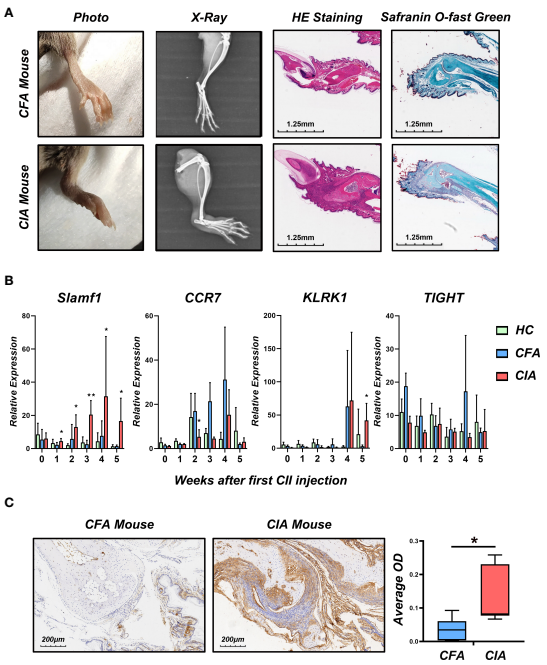

FIGURE 4
Construction collagen-induced arthritis and validation of candidate gene in the model. **(A)** Photographs, X-ray, H&E and Safranin O-fast green staining images of the hind limbs of CIA and HC 5 weeks after the first CII injection. **(B)** Realtime-PCR analysis for gene expression of *CCR7, KLRK1, TIGIT and SLAMF1* in the PBMC of CIA, CFA control and healthy control (HC) mice every week. **(C)** Immunohistochemical detection of *SLAMF1* expression in joint synovial samples from CIA and CFA mice. Data shown are the mean ± SEM with 6 mice per group and are from one experiment, representative of two performed. *p < 0.05 and **p < 0.01 (Wilcoxon rank-sum test).

the gene expression profiles of GSE89408, and GSE45291 downloaded from the GEO database and identified 1,885 up-regulated and 1,899 down-regulated overlap DEGs. According to the GO functional enrichment analysis, the RA-related DEGs were primarily enriched in the chemokine-mediated signaling pathway, T cell activation and regulation of T cell activation in BP; external side of the plasma membrane, collagen-containing extracellular matrix, and collagen trimer in CC; and immune receptor activity, chemokine activity and cytokine activity in MF. KEGG pathway analysis showed that the DEGs were enriched in cytokine-cytokine receptor interaction and chemokine signaling pathway viral protein

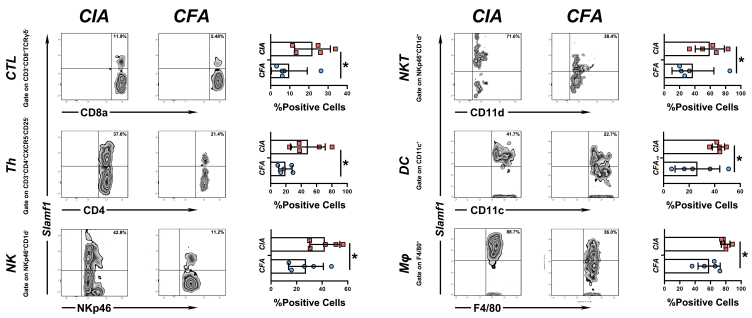

FIGURE 5
Expression level of slamf1 on different immune cell subsets PBMC from CIA and CFA mice were stained with respective surface markers and grouped into different immune cell subsets, the expression of *SLAMF1* were detected by flow cytometry and analysis by mean fluorescence intensity (MFI). Data shown are the mean ± SEM with 6 mice per group and are from one experiment, representative of two performed. *p <0.05, **p < 0.01 and ***p < 0.001 (Wilcoxon rank-sum test).

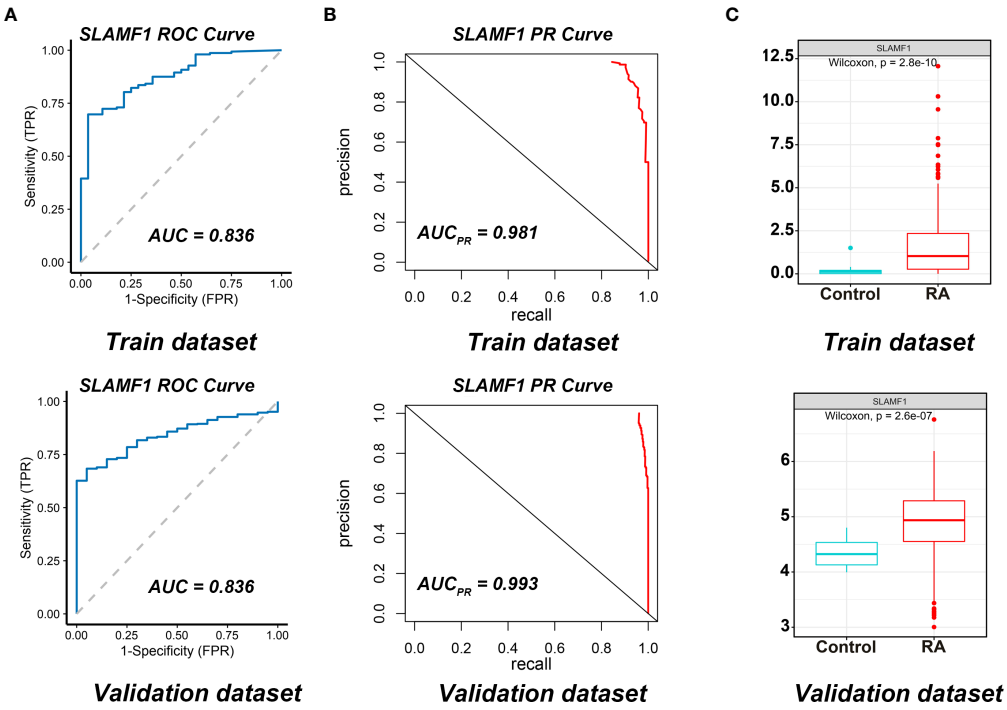

FIGURE 6
Diagnostic effect of *SLAMF1* on RA. **(A)** ROC curve and **(B)** Precision-recall evaluation of the diagnostic effectiveness of SLAMF1 biomarkers in Train Dataset (GSE89408) and Validation Dataset (GSE45291). **(C)** The expressions of *SLAMF1* in Train Dataset (GSE89408) and Validation Dataset (GSE45291).

interaction with cytokines. These functional enrichment analyses indicated that the DEGs were enriched in immune response and inflammation, which play an essential role in the pathogenesis of RA (20).

Increasing evidence shows that various immune cells play pathogenic roles in RA (19, 30). This study mapped the roles of various immune cells in the pathological process of RA. We analyzed the infiltration profiles of 28 infiltrating immune cells using ssGSEA and found that 23 of them have a significant increase compared to healthy controls. Next, we performed WGCNA to screen genes associated with differentially infiltrating immune cells and obtained 603 immune-related genes in one module. By intersecting DEGs and immune-related modules, we screened 270 DEIRGs and finally identified *CCR7, KLRK1, TIGIT*, and *SLAMF1* as candidate RA biomarkers using literature research.

To verify the role of these genes in RA, we employed a mice CIA model to dynamically detect the expression of candidate genes in both peripheral blood mononuclear cells and pathological joint tissue. We proved that *SLAMF1* has a perfect fitting curve with the CIA disease process. We detected the expression of *SLAMF1* on different immune cells using flow cytometry. We demonstrated a significantly elevated expression of *SLAMF1* on CTLs, Th cells, NK cells, NKT cells, cDC, and Mφ. Therefore, *SLAMF1* may be involved in the inflammatory response mediated by these infiltration immune cells in the pathogenesis of RA. Finally, the ROC curves indicated that *SLAMF1* had a particular reference value in the diagnosis of RA patients, prompting that *SLAMF1* may be an essential biomarker for RA.

Nevertheless, there are several limitations to this study. Firstly, the samples in datasets GSE89408 and GSE45291 were from joint synovial biopsies and peripheral blood mononuclear cells respectively. Different specimen sources may have some impact on the subsequent analysis results. Secondly, the relationships between the biomarkers and immune activation in rheumatoid arthritis were only verified by the expression. Moreover, although the datasets we analyzed were from clinical samples, our validation work was carried out only in mouse models, we will validate these findings in clinical patient samples in future work. Finally, additional *in vitro* and *in vivo* experiments are necessary for exploring the in-depth mechanisms of how the *SLAMF1* regulates inflammatory responses in RA.

In summary, the present study revealed that *SLAMF1* might play a key role in the development and progression of RA, which might provide new insight for exploring the pathogenesis of RA, a basis for finding auxiliary diagnostic biomarker and potential therapeutic targets for RA.

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by Animal ethics committee of First Affiliated Hospital of Huzhou University.

Author contributions

AL and ZZ: Methodology, Data Curation and Writing - Original Draft. XR, YYi and XL: Investigation. JQ and JW: Validation. XY: Resources. YYa: Conceptualization, Supervision and Writing - Review & Editing.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.961129/full#supplementary-material

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Environmental factors influencing the risk of ANCA-associated vasculitis

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Antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is a group of diseases characterized by inflammation and destruction of small and medium-sized blood vessels. Clinical disease phenotypes include microscopic polyangiitis (MPA), granulomatosis with polyangiitis (GPA), and eosinophilic granulomatosis with polyangiitis (EGPA). The incidence of AAV has been on the rise in recent years with advances in ANCA testing. The etiology and pathogenesis of AAV are multifactorial and influenced by both genetic and environmental factors, as well as innate and adaptive immune system responses. Multiple case reports have shown that sustained exposure to silica in an occupational environment resulted in a significantly increased risk of ANCA positivity. A meta-analysis involving six case-control studies showed that silica exposure was positively associated with AAV incidence. Additionally, exposure to air pollutants, such as carbon monoxide (CO), is a risk factor for AAV. AAV has seasonal trends. Studies have shown that various environmental factors stimulate the body to activate neutrophils and expose their own antigens, resulting in the release of proteases and neutrophil extracellular traps, which damage vascular endothelial cells. Additionally, the activation of complement replacement pathways may exacerbate vascular inflammation. However, the role of environmental factors in the etiology of AAV remains unclear and has received little attention. In this review, we summarized the recent literature on the study of environmental factors, such as seasons, air pollution, latitude, silica, and microbial infection, in AAV with the aim of exploring the relationship between environmental factors and AAV and possible mechanisms of action to provide a scientific basis for the prevention and treatment of AAV.

KEYWORDS

AAV (ANCA-associated vasculitis), ANCA, air pollution, environmental risks, etiology, vasculitis

Introduction

Systemic autoimmune rheumatic diseases (SARDs) are a group of chronic autoimmune diseases that attack joints, bones, muscles, blood vessels, and related soft or connective tissues. Common SARDs include rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), primary Sjögren's syndrome (pSS), systemic sclerosis (SSc), polymyositis (PM), dermatomyositis (DM), mixed connective tissue disease (MCTD), and systemic vasculitis. The onset of these diseases is more insidious. The course of these diseases are longer and require lifelong treatment, which severely threatens the physical and mental health of patients and has become an important public health problem (1–3). Systemic vasculitis, one of the most complex and challenging SARDs, is classified into large, medium, and small vessel vasculitis, mainly based on the size of the affected vessels (2022ACR/EULAR) (4). Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is an important part of the classification of vasculitis. AAV can affect many vital organs throughout the body, including the skin, kidneys, lungs, and brain. Additionally, untreated vasculitis progresses rapidly, causing irreversible damage to vital organs in the body and even death. Therefore, exploring the etiology and pathogenesis of AAV is crucial for early diagnosis and timely treatment.

AAV is a multisystem autoimmune disease that primarily involves small blood vessels throughout the body, and it is associated with the presence of ANCA in the serum (5, 6). ANCA, which was first identified by Davies in patients with necrotizing glomerulonephritis, is divided into two main types: cytoplasmic (C-ANCA) and perinuclear (P-ANCA), whose target antigens are proteinase 3 (PR3) and myeloperoxidase (MPO), respectively. Growing evidence confirms the pathogenic role of ANCA in AAV. Transfer of splenocytes from MPO-deficient mice immunized with mouse MPO into wild-type mice resulted in hyperimmune systemic vasculitis (7). Pendergraft et al. (8) demonstrated that complementary proteinase-3 (cPR3) antibodies may induce PR3-ANCA. Additionally, a new ANCA-targeting human lysosome-associated membrane protein-2 (LAMP-2) has been described as a sensitive and specific marker for renal limited vasculitis (RLV). Rats produce LAMP-2 and induce crescentic glomerulonephritis when immunized with the adhesin FimH, which has strong homology with human LAMP-2 (9, 10).

The incidence of AAV has been increasing with the introduction of ANCA testing (11, 12). The prognosis of patients with AAV has improved since the introduction of immunosuppression in the 1960s. However, some severe cases with cumulative renal and pulmonary disease remain aggressive. The exact etiology of AAV is unknown, and a complex association exists between factors, such as polygenic genetic susceptibility (13, 14), epigenetic influences (15), and environmental factors (16), and AAV. AAV is probably not caused by any single factor, but the interaction and combination of multiple factors ultimately lead to the occurrence of this disease (17, 18). ANCA-associated necrotizing glomerulonephritis has been reported in two sets of identical twins, suggesting that genetic factors may be involved in disease pathogenesis (19). Two genome-wide association studies (GWAS) in European and North American populations have identified disease susceptibility loci in AAV. The genetic background of different clinical subtypes of AAV is different (13). GPA, MPA, and EGPA are associated with HLA-DP1, HLA-DQ, and HLA-DRB4, respectively. Additionally, genetic variants in non-MHC regions, such as CTLA-4, FCGR2A, PTPN22, SERPINA1, and TLR9, were significantly associated with AAV. These findings help to elucidate the etiology of AAV and develop new biomarkers for diagnosis and targeted therapy.

In recent years, increasing research evidence has emphasized that environmental factors are involved in the occurrence and development of AAV. Many environmental factors, including silica exposure, season, latitude, and microbial infection, have been reported to be associated with AAV. Several studies (20, 21) have shown that sustained exposure to silica in an occupational setting results in a 3.4–7-fold increased risk of positive ANCA. Additionally, season and latitude have different effects on the incidence of different subtypes of AAV. Generally, AAV tends to occur during winter (22, 23). The incidence of GPA and EGPA increased with increasing latitude and decreasing environmental ultraviolet radiation, whereas the incidence of MPA did not change significantly with changes in latitude and ultraviolet radiation (24). Kronbichler et al. (25) suggested that nasal *staphylococcus aureus* infection may be an important risk factor for the onset and recurrence of AAV. This review summarized the environmental risk factors and possible mechanisms of AAV to provide a scientific basis for the prevention and treatment of AAV.

Classification of AAV

Clinically, AAV is classified into three types: granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA), and eosinophilic GPA (EGPA) (4, 26). This type of disease is characterized by necrotizing small vessel vasculitis. AAV has a predilection for the kidney, with more than 75% of patients having renal involvement. Vasculitis confined to the kidney is known as RLV, and it is characterized clinically by rapidly progressive glomerulonephritis. ANCA is an autoantibody against neutrophil granules and monocyte lysosomal components. The serological marker for AAV is ANCA positivity. All the above diseases are usually associated with circulating ANCA, and accurate ANCA testing is important for diagnosis, treatment, and prognosis. The main target antigens for C-ANCA and P-ANCA are PR3 and MPO, respectively. Additionally, due to the significant overlap in the clinical features of GPA and MPA, CHCC2012 recommended adding

a prefix to the clinical phenotype of patients with established AAV (classified as PR3-ANCA disease and MPO-ANCA disease based on ANCA specificity) (26). MPA was associated with PR3-ANCA in 26% of cases and MPO-ANCA in 58% of cases (27). Whereas GPA was characterized by PR3-ANCA in 66% of patients and MPO-ANCA in 24% of patients. Studies have shown a higher rate of disease recurrence in PR3-ANCA and a higher mortality rate in MPO-ANCA disease. As an important clue to disease diagnosis, a positive ANCA does not necessarily confirm the diagnosis of AAV, whereas a negative ANCA does not exclude the diagnosis of AAV. For example, the presence of ANCA is absent in 40%–50% of patients with EGPA (28). Therefore, clinicopathological findings are the gold standard for the diagnosis of AAV.

Epidemiology of AAV

The introduction of ANCA testing in the 1990s has led to a marked increase in the incidence of AAV in recent years (12, 29). Currently, the prevalence of AAV is approximately 300/million, with an annual incidence of 13–20/million (11, 12). In Norway, the annual incidence of AAV is as high as 24.7/million. The incidence of adult GPA, MPA, and EGPA are 15.6/million, 6.5/million, and 2.7/million, respectively (12, 29). The overall incidence of AAV is increasing in Spain, Germany, and the UK (29, 30). Compared to incidence studies, relatively few studies on AAV prevalence have been reported. The overall prevalence of AAV per million adults reported in Norway in 2013 was 351; the prevalence of GPA, MPA, and EGPA were 261, 58.2, and 32.9, respectively (29). The increased prevalence of AAV may be related to factors, such as increased incidence, improved disease definition, and improved vasculitis registry systems.

Unlike other autoimmune diseases, AAV tends to develop in older and male patients. Studies in both the UK and New Zealand have confirmed that the peak incidence of AAV is at the age of 60–79 years (31–33). The reason for the tendency of AAV to develop in patients of advanced age is unclear. This may be related to advances in ANCA testing that have led to the detection of previously unrecognized AAV. Additionally, AAV is more common in men than in women (31, 34). Studies have shown a male prevalence to female prevalence ratio between 1.07:1 and 1.48:1 (31, 35–37). In Germany and New Zealand, no significant gender differences are present in the incidence of AAV. The reasons for the above occurrence are not clear.

Immunology of AAV

The mechanisms of the AAV autoimmune response have not been fully elucidated, but molecular mimicry and dysregulation of B and T lymphocytes have dominated the disease process. Activated B lymphocytes can produce pathogenic ANCA. Regulatory B (B reg) cells induce T cell differentiation into regulatory T (T reg) cells away from T helper 1 (TH1) and TH17 phenotypes and reduce B cell production of ANCA (38). Neutrophils are both targets of ANCA and mediators of endothelial injury. When exposed in response to infection or inflammation, the ANCA antigen-binding site can bind and activate neutrophils, leading to their degranulation and production of reactive oxygen species (ROS). It subsequently mediates vascular endothelial cell damage (18). Concurrently, intracellular signaling pathways are activated, resulting in changes in the expression and conformation of adhesion molecules, which promote the adhesion and migration of neutrophils in the vascular endothelium (39). Activated neutrophils undergo a specific form of cell death (NETosis), releasing neutrophil extracellular traps (NETs). NETs can mediate direct damage to the endothelium, transfer MPO/PR3 to the vascular endothelium and dendritic cells for antigen presentation, and activate the alternative pathway of complement. Tissue deposition of chemokines, PR3, and MPO lead to the recruitment of autoreactive T cells and monocytes, thereby aggravating vascular tissue damage. The therapeutic targets of NETs in different diseases mainly depend on the components of NETs. AAV-induced NETs were enriched in citrullinated histones, whereas SLE-induced NETs were enriched in oxidized mitochondrial DNA (40).

GPA is characterized by granuloma formation. Early granulomas are characterized by activated neutrophils forming microabscesses and scattered multinucleated macrophages. These macrophages release pro-inflammatory cytokines that promote the recruitment of neutrophils and monocytes from the blood to the lesion site. Recruited neutrophils release lytic enzymes and ROS upon encountering microorganisms and undergo lysis, leading to the formation of a necrotic core of the lesion. Advanced granulomas consist of a central area of necrosis with multinucleated giant cells at the margin, surrounded by dendritic cells, T lymphocytes, B lymphocytes, and plasma cells, forming a follicular structure of ectopic lymphoid tissue (41, 42). Lymphangiogenesis, defective transport capacity, and formation of ectopic lymph node-like structures are important mechanisms for the development of acquired immunity. Granuloma formation may be driven by B and T lymphocytes (43, 44). In patients with EGPA, elevated levels of TH2 cytokines, such as IL-4 and IL-5, are associated with eosinophilia. Eosinophils infiltrating tissues secrete eosinophilic granules, including major basic protein, eosinophilic neurotoxin, and eosinophilic cationic protein, that destroy vascular tissues.

Environmental risk factors associated with AAV

Seasons

Many studies have confirmed that the onset of AAV is strongly associated with seasonal changes, but the specific

results are inconsistent. Most studies (22, 23) report a higher number of patients with AAV hospitalized in winter, with a peak incidence during winter, and demonstrate a higher incidence of kidney damage in patients with AAV during winter. However, Mahr et al. (45) suggested that the incidence of AAV is significantly higher during summer, particularly in August. In studying the factors related to AAV relapse, Kemna et al. (46) showed that AAV is prone to relapse during autumn, accompanied by increased titers of ANCA-related immune markers. In contrast, no significant seasonal variation was found regarding the timing of symptom onset in a study of 445 patients with GPA (47). These findings cannot be merely limited to seasonal changes but also need to be extrapolated to specific causes or triggers.

The possible mechanisms that affect the incidence of AAV in different seasons may be different. Winter is a high incidence period for respiratory-related diseases, and infection may trigger the occurrence of AAV (48). Additionally, the level of vitamin D is an important factor affecting the pathogenesis of AAV. The active form of vitamin D is 1,25-dihydroxy vitamin D_3 (1,25 $(OH)_2$ D_3), which is an immunomodulator. Vitamin D and vitamin D-activating enzymes are widely present in various tissues, especially immune-related cells (Figure 1). The concentration of vitamin D in the body fluctuates with seasonal variations, and the concentration is the lowest during winter (49, 50). Kälsch et al. (50) reported that patients with AAV had significantly lower serum vitamin D levels than healthy controls. Immune dysfunction caused by vitamin D deficiency may be involved in the development of AAV. The high incidence of AAV during summer may be caused by exposure to sunlight or air pollutants. Spring and summer are common seasons for various allergy-related diseases. Furthermore, AAV-related nasal disease may be caused by an immune response driven by Th2 cells. However, more studies are needed to confirm these speculations (30, 51, 52). Seasonal inconsistency may be due to differences in AAV disease subtypes, geographic regions, patient records, onset time deviations, and regional differences in medical levels in each study.

Air pollution

Air pollution has become a serious environmental problem, severely endangering public health (53–55). Air pollution is composed of a variety of gases and particles, including carbon monoxide (CO), sulfur dioxide (SO_2), nitrates (NOX), ozone (O_3), lead, toxic by-products of tobacco smoke, and particulate matter (PM). Fuel combustion is a major source of ambient air pollution. Combustion releases various pollutants, such as carbon oxides,

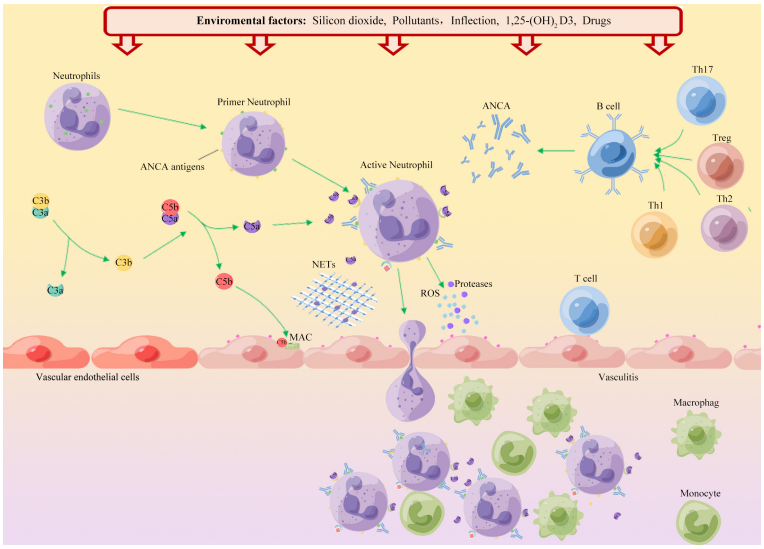

FIGURE 1
Schematic diagram of the environmental factors in the onset of AAV. ANCA autoantigens (PR3 and MPO) are usually hidden in the primitive granules of neutrophils. Environmental factors such as silica, air pollution, and infection, lead to neutrophil initiation and PR3 and MPO movement to the cell surface. Binding of ANCA to these autoantigens leads to activation of neutrophils, which adhere to the vascular endothelium. Neutrophil degranulation leads to the release of reactive oxygen species (ROS), proteases and neutrophil extracellular traps (NETs), which in turn destroy endothelial cells. Chemokines and tissue deposition of PR3 and MPO lead to increased tissue damage by recruitment of autoreactive T cells and monocytes. Additionally, ANCA binds to cell surface autoantigens, leading to neutrophil activation and release of factors that activate the complement replacement pathway. The production of the allergenic toxin C5a further attracts neutrophils and enhances neutrophil initiation and activation upon binding to cell surface C5a receptors, thereby promoting vascular inflammation.

sulfur oxides, nitrogen oxides, polycyclic aromatic hydrocarbons (PAHs), and PM, and harmful metals, such as lead and cadmium, into the atmosphere. Additionally, transportation is an important source of ambient air pollution, which can produce a large amount of pollutants, such as PM, nitrogen oxides, CO, and polycyclic aromatic hydrocarbons. Studies have shown that air pollution is associated with various rheumatic immune diseases. Air pollutants may be involved in the induction of systemic inflammation and enhancement of autoimmunity, thereby inducing or aggravating autoimmune rheumatic diseases (56–58). For example, changes in the concentrations and types of air pollutants may affect disease activity in patients with SLE. In recent years, some studies have shown that air pollution may be related to the occurrence and development of AAV. Data from a survey on the prevalence of AAV disease in China (22) showed that CO exposure was positively correlated with AAV incidence, but air pollutants (PM2.5, PM10, other inhalable particulate matter, NO_2, and SO_2) had no significant correlation with AAV incidence (Table 1). Previous studies have found that CO has anti-inflammatory effects; therefore, the harmful effect of CO on vasculitis needs to be further explored (77). Nuyts et al. (65) found that exposure to hydrocarbons was not a risk factor for GPA and found no significant association between lead, cadmium, and GPA. In contrast, Pai et al. (66) found significantly higher mean hydrocarbon exposure in GPA and MPA cases. Albert et al. (51) found that heavy metal exposure can significantly increase the risk of GPA; these heavy metals are mainly cadmium, lead, and mercury. Subsequently, they found that the GPA population may be exposed to high levels of industrially generated contaminants, including trichloroethylene (TCE), vinyl chloride, methyl tertiary-butyl ether (MTBE), dichloroethene (DCE), and chromic acid (67).

AAV disease is an occupational hazard of agriculture, and the reason may be related to exposure to pollutants. Lane et al. (69) found that a history of organic solvent exposure may be associated with AAV, especially GAP. The same results were obtained in two other studies (70, 71). Studies in Scotland, Germany, and Canada showed that the incidence of AAV in rural areas is higher than that in cities. This may be related to environmental pollutants and pesticide exposure in remote areas (68, 72, 78, 79). Additionally, a large Swedish case-control study (73) found no association between occupation and GPA (Table 1). Unfortunately, these studies only reported the association between pollutants and AAV disease but did not investigate the mechanisms that influence disease.

Previous studies found that tobacco smoking is associated with the development of RA and SLE (80–84). However, findings on the relationship between smoking and AAV have been inconsistent. McDermott et al. (76) proposed that smoking is a risk factor for AAV disease, especially with MPO-ANCA. Yamaguchi et al. (75) found that current smoking status was associated with recurrence (Table 1). However, Haubitz et al. (85) found that smoking may have a potential protective effect against AAV disease. Additionally, studies have linked exposure to silica, tillage, or organic solvents to an increased risk of EGPA, whereas smoking is associated with a lower risk (74). The immunosuppressive effects of nicotine have been suggested as a potential explanation for these findings (86). A series of studies could not elucidate the effect of smoking on AAV disease (69, 70, 87). Current research on smoking and AAV risk has produced conflicting results, and further research is needed to examine the link between smoking and AAV disease progression.

Silicon dioxide

Silica is one of the most abundant minerals on earth, and exposure to silica dust has been identified as a risk factor for many SARDs, including SS, RA, SLE, and AAV (60, 88). Individuals working in agriculture, mills, drilling, painting, and textiles have been identified to have a greater risk of developing AAV disease (89). Multiple case reports (20, 21) have shown that continuous exposure to silica increases the risk of positive ANCA. Several studies have described cases of silica exposure and AAV. A 74-year-old patient with AAV developed fever and malaise after prolonged exposure to silica (90). Main and Wroe (91) described three cases of silica-exposed patients with AAV, two of whom still required dialysis after treatment. Analysis of the occupational histories of 16 patients with AAV revealed that patients with vasculitis were more likely to be exposed to silica than controls (61). Previous surveys on post-earthquake disease prevalence, such as the Kobe earthquake in Japan, the Great East Japan earthquake, and the Yunnan earthquake in China, showed that the incidence of AAV was higher than before (62–64). The change was attributed to the harmful effects of air pollution on the human body due to increased atmospheric levels of silica from the earthquake. Studies have confirmed the dose-related effects of silica exposure. A meta-analysis (60) showed that silica exposure was positively associated with AAV. A case-control study (59) suggested a 3.4-fold increased risk of ANCA serology positivity in individuals with occupational silica exposure. Only a few studies (92, 93) have proposed a relationship between sustained exposure to silica and AAV. However, research on the relationship between sustained exposure to silica and severity of AAV remains inadequate.

The mechanism by which silica causes AAV is unclear. A previous study (91) found that silica does not have a direct toxic effect on genetically susceptible individuals but rather enhances the immune response non-specifically, activates T cells and Treg cells, and leads to autoimmune dysfunction (Figure 1). With continued exposure to crystalline silica, the body produces inflammatory cytokines, including interleukin-1(IL-1) and tumor necrosis factor-beta (TNF-β), leading to inflammation and eventual fibrosis (60). Silica can induce apoptosis of neutrophils, macrophages, and monocytes, and damaged cells release many proteolytic enzymes, leading to chronic

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TABLE 1 Study on the relationship between environmental pollutants and AAV.

Environmental factors	Year	Region	Study design	Participants	Main conclusions
SiO_2					
Beaudreuil et al. (59)	2005	France	Case-control study	Patients with AAV	Silica exposure is dose-dependently associated with ANCA positivity.
Gomez-Puerta et al. (60)	2013	USA	Systematic review and meta-analysis	Six studies	Exposure to silica increases the risk of AAV by 2.57 times.
Gregorini et al. (61)	1993	Italy	Hospital-based case-control study	Patients with AAV	Seven of the 16 cases and one of the 32 controls had positive histories of jobs with exposure to silica dust.
Gupta et al. (20)	2019	India	Case report	Patients with MPA	In a tuberculosis-endemic country, for patients presenting with diffuse alveolar hemorrhage (DAH), with history of silica exposure, differential diagnosis of ANCA-associated vasculitis must be considered.
Rao et al. (21)	2020	Australia	Case report	Patients with AAV	The relevance of occupational exposures in renal disease and the immune-stimulatory effect of silica.
Earthquake-related environmental exposures					
Yashiro et al. (62)	1999	Japan	Case series	Patients with AAV	The frequency of MPO-AAV cases in the Kobe area has more than doubled each year since the earthquake.
Takeuchi et al. (63)	2017	Japan	Retrospective population-based cohort study	Patients with MPO	The annual incidence of MPO-AAV doubled after the earthquake.
Farquhar et al. (64)	2017	New Zealand	Retrospective cohort study	Patients with AAV	No statistically significant difference in the incidence of AAV existed before and after the earthquake.
Other pollutants					
Li et al. (22)	2018	China	Retrospective cohort study	Patients with AAV	Carbon monoxide exposure was positively correlated with the frequency of AAV.
Nuyts et al. (65)	1995	Belgium	Case-control study	Patients with GPA AAV	The association between lead and cadmium and GPA was not significant. Exposure to hydrocarbons and welding fumes were not risk factors for GPA.
Pai et al. (66)	1998	UK	Case-control study.	Patients with AAV	The mean hydrocarbon exposure was significantly greater in cases than in controls.
Albert et al. (51)	2004	USA	Case-control study	Patients with GPA	Mercury was associated with GPA. The association between CO and GPA approached statistical significance.
Albert et al. (67)	2005	USA	Case series	Patients with GPA	This cluster of patients with GPA were potentially exposed to high levels of industrially generated contaminants.
Chung et al. (68)	2022	Australia	Retrospective study	Patients with AAV	No significant relationship existed between region and exposure to silica, solvents, metal, dust, farming, gardening, or sunlight.
Agriculture					
Lane et al. (69)	2003	UK	Case-control study	Patients with AAV	Farming exposure was associated with risk of GPA and MPA but not EGPA. High occupational silica exposure in the index year was a risk factor for AAV. The risk of MPA rises with occupations at intermediate or high silica exposure.
Stamp et al. (70)	2015	New Zealand	Case-control study	Patients with GPA	Farming was associated with an increased GPA risk.
Willeke et al. (71)	2015	Germany	Case-control study	Patients with AAV	Regular farm, cattle, and pig exposure were strongly associated with AAV.
Aiyegbusi et al. (72)	2020	UK	National cohort study	Patients with AAV	GPA (but not MPA) was positively associated with rurality.
Knight et al. (73)	2010	Sweden	Population-based case-control study.	Patients with GPA	No general association existed between 32 selected occupations and GPA.
Smoking					
Haubitz et al. (74)	2005	Germany	Cross-sectional cohort study	Patients with AAV	The prevalence of GPA/MPA among smokers was lower than among the general population.

(Continued)

TABLE 1 Continued

Environmental factors	Year	Region	Study design	Participants	Main conclusions
Yamaguchi et al. (75)	2018	Japan	Multicenter retrospective cohort study	Patients with AAV	Current smoking status was associated with recurrence. Smoking was significantly associated with relapse in MPA, in a dose-dependent manner.
McDermott et al. (76)	2020	USA	Case-control study	Patients with AAV	Patients with AAV were more likely to be former or current smokers; a dose-response relationship existed according to pack-years of exposure. These associations were especially strong among participants with MPO-ANCA-positive disease.
Maritati et al. (69)	2021	UK	Case-control Study	Patients with EGPA	Exposure to silica, farming, or organic solvents is associated with an increased risk of EGPA, whereas smoking is associated with a lower risk. These exposures seem to have distinct effects on different EGPA subsets.

AAV, Antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis; GPA, granulomatosis with polyangiitis; MPA, microscopic polyangiitis; EGPA, eosinophilic GPA.

inflammation and tissue fibrosis (94). Another study (95) suggested that silica can induce the expression of MPO in the cell membrane of neutrophils and monocytes, causing ANCA-related autoimmune responses.

Latitude

A previous study (33) found that the incidence of AAV varies significantly with latitude, further supporting the influence of geographical region on AAV disease. Epidemiological studies (33, 34) have shown that the risk of GPA is high in the northern hemisphere of the earth, whereas the risk of MPA is high in the southern hemisphere. Quantitative changes showed marked changes, while the incidence of GPA and EGPA increased with increasing latitude and decreasing ambient UV radiation levels (24). Similarly, related studies have confirmed that the positive rate of PR3-ANCA decreases with increasing latitude and ultraviolet radiation intensity (96).

UV radiation is a sensitive factor that varies with latitude, and related studies have found a close relationship between UV radiation and immune diseases (46). UV radiation, which changes with latitude, is considered to be the actual cause of AAV. UV radiation is necessary for the skin's synthesis of 1,25 $(OH)_2$ D_3, which regulates immune system homeostasis. UV irradiation of the skin induces vitamin D synthesis, which in turn inhibits the proliferation of Th1 and Th17 cells and the production of cytokines. These changes cause the immune system to differentiate into Th2 cells, thereby enhancing the activity of CD24+, CD25+, and CD8+ cells. This is consistent with a mechanism mediated by Th1 and/or Th17 cells in the pathogenesis of GPA (97–99). This may explain why the association between MPA and UV light is not strong, since granulomas are not present in MPA. However, accurate estimation of the average amount of UV radiation in a region is challenging. The influence of immigration, clothing characteristics, skin color preferences, religious and cultural beliefs, and other factors need to be excluded, as well as the influence of dietary intake of vitamin D, related drugs, and other environmental factors on the final serum vitamin D level in each region. These challenges should be addressed in future studies.

Microbial infections

Staphylococcus aureus

Microbial infection is considered to be an important risk factor for the development of AAV. Intranasal *staphylococcus aureus* (*S. aureus*) infection is most closely associated with AAV (25). The early symptoms of patients with GPA are mainly runny nose, nosebleeds, and other symptoms, because the most prominent feature of the disease is the granulomatous inflammation of the respiratory tract. *S. aureus* infection that colonizes the respiratory tract may trigger GPA disease activity (100). Previous studies (101) have found that the detection rate of *S. aureus* in patients with GPA is significantly higher than that in healthy individuals, and patients with GPA with chronic *S. aureus* infection have a significantly increased risk of recurrence. A randomized controlled trial (102) in the Netherlands showed that patients treated with trimethoprim/sulfamethoxazole (T/S, 960 mg three times a week) had a decreased recurrence rate by 66%. In contrast, prophylactic treatment of chronic *S. aureus* carriers with T/S did not reduce the risk of relapse (101). This may be related to factors, such as drug dosage and different bacterial detection methods (102). Further studies found that the imbalance in the proportion of various bacteria colonized in respiratory tract may contribute to the incidence of AAV. The proportion of *S. aureus* colonization in nasal samples of patients with GPA increased, but the diversity of the microbiome decreased (103–105). Current studies indicate that *S. aureus* is only related to the pathogenesis of GPA, but no obvious relationship seems to exist between *S. aureus* and the pathogenesis of MPA and EGPA.

The role of *S. aureus* in the pathogenesis of AAV may be as follows: (1) Superantigens of *S. aureus* directly stimulate B cells and T cells. Among them is the polyclonal activation of B cells by *S. aureus* cell wall components. Additionally, *S. aureus* may directly initiate neutrophils, leading to surface expression of PR3 (106). (2) *S. aureus* contains a highly homologous complementary form of the protein in humans. cPR-3 (105–201) acts as a protein complementary to the human autoantigen PR3 and elicits an autoimmune response (8). (3) The CpG motif of *S. aureus* may trigger B lymphocytes in the peripheral blood of patients in remission, leading to the production of ANCA and relapse of AAV (107). (4) The polypeptide 6-phosphogluconate dehydrogenase (6PGD) 391–410 encoded by the *S. aureus* plasmid is homologous to the previously determined immunodominant MPO-T cell epitope, and it is immunogenic in humans. Studies have shown that 6PGD induces MPO-related nephritis (108). (5) *S. aureus*-derived extracellular adhesion protein (EAP) and Staphylococcus peroxidase inhibitor (SPIN) can induce the body to produce ANCA (109). (6) *S. aureus* is an effective inducer of NETs, DNA extracellular complex, and antibacterial factors secreted by neutrophils. Exposure of ANCA antigens to the immune system can initiate an autoimmune response to AAV (110, 111).

Viruses

Epstein-Barr virus (EBV) infection is most closely related to various SARDs (112–116). Multiple case reports found that patients with AAV may develop anti-MPO antibodies following EBV infection. Treatment with glucocorticoids combined with ganciclovir can significantly relieve clinical symptoms and reduce viral load (117–119). Lidar et al. (120) found that anti-EBV capsid antigen antibodies and anti-EBV early antigen antibodies were significantly higher in the sera of patients with AAV than in healthy individuals. Treatment with glucocorticoids in combination with ganciclovir significantly relieved clinical symptoms and reduced viral load. Hepatitis B virus (HBV) and hepatitis C virus (HCV) may be triggers for SARDs. An Egyptian study (121) found 62.7% hepatitis C virus infection in 42 patients with AAV, and C-ANCA levels were significantly correlated with hepatitis C virus antibody levels. Lee et al. (122) found a significantly higher risk of relapse in anti-HBc-positive patients with EGPA. Resolved HBV infection may have an important impact on vasculitis activity at diagnosis and subsequent relapse after remission in patients with EGPA. Recently, ANCA has been identified in patients with coronavirus disease 2019 (COVID-19) infections, but relatively few cases have been reported (123, 124). Studies have proposed the involvement of the parvovirus B19, human herpesvirus, and hantavirus in the occurrence of AAV (122, 125, 126). However, these studies are few and have not found a significant correlation between these viruses and the development of AAV.

Other microorganisms

Few studies have been conducted on other microorganisms in AAV. A Japanese study (127) reported that Aspergillus infections, including Candida, Candida, and Fusarium, were found in patients with both allergic bronchopulmonary mycosis (ABPM) and EGPA. Kuwabara et al. (128) found that *Mycobacterium tuberculosis* infection and anti-tuberculosis drugs may be related to AAV. Fujita et al. (129) found that the positive rate of *Chlamydia pneumoniae* in patients with MPO-AAV was 33%. A Japanese report (130) described a woman who underwent total thyroidectomy, developed PR3-ANCA 3 months after surgery, and had a chronic infection with *Tsukamurella pulmonis*. GPA often occurs in gastrointestinal mucosal lesions, and the study detected 25 cases of *Helicobacter pylori* infection among 36 patients with GPA (131). Currently, the effect of these microorganisms on AAV is only speculative, and further large-scale studies are needed to verify.

Other environmental risk factors

Drugs

Drug-induced small vessel vasculitis is a small group of AAV disorders that still do not have a precise definition. Drugs that may be associated include hydralazine, allopurinol, propylthiouracil, phenothiazine, nitrofurantoin, methimazole, minocycline, phenytoin sodium, penicillamine, lorazepam, levamisole, cocaine, isoniazid, montelukast, erlotinib, and tofacitinib (86, 89, 128, 132–134). Among them, the incidence of AAV caused by antithyroid drugs is higher, especially propylthiouracil. The clinical manifestations of propylthiouracil-induced AAV disease are similar to those of primary AAV, whereas the disease severity is less severe and prognosis is better. After cessation of antithyroid drug use, symptoms of patients with AAV gradually resolve and ANCA titers decrease significantly (135). Treatment strategies for drug-induced AAV differ from those for primary AAV (136). In patients with mild symptoms, immediate discontinuation of the relevant drug can lead to disease remission. Patients with severe diseases should be treated aggressively. However, immunosuppressive maintenance therapy is often unnecessary (137). The mechanism of drug-induced AAV disease may be related to NETs (138). However, further studies are needed to verify the exact mechanism (132). NETs are associated with inflammation in various ways. NETs can directly induce endothelial damage and activate alternative complement pathways (139). Additionally, they are a major component of thrombosis. The relationship between NETs and ANCAs seems to be bidirectional, a vicious circle (111, 140, 141).

Vaccines

The efficacy of vaccines is based on the ability of the host immune response to the antigen to elicit a memory T-cell response over a period of time. The influenza vaccine is

generally considered safe and effective. However, in recent years, the population after influenza vaccination has developed various autoimmune phenomena, such as Guillain-Barré syndrome, RA, pemphigus vulgaris, psoriasis, giant cell arteritis, and AAV (142, 143). Several AAV cases associated with influenza vaccination have been reported (144, 145), but influenza vaccination does not increase the recurrence rate of AAV disease. The exact etiology of AAV induced by influenza vaccination is unclear and may be related to molecular mimicry and autoimmune/inflammatory syndrome induced by adjuvants (ASIA syndrome) (146–148). Recent studies (149, 150) have found that AAV may occur after receiving the COVID-19 mRNA vaccine, and patients with existing AAV may experience recurrence after receiving the COVID-19 mRNA vaccine. The mechanism of new or recurrent AAV after vaccination is still a mystery and may be similar to the mechanism of AAV caused by influenza vaccine. Additionally, the enhanced immune response and presence of monocytes after vaccination may cause MPO-ANCA and PR3-ANCA (151). However, this evidence originates from individual case reports, and no specific mechanism has been explored.

Conclusion

Studies to identify modifiable environmental risk factors for AAV can provide insights into disease pathogenesis and can facilitate the development of preventive strategies, especially in those individuals at high risk. The current consensus is that multiple environmental and epigenetic factors interact in a complex manner. Different triggers and extent of their roles in disease activity may vary by subgroups (e.g., ANCA subtype, geographic region). Numerous epidemiological studies support the relationship between exposure to various environmental pollutants, UV radiation deficiency, and microbial infections and the risk of developing AAV. Other environmental factors, including seasonal changes, latitudinal changes, medications, and vaccinations may be associated with an increased risk of AAV. Further studies are needed to confirm these findings. Additionally, future studies on environmental factors and AAV susceptibility subgroups need to be advanced, and exposures throughout the life course should be considered comprehensively.

Author contributions

W-MZ, Z-J W and D-GW were part of the organizing committee of the workshop. W-MZ wrote the manuscript. Z-JW, RS, Y-YZ, SZ, and R-FW contributed to the revision of the initial draft of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Composition and regulation of the immune microenvironment of salivary gland in Sjögren's syndrome

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Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease characterized by exocrine gland dysfunction and inflammation. Patients often have dry mouth and dry eye symptoms, which seriously affect their lives. Improving dry mouth and eye symptoms has become a common demand from patients. For this reason, researchers have conducted many studies on external secretory glands. In this paper, we summarize recent studies on the salivary glands of pSS patients from the perspective of the immune microenvironment. These studies showed that hypoxia, senescence, and chronic inflammation are the essential characteristics of the salivary gland immune microenvironment. In the SG of pSS, genes related to lymphocyte chemotaxis, antigen presentation, and lymphocyte activation are upregulated. Interferon (IFN)-related genes, DNA methylation, sRNA downregulation, and mitochondrial-related differentially expressed genes are also involved in forming the immune microenvironment of pSS, while multiple signaling pathways are involved in regulation. We further elucidated the regulation of the salivary gland immune microenvironment in pSS and relevant, targeted treatments.

KEYWORDS

immune microenvironment, salivary gland, Sjögren's syndrome, senility, chronic inflammation, hypoxia

1 Introduction

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease that occurs mostly in middle-aged women and is characterized by impaired glandular function and the appearance of autoantibodies caused by infiltrating exocrine glands with lymphocytes, with an estimated prevalence of 0.3-3/1000 in the general population (1, 2). PSS is a heterogeneous disease; approximately 5% to 35% of the population has dry eye, approximately 20% of patients have dry mouth, and up to 34% of patients have

parotid gland swelling (3–5). In addition to glandular involvement, arthritis occurs in approximately 50% of patients, lung involvement occurs in 9-12% of patients, skin lesions occur in 10% of patients, kidneys involvement occur in 5% of patients, and sensory neuropathy occurs in 10-25% of patients (6–8). In blood samples, 40%-75% of pSS patients have anti-RO/SSA, and 23%-52% have LA/SSB antibodies (9).

The etiology and pathogenesis of SS are still unclear, and it is generally believed that genetic susceptibility related to environmental factors is an important cause of the occurrence of Sjogren's syndrome (10). Currently, genome-wide association studies on pSS have been completed, among which HLA genes have the strongest association signal (11). Epigenetic mechanisms such as DNA methylation, histone modification, and noncoding RNA play a role in the pathogenesis of pSS by regulating gene expression and may form a dynamic link between the genome and phenotypic expression. Bacteria and viruses are essential components of environmental factors. Bacteria can cause autoimmune diseases through various mechanisms, such as pathogen persistence, epitope spread, molecular mimicry, epigenetic changes, and Toll-like receptor activation. Type I IFN is a critical immune mediator involved in viral defense and immune response activation, which suggests the important role of viral infection in the pathogenesis of the disease. A recent analysis of the gene expression of SGECs showed that the IFN signaling pathway and genes involved in the immune response (HLA-DRA, IL-7, and B-cell activator receptor) in pSS were upregulated (12). Other studies found dysregulation of the IFN signaling pathway in SG and peripheral blood of some patients with SS (13), especially the upregulation of type I IFN-induced genes. Various factors, such as infection and hypoxia, induce the activation of SG epithelial cells, leading to lymphocyte infiltration (especially CD4+ T cells) and the release of inflammatory factors. New cell populations, such as follicular T cells, TH17 cells, dendritic (IFN-producing) cells, and B lymphocytes, gradually develop into B lymphocyte-dominated ectopic germinal centers (GCs) with autoantibody production.

The occurrence and development of pSS is a complex process involving many kinds of cells. The salivary gland, as the most commonly affected organ, has attracted increasing attention. Early SG lesions in pSS are rarely reported, which may be related to the delayed diagnosis of pSS. However, many studies have attempted to find targeted therapies for pSS by intervening in the inflammatory process of pSS. Among them, the improvement of dry mouth symptoms caused by impaired salivary gland function is an important goal of treatment. Autoantibodies in the salivary glands induce abnormal immune responses, which together with a large number of infiltrating inflammatory cells destroy normal salivary gland cells, atrophy of salivary gland cells and disappearance of salivary duct. As time goes on, the normal secretory function of salivary glands can be affected, resulting in dry mouth (14). Salivary gland cells, inflammatory cells, inflammatory mediators, autoantibodies and cytokines produced by each of them or by each other constitute the unique immune microenvironment of salivary gland cells. Changes in the immune microenvironment may cause changes in glandular function. Therefore, obtaining more salivary gland tissue samples, analyzing the potential differences between peripheral blood and salivary gland tissue, and further understanding the composition and regulation of the salivary gland immune microenvironment can help us find more targeted treatments for pSS xerostomia. In addition, the salivary gland immune microenvironment of pSS, a type of autoimmune epithelitis, can provide a model for the study of other autoimmune epithelitis (celiac disease, primary biliary cirrhosis, etc.).

2 Characteristics of the salivary gland microenvironment

The salivary gland microenvironment directly affects salivary gland secretion function and is very important for the occurrence and development of pSS. It generally has three characteristics: hypoxia, chronic inflammation, and senescence.

2.1 Hypoxia

Hypoxia is a state of reduced available oxygen caused by reduced blood flow, anemia, metabolic changes, and inflammation (15, 16). Hypoxia has been shown to accelerate cell apoptosis in the renal epithelium (17) while downregulating Cl- ion secretion in the intestinal epithelium, resulting in decreased epithelial fluid transport activity and destruction of tight connections between epithelial cells (18). Hypoxia can also lead to macrophage polarization, regulatory T-cell aggregation, and inhibitory T-cell maturation, leading to immune tolerance and tissue damage. A recent study of minor salivary glands in pSS patients found that hypoxia and IFN-related genes were closely associated with the expression of interleukin (IL)-21 signaling genes, which were significantly increased in pSS patients (19), suggesting a correlation between hypoxia and pSS morbidity. Hypoxia-inducible factor 1α (HIF1α), a transcription factor, is a major regulator of oxygen homeostasis and can be regarded as a hypoxia marker. HIF1α is also a key player in integrating the T-cell receptor (TCR) and cytokine receptor-mediated signals of CD4+ helper T cells (20). In addition, HIF1α enhances Th17 development through direct transcriptional activation of RAR-related orphan receptor gamma t (RORt) (21). This subpopulation is highly increased in salivary gland tissue of patients with SS and a mouse model of SS (22). Longyun Ye et al. cultured mouse submandibular glands (SMGs) *in vitro* and showed that hypoxia (5% O2) induced HIF-1α, glucose transporter 1 and VEGF expression, while BAY 87-

2243-mediated HIF-1α inhibited salivary gland development (23). There are also differing viewpoints. Recent studies have shown that HIF1α expressed in epithelial cells protects against hypoxia-induced tight junction integrity loss and epithelial secretory function loss. The genotype and the allele of the HIF1A Pro582Ser polymorphism were associated with a reduced risk of pSS, suggesting that HIF1α activity may be involved in the development of pSS disease (24). Such different results may be related to different degrees of hypoxia. Antonela Romina Terrizzi et al. performed a comparative analysis of adult Wistar rats exposed to persistent or intermittent hypoxia over 90 days (25). The results suggested that salivary secretion decreased and prostaglandin E2 (PGE2) content increased in animals exposed to hypoxia. The persistent hypoxia group showed higher HIF-1α staining. This suggests that PGE2 plays a negative role during gland adaptation to hypoxia.

2.2 Chronic inflammation

Chronic inflammation is another feature of salivary gland involvement in pSS patients, mainly manifested by periductal lymphocytic infiltration. The lesions are mainly T and B lymphocytes, with a few monocytes, including macrophages, myeloid cells, plasmoid dendritic cells, and follicular dendritic cells (FDC) (26). The corresponding plasma cells attack normal tissues and organs, including salivary glands, and cause tissue damage when they produce autoantibodies. It has been reported that in a mouse salivary gland inflammation model, the degree of salivary gland inflammation is related to the titer of antinuclear antibodies (27). Some authors have described the presence of anti-Ro/SSA and anti-La/SSB autoantibodies in the saliva of pSS patients but no circulating antibodies in serum, suggesting that the salivary glands of pSS patients can specifically produce these autoantibodies (28). In addition to anti-Ro/SSA and anti-La/SSB autoantibodies, anti-salivary gland protein 1, anti-carboxylase 6, and anti-parotid secreted protein autoantibodies have also been reported, which recognize salivary gland- and lacrimal gland-specific antigens (29). Moreover, IL-2, IL-6, IL-21, BCL6, Foxp3, and other cytokines and transcription factors were detected in the salivary glands, which further proved the persistence of inflammation (30–33).

2.3 Senescence

Senescence is a permanent state of cell cycle arrest, with the upregulation of antiapoptotic pathways (34). Using organoid culture techniques, some researchers found that salivary gland progenitor cells (SGPCs) in pSS patients showed insufficient self-renewal ability and differential ability compared with the control group. The telomeres of pSS SGPCs were shorter than normal, suggesting the existence of an aging phenotype (35). In addition, p16+ expression increased in the basal striated duct cell (BSD) progenitor cell niche and the whole parotid epithelium, a marker of aging (36). Senescent cells express and secrete proinflammatory cytokines (i.e., senescence-related secretion phenotypes, SASP) that play a role in spreading senescence and promoting tissue inflammation (37).

In conclusion, pSS SGPCs tend to be senescent, and SASPs maintain the senescent SG microenvironment. The salivary function of pSS patients could not recover after the improvement of salivary gland inflammation, which proved the existence of senescence from another aspect. Mie Kurosawa et al. found an accumulation of senescence-associated T cells (SA-TS) in salivary glands of PSs model mice, which were involved in the pathogenesis of SS-associated sialadenitis through upregulation of the epithelial chemokine CXCL13 (38), and they may become another target for pSS treatment.

3 Composition of salivary gland immune microenvironment

3.1 Gland cells

First, let us review the SGs. The salivary gland is the general name for the exocrine gland opening in the mouth through the duct because its secretions are discharged into the mouth and mixed into saliva. There are three pairs of SGs: parotid, sublingual and submandibular, and the largest pair is the parotid gland. SGs are composed of repeatedly branching ducts and terminal acinus forming the gland parenchyma. The acinar is divided into serous, mucinous, and mixed acinar, producing and secreting watery or mucus-rich saliva from serous and mucinous acinar cells. The secreted saliva passes through intercalated ducts into striate tubes of basal cells and lumen cells and finally into the mouth through larger excretory ducts. Luminal striated duct cells, basal striated duct cells, intercalated ducts, acinar cells, and myoepithelial cells constitute the salivary gland's epithelial cells (SGECs). Salivary progenitor cells reside in striatal canals and proliferate and differentiate to maintain gland homeostasis.

SGECs are not only a critical immune target of pSS but also play an essential immune function in the pathogenesis of pSS, mediating the initiation and persistence of inflammation and autoimmune response. Human pSS SG epithelial cells show increased proapoptotic molecules (such as Fas and Bax) and decreased antiapoptotic molecules (Bcl-2) compared with healthy individuals (39–41). Endoplasmic reticulum stress leads to autophagy and apoptosis, which may lead to redistribution of Ro/SSA and La/SSB autoantigens, initially on the cell surface and eventually in apoptotic blisters (42). These autoantigens are upregulated in pSS SGECs and regulated by the TLR/IFN TYPE I signaling pathway (43). SGECs are not only

important sources of pSS autoantigens Ro/SSA and La/SSB but also express MHC class I and II and T-cell costimulatory molecules (CD80/CD86), enabling them to function as autoantigen presenting cells (44–46). SGECs have been shown to express not only virus-associated toll-like receptors (TLRs) (3 and 7) but also bacterial infection-associated TLRs (1, 2, 4) (47). SGECs bind to multiple pathogen-associated molecular patterns (PAMPs) through the expression of TLRs 1, 2, 3, 4, and 7. In addition to the B-cell activator CD40, SGECs also express CXCL10, CXCL12, CXCL13, IFNα, IFNβ, IFNλ, TNFα, and other receptors. They also produce a variety of cytokines/chemokines under various stimulus conditions, including IL-18, IL-21, IL-1, IL-6, TNFα, B-cell activating factor (BAFF), CXCL-10, CXCL-12, CXCL-13, and CCL-21 (47–59). IL-6 and the costimulatory molecule ICOSL contribute to follicle-assisted T-cell induction, which is critical for B-cell activation and differentiation (60). IFNλ stimulation of SG epithelial cells also induces the expression of BAFF and CXCL10, suggesting that type III IFN plays a role in developing SG pathology in pSS (61).

Abundant evidence suggests that SGECs can drive the activation, differentiation, and survival of B cells through direct interaction and cytokine production and promote the pathogenesis of SS (12, 62, 63). *In vitro*, culture results showed that SGECs from pSS patients promoted the differentiation of B cells into mature B-cell phenotypes and improved the survival rate (12, 63). SGECs may also indirectly induce B-cell differentiation. SGECs have been reported to promote T follicular helper cell differentiation and IL-21 production (60), which may further enhance B-cell hyperactivity in salivary glands in pSS patients. In the salivary glands of pSS patients, high levels of CXCL12 were detected in ductal epithelial cells (64), and CXCL12 expression and IL-6 were associated with high focusing scores and high levels of CD138+ plasma cell infiltration (51). Riviere et al. showed the presence of IL-7/IFNγ amplification loops involving SGEC and T cells in primary SS (65). They stimulated primary cultures of SGECs from control and primary SS patients with poly (I-C), interferon α, or interferon γ. SG explants were cultured with an anti-IL-7 receptor (IL-7R) antagonist antibody (OSE-127) for 4 days, and transcriptome analysis was performed using the NanoString platform. The results suggested that the expression of IL7R was decreased in T cells. Il-7 is secreted by SGECs stimulated by poly (I-C), IFNα, or IFNγ. IL-7 stimulation increases T-cell activation and IFNγ secretion. Transcriptome analysis of SG explants showed a correlation between IL7 and IFN expression, and explants cultured with anti-IL-7R antibodies showed reduced IFN-stimulated gene expression. These results indicate the presence of IL-7/IFNγ amplification loops involving SGEC and T cells in primary SS. Il-7 is secreted by the SGEC stimulated by type I or TYPE II IFN, which in turn activates T cells that secrete type II IFN.

The stromal component of the salivary glands is composed of mesenchymal stromal cells (MSCs), which provide tissue-homeostatic properties, including regeneration, repair, and immune regulation. It has been shown that human bone marrow mesenchymal stem cells (hMSCs) cocultured with purified immune cell subpopulations alter the cytokine secretion profile of dendritic cells (DC), primary and effector T cells (Th1 and Th2), and natural killer cells (NK) to induce a more anti-inflammatory or tolerant phenotype (66). This suggests that MSCs may reduce inflammation by acting as immunomodulators and promoting tissue regeneration. In recent studies, IFNγ stimulated cultured resident MSG-derived MSCs (MSG-Mscs) isolated from the small salivary glands of pSS patients, and the protein levels of indoleamine 2,3-dioxygenase (IDO), programmed death ligand 1 (PD-L1), and intercellular adhesion marker 1 (ICAM-1) increased. These results suggest that MSG-Mscs have normal immunomodulatory functions in small salivary glands. In addition, MSG-Mscs inhibited T-cell proliferation in a dose-dependent manner and were not associated with 17-β-estradiol exposure (67). In addition, follicular dendritic cells (FDCs) are stromal cells located in primary follicles and germinal centers (GCs) of secondary and tertiary lymphoid organs and have the unique ability to retain natural antigens in B-cell follicles for several months (68).

3.2 Multiple participating cells

With the progression of epithelial cell activation and disease, new cells appear in the salivary gland, such as follicle T cells and Th17 cells, dendritic cells (producing IFN), macrophages (MFs), natural killer cells, and B lymphocytes (T cells are usually found in mild lesions, and B cells and MFs dominate in the most severe lesions). Furthermore, they gradually develop into ectopic germinal centers (69, 70).

3.2.1 B cells

There are many different types of B cells in salivary gland tissue. FcRL4+B cells were found in or near the ductal epithelium of the inflammatory salivary gland tissue of pSS. FcRL4 is closely related to lymphoma and is expressed in almost all MUCOSAL-associated lymphoid tissue (MALT) B-cell lymphomas associated with pSS, especially in the parotid gland. RNA sequencing of FcRL4+ B cells isolated from parotid cell suspensions from pSS patients showed that FcRL4+ B cells were not enriched in the blood of pSS patients compared with non-SS-sicCA patients, but these cells generally displayed a proinflammatory phenotype. Genes encoding CD11c (ITGAX), T-BET (TBX21), TACI (TNFRSF13B), Src tyrosine kinase and NFκB pathway-related genes were significantly upregulated in glandular FcRL4+B cells compared with FCRL4-B cells. Therefore, FcRL4+B cells in pSS exhibit many characteristics of chronic activation and proinflammatory B cells (71). Some researchers found that through immunohistochemistry and

mRNA analysis, the expression level of FcRL4 mRNA in parotid MALT lymphoma was increased compared with the parotid tissue of pSS patients without lymphoma, which may explain why MALT lymphoma in pSS patients preferentially occurred in this specific site (15). In addition, MZB cells were detected in saliva and lacrimal glands in both patients with salivary gland disease and mouse models. The C57BL/6. Nod-aec1aec2 mouse model, as well as several SS gene knockout mouse models, showed that B lymphocytes, especially peripheral zone B (MZB) cells, are necessary for the development of clinical manifestations and pathogenesis, although destruction of lacrimal and salivary gland cells involves a typical T-cell-mediated autoimmune response. Peck et al., through *in vitro* temporal global RNA transcriptomic analysis, showed that MZB cells from C57BL/6. Nod-aec1aec2 mice were recruited by the upregulated Cxcl13 chemokine to the exocrine gland, where they recognized complement-modified antigens through their sphingosin-1-phosphate and B-cell receptors (72). BAFF transgenic (TG) mice developed autoimmune diseases characterized by autoantibody production, leading to salivary gland destruction (salivary adenitis), which was associated with enlargement of the B-cell compartment in the marginal region (MZ) and abnormal presence of MZ-like B cells in blood and inflamed salivary glands (15). In the IL14aTG mouse model, elimination of MZB from mice by B-cell-specific deletion of RBP-J resulted in complete elimination of all SS disease manifestations (73). Daridon and others, through classification and reverse transcription polymerase chain reaction analysis of salivary gland specimens in the presence of B cells and its polyclonal form in 18 patients, found that pSS patients with heterotopic salivary glands in GC sample structure transition - 2 B-cell amplification, locally produce autoantibodies, which may help and influence subsequent epithelial damage (74). Immunoglobulin rearrangement in single parotid B cells isolated from the parotid gland was analyzed by fluorescence-activated cell sorting, and the results showed that compared with peripheral blood, most parotid B cells in pSS showed the mutant status and phenotype of memory B cells, which accumulated in the salivary glands of pSS patients (75). Hansen et al. analyzed chemokine receptor expression in CD27- naive and CD27+ memory B cells from primary SS patients and healthy controls using flow cytometry, single-cell reverse transcription polymerase chain reaction (RT–PCR), and migration assays. The results showed that CD27+ memory B cells overexpressed the chemokine receptors CXCR4 and CXCR5, which may promote the infiltration of memory B cells into inflammatory glands through the chemokine receptors CXCL12 and CXCL13 from epithelial cells (76). Similar to transition B cells, CD27+ memory B cells seem to promote the formation of ectopic GC-like structures in the exocrine glands of pSS patients (77). Skarstein et al. found that in pSS patients, the increase in CD138+ plasma cells and CD20+ B cells is associated with fat infiltration and focal infiltration, suggesting that they are actively involved in promoting inflammation (78). Szyszko et al. performed single, double, and triple immunohistochemical and immunofluorescence staining of small salivary gland tissues from pSS, chronic inflammatory, and normal subjects, suggesting that plasma cells were located near CXCL12- and IL-6-expressing cells. A salivary gland environment with a high focus score provided a critical factor for plasma cell survival (51). In addition, Cui et al. developed an enzyme-linked immunosorbent assay for autoantibodies with good quantitative ability and found that the expression levels of saliva anti-Cofilin-1, anti-α enolase and anti-RGI2 in parotid gland tissues of pSS/MALT patients were significantly higher than those of healthy controls (79). These results suggest the promoting role of plasma cells in MALT lymphoma.

3.2.2 T cells

In the salivary glands, T cells mainly assist B cells. Th1 cells are believed to play a major role in pSS and are the most relevant CD4 + cell population infiltrating inflammatory SGs (80). Th2 cytokine levels are closely associated with SG lymphocyte infiltration (30). Th17 cells also play a key role in pSS. Th17 cells in SGs can develop into Th17.1 cells and produce IL-17 and IFN-γ, which are involved in the pathogenesis of pSS (81). It has been reported that Tfh cells selectively accumulate in the SGs of pSS patients (82, 83). Tfh cells appear as a unique subpopulation of CD4+ T helper cells that promote the development and activation of B cells. Tfh cells express CXCR5, which migrates and localizes in B-cell follicles and induces the expression of T-cell costimulatory (ICOS) molecules, coinhibitory programmed cell death protein-1 (PD-1), and the transcription factor Bcl6 (84). Tfh cells release large amounts of IL-21, a key cytokine that activates the molecular mechanism of somatic excessive mutation and analog switching of B cells (85, 86). Another subpopulation of CD4+ T cells, follicular regulatory T cells (Tfr), also express CXCR5 but have the typical inhibitory function of regulatory T cells, negatively regulating GC responses to prevent abnormal GC responses (87). In addition, pSS patients showed a high degree of infiltration of pathogenic T peripheral helper cells (Tph) in SGs, which lacked typical Tfh markers such as CXCR5 and Bcl6 but could assist homologous B cells through IL-21 and CD40-L (82, 88). By studying the peripheral blood of pSS patients, Dupre et al. found that Tfh and Tph were amplified in the peripheral blood of patients and correlated with disease activity and B-cell marker (RF and anti-SSB) levels (89). Pontarini et al. performed transcriptome (microarray and quantitative PCR) analysis, FACS T-cell immunophenotyping and intracellular cytokine detection, polychromatic immunofluorescence microscopy and *in situ* hybridization. It was found that damaged CD4+CD45RO+ICOS +PD1+ cells selectively infiltrated ELS+ tissues in SG and

amplified abnormally in parotid malt-L. In ELS+SG and MALT-L parotid glands, the traditional CXCR5+CD4+PD1+ICOS+ FOXP3-TFH cell population and the uniquely enlarged CXCR5-CD4+PD1HIICOS+ Foxp3-TPH cell population showed frequent IL-21/interferon dual production. The results highlight Tfh and Tph cells and IL-21 and ICOS costimulation pathways as key pathogenic factors in SS immunopathology (90). Cytotoxic T lymphocytes (CTLs) can specifically recognize and lyse their targets. Recently, antigen-specific cytotoxicity expressed by cytotoxic T cells *in vitro* and *in vivo* has been shown to be Fas based (91). Kong et al. used immunohistochemical staining and reverse transcription polymerase chain reaction *in situ* to detect the expression of Fas and Fas ligand (FasL) in salivary gland biopsy materials and evaluated the DNA fragments in apoptotic cells by enzymatic incorporation of labeled nucleotide (digoxin -dUTP). The results showed that the acinar epithelial cells of SS were Fas+ and FasL+, and the cells died by apoptosis. Fas+ and Bcl-2+ were the dominant infiltrating lymphocytes in SS, and FasL was expressed in a few lymphocytes. *In situ* detection of apoptosis showed minimal cell death of lymphocytes, especially in dense periductal lesions. These results suggest that the Fas pathway may be an important mechanism of SS gland destruction (39).

3.2.3 Other cells

Dendritic cells (DCs) can be divided into antigen presenting myeloid DCs (MDCs), which are effector cells, and plasma cell DCs (PDCs), which mainly produce type I interferon. Among them, plasmacytoid dendritic cells (PDCs) produce type I interferon (IFN) and contribute to the pathogenesis of various autoimmune diseases. PDC and type I IFN activity are elevated in the salivary glands of SS patients. Zhou et al. applied pdC-consuming anti-BST2/CD317 antibodies to female NOD mice aged 4 to 7 weeks at the early stage of SS and assessed the pathology of SS at 10 weeks of age. The results suggested that PDC treatment inhibited the development of inflammation and secretory dysfunction of SMG and significantly reduced the number of type I interferon mRNA, total white blood cells, T lymphocytes and B lymphocytes in SMG. This suggests a role for PDC in the pathogenesis of pSS (92). In patients with pSS, immature myeloid dendritic cells (DCS) are reduced in the blood, and mature myeloid dendritic cells accumulate in the salivary glands. As the duration of pSS syndrome increases, the reduction in myeloid dendritic cells in the blood spontaneously recovers. Myeloid DCs may play an important role in the pathogenesis of pSS by initiating the helper T-cell immune response (93).

Fibroblasts are an extremely heterogeneous population of cells with a spindle shape, oval nuclei, and the ability to adhere to collagen fibers. In addition to synthesizing and reshaping the structure and function of the extracellular matrix, they also have the ability to secrete cytokines, chemokines and growth factors. Furthermore, immune fibroblasts affect the homeostasis of immune cells, and are one of the important stromal cells constituting the tertiary lymphoid structures (TLS) (94). Nayar et al. showed that immune fibroblast activation and expansion were observed during TLS formation in wild-type (WT) mice that induced salivary gland inflammation. In Dm2 mice, the loss of $PDPN^+/FAP^+$ fibroblasts disrupted the establishment of TLS and impaired the establishment of local pathology. Meanwhile, in salivary glands of pSS patients, the phenotype and proliferation of TLS immune fibroblasts are regulated by IL-13 and IL-22 (95). In addition, Korsunsky et al. performed single-cell RNA sequencing of fibroblasts and found that $CXCL10^+CCL19^+$ immune-interacting and $SPARC^+COL3A1^+$ vascular-interacting fibroblasts were expanded in a variety of inflammatory tissues (salivary glands of pSS, synovium of RA, colon of ulcerative colitis, etc.) (96). In ulcerative colitis, fibroblasts are the main source of IL-6, and many cytokines and inflammatory mediators have been found to significantly induce IL-6 expression in fibroblasts, including TNF, IL-17, IL-1, LPS, and IFN (97).

3.3 Extracellular matrix

SS is essentially a kind of epithelial inflammation, and the integrity, structure, and function of epithelial cells largely depend on the homeostasis of the extracellular matrix (ECM). The ECM is a network of many components, including fibrin, glycosaminoglycan, growth factor, protease, and inhibitors. An increasing number of studies have shown that changes in the morphology and function of acini and ducts, accompanied by the degradation and remodeling of ECM, are critical events in salivary gland changes in pSS patients. ECM not only supports glandular cells, but its components are also important components of damage-related molecular patterns (DAMPs). DAMPs are potential endogenous inflammatory sources that drive autoimmunity by activating pattern recognition receptors (98). When the glandular tissue of pSS patients is damaged by internal and external environmental factors, the ECM releases soluble DAMPs (Biglycan, decorin, etc.) under the action of matrix metalloproteinases (MMPs). Soluble DAMPs activate homologous receptors that mediate inflammation (e.g., MyD88-dependent TLRs), leading to aseptic inflammation and enhancing pathogen-mediated inflammation (99–101). The salivary mucins MUC1, MUC7, and MUC5B secreted into the intercellular space have been reported to activate proinflammatory molecules and Toll-like receptors, which are also involved in inflammatory responses (99). Aberrant decorin levels (DCN) induce damage to human salivary gland epithelial cells and the polarization of macrophages (102). The increased level of DCN in the parotid gland of pSS patients was positively correlated with several chemokines (CXCL13, CXCL9, and CCL20), IL-1ß, and caspase3 but negatively correlated with the proliferation-related gene MKI67. DCN induces apoptosis of A253 cells and

differentiation of macrophages into the M1 phenotype, which is characterized by the expression of proinflammatory cytokines (102).

4 Special structure

The salivary glands of pSS patients are characterized by chronic inflammation, and the lesions are mainly composed of T and B lymphocytes (26). In the initial stage of disease, lymphoid tissue initiator or inducer (LTi) cells, induced by precursors such as Epstein-Barr virus or cytomegalovirus, produce lymphotoxin, which promotes the expression of NF-κB signals in lymphoid tissue organizer cells by lymphotoxin-β receptors. This results in the enhanced expression of homeostatic chemokines and cytokines (CXCL13, CCL19, CCL21, RANKL, IL-17 and IL-22) (103, 104). At the same time, the virus induced the expression of interferon-γ and stimulated the expression of CXCL9 and CXCL10 in ductal epithelium. These cytokines and chemokines are involved in attracting T lymphocytes, B lymphocytes, and other immune cells to the site of inflammation and promote the formation and maintenance of organized lymphoid tissue (105–107). In addition, prolonged gland activation leads to the formation of FDC networks and the separation of T and B cells, and finally to the formation of ectopic lymphoid structures (ELS) with ectopic germinal centers as the core, T and B cell separation areas surrounding, and the formation of high endothelial venules (HEVs) as exchange channels with peripheral blood lymphocytes (103, 104, 108). The germinal center consists of a light zone and a dark zone. There are rapidly proliferating central blasts in the dark zone, and their Ig variable region genes can undergo somatic hypermutation, thereby protecting them from apoptosis. In the light zone, the central cells transformed from the central blast cells competitively bind to the antigen presented by FDC. The central B cells with high affinity BCR bound to the antigen were positively selected, and the cells that did not receive the antigen underwent apoptosis. Tfh cells regulate the apoptosis of positively selected central B cells or further development into memory B cells or plasma cells through Fas-FasL and CD40-CD40L (109). Tph further induces chemotaxis and conversion of memory B cells into plasma cells by producing IL-21 and CXCL13. Initiation and maintenance of ELS in pSS requires ectopic expression of lymphoid chemokines, including CXCL12, CXCL13, CCL19, and CCL21. These chemokines regulate lymphocyte trafficking and tissue localization by interacting with their unique receptors CXCR4 (for CXCL12), CXCR5(for CXCL13), and CCR7(for CCL19 and CCL21). CXCL12 is mainly produced by follicles and ducts, and its receptor is expressed in plasma cells (110). CXCL13 is mainly produced by stromal cells, memory CD4+ T cells, and non-immune cells (ductal epithelial and endothelial cells) in the FDC network. CCL21 is released from myofibroblast-like stromal cells and is closely associated with HEV formation (Figure 1).

5 Regulation of the immune microenvironment

5.1 Gene regulation

5.1.1 Genes

In the SG of pSS, upregulated genes are associated with lymphocyte chemotaxis, including IFN-induced chemokines such as CXCL10, and lymphocyte activation, such as TCR β-sites, which play a central role in T-cell activation. The MHC genes HLA-DR and HLA-DQ, which are related to antigen presentation, were also highly expressed in pSS. CXCL13 and CD3D genes were expressed in >90% of pSS patients and <10% of controls, confirming lymphocyte chemotaxis and activation in the SG of pSS patients. Lymphocyte-β (LTβ) is involved in ELS formation in inflammatory tissues and is one of the top 50 differentially expressed genes (DEGs) (111). Shimoyama et al., using single nucleotide polymorphism (SNP)-specific sequencing, found that the risk allele of human salivary gland GTF2I SNPs increased GTF2I expression and enhanced nuclear factor κB (NFκB) activation in human salivary gland cells *via* the NFκB P65 subunit (112). Inamo et al. used microarray technology to detect peripheral blood B cells of pSS patients and healthy controls and identified LINC00487 and SOX4 as key genes of B-cell disorder in pSS patients by the WGCNA algorithm (113). Many type I IFN genes associated with the response to viral infection were found in the first 200 genes with increased expression in pSS (114). These include IFNα-inducible protein 27, 9-27 (IFITM1), IFN stimulates gene 12 (ISG12), GBP2, and IFN regulator 8 (IRF8). Furthermore, the EBV-induced ligand (CCL19) and its receptor CCR7 genes were upregulated in pSS SGs. These two genes are involved in the activation of B and T cells.

In addition to infection, oxidative stress is an important cause of Sjögren's syndrome, and reactive oxygen species (ROS) in oxidative stress mainly come from mitochondria. Mitochondria are organelles necessary to maintain homeostasis in cells, and their function is maintained by dynamic fine-tuning. Damaged mitochondria produce more ROS than healthy mitochondria. Changes in mitochondrial-endoplasmic reticulum contact sites (MERCs) can increase inflammatory signals and regulate stress responses and intracellular homeostasis, ultimately affecting cell fate (115). Damaged mitochondria produced more reactive oxygen species than healthy mitochondria, and the presence of mitochondria in pSS salivary gland cells resulted in severe ultrastructural changes (115). Recent scientific studies have shown that mitochondria-related differentially expressed genes

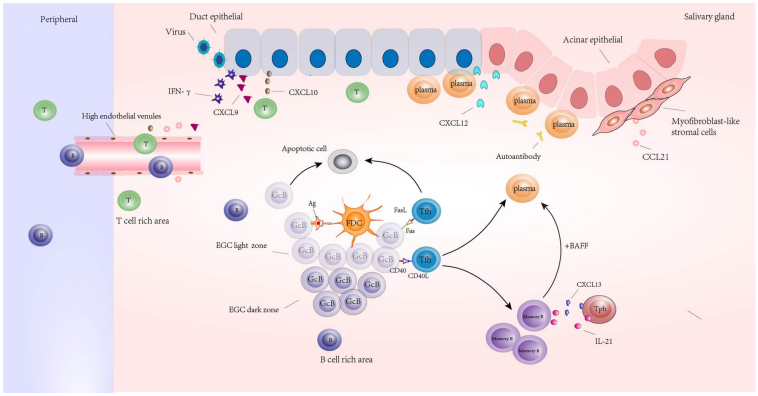

FIGURE 1
Ectopic lymphoid structures (ELSs) formed around the glands in the salivary glands of a patient with Sjogren's syndrome. Its core is the ectopic germinal center (EGC), including the bright and dark regions; Germinal center B cells (GcB) were screened by follicular dendritic cells (FDC) in the bright region. The GcB cells that did not receive antigen presentation were apoptotic, and the GcB cells that received antigen presentation were transformed into memory B cells and plasma cells under the assistance of follicular helper T cells (Tfh) or were regulated by Tfh cells to undergo apoptosis. Under the regulation of IL-21 and CXCL13 produced by Tph, memory B cells were transformed into plasma cells under the combined action of BAFF and other factors. Plasma cells infiltrated around ducts and acinar epithelial cells expressing CXCL12 and produced autoantibodies. There are B cell rich areas and T cell rich areas around EGC. High endothelial venules (HEVs) appear in the periphery of lymphoid aggregates in T cell-rich areas, and HEV formation is influenced by CCL21 produced by myofibroblast-like stromal cells; Under the stimulation of virus and other inducements, tissues expressed IFN-γ and induced the release of CXCL9 and CXCL10; CXCL9 and CXCL10 promote the accumulation of peripheral T and B lymphocytes that enter the gland through HEV to the site of inflammation.

(CD38, CMPK2, TBC1D9, and PYCR1) are closely related to the immune cell infiltration of salivary glands in pSS patients through real-time quantitative PCR (116).

5.1.2 Epigenetics

Moreover, epigenetics, which includes DNA methylation, noncoding RNA and histone modifications, are involved in the regulation of inflammatory signals in pSS.

5.1.2.1 DNA methylation

At present, there are many literature reports on DNA methylation and pSS (117). DNA methylation is catalyzed by DNA methyltransferase (DNMT) and refers to the presence of methyl radicals (methyl) in CpG dinucleotides from 5-methylcytosine (5-MC). A study on salivary gland methylation showed that the level of salivary gland epithelial cell methylation in pSS patients was lower than that in healthy individuals through DNA global methylation and real-time PCR detection (118). Coculture of human salivary gland cell lines established from irradiated tumor epithelial duct cells and Ramos human B-cell lines suggested that DNA demethylation is associated with lymphocyte infiltration, especially B-cell infiltration, and that the protein kinase C δ - extracellular signal-regulated kinase DNA methyltransferase 1 pathway may be involved in this phenomenon (118). However, DNA methylation is an epigenetic mechanism that includes the adjustment of gene expression and is heritable and reversible without modifying the DNA sequence. This provides another direction for the treatment of pSS.

5.1.2.2 MiRNAs

MicroRNAs (miRNAs) are small noncoding RNAs (sRNAs) that alter gene expression by binding to target messenger RNAs (mRNAs) and inhibiting translation. A researcher (119) conducted sRNA analysis based on global next-generation sequencing (NGS) for pSS labial salivary glands (LSGs) and sicca control groups, and the results suggested that 30% of sRNA in pSS LSG was miRNA, and the miRNA with the most significant change was HSA-Mir-181D-5p compared with the control group. The mRNA level of TNF-α, a direct target of HSA-miR-181D-5p, was significantly increased and negatively correlated with the presence of HSA-miR-181D-5p. Downregulation of HSA-miR-181D-5p in the LSG of SS patients may promote the adenoinflammatory environment by deregulating its direct target TNF-α.

5.1.2.3 Histones

The N-terminal tail of histones protrudes from the nucleosome and is subject to various covalent posttranslational modifications, including acetylation, methylation, phosphorylation, ADP ribosylation, protein conjugation, β-N-acetylglucosamination, deimination/citrullination and ubiquitination/sumoylation. A variety of enzymes are involved in histone modification, such as histone deacetylases (HDACs), histone acetyl transferases (HATs) and histone methyl

transferases (HMTs). It has been widely reported that HDACs are involved in immune reactions. HDAC4 negatively regulates the polarization of naive CD4+ T cells toward the Th17 phenotype (120), while HDAC6 induces IL-13 expression through AP-1, leading to the polarization of M2 macrophages (121). Sirtuin 2 (SIRT2), a member of the NAD+-dependent histone deacetylase family, promotes the deacetylation of p70S6K, activates the mTORC1/HIF-1α/RORγ T pathway, inhibits the production of IL-2 by CD4+ T cells, and promotes their differentiation into Th17 cells (122). Histone deacetylase inhibitors (HDACi) modulate the inhibitory T lymphocyte subsets of regulatory T cells (Tregs) and enhance FoxP3 acetylation, thereby protecting transcription factors from proteasome degradation. *In vitro* T-cell culture experiments in mice showed that HDACi reduced the proliferation of effector T cells and enhanced the inhibitory function of Tregs in coculture with effector T cells (123). HDAC6 inhibitors inhibit Th17-cell differentiation through the PKM2/STAT3 axis (124), while trastatin A (TSA), an HDAC inhibitor, inhibits dendritic cell maturation through downregulation of NF-κB (P65) (125). HDAC also plays an important role in B cells. Studies have shown that the development and survival of plasma cells depend on HDAC11, and the number of plasma cells in the peripheral blood of mice lacking HDAC11 is significantly reduced. In B cells lacking functional HDAC11, the differentiation of plasma cells *in vitro* is blocked, and HDAC11 is involved in the deacetylation of IRF4 at lysine 103 (126).

5.2 Signal transduction

5.2.1 BAFF

BAFF is a crucial cytokine of B cells, promoting B-cell maturation, proliferation, and survival. In pSS patients, salivary gland epithelial cells and innate immune cells can secrete BAFF (127, 128). Elevated LEVELS of BAFF were detected in salivary glands (127). Epithelial cells promote B-cell activation through BAFF, and BAFF may also promote epithelial cell survival through a member of the TNF receptor superfamily 13C (also known as the BAFF receptor) (129). BAFF is induced by type I and type II IFN (130). Therefore, TNFSF13B, encoding BAFF, can be considered the gene stimulated by IFN. IFN regulatory factors (IRFs) control the transcription of TNFSF13B: IRF1 and IRF2 are induction factors of TNFSF13B transcription, while IRF4 and IRF8 are inhibition factors (131). TNFSF13B transgenic mice overexpressing BAFF first developed a lupus-like phenotype and then acquired pSS characteristics, including reduced salivation. This finding supports the role of BAFF in promoting the pathogenesis of pSS.

5.2.2 EGF

The SG epithelium relies on a variety of signaling pathways to maintain homeostasis. One pathway is epithelial growth factor (EGF) signal transduction. For TLR signal transduction, EGF receptor (EGFR) activation has also been required for TLR3 signal transduction in the epithelial cell line and TLR-4-mediated downstream NFκB pathway activation, although in the cancer cell model system (132, 133). Conversely, TLR4 signaling also activates EGFR signaling, epithelial cell proliferation, and EGFR ligand expression (134).

5.2.3 NFκB

The NFκB family is a group of transcription factors that activate a range of inflammatory downstream targets when they translocate to the nucleus. The NFκB pathway has been well demonstrated to be active in pSS SGECs. Phosphorylated IKKϵ (pIKKϵ), pIκBα, and pNFκB were highly expressed in the ductal epithelium of small SGs in pSS patients (135). In pSS SGECs, the expression of the NFκB inhibitor IκBα was significantly lower than that in healthy controls (135, 136), and IκBα inhibited NFκB activity by masking nuclear localization signals. Stimulation of TLR2 receptors in SGECs induces IL-2 production through the NFκB pathway in pSS SGECs (137, 138). In the human SG cell line, IL-6 upregulation is regulated by a set of pathways, including NFκB (139). Knockout of the natural NFκB inhibitor A20 in K14+ epithelial cells (thereby activating the constitutive pathway) is sufficient to trigger the initial stages of pSS, including reduced saliva production and lymphocyte invasion of SGs (54).

5.2.4 EMT

The transdifferentiation of epithelial cells into motile mesenchymal cells, a process known as epithelial-mesenchymal transformation (EMT), is essential for development, wound healing, and stem cell behavior and contributes pathologically to fibrosis and cancer progression (140). In primary epithelial tumors, the interaction between cells and the extracellular matrix is reshaped during EMT, resulting in the separation of epithelial cells from each other and the underlying basement membrane and the formation of migratory mesenchymal cells that migrate to different sites with blood flow (141). In pSS, the EMT process mainly involves fibrosis of epithelial cells. During fibrosis, EMT responds to the triggering of TGF-β1, and TGF-β family receptors mediate intracellular signaling cascades that activate SMAD family members through SMAD 2/3 phosphorylation (142). Sisto et al. exposed cultures of healthy salivary gland epithelial cells (SGECs) from healthy donors to TGF-β1 treatment. Semiquantitative RT–PCR, quantitative real-time PCR and Western blot analysis were performed to compare the related gene and protein levels of Smad2/3/4, Snail, e-cadherin, Vimentin and type I collagen (143). They observed higher expression of SMAD2, 3, and 4 and Snail in TGF-β 1-exposed SGECs than in untreated healthy SGECs at both the genetic and protein levels. Snail is the transcriptional repressor and

promoter of EMT. Furthermore, compared with untreated SGEC, we found a significant decrease in the epithelial phenotypic marker e-cadherin and a significant increase in the mesenchymal phenotypic marker vimentin and type I collagen in the TGF-β 1-treated samples. This finding suggests that TGF-β1 induces EMT through the TGF-β1/SMAD/Snail signaling pathway, further confirming the existence of EMT in SGs. Concurrent use of the specific TGF-β1 inhibitor SB-431542 in healthy SGECs treated with TGF-β1 significantly reduced the fibrosis markers vimentin and type I collagen, while the epithelial marker e-cadherin returned to levels similar to those of untreated healthy SGECs. This further confirms that TGF-β1 plays an important role in EMT-dependent fibrosis. IL-17 and IL-22 play an important role in EMT. Through the study of salivary glands of pSS patients, researchers found that the expression of the epithelial marker E-cadherin was negatively correlated with the increase in tissue inflammation in pSS SG specimens, while the expression of mesenchymal vimentin and type I collagen was positively correlated. At the same time, they assessed the effect of IL-17 and IL-22 treatment on EMT-dependent SG fibrosis in primary human salivary gland epithelial cells (SGECs) isolated from healthy subjects. The results suggest that vimentin and type I collagen are upregulated after interleukin treatment, while e-cadherin expression is decreased, and the cooperation between IL-17 and IL-22 is required to induce EMT (144).

5.2.5 JAK/STAT

Recent results show that IFN-γ specifically inhibits the early steps of TGF-β-induced SMAD3 activation through the JAK/STAT pathway while inducing a rapid increase in SMAD7 expression (145, 146). SMAD7 binds to the TFG-β-receptor complex to inhibit TGF-β-mediated phosphorylation of SMAD3 and block TGF-β signaling (35), promoting SG precursor cell differentiation and saliva production (147). Pringle et al. recently demonstrated that SG progenitor cells respond to proinflammatory cytokines through proliferation and apparent cell death, likely through the JAK/STAT signaling pathway, suggesting that JAK/STAT signaling pathway inhibitors may interfere with SG epithelial homeostasis (35).

5.2.6 IFN

Microarray and real-time quantitative polymerase chain reaction (RT-qPCR) studies showed that IFN-stimulating genes were significantly upregulated in small salivary glands (MSG) in pSS patients compared with healthy controls (148, 149). Studies have shown that specific type I IFN-associated transcripts (IFIT-3) and type II IFN-associated transcripts (GFP-2) are expressed in MSGs, and IFIT-3 is mainly located in the duct epithelial cells of salivary glands. Gbp-2 is simultaneously located in ductal epithelial cells in lymphocyte aggregates and inflammatory cell infiltrates (150). Animal experiments demonstrated type I IFN dependence on SS development in female NOD mice and elevated pDC (the main producer of type I IFN) TYPE I IFN in their submandibular gland (SMG). After injection of pDCs consuming anti-BST2/CD317 antibodies into female NOD mice aged 4 to 7 weeks, the lack of pDCs hindered the development of SMG inflammation and secretion dysfunction and significantly reduced the number of TYPE I IFN mRNA, white blood cell count, and T and B lymphocytes in the SMG. The expression of IL-7, BAFF, TNF-α, IFN-γ, CXCL9, CXCL11, CD40, CD40 L, Lt-α, Lt-β and NOS2 decreased (92). A study confirmed that overexpression of both type I and type II interferon-induced genes (IFIG) was simultaneously observed in peripheral blood and MSG tissues of patients with pSS (13). Recent studies have suggested that type III IFN (also known as IFN-λ) may be involved in the pathogenesis of pSS. Epithelial IFN-λ2/IL-28a expression was increased in the MSGs of pSS patients compared with non-PSS controls (151). These results suggest the role of the IFN pathway in the pathogenesis of pSS.

5.2.7 LAMP3/HSP70/BMP6

BMP6 is a central cytokine that induces pSS-related secretion dysfunction. BMP6 can inhibit the water permeability of the salivary gland epithelial cell membrane by downregulating aquaporin 5 (AQP5), while local overexpression of BMP6 in the salivary gland or lacrimal gland can lead to loss of body fluid secretion in mice (152). HSP70 is an endogenous natural TLR4 ligand (TLR4 is an upstream regulator of BMP6) that stimulates BMP6 expression in pSS. The release of HSP70 from salivary epithelial cells may be triggered by overexpression of lysosome-associated membrane protein 3 (LAMP3). RT–PCR of small salivary gland RNA in pSS patients confirmed a positive correlation between BMP6 and LAMP3 expression. However, LAMP3 overexpression can induce BMP6 expression and a pSS phenotype in murine monocytes. The newly discovered LAMP3/HSP70/BMP6 axis provides an etiological model for SS gland dysfunction and autoimmunity (153).

5.2.8 Pro-resolving mechanism

Salivary gland inflammation in pSS is generally triggered by viral and bacterial infections in susceptible individuals, leading to initial tissue loss. Neutrophils and M2 macrophages clear the site of injury or infection when the decomposition mechanism works appropriately (154). However, when this mechanism is abnormal, dead cells are not cleared in time, leading to the formation of their antigens, increased levels of cytokines and chemokines, and lymphocyte infiltration (155). Specific pro-resolving mediators (SPMs, including liposomes, resolvins, marisins, and protectin) and their aspirin-triggered (AT) forms act as inflammatory mediators, promoting tissue regeneration by limiting uncontrolled inflammation while promoting its termination (156). Odusanwo et al. found that

the RvD1 receptor ALX/FPR2 was present in fresh, isolated salivary gland cells and salivary-derived cell lines of 16-week-old C57BL/6 mice in animal experiments. RvD1 receptor activation eliminates tight junctions and cytoskeletal disruption caused by TNF-α by modulating the phosphatidylinositol 3-kinase (PI3K)/AkT signaling pathway, enhances the migration and polarity of salivary epithelial cells, and promotes inflammation regression and tissue repair in salivary epithelial cells (157). Parashar et al. showed that the gene expression of enzymes involved in SPM biosynthesis was changed in the submandibular glands of NOD/ShiLtJ female mice, in which 5-LOX and 12/15-LOX were downregulated and upregulated, respectively. Specific predecomposition mediator (SPM) lysosomal D1 (RvD1) promotes the breakdown of salivary gland inflammation, and mice lacking the RvD1 receptor ALX/FPR2 exhibit congenital and adaptive immune deficiencies in salivary glands. Female ALX/FPR2 KO mice showed increased autoantibody production and loss of salivary gland function with age. This suggests that underlying SPM maladjustment may lead to SS progression (158).

6 The role of key inflammatory factors

6.1 TNF

In the salivary glands of patients with Sjogren's syndrome, upregulated TNF-α induces apoptosis of epithelial cells and disrupts barrier function controlled by tight junction proteins such as the Claudin superfamily, resulting in reduced salivary secretion and gland atrophy (159). TNF-induced apoptosis occurs through the binding of TNF type I receptors (TNFR1), which contain death domains that transmit apoptotic signals through caspase activation (160). In addition, TNFα significantly increased the levels of caspase 3, 8, 9 and cytochrome C, leading to a decrease in the level of Bcl-2 and induced apoptosis of SMG-C6 cells and human SMG tissues (161). Caspase 3 is considered the most important executor of apoptosis, and caspase 8 initiates the death receptor pathway of apoptosis. Caspase 9 is a key player in the mitochondrial pathway and is involved in various stimuli. Cytochrome C is released from damaged mitochondria and plays a key role in inducing apoptosis. miRNAs regulate the expression of target genes at the posttranscriptional level, and many miRNAs are involved in the regulation of apoptosis. To determine the role of these miRNAs in TNFα -induced acinar cell apoptosis, real-time PCR was used to measure their expression levels after cells were incubated with TNFα for a specified period of time. The results showed that TNFα could induce significantly increased levels of Mir-34a-5p, Mir-34a-3p, Mir-200b-5p and Mir-200b-3p simultaneously, while leT-7a-5P expression remained unchanged (161).

6.2 Interleukin

Interleukin-2 (IL-2) and high-affinity IL-2 receptor (IL-2R) are essential for the survival of regulatory T cells (Tregs), which are major players in immune tolerance and prevention of autoimmune diseases. Elevated IL-2R levels were found to be positively correlated with SS severity, as reflected by pathologically low salivary flow. Due to the impaired IL-2/IL-2R signaling ability in pSS patients, the immunosuppressive function of Tregs in SS patients was weakened, which may induce salivary gland infiltration of lymphocytes and induce and aggravate pSS (162).

Recent studies have found that interleukin-6 is significantly higher in pSS patients than in HCs patients and is associated with mononuclear cell infiltration in salivary gland tissues in these patients (163). Salivary gland epithelial cells are the primary cellular source of increased IL-6 secretion in these patients. In addition, IL-6 can induce the transformation of SGECs from morphological and phenotypic to mesenchymal phenotypes in a dose-dependent manner. Recent studies have shown that IL-6-treated SGECs have decreased e-cadherin expression and increased vimentin and type I collagen expression compared to control cells. The results confirmed that IL-6 dysregulation may lead to EMT-dependent fibrosis (164).

IL-7 is a 25 kDa soluble globular protein produced and secreted by nonhematopoietic cells such as stromal cells, epithelial cells and endothelial cells. IL-7R is widely expressed in T and B cells, and IL-7/IL7R signaling is critical for the development and maintenance of the entire lymphoid compartment. *In vitro* experiments have shown that IL-7 induces the production of Th1- and Th2-related cytokines, including IFNγ, monocytes induced by IFNγ (MIG), IFNγ-inducible 10-KD protein (IP-10) and IL-4 (165). Another *in vitro* cell study showed that IL-7 stimulation induced higher IFN-γ, IL-4, IL-17 and IL-21 production in CCR9+ Th cells and CXCR5+ Th cells (166).

Katsifis et al. showed that the expression of IL-17 protein in salivary glands increased gradually with increasing biopsy lesion score. Transforming growth factor β, IL-6, and IL-23 are essential promoters of Th17 differentiation and are abundant compared to the amounts in control tissues (167). Animal experiments showed that IL-17 inhibited acetylcholine-induced calcium migration and downregulated transient receptor type 1 expression in SG epithelial cells by promoting Nfkbiz mRNA stabilization. In addition, local IL-17 neutralization in SGs significantly reduced salivation and improved tissue

inflammation in mice (168). These results suggest that IL-17 may lead to salivary gland dysfunction in Sjogren's syndrome by inhibiting TRPC1-mediated calcium movement.

IL-21, a member of the recently discovered type I cytokine family, is mainly secreted by Tfh and Tph cells. IL-21R is expressed in B cells and activated CD4+ T cells. IL-21 costimulates B cells with BCR to promote their differentiation into plasma cells, which is also necessary for the formation of normal germinal centers (GCs). The addition of IL-21 to the coculture system blocked 90% of B cells from differentiating into plasma cells. Animal experiments showed that IL-21R knockout mice completely eliminated spontaneous accumulation of GC B cells and plasma cells in blood (169). In addition, IL-21R is required for T_{hef} cell development. In general, IL-21R signaling is necessary for spontaneous accumulation of B and T-cell effector populations.

7 Treatment

As a target organ most frequently involved in pSS patients, the impairment of salivary gland function can lead to an imbalance in oral microecology and severe discomfort for patients. An increasing number of treatments are available with further research on the pathogenesis of pSS. In the following sections, we will review the treatment of pSS from the perspective of the immune microenvironment.

7.1 Resistance to hypoxia

As mentioned above, the salivary glands of pSS patients are chronically hypoxic, and blocking hypoxia development may be a potential treatment option. DMOG and FG-4497 hypoxic stabilizers have shown promising results in inflammatory bowel disease (IBD), reducing inflammation, reducing intestinal epithelial cell apoptosis, and enhancing intestinal barrier function (170–172). These may be potential drugs for improving salivary gland hypoxia. Moreover, drugs that inhibit PGE2, such as nonsteroidal drugs, may be equally effective in improving salivary gland function because of their role in hypoxia. In *in vitro* culture of human tubular HK-2 cells, cell death was mediated by COX-2-dependent PGE2 production, and the COX-2 inhibitor cefoxib prevented hypoxia-induced cell death (173). In another study of human retinal pigment epithelium, celecoxib also showed inhibition of HIF-1α under hypoxia (174)

7.2 Senolytics

Senescence makes salivary gland progenitor cells lose their ability to increase their value and differentiate, and the damaged salivary gland does not have enough regeneration potential to fully restore its function. Therefore, it is beneficial for pSS SGs to consume senescent cells and prevent the spread of senescence. Senolytics are a group of drugs that selectively eliminate senescent cells (175). In addition, pro-aging agents (such as navitoclax, dasatinib, and quercetin) work by inhibiting pro-survival pathways (such as Bcl-2 and Bcl-XL) to promote senescent cell death, thereby rejuvenating glandular cells and restoring glandular function (176). Selective removal of p16Ink4a-positive cells by ganciclovir or the antiaging drug ABT263 can eliminate senescent cells and improve the self-renewal ability of stem cells, thereby improving salivary gland function (177). Repressing cellular senescence contributes to the rescue of IR-induced hyposalivation by transient activation of Hh signaling, which is related to enhanced DNA repair and decreased oxidative stress in SMGs (178). Agonists of the Hh signaling pathway may be new targets for treating dryness. In addition, in an *in vitro* culture experiment of SGSCs after passage culture, the ROCK inhibitor Y-27632 inhibited the expression of senescence-related proteins and promoted cell proliferation (179). Another study showed that in C57BL/6 mice, loss of salivary function is closely related to cellular senescence, and radiation-induced loss of salivary gland function is dependent on IL-6, but IL-6 preconditioning can also prevent senescence and salivary gland hypofunction by enhancing DNA damage repair mechanisms (180). This suggests that IL-6 may play a dual role in Sjogren's syndrome. A 6-month multicenter, double-blind, randomized placebo-controlled trial showed no improvement in systemic involvement and symptoms with toclizumab compared with placebo in patients with pSS (181). Currently, only toclizumab has been reported in the treatment of pSS with myelitis or refractory interstitial pneumonia (182, 183). However, the application of IL-6 in Sjogren's syndrome remains to be explored.

7.3 Anti-inflammatory drugs

7.3.1 Treatment of B cells and related factors

As a novel small molecule immunomodulator, iguratimod was confirmed to inhibit B cells by reducing immunoglobulin production and various inflammatory cytokines, including IL-1, IL-6, IL-8, and TNF (184). Clinical studies have validated that iguratimod improved some dryness symptoms and disease activity in pSS patients, reducing BAFF and the percentage of plasma cells over 24 weeks. It can also inhibit PGE2 production by selectively inhibiting COX-2 and the NFκB pathway (185). In animal studies, iguratimod improved inflammatory infiltration of the submandibular gland in mice (186).

Rituximab (RTX) is a monoclonal antibody that targets CD20 on B cells. CD20 is involved in the regulation of B lymphocyte growth after activation. In their open study using

RTX, Carubbi et al. (179, 187) found that RTX treatment reversed specific focal lymphocytic sialoadenitis into a nonspecific chronic sialoadenitis mode by depleting B cells, resulting in complete recovery of small salivary gland structure in patients with residual SG function. However, other studies suggested that RTX anti-CD20 treatment might not deplete B-cell infiltration of pSS MALT sites (188). Gong et al. demonstrated in a mouse model that the local production of BAFF is a key local factor in MALt-mediated anti-RTX-depleting B cells (189). B-cell depletion can be achieved only when anti-BAFF is combined with anti-CD20.

Belimumab inhibits soluble BAFF. A one-year open-label trial on belimumab showed that the reduction in B-cell activation biomarkers observed at week 28 continued to week 52, but there was no change in salivary flow, Schirmer test, or salivary biopsy lesion scores (190). Immunobiological evidence supports a sequential regimen of RTX prebelimumab administration designed to target microenvironment BAFF first to improve the success rate of subsequent rituximab depletion therapy in MALT pathological tissues (189). Ianalumab is a monoclonal antibody that consumes B cells and blocks the B-cell activator receptor. In a double-blind, placebo-controlled phase II single-center study, ianalumab (VAY736) resulted in rapid and sustained B-cell depletion and improved ESSDAI and ESSPRI scores, but the variability in salivation flow rate was high enough to make any comparison difficult (191)

In pSS mouse models, labial gland mesenchymal stem cell-derived Exos (LGMSC-EXOS) reduced inflammatory infiltration and restored salivary secretion in salivary glands (192). LGMSC EXO-derived microRNA-125B affects the plasma cells of pSS by directly binding to its target gene, PRDM1 (PR domain zinc finger protein 1, also known as BLIMP1), which may be developed as a target gene for the treatment of pSS.

7.3.2 Treatment of T cells and related factors

Cyclosporine A inhibits the IL-2 activity of T cells by interfering with calcineurin required for IL-2 gene transcription (193, 194). Hydroxychloroquine (HCQ) reduces the production of type I IFN and blocks the activation of TLR7 and TLR9 receptors (195), thereby interfering with antigen processing and blocking T-cell activation (196). However, in randomized, double-blind controlled trials in patients with pSS, HCQ did not improve disease symptoms despite inhibiting type I IFN-induced gene expression (188). The effect of HCQ alone on improving glandular function remains controversial.

Abatacept (CTLA4-Ig) binds to the costimulatory molecule CD80/CD86 and blocks the binding of these molecules to CD28 on T cells (197). A recent 48-week trial of abatacept in patients with pSS showed significant improvement in clinical and dry eye symptoms but not in stimulated whole salivary flow (198). Studies have shown a reduction in GCs in lymphocytic lesions and SG lip biopsies after abatacept treatment (199, 200), but salivary and lacrimal gland function remained stable (201).

In a recent clinical trial, prezalumab (a nondepleting monoclonal antibody against ICOSL) had a significant biological effect on SG inflammation, with a significant reduction in the number of CD4+ICOS+Tfh-like cells compared with placebo, despite the failure of the primary endpoint. demonstrated the biological efficacy of targeting the ICOS/ICOS-L pathway in pSS (202).

Other researchers have mitigated pSS by blocking MHC class II IAg7 antigen presentation in NOD mice to prevent pathogenic T cells from recognizing their antigens. The results showed that tetraazatricyclo-dodecane (TATD) and 8-azaguanine (8-AZA) alleviated symptoms by improving saliva and lacrimal gland secretion, reducing autoantibody levels, and reducing the severity of lymphocyte infiltration in saliva and lacrimal glands (203).

7.3.3 Other anti-inflammatory drugs

Glucocorticoids are a widely used drug for chronic inflammatory autoimmune diseases. They bind to glucocorticoid receptors, resulting in increased transcription of anti-inflammatory genes, such as IL-10, and anti-inflammatory proteins that inhibit the expression of inflammatory genes. Studies have shown that glucocorticoid administration for 6 weeks improves saliva flow in patients but generally does not improve histological or functional parameters of SGs (204). However, a four-year long-term prospective study showed the opposite result: early pSS is characterized by a decline in salivary gland function, with or without steroid use, and a further decline in salivary gland function over time. Reduced salivary gland flow was not associated with corticosteroid use (205).

Leflunomide (LEF) inhibits pyrimidine biosynthesis and decreases naive and memory CD4+ T-cell and B-cell proliferation and NFκB activation (206, 207). In a phase II clinical trial involving 15 patients with early active PSS for 24 weeks, LEF treatment did not improve salivary flow (208). However, the combination of leflunomide and HCQ has been reported to increase salivary gland unstimulated significantly and stimulate total salivary production of pSS at certain time points (209, 210).

Drugs that treat pSS through the NF-κB signaling pathway, such as the novel synthetic DMARD drug iguratimod and the Syk signaling blocker GS-9876 (the Syk signaling pathway is upstream of IKK activation, and its blocking improves the release of NF-κB by its inhibitory complex), are currently in clinical trials. Their effect on the glands has yet to be tested.

In mouse models, Harim Tavares Dos Santos et al. found that hemolysin D1 (RvD1) and its aspirin-triggered AT-RVD1

effectively reduced inflammation and restored saliva flow before and after the onset of pSS. Resolvins are special proresolving mediators (SPMs) that can actively regulate inflammation. Furthermore, the expression of various SPM receptors (ALX/FPR2, BLT1, and CMKLR1) was found in human salivary glands, which may be a potential target for treating pSS patients (156).

CD40 is a transmembrane type I glycoprotein composed of 277 amino acids that belongs to the tumor necrosis factor (TNF) gene superfamily. The ligand CD40L/CD154 is a type II transmembrane protein and exists in a soluble (scd40L) or membrane-bound form. It is present on activated T cells, B cells, endothelial cells and epithelial cells (190). Compared with the control group, NOD mice treated with the CD40 DNA vaccine showed reduced lymphocyte infiltration and increased salivary secretion in salivary glands. At the same time, the expression levels of TNF-α and IL-6 in salivary glands decreased, the number of dendritic cells and plasma cells decreased, and the ANA level decreased (211). Iscalimab, an anti-CD40 antibody, has been shown to be safe and well tolerated at all doses in phase I clinical studies, with no clinically relevant changes in any of the safety parameters, including no evidence of thromboembolic events (212). However, its role in pSS patients remains to be further evaluated.

PSS patients have elevated levels of IL-7 and its receptor in salivary glands. Animal experiments showed that intraperitoneal injection of a blocking antibody against IL-7 receptor α chain (IL-7Rα) for 3 weeks in 10-week-old female NOD mice significantly improved characteristic SS pathology, including reduced salivary secretion and infiltration of leukocytes in the submandibular gland (SMG). Anti-IL-7r α treatment significantly reduced the amount of TNF-α in SMGs and increased the levels of Claudin-1 and aquaporin 5, two molecules essential for normal salivation (213). In phase I clinical trials of the monoclonal antibody GSK2618960 against interleukin-7 receptor α subunit (CD127), GSK2618960 was well tolerated and blocked IL-7 receptor signaling when fully targeted (214). This may be a new target for the future treatment of Sjogren's syndrome.

Conclusion

Oral salivary gland reduction is one of the most common clinical manifestations of pSS, a disease that directly affects exocrine function. The onset of the disease is genetically susceptible in cells, viruses, and other environmental factors under stimulation through chronic hypoxia, cell senescence, local inflammation, and the production of autoantibodies and other pathways in salivary gland cells, so that their function is impaired. This paper systematically reviews the characteristics and regulatory pathways of the salivary gland microenvironment, hoping that more targeted treatments can be developed to restore gland function and improve dry mouth symptoms through an in-depth understanding of the local immune microenvironment.

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.

Author contributions

ZT wrote the manuscript. LW and XL participated in the modification. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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The commonness in immune infiltration of rheumatoid arthritis and atherosclerosis: Screening for central targets *via* microarray data analysis

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Background: Although increasing evidence has reported an increased risk of atherosclerosis (AS) in rheumatoid arthritis (RA), the communal molecular mechanism of this phenomenon is still far from being fully elucidated. Hence, this article aimed to explore the pathogenesis of RA complicated with AS.

Methods: Based on the strict inclusion/exclusion criteria, four gene datasets were downloaded from the Gene Expression Omnibus (GEO) database. After identifying the communal differentially expressed genes (DEGs) and hub genes, comprehensive bioinformatics analysis, including functional annotation, co-expression analysis, expression validation, drug-gene prediction, and TF-mRNA-miRNA regulatory network construction, was conducted. Moreover, the immune infiltration of RA and AS was analyzed and compared based on the CIBERSORT algorithm, and the correlation between hub genes and infiltrating immune cells was evaluated in RA and AS respectively.

Results: A total of 54 upregulated and 12 downregulated communal DEGs were screened between GSE100927 and GSE55457, and functional analysis of these genes indicated that the potential pathogenesis lies in immune terms. After the protein-protein interaction (PPI) network construction, a total of six hub genes (*CCR5, CCR7, IL7R, PTPRC, CD2*, and *CD3D*) were determined as hub genes, and the subsequent comprehensive bioinformatics analysis of the hub genes re-emphasized the importance of the immune system in RA and AS. Additionally, three overlapping infiltrating immune cells were found between RA and AS based on the CIBERSORT algorithm, including upregulated memory B cells, follicular helper T cells and γδT cells.

Conclusions: Our study uncover the communal central genes and commonness in immune infiltration between RA and AS, and the analysis of

six hub genes and three immune cells profile might provide new insights into potential pathogenesis therapeutic direction of RA complicated with AS.

KEYWORDS

atherosclerosis, rheumatoid arthritis, immune infiltration, hub genes, memory B cells, follicular helper T cells, γδT cells

Introduction

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disorder characterized by articular and extra-articular involvement, with a global prevalence of 0.24% (1, 2). RA patients were reported to have higher cardiovascular morbidity and mortality (3, 4). Atherosclerosis (AS) is defined as the formation of fibrofatty lesions in the artery wall, commonly found in the cardiovascular and cerebrovascular systems, that has been the major cause of disability and death worldwide (5, 6). Clinic evidence has shown that patients with RA had a 68% increased risk of myocardial infarction (MI), and the risk of developing silent MI was twofold higher in RA patients compared with normal (7, 8). Even in groups without a history of coronary heart disease, patients with RA had more coronary plaques and were more likely to form vulnerable plaques (7, 9, 10). The association between the two diseases has not been only reported from a clinical perspective. Nearly 10 years of research have also found that RA and AS have similar pathological processes and shared risk factors (11, 12), the most pivotal among which are chronic inflammation and immune activation (13, 14). Innate and adaptive immune system activation promotes higher cumulative inflammation that involves cytokines, immune cells, and non-immune cells, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) production, T and B cells activation, and the increase in epithelial cells, fibroblasts, and smooth muscle cell, etc. (11, 15, 16). In addition, chronic inflammation not only increases the traditional risk factors but also interacts with their mechanisms (11). The above-complicated biological process leads to endothelial dysfunction, arterial stiffness, and atherosclerotic plaque formation and progression, ultimately accelerating the atherogenic process in RA (12, 13, 17, 18). However, the exact biological pathway and molecular mechanism behind the above-mentioned biological process between RA and AS is still far from being elucidated. Meanwhile, some biological medications, such as anti-TNF medications and IL-1 inhibitors, have been reported to improve vascular function and reduce the risk of MI by easing the inflammatory burden to some extent (9, 19, 20). Hence, there is an urgent need to uncover more and exact biological targets in patients with RA to prevent AS occurrence and development.

Microarray technology is an effective analysis that is widely adopted to compare genes that are differentially expressed in biological models or patients under different diseases state (21, 22). Microarray technology is also used to better understanding gene association, mapping, expression, and linkage studies. At present, some articles have analyzed the RA or AS datasets from a single disease perspective, and dig out the potential targets and regulatory biological pathway of RA or AS respectively (21, 23). These studies provide new insights and research directions for single diseases of RA or AS, yet there is still a lack of studies to systematically explore the commonality of RA and AS and provide exact research directions. The common transcription signatures may indicate new insights into the common pathogenesis and immune mechanism of RA and AS. Hence, the purpose of this research was to uncover the biological mechanism and relevant immune pathway of RA complicated with AS, and more significantly, to explore the potential biomarkers and therapeutic directions of the two diseases. In this study, GSE55457 and GSE100927 were downloaded from the Gene Expression Omnibus (GEO) database to identify the communal differentially expressed genes (DEGs) between RA and AS. Subsequently, we construct the protein-protein interaction (PPI) network of communal DEGs to determine hub genes *via* STRING database and Cytoscape software. Comprehensive bioinformatics analysis, including functional annotation, co-expression analysis, expression validation, drug-gene prediction, and TF-mRNA-miRNA regulatory network construction, was performed to uncover the biological characteristic of hub genes. Additionally, we analyzed and compared the immune infiltration of RA and AS based on the CIBERSORT algorithm and evaluated the correlation between hub genes and infiltrating immune cells. Finally, a total of 66 communal DEGs, six hub genes, and three infiltrating immune cells that might indicate new insights into the relevant immune mechanisms and therapeutic directions of RA complicated with AS, were identified.

Materials and methods

Data source

GEO (http://www.ncbi.nlm.nih.gov/geo) is a microarray and high-throughput sequencing database created by NCBI (24). RA and AS were used as keywords to screen for qualified gene datasets based on strict inclusion/exclusion criteria. The inclusion criteria were as follows: (1) Sporadic RA or AS; (2) datasets containing patients and healthy controls and including the largest possible sample size; (3) the test specimens in datasets derived from human tissues. Exclusion criterium was: patients who participated in a clinical trial for drugs or other treatments. After filtering and comparing, four gene datasets were chosen from GEO based on strict inclusion/exclusion criteria. GSE55457 (13 RA patients and 10 controls) and GSE55235 (10 RA patients and 10 controls) were screened as RA datasets (25). Meanwhile, in the AS group, GSE100927 (69 AS human tissues and 35 controls) and GSE28829 (13 early atherosclerotic plaques and 16 advanced atherosclerotic plaques) were selected from GEO (26, 27). GSE55457 and GSE100927 were used to screen DEGs and perform Immune Infiltration analysis, while GSE55235 and GSE28829 were used for hub gene expression validation.

Identification and enrichment analyses of overlapped DEGs

GEO2R (www.ncbi.nlm.nih.gov/geo/ge2r) is a network tool that works *via* the Limma package and GEOquery (28). DEGs of RA and AS were respectively identified using GEO2R with the condition of P-value < 0.05 and |logFC| > 1. After that, the overlap DEGs between RA and AS were detected and visualized by constructing VENN diagrams.

Based on the DAVID database (https://david.ncifcrf.gov/), Gene Ontology (GO) enrichment was conducted to analyze the biological characteristics of the overlap DEGs at the functional and molecular levels (29). The enrichment results are sorted separately by P-value and gene count in order to reveal the more meaningful biological processes. In addition, we performed pathway enrichment analysis of overlap DEGs from five pathway databases (KEGG PATHWAY, PID, BioCyc, Reactome and Panther) *via* network platform KOBAS 3.0 (30). P-value < 0.05 was considered statistically significant.

PPI network construction, hub genes selection and analyses

A PPI network could reveal protein interactions and unearth the core protein genes. Upon the condition of interaction combined score > 0.4, the PPI network of overlap DEGs was constructed based on the STRING database (http://string-db.org) and visualized using Cytoscape software (31, 32).

CytoHubba is a Cytoscape plugin that could calculate the core protein genes of the PPI network (33). There are up to 12 algorithms in CytoHubba, all of which are already proven to be effective in screening hub genes. Five of the 12 algorithms were selected randomly in CytoHubba to calculate the top 10 core genes and determine the hub genes by taking the intersection of five algorithms running outcomes.

Metascape (https://metascape.org) platform was adopted here to perform functional annotation analysis of hub genes, and further pathway enrichment was performed by KOBAS 3.0 (34) from five above-mentioned pathway databases. Subsequently, we constructed and analyzed the co-expression network of hub genes *via* the GeneMANIA (http://www.genemania.org/) platform which is a reliable network tool for analyzing gene list function and exploring internal associations (35). Additionally, we predicted the drug-gene pairs of hub genes using DGIdb 3.0 (http://www.dgidb.org) database based on the condition that a predicted drug was FDA-approved (36). After the prediction, the network map of drug-gene pairs was visualized *via* Cytoscape software.

Validation of hub gene expression

The expression of hub genes was verified in GSE55235 (10 RA patients and 10 controls) and GSE28829 (13 early atherosclerotic plaques and 16 advanced atherosclerotic plaques) respectively, and the comparison between the two sets of data was conducted with the T-test. A P value of < 0.05 was considered statistically significant.

Construction of the TF-mRNA–miRNA regulatory network

Mirwalk is a credible database basically focused on miRNA-target interactions (37). In this research, we predicted the miRNA of hub genes *via* the Mirwalk database under the strict condition that the predicted interactions could be verified by experiments or other databases. Transcriptional Regulatory Relationships Unraveled by Sentence-based Text mining (TRRUST) database consisting of the target genes corresponding to TFs and the regulatory relationships between TFs, was adopted to predict the TFs that regulate the hub genes (38). After the prediction, the regulatory network of TF-mRNA-miRNA was constructed and visualized using Cytoscape software.

Evaluation of immune cell infiltration and correlation analysis

On the ground of the CIBERSORT algorithm, we analyzed the relative percentage of 22 immune cell subpopulations in AS and control samples from the GSE100927. Besides, violin diagrams were generated to compare and visualize the difference in the immune cell between AS and control *via* the ggplot2 package. Consistent with the GSE100927, the percentage of immune cells and the difference between RA and control from GSE55457 were analyzed and visualized in the same way.

Person correlation analysis was carried out on hub genes and infiltrating immune cells by the ggstatsplot package, and the results were visualized by ggplot2 package.

Result

Identification and enrichment analyses of overlapped DEGs

The research design flow chart is shown in Figure 1. After standardizing the microarray results, DEGs of RA and AS were screened respectively. In the RA group, there were 1163 genes (447 upregulated and 716 downregulated genes) in GSE55457 identified as DEGs (Figure 2A). Meanwhile, 565 genes (418 upregulated and 147 downregulated genes) in GSE100927 were screened as DEGs in AS patients compared with controls (Figure 2B).After taking the intersection of the DEGs in GSE100927 and GSE55457, we obtained the communal DEGs (54 upregulated and 12 downregulated genes) between RA and AS (Figures 2C, D).

We performed GO enrichment analysis using the David database to unearth the biological characteristic of the communal DEGs, and the result was divided into three functional parts consisting of biological processes (BP), cell component (CC), and molecular function (MF) (Figures 3A, B). The enrichment results of the overlap DEGs are mainly ranked in terms of enrichment P-value in three functional parts respectively. In the BP category, DEGs were mainly enriched in immune response (GO:0006955), positive regulation of T cell proliferation (GO:0042102), chemotaxis (GO:0006935) and positive regulation of T cell activation (GO:0050870). Regarding the CC category, DEGs were significantly involved in external side of the plasma membrane (GO:0009897), immunological synapse (GO:0001772), cell surface (GO:0009986) and clathrin-coated endocytic vesicle membrane (GO: 0030669). As for the MF category, DEGs were significantly

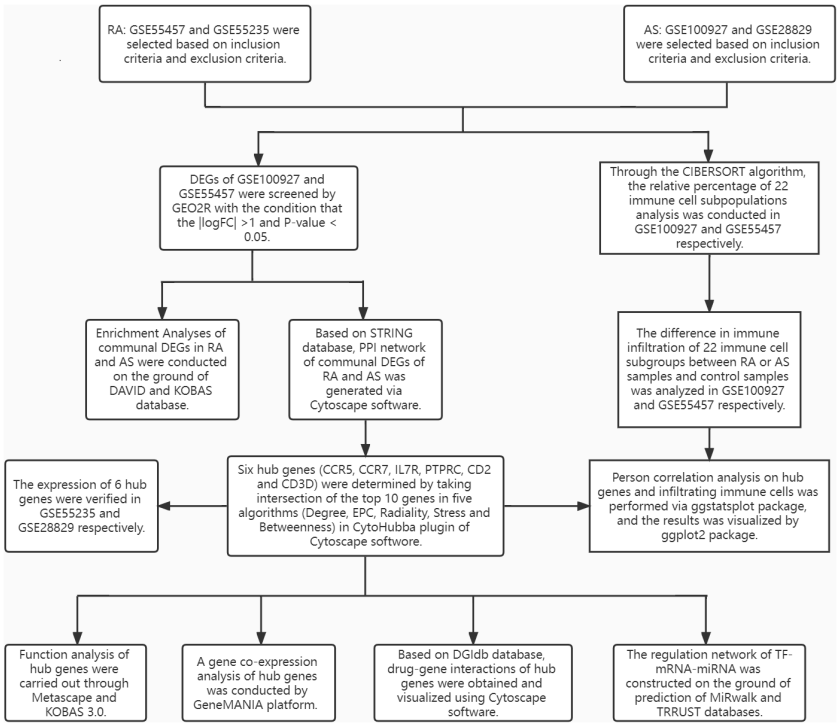

FIGURE 1
The research design flowchart.

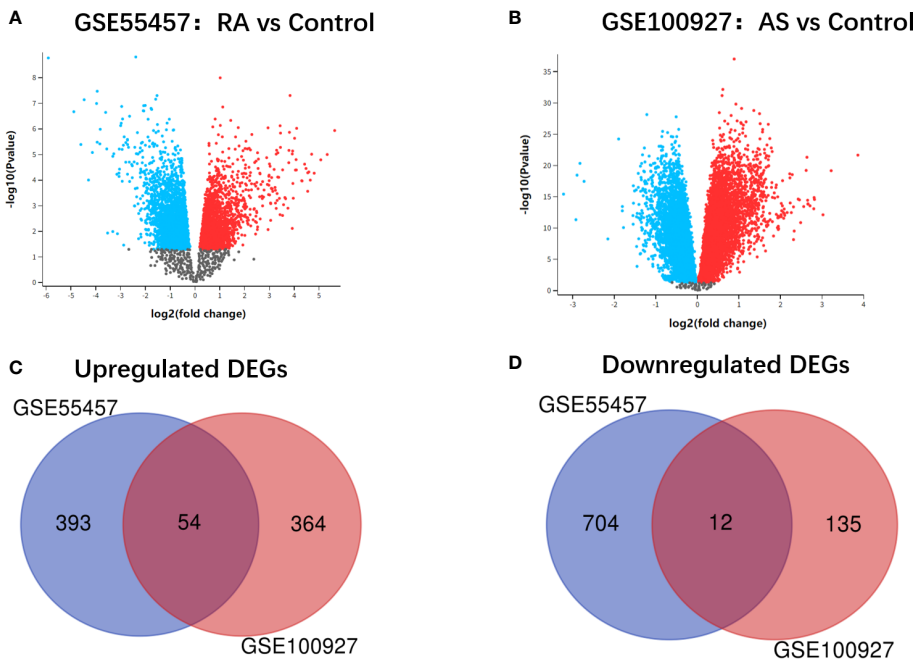

FIGURE 2
(A) Volcano plot of GSE55457. **(B)** Volcano plot of GSE100927. Upregulated genes are marked in red; downregulated genes are marked in bule. **(C)** Venn diagram of the 54 upregulated communal DEGs between RA and AS. **(D)** Venn diagram of the 12 downregulated communal DEGs between RA and AS.

involved in C-C chemokine receptor activity (GO:0016493), MHC class II protein complex binding (GO:0023026), CCR chemokine receptor binding (GO:0048020) and interleukin-7 receptor activity (GO: 0004917). Moreover, according to KOBAS 3.0, the pathway enrichment analysis of overlapped DEGs showed that the genes were mainly enriched in the Immune System, Cytokine Signaling in the Immune system, Cytokine-cytokine receptor interaction(Figure 3C).

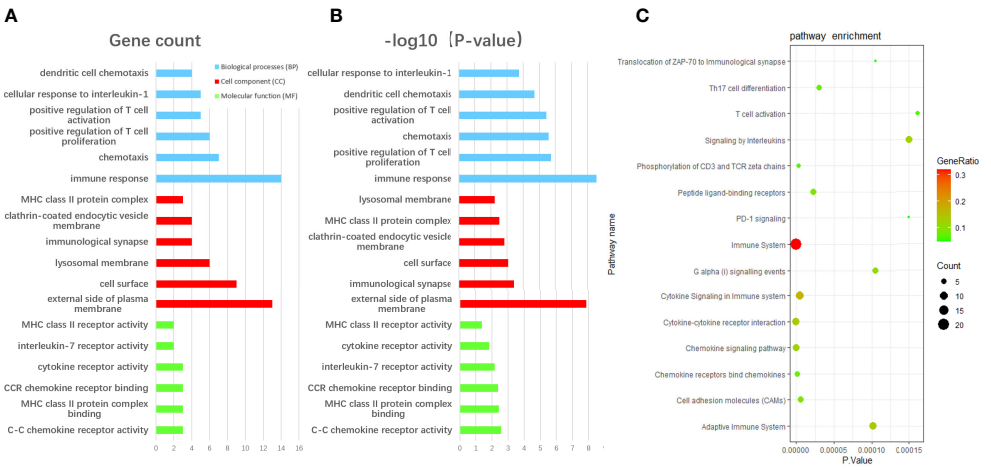

FIGURE 3
The enrichment analysis of communal DEGs between RA and AS. **(A)** The GO enrichment analyses of communal DEGs with gene count and **(B)** P-value based on the DAVID database. **(C)** The pathway enrichment analysis of communal DEGs on the ground of KOBAS 3.0. P-value < 0.05 was considered significant.

PPI network construction and hub gene selection

On the ground of the STRING database, the PPI network of overlap DEGs was constructed using Cytoscape with the condition of combined scores of >0.4 points, consisting of 45 nodes and 162 edges (Figure 4). After that, five algorithms (Degree, EPC, Radiality, Stress, and Betweenness) in the CytoHubba plugin were selected to identify hub genes. The top 10 genes calculated by the five above-mentioned algorithms are listed in Table 1. Finally, by taking the intersection of the top 10 genes in five algorithms, six central genes (*CCR5, CCR7, IL7R, PTPRC, CD2*, and *CD3D*) were determined as hub genes (Figure 5). All six hub genes were upregulated genes.

Hub gene analyses and validation

We conducted the functional annotation analysis of hub genes *via* Metascape to better uncover the biological characteristics and mechanism of the six hub genes (Figure 6A). Hub genes were mainly involved in T cell activation (GO:0042110) and thymic T cell selection (GO:0045061). Besides, further pathway enrichment analysis was performed by KOBAS 3.0, and hub genes were significantly involved in the Immune System, Primary immunodeficiency, and Cytokine-Cytokine receptor interaction (Figure 6B). Similar to the analysis outcome of overlap DEGs, the gene annotation analysis revealed that hub genes were associated with the process of the immune system reacting. Subsequently, the network of the hub genes and their co-expression genes were generated on the ground of the GeneMANIA platform (Figure 6C). Six hub genes showed the complex PPI network with the Co-expression of 69.90%, Physical interactions of 10.20%, Pathway of 9.28%, Co-localization of 5.83%, Shared protein domains of 3.22% and Predicted of 1.57%. As expected, the biological function and roles of the hub genes re-emphasized the importance of the immune system in RA and AS. In addition, on the grounds of the DGIdb database, 15 drug-gene pairs were obtained with the condition that the drug should be FDA-approved, including 4 hub genes (PTPRC, CD3D, CCR5 and CD2) and 15 drugs. The categories of predicted drug are varied and all are FDA-approved drugs. Among them, there were a total of 10 potential drugs that

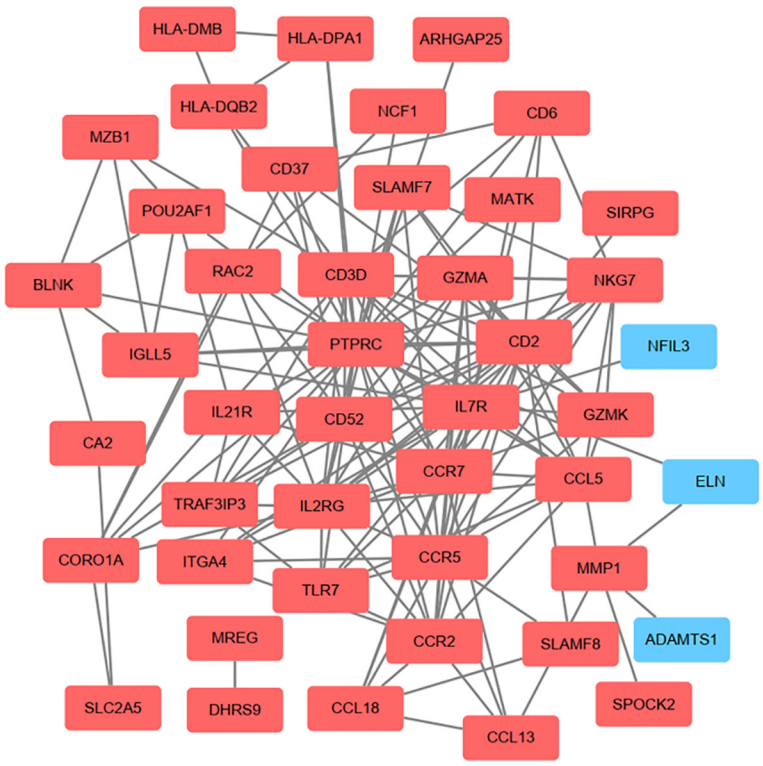

FIGURE 4
On the ground of STRING database, PPI networks of the communal DEGs were constructed by Cytoscape software. The red round rectangle represents upregulated genes and the blue round rectangle represents downregulated genes.

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TABLE 1 The top 10 genes in 5 algorithms.

Degree	EPC	Radiality	Stress	Betweenness
PTPRC	PTPRC	PTPRC	PTPRC	PTPRC
CD2	CD2	CD2	CD2	CD2
IL7R	IL2RG	IL7R	CCL5	MMP1
CCR5	IL7R	CD3D	IL7R	CCL5
CD3D	CCR5	IL2RG	CD3D	IL7R
IL2RG	CCR7	CCR7	MMP1	CD3D
CCR7	GZMA	CCR5	CCR5	CORO1A
CD52	CD3D	CCL5	CCR7	BLNK
CCL5	CD52	GZMA	IL2RG	CCR5
GZMA	NKG7	CD52	CORO1A	CCR7

could have effect on PTPRC, yet no drug was found to interact with multiple genes at the same time. These outcomes might reveal clues of potential therapeutic direction (Figure 7).

In order to ensure the reliability and accuracy of bioinformatics analysis results, GSE55235 and GSE28829 were adopted to verify the expression of hub genes in RA and AS samples by independence testing analysis respectively (Figure 8). Encouragingly, compared with controls, all 6 hub genes were significantly upregulated in AS and RA samples.

Construction of the TF-mRNA–miRNA regulatory network

With the strict condition predicting that miRNA of hub genes could be verified by experiments or other databases, a total of 162 miRNAs were screened based on the MiRwalk database. Meanwhile, on the ground of the TRRUST database predictions of six hub genes, up to 13 TFs that could regulate hub genes were obtained. Then, based on the outcome of the prediction, the

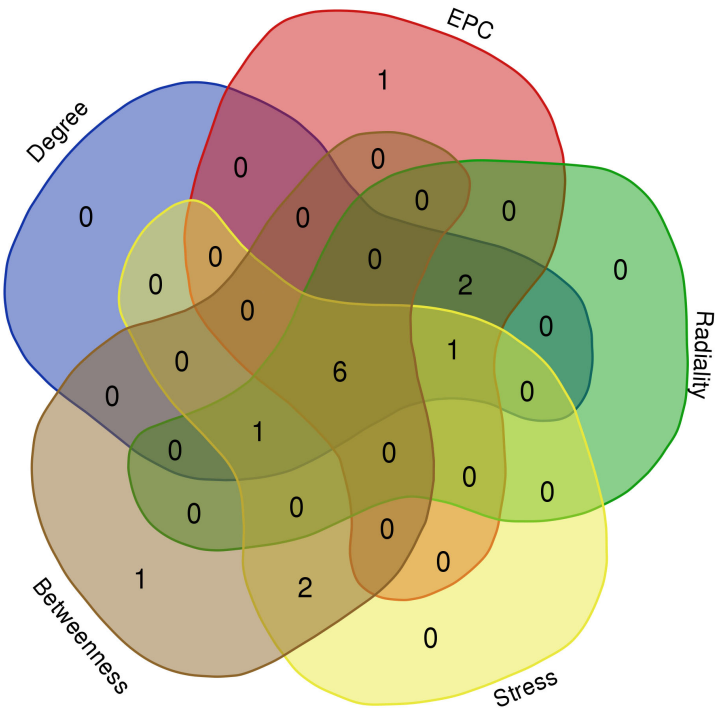

FIGURE 5
Six hub genes were determined by taking the intersection of the first 10 genes in the five algorithms of cytoHubba plugin (Degree, EPC, Radiality, Stress, and Betweenness).

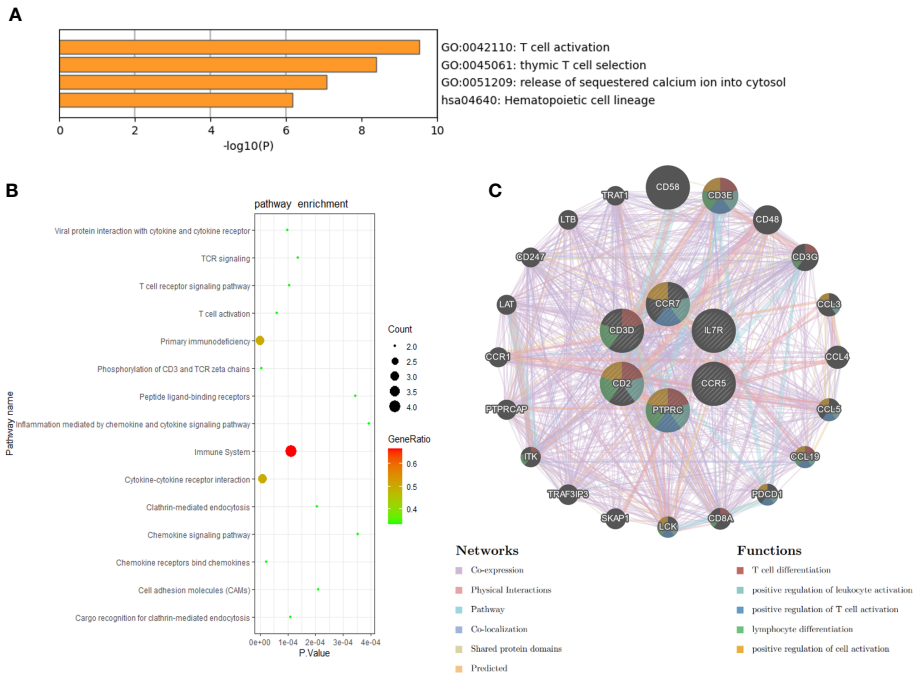

FIGURE 6
Biological process, pathway, and co-expression analysis of the hub genes. **(A)** The functional annotation analysis of hub genes *via* Metascape. **(B)** The further pathway enrichment analysis of hub genes by KOBAS 3.0. P-value < 0.05 was considered significant. **(C)** The network of hub genes and their co-expression genes were constructed and analyzed by GeneMANIA.

regulatory network of TF-mRNA-miRNA was constructed using Cytoscape software (Figure 9).

Evaluation of immune cell infiltration

Through the CIBERSORT algorithm, the relative percentage of 22 immune cell subpopulations analysis was conducted in GSE100927 and GSE55457 respectively (Figures 10A, 11A). Subsequently, we analyzed 22 immune cell subgroups difference between disease samples and control samples. In terms of AS, there were more memory B cells, CD4+ naïve T cells, γδT cells, follicular helper T cells, M0 macrophages, and mast cells activated in AS tissue compared to control tissue, but fewer B naïve cells, plasma cells, CD4+ activated memory T cells, monocytes, M1 macrophages, M2 macrophages, activated

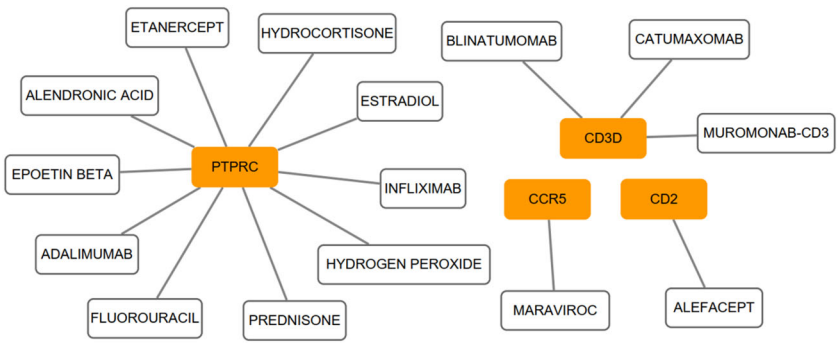

FIGURE 7
On the ground of the DGIdb database, the drug-gene pairs network was generated *via* Cytoscape, consisting of 4 hub genes (PTPRC, CD3D, CCR5 and CD2) and 15 drugs. Yellow circle indicates the hub gene and blank square indicates the drug.

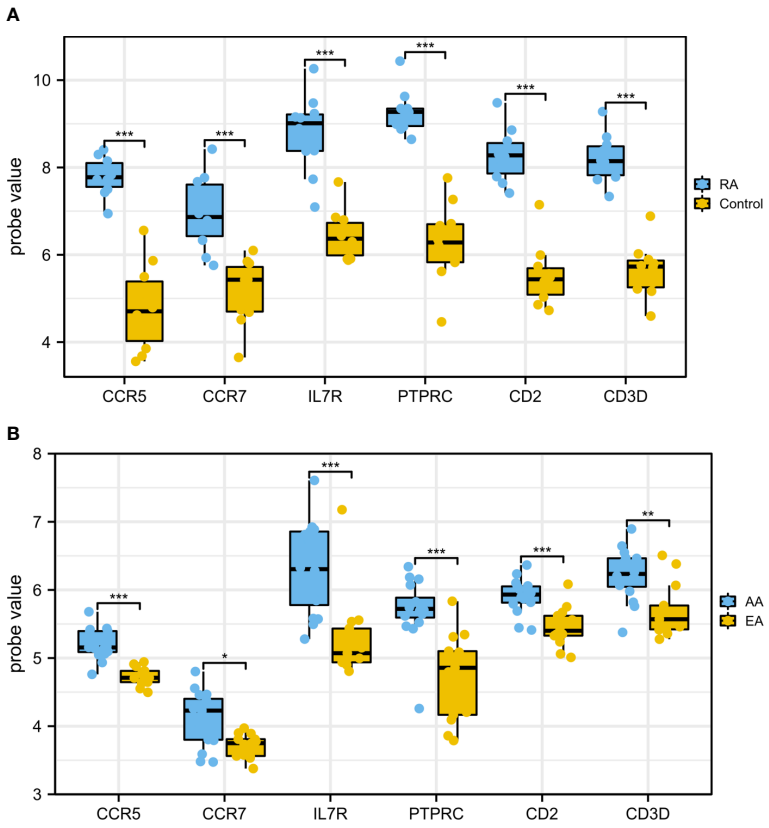

FIGURE 8
The expression of hub genes in **(A)** GSE55235 and **(B)** GSE28829. The comparison between the two datasets used the mean t-test. P < 0.05 was considered to be statistically significant. EA, early atherosclerotic plaques; AA, advanced atherosclerotic plaques. *P < 0.05; **P < 0.01; ***P < 0.001.

dendritic cells, and resting mast cells (Figure 10B). As for RA, the violin chart showed that compared with the normal control sample, there were more memory B cells, plasma cells, CD8+ T cells, follicular helper T cells, γδT cells, and M1 macrophages in the RA samples, but fewer CD4+ resting memory T cells, activated NK cells, and resting dendritic cells (Figure 11B). Comprehensive analysis of immune infiltration outcome revealed that upregulated memory B cells, follicular helper T cells, and γδT cells could be the common immune process and mechanism between RA and AS.

Correlation analysis of hub genes and infiltrating immune cells

The correlation heatmap of 22 types of immune cells showed that γδT cells had a significant positive correlation with follicular helper T cells and memory B cells, but the correlation between memory B cells and follicular helper T cells is statistically insignificant in AS (Figure 12A). As for RA, the correlations between γδT cells, follicular helper T cells and memory B cells were all significantly positive (Figure 13A).

In the AS group, the correlation analysis showed that all six hub genes were positively correlated with memory B cells and γδT cells, yet only CCR5 was positively correlated with follicular helper T cells. Additionally, and the correlation between other hub genes and follicular helper T cells was statistically insignificant (Figure 12B). As for RA, *CCR7, IL7R, PTPRC, CD2*, and *CD3D* were positively correlated with memory B cells and γδT cells, and *CCR7, CD2*, and *CD3D* were positively correlated with follicular helper T cells (Figure 13B).

Discussion

In this study, we identified 54 upregulated and 12 downregulated communal DEGs in RA and AS. Enrichment analyses showed that these genes were significantly involved in the immune system and related immune signaling pathways. Subsequently, six hub genes (*CCR5, CCR7, IL7R, PTPRC, CD2*, and *CD3D*) were determined in the PPI network based on five

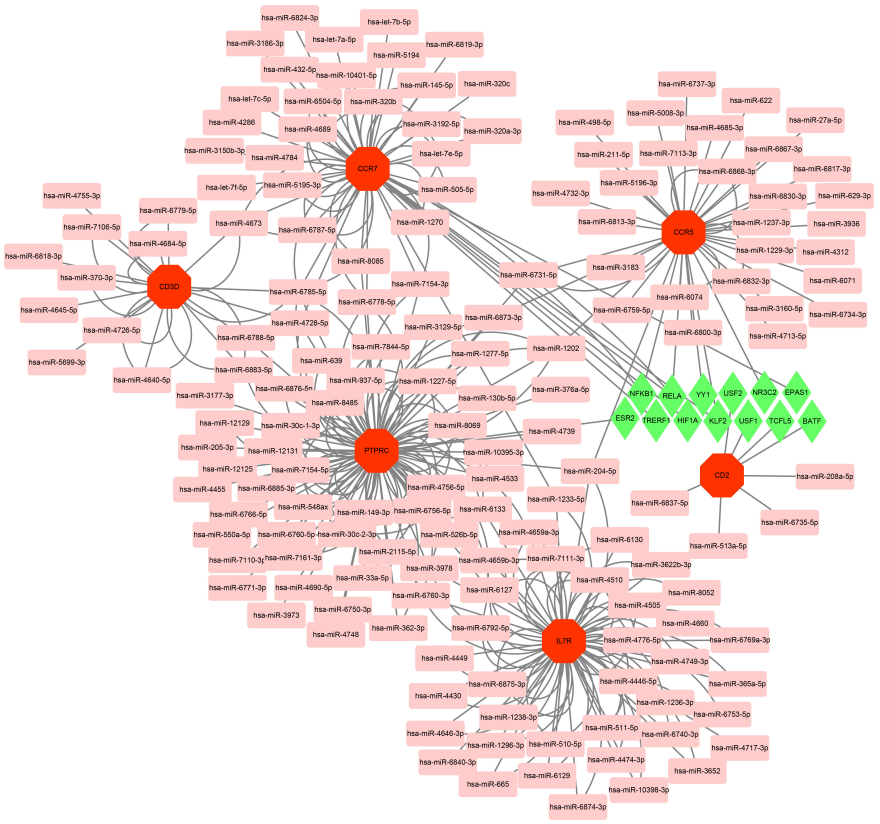

FIGURE 9
Based on MiRwalk and TRRUST database, TF-mRNA–miRNA regulation network of hub genes was constructed using Cytoscape. Hub genes were marked in red octagon; miRNAs were marked in pink round rectangle; TFs were marked in green diamond.

algorithms (Degree, EPC, Radiality, Stress, and Betweenness) in the Cytohubba plugin. The function annotation analysis of hub genes and analysis of the co-expression network re-emphasized the importance of the immune system in RA and AS. Besides, we also predicted the drug-gene pairs and constructed the TF-mRNA-miRNA regulatory network to further elucidate the potential biological role of hub genes. Finally, the immune infiltration of RA and AS was analyzed and compared based on the CIBERSORT algorithm, and the correlation between hub genes and infiltrating immune cells was evaluated to reveal the relevant immune mechanisms.

Based on the results of the immune infiltrating landscape between AS and RA, we can dig out that the B memory cells, γδT cells, and follicular helper T cells were both higher in the disease group. Thus, we hypothesize that the pathogenesis between AS and RA shared some commonness, which was correlated with memory B cells and γδT cells. B cells play an essential role in RA pathogenesis. On the one hand, B cells can locally infiltrate the affected joints synovial membranes with autoantibodies, such as the rheumatoid factor (RF) and anti-cyclic citrullinated peptide (ACPA) (39). On the other hand, B cells serve as the antigen presentation cell, various inflammatory cytokines, and chemokines producers and CD4+T cells co-stimulator (40–42). However, B cells in the RA patient synovial tissue express memory B cell marker CD27 rather than naïve B-cell markers (43). Moreover, RA patients have an expansion of memory B cell subsets at the time of clinical episodes, which has been associated with worse long-term clinical outcomes (44). For the AS, both cellular and humoral B cell immunity play an important role in atherosclerotic plaque formation (45). Clinical studies have demonstrated a negative association between atherosclerotic outcomes and the unswitched memory B cells, which mainly express immunoglobulin (Ig)M antibodies. Interestingly, the switched memory cells mainly express IgG or IgA antibodies, and univariate analysis uncovered that serum level of IgG has been positively associated with atherosclerosis (46). Additionally, Hamze et al. has identified that B cells secreting IgG and IgA are present in the human atherosclerotic plaques located in the vascular wall (47). However, the characteristics of memory B cells in AS are intricacy, and memory B cells may participate in AS and RA development. Different from B memory cells, there was still lack direct evidence of the

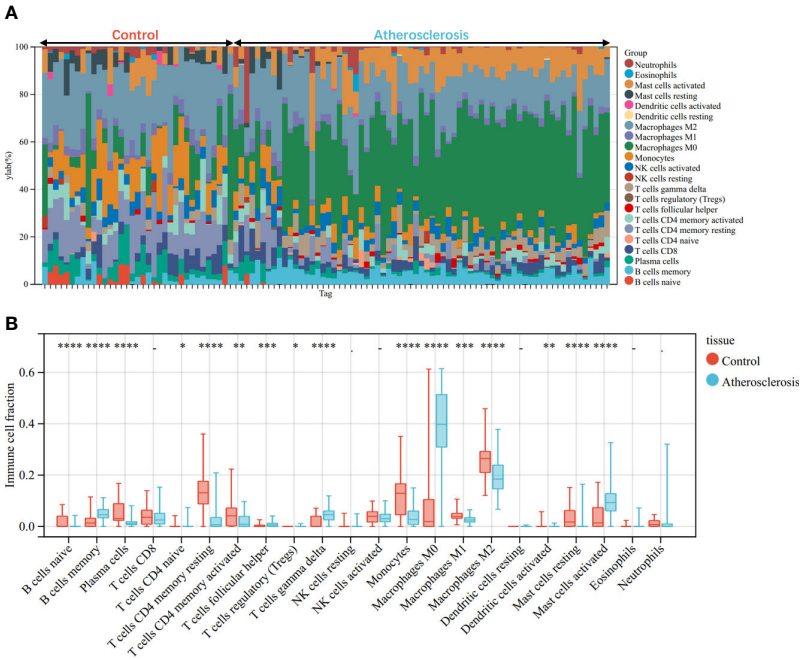

FIGURE 10
Immune cell infiltration mode in AS. **(A)** The relative percentage of 22 immune cell subpopulations out of 104 samples from the GSE100927. **(B)** The ratio of 22 immune cells between AS and control in GSE100927. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

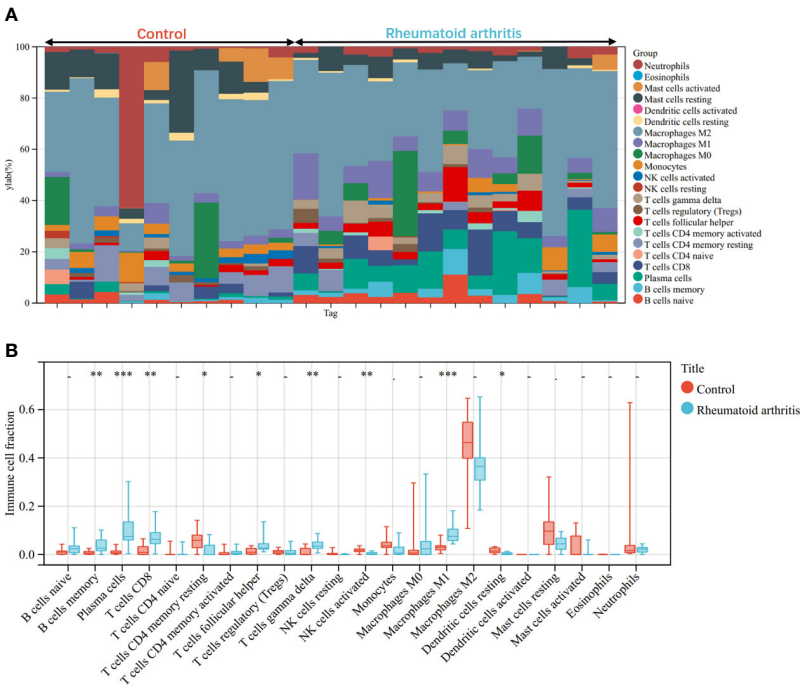

FIGURE 11
Immune cell infiltration mode in RA. **(A)**The relative percentage of 22 immune cell subpopulations out of 23 samples from the GSE55457. **(B)** The ratio of 22 immune cells between RA and control in GSE55457. *p < 0.05; **p < 0.01; ***p < 0.001.

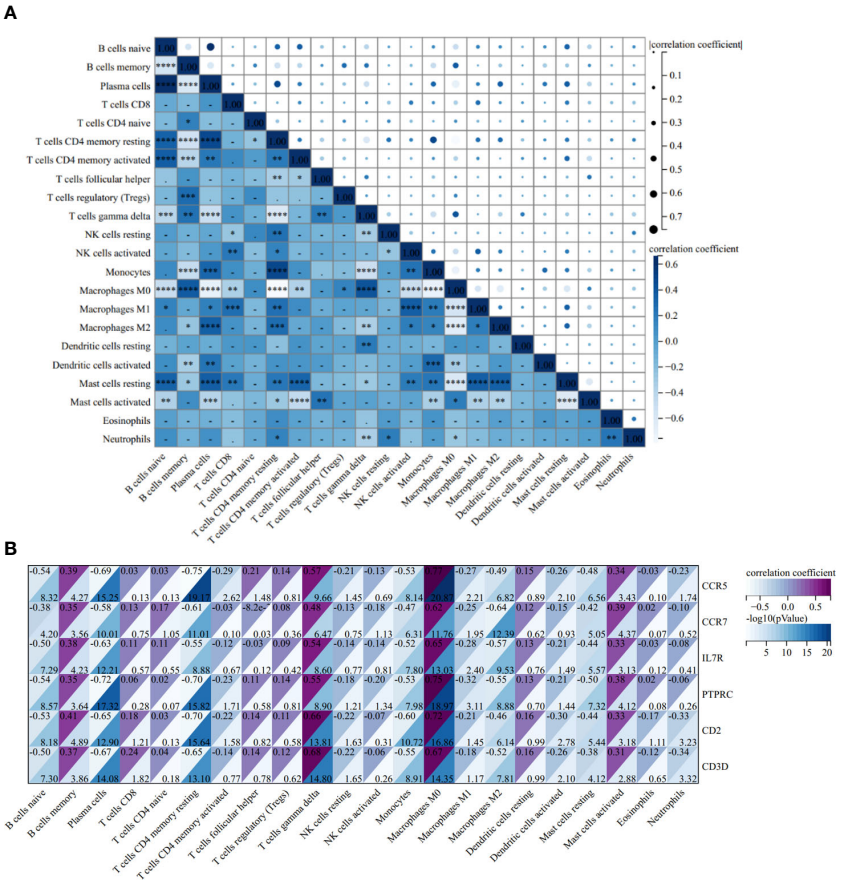

FIGURE 12
Correlation Analysis in AS. **(A)** Related heat maps of 22 immune cells in GSE100927. **(B)** Correlation heat map between hub genes and immune cells in GSE100927. *p < 0.05; **p < 0.01; ***p < 0.001, ****p < 0.0001.

position and role of γδT cells in two diseases. γδT cells, a subtype of T cell, are different from αβ T cells because they cannot recognize specific antigens (48). Despite little evidence on the role of γδT cells in AS, γδT cells could modulate AS *via* IL-17 production owing to it being a rich source of IL-17. IL-17 was known related to favoring plaque stability (49), and increased IL-17 is one of the key molecules in RA pathogenesis (50). Notably, studies have shown that γδT17 cells are the main innate cellular source of IL-17 in collagen-induced arthritis models (51, 52). Meanwhile, the number of γδT17 cells is equal to regular T-helper 17 (Th17) cells in mice synovium, and the proportion of γδT17 cells in the joints has been more dramatically increased than Th17 cells (53). Thus, the mechanism of γδT cells between RA and AS remains unclear, and more basic and clinical research is needed to understand the mechanism. In addition, correlation analysis indicated that all 6 hub genes are positively correlated with at least one communal high-expressed immune cell in both diseases. Meanwhile, both the functional annotation analysis of hub genes and analysis of co-expression network point to the immune system and associated biological pathway. Based on the above analysis, we speculate that hub genes may act as mediator in associated immune pathways and affect immune cell action.

CCR5, a seven-transmembrane-spanning G protein-coupled receptor, is the chemokine receptor for CCL3, CCL4, CCL5, CCL8 and CCL3L1 (54, 55). Strikingly, although CCR5 is predominantly expressed on stimulated macrophages, it is also expressed on osteoclasts and vascular smooth muscle cells (56, 57). In RA pathogenesis, CCR5 induces osteoclast formation to influence osteoclast function. Clinically, CCR5 loss function decreased incidence and/or severity of human RA (58–62), and CCR5 intervention has been negatively related to human RA (63). In AS animal models, inhibiting CCR5 has been identified as a safeguard for lesion size, macrophage infiltration and plaque stability (54). In clinical practice, CCR5Δ32 polymorphism, causing a truncated nonfunction receptor, has been demonstrated as a protective influence on the risk of cardiovascular disease (64). Therefore, CCR5 may be the risk factor for both RA and AS and is involved in both RA and AS development. CC-chemokine receptor 7 (CCR7), also a

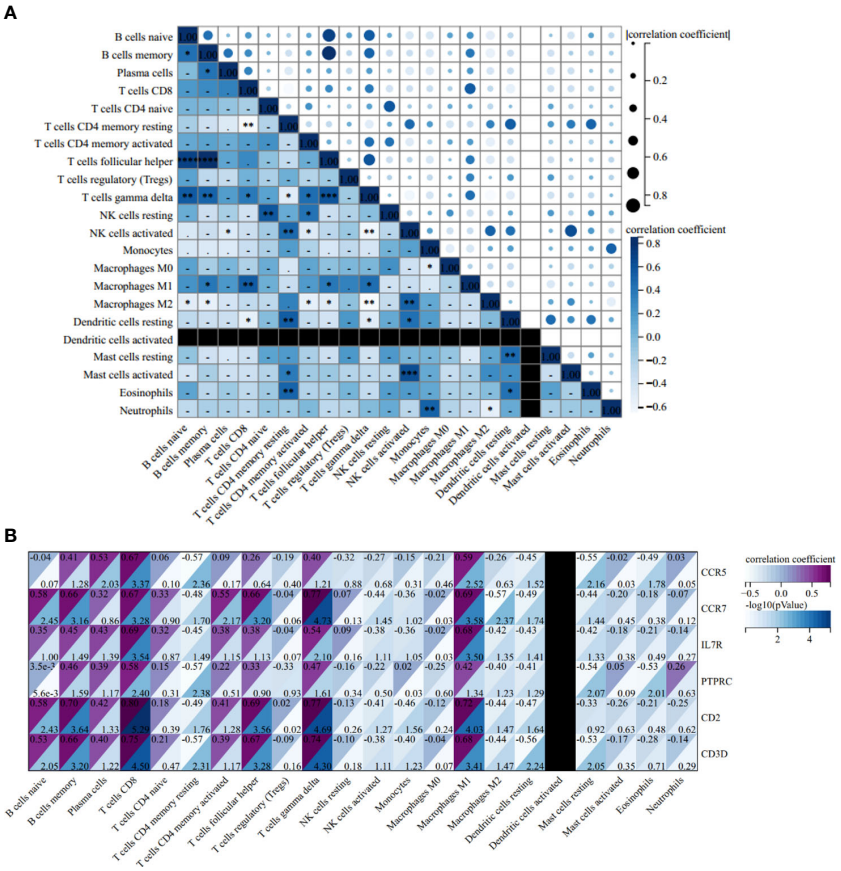

FIGURE 13
Correlation Analysis in RA. **(A)** Related heat maps of 22 immune cells in GSE55457. **(B)** Correlation heat map between hub genes and immune cells in GSE55457. *p < 0.05; **p < 0.01; ***p < 0.001.

G protein receptor, is the sole receptor for CCL19 and CCL21 (65). In RA, CCL21 and CCR7 are highly co-expressed and play a role throughout RA pathogenesis (66). CCR7 is overexpressed on RA dendritic cells, which has been closely related to levels of RF and C-reactive protein (67). What's more, CCR7 expression is also elevated in RA synovial tissue macrophages, fibroblasts, and endothelial cells (66, 68). Intriguingly, activated CCR7 in these cells can directly or indirectly facilitate RA angiogenesis (69). Meanwhile, for AS, CD68+ macrophages elevate CCR7 expression-promoted low-density lipoprotein (LDL) binding and foam cell formation in both asymptomatic and symptomatic carotid plaques (70). CCR7 plays a critical part in cell migration; therefore, the deficiency of CCR7 reduces atherosclerotic plaque content and disturbs the T cells entry or exit in the inflamed vessel wall (71). Nevertheless, considering the pleiotropic nature of CCR7 in RA and AS, therapy targeting CCR7 would be a potential strategy to ameliorate both RA and AS.

IL-7R, a heterodimeric complex, is a receptor for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. Pickens et al. have discovered that the proportion of M1 macrophages is increased in RA synovial fluid, with elevated IL-7R expression (72). IL-7 provokes RA-naïve myeloid cells to remodel into M1 macrophages, which are known as pro-inflammatory immune cells. Meanwhile, IL-7 induces osteoclast formation in M1 macrophages that express high IL-7R levels and are more responsive than naïve and M2 macrophages (73). Moreover, IL-7R ligation by IL-7 can also maintain T cell homeostasis and promote T cell proliferation, selection, activation, and cytokine production. Consistently, blocking IL-7 or IL-7R function can attenuate collagen-induced arthritis monocyte recruitment and osteoclast differentiation (74). Orchestrally, IL-7R antibody treatment in ApoE-/- mice has significantly reduced monocyte/macrophage cell infiltration and lipid content in the atherosclerotic plaque (75). Hence, the increased IL-7R expression may be required for AS and RA, and IL-R blocking would be valuable to hold back the development of AS and RA.

PTPRC, also known as CD45, encodes a protein that is a member of the protein tyrosine phosphatase (PTP) family. PTPs are known to be signaling molecules that regulate various cellular

processes, and has been confirmed to be an essential regulator of T-cell and B-cell antigen receptor signaling (76, 77). Previous numerous studies have reported that PTPRC was densely associated with the anti-TNF therapy response in RA patients, meaning that PTPRC could effectively predict and guide personalized medicine in RA therapy (78–80). Consistent with our result, Xia et al. have demonstrated that PTPRC could act as a regulatory T cell (Treg)-related gene in the progression of atherosclerosis in previous bioinformatics analysis (81), yet there is still a lack of directly experiment evidence that PTPRC is correlated with atherosclerotic plaque evolution. The protein encoded by CD2 is a surface antigen found on all peripheral blood T cells. CD2 interacts with LFA3 (CD58) on antigen-presenting cells to optimize immune recognition (82, 83). Based on the GRAIL2 computational method, CD2/CD58 has been predicted to be new RA risk loci (84). Meanwhile, Fernandez Lahore G et al. found that the expression of CD2 was obviously upregulated in RA synovial tissue compared with osteoarthritis or healthy synovium and speculated that CD2 is densely involved in joint inflammation and CD2 polymorphisms because affecting its expression led to the development or perpetuation of joint autoimmunity (85). The interaction between CD2 and CD58 could promote T cells and macrophages to secrete chemokines and cytokines establishing the inflammatory environment, resulting in the formation of atherosclerotic plaque in psoriasis patients, yet the role of CD2/CD58 in the communal pathogenesis of RA and AS still lacks relevant evidence and needs to be further explored (86). CD3D encodes a part of the T-cell receptor/CD3 complex that takes part in T-cell development and signal transduction (87). Nowadays, for the association between CD3 and two diseases only bioinformatic analysis evidence exists, and more experiments are needed to uncover and verify the potential mechanism (88).

At present, drug treatment options for RA contains nonsteroidal anti-inflammatory drugs (NSAIDs), corticosteroids, disease-modifying antirheumatic drugs (DMARDs) and biologics (2, 89, 90). Cause the heterogeneous factors and complex pathological mechanisms in RA, there are still many patients have an unsatisfactory or poor clinical response under above therapy (90). In addition to new biologics and DMARDs with low side effects keeping in continuous developing states, some novel therapy directions such as GPCR-targeted drug and RNA therapeutics are positively explored and developed (89, 91). The potential therapy target of RA complicated with AS could lies in above explored directions. On the ground of our analyze outcome, the functional annotation analysis of DEGs and hub genes both indicated the role of chemokine receptors in two diseases. Chemokine receptors are a class of GPCRs that regulate immunity, and two above-mentioned hub genes (CCR5 and CCR7) are one type of this class (92). Currently, chemokine receptors are the most intensively studied GPCRs in RA, and the two main drug strategies of RA in chemokines and chemokine receptors as follow: corresponding ligands that can be selected to inhibit chemokine receptors and direct inhibition of chemokine receptors (1, 89). Because the chemokine is widely expressed in numerus human cells and the expression of chemokine might have diverse functional roles at different disease stages of RA, the development of GPCRs was still full of challenge (89). Nowadays, the main aspect of RNA therapeutics is RNA interference (RNAi). RNAi is an endogenous mechanism of mRNA silencing involving miRNA, and siRNA-based Interventions have revealed promising prospective in treatment of RA (93, 94). Based on the MiRwalk database and TRRUST database, the TF-mRNA–miRNA regulatory network of hub genes was predicted and constructed and might provide the potential exploring clues for RNA therapeutics.

The highlight of this article is to explore the communal immune pathway and identify the hub gene and immune infiltration profiles in RA and AS. However, our research also had some limitations as follows: First, although the sample size was slightly large and the hub gene was successfully verified in other gene datasets, this article is a retrospective study that still requires external verification to further verify findings. Second, the drug-genes interaction pairs and TF-mRNA–miRNA regulatory network could provide the primary clues for further exploring core genes, yet the exact and truly association and effect still needs more experiment to uncover. Third, the function of hub genes and immune cells needs to be validated and further explored in *in vitro* and *in vivo* models. The above-described experiments will be the focus of our future work.

Conclusion

In summary, a total of 66 communal DEGs, six hub genes and three infiltrating immune cells were screened based on the comprehensive biological analysis. The evidence of overlapping pathogenesis between AS and RA points to relevant immune pathways, which might be mediated by specific hub genes and infiltrating immune cells. Furthermore, the analysis of these hub genes and immune cells may indicate new insights in potential therapeutic direction of RA complicated with AS.

Data availability statement

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

Author contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for

important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

Conflict of interest

Author WS was employed by China National Nuclear Corporation 416 Hospital.

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1013531/full#supplementary-material

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Using genetic instruments to estimate the causal effect of hormonal reproductive factors on osteoarthritis

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Objectives: Hormonal reproductive factors have been considered to play an important role in the etiology of osteoarthritis (OA). We performed Mendelian randomization (MR) to examine whether a causal effect existed between them.

Methods: MR was performed by using publicly released genome-wide association study (GWAS) summary statistics to estimate the causal associations of three relevant exposures, including age at menarche (AAM), age at natural menopause (ANM) and age at first birth (AFB), with the risk of OA. We employed several MR methods, including inverse-variance weighted (IVW), MR-Egger regression, weighted median and weighted mode, to estimate the causality. We performed a sensitivity analysis by manually pruning pleiotropic variants associated with the known confounder body mass index (BMI).

Results: The instrumental variables that achieved genome-wide significance, including 349 AAM single nucleotide polymorphisms (SNPs), 121 AAM SNPs, 54 ANM SNPs, and 10 AFB SNPs, were incorporated into the operation. IVW analysis indicated that each additional year in AFB was associated with a decreasing risk of hip and/or knee OA and overall OA (hip and/or knee OA: OR = 0.79, 95% CI: 0.64–0.93, $P = 1.33 \times 10^{-3}$; overall OA: OR = 0.80, 95% CI: 0.68–0.92, $P = 1.80 \times 10^{-4}$). In addition, our results suggested that AAM exerted a causal effect on knee OA in an unfavorable manner (OR = 0.86, 95% CI: 0.76–0.95, $P = 1.58 \times 10^{-3}$). After accounting for the effect of BMI, the causal effect association between AFB and hip and/or knee OA was also examined (IVW: OR = 0.78, 95% CI: 0.66–0.92, $P = 3.22 \times 10^{-3}$).

Conclusion: Our findings add a growing body of evidence surrounding the unfavorable effects of early AFB on OA risk, suggesting the essential for relevant health problem management in susceptible populations.

KEYWORDS

osteoarthritis, hormonal reproductive factors, Mendelian randomization, causality, GWAS

Introduction

Osteoarthritis (OA) is the most common form of joint disease around the world, and it is estimated that approximately 302 million individuals suffer from OA (1). Numerous studies have shown that OA has been ranked as the leading cause of disability and accounts for a heavy burden of disease (2, 3). As the aging population and obesity pandemic increase, OA is more prevalent and has become a major public health concern (4). However, the exact mechanism underlying the pathogenesis of OA has not been fully clarified.

Epidemiological studies indicate a distinct sexual disparity in the incidence of OA in which females develop the disease more frequently and more severely than males, particularly after menopausal age (5). Individuals who had undergone postmenopausal hormone therapy exhibited a higher risk of developing OA than those who did not, revealing that hormonal reproductive factors may play a critical role in the initiation and progression of OA. There is a wealth of data concerning the associations of hormonal reproductive factors, including age at menarche (AAM), age at nature menopause (ANM) and age at first birth (AFB), with incident OA. For example, findings from a prospective cohort study suggested that an early AAM increased the risk of hip and knee replacement for OA, while the ANM was not associated with the risk (6). Another cross-sectional study demonstrated that women with ANM <45 years were associated with a 2.60-fold risk of developing OA compared with those with an ANM $\geq$ 45 years (7). These discrepancies may be due to potential confounding factors (such as obesity and age) and reverse causality. Reproductive behavior is shaped by biology and environment, while AFB represents an accurate measure of complex reproductive outcomes, are frequently recorded and consistently measured. It is indicated that the heritability of AFB shifted from 9% for women born in 1940 to 22% in 1965 (8).

Mendelian randomization (MR) is an epidemiological method that utilizes genetic variants robustly associated with exposure as instrumental variables (IVs) to estimate the causal effect of exposure on an outcome (9, 10). Because of the bias from confounders, reverse causation and measurement error, even there was a significant statistical association between the exposure and outcome, traditional analyses have limitations for the assessment of causality, while MR offers an alternative way to probe it. It is considered important to target the management of relevant health problems in OA susceptible individuals, which requires us to first clarify the root causes of OA. In this study, confounders including environmental factors and BMI could be excluded due to the application of MR. To the best of our knowledge, the MR study focusing on this topic do not exist. Therefore, it is necessary to do the MR design to investigate it.

Abbreviations: OA, osteoarthritis; MR, Mendelian randomization; GWAS, genome-wide association studies; AAM, age at menarche; ANM, age at natural menopause; AFB, age at first birth; IVW, inverse-variance weighted; BMI, body mass index; SNPs, single nucleotide polymorphisms; MR-PRESSO, MR pleiotropy residual sum and outlier; TKR, total knee replacement.

Materials and methods

Study design and data source

In our current study, a standard two-sample framework was applied to explore the effect of three female hormonal reproductive factors (AAM, ANM and AFB) on hospital-diagnosed OA and its subtypes (OA at any site, hip OA, knee OA, hip and/or knee OA). Individuals were restricted to European ancestry to decrease the bias from population stratification. As a milestone in the development of female pubertal development, age at menarche varies markedly among females. GWAS have identified tens of thousands of sequence variant on a genome-wide scale in humans and from which to determine the effect size of genetic variants statistically in order to identify the risk factors of disease etiology in different ethnic populations. GWAS gives us the opportunity to research complex diseases by comparing SNPs loci detected genome-wide in patients to controls for all variant allele frequencies, obviating the need to presuppose causative genes as in a candidate gene strategy. Genetic associations with AAM were obtained from two large GWAS meta-analyses, including a total of 329,345 individuals in AAM (11) and 182,416 in AAM (12). Summary level statistics for ANM were derived from the GWAS of 69,360 women, identifying 44 genomic regions containing 54 independent signals, most of which were associated with one or more DNA damage response pathway genes (13). Biological processes, such as AFB, are indicated to partly cause reproductive behavior. A recent GWAS of 251,151 women examined the genetic architecture of reproductive rhythms defined by AFB, and 10 AFB-associated loci were identified (14). The full OA (OA at any site, hip OA, knee OA, hip and/or knee OA) summary statistics were obtained from the largest release GWAS meta-analysis across 16.5 million derived from the UKB resource (15). Accounting for the confounding effects of other traits that were genetically correlated with sleep phenotypes, sensitivity analysis was performed after adjusting for BMI-related genetic disorders. Summary statistics of BMI were downloaded from a GWAS including 806,834 individuals (16). A more detailed description of the included data sources is available in Supplementary Table 1. No ethical approval was required in this work, as all the data analyzed were publicly available.

Selection of the genetic instruments

A valid IV estimator should meet the following three assumptions: (1) reliably and strongly associate with the risk

factor for interest (relevance assumption); (2) no unmeasured confounders of the associations between genetic variants and outcome (independence assumption); and (3) independent of the outcome (exclusion restriction) (17). The qualified IVs were selected as follows: After removing SNPs with missing information, a list of SNPs passing the threshold of significance $P < 5 \times 10^{-8}$ was first screened using a distance-based metric. We performed PLINK to calculate r^2 between all selected SNPs in European ancestry samples from the 1000 Genomes Project (18). To ensure that all the selected SNPs obeyed the independence assumption, only those with the smallest P value were retained among all pairs of SNPs with $r^2 > 0.01$. A proxy SNP in strong LD ($r^2 > 0.8$) was included where a specific instrument SNP was not available in the look-up GWAS dataset. To ensure that all corresponding risk factors and outcome alleles were on the same strand, we harmonized the effect of these instrumental SNPs where possible. The equation R^2 = 2*Beta^2*EAF*(1-EAF)/(2*Beta^2*EAF*(1-EAF)+2*SE^2*SampleSize*EAF*(1-EAF), F = R^2(SampleSize-2)/(1 – R^2) was used to calculate the F-statistic for all selected instrument SNPs separately and synthetically to reject the weak instruments with an F-statistic lower than 10 (19). R^2 in the equation represents the individual exposure variance explained by each IV.

Mendelian randomization analysis

Subsequently, MR analyses were conducted with inverse variance weighted (IVW), MR Egger regression, weighted median and weighted mode. The primary calculation was run by inverse variance weighted, which estimates the ratio from several instruments. This method assumed that all SNPs were valid instruments or were invalid with zero overall bias. However, IVW may be overpraised in the presence of heterogeneity that can occur due to, among other factors, horizontal pleiotropy or, more simply, off-target genetic effects.

Consistency in results across methods builds confidence in the obtained estimates, as they depend on different assumptions and models of horizontal pleiotropy. MR-Egger deemed uncorrelated associations between SNP exposure and horizontal pleiotropic effects, which indicates instrument strength independent of the direct effects assumption. MR-Egger regression analysis, whose slope represents the causal effect estimate, is robust to invalid instruments against directional pleiotropy (20, 21). A weighted median requires the weight of each SNP in the overall estimate to depend on the precision of its ratio estimate, which differs from a simple median estimate. More specifically, 50% of the weights come from valid IVs smaller than or equal to the weighted median in this analysis (22), while the weighted mode requires that the largest subset of instruments which identify the same causal effect to be valid instruments (23).

Pleiotropy and sensitivity analysis

Although MR is a potentially powerful technique for strengthening causal inference, several issues, including disequilibrium, pleiotropy and epigenetic effects, could disturb instrumental variable assumptions. Funnel plots were used as a visual test for horizontal pleiotropy, where symmetry is indicative of a lower probability of pleiotropy (24). As an additional control for pleiotropy, we applied the global test, outlier test, and distortion test using the MR pleiotropy residual sum and outlier (MR-PRESSO) to identify and correct for outliers in IVW linear regression (25). Furthermore, MR-Egger regression provides an estimate of the average pleiotropy effect, and an intercept of the regression equation of 0 proves the evidence of pleiotropy (26). In the regression model, regression coefficients are highly susceptible to an individual datapoint. Leave-one-out sensitivity analysis was performed to identify whether the association was disproportionately influenced by a single SNP. An increased BMI is a well-known risk factor for OA (15). To minimize the possibility of spurious causal associations due to confounding factor BMI, we performed a sensitivity analysis by manually pruning pleiotropic BMI-associated instrumental variables.

Statistical analysis

We employed the packages “Two Sample MR” (24) and “Mendelian Randomization” (27) to perform MR analysis. Forest plots were produced using the “forestplot” package. The Bonferroni method was utilized in the primary analysis to indicate multiple comparisons. Correcting for 3 exposures and 4 outcomes, P value below 0.004 indicated strong evidence of associations (0.05/12 = 0.004). All statistical analyses were implemented in R project version 3.6.1.

Results

In total, after implementing the pruning strategy previously described, there were 349 SNPs achieved genome-wide significance for AAM (11) and 121, 54 and 10 IVs for AAM (12), ANM and AFB, respectively. F-statistic values for individual instrumental SNPs were all above the threshold 10, with means of 64.27, 58.00, 68.14 and 36.49 for AAM (11), AAM (12), ANM and AFB, respectively (Table 1). SNPs were excluded or substituted with highly correlated ($r^2 > 0.8$) proxy SNPs due to unavailability in outcome

TABLE 1 Univariable MR results of hormonal reproductive factors on risk of OA and subtypes.

Exposure	Outcome	No. of SNPs	F-Statistic	OR (95% CI)	*P*
AAM (11)	Overall OA	336	64.27	0.91 (0.85–0.98)	5.95E-03
	Hip OA	337		1.01 (0.89–1.13)	8.56E-01
	Knee OA	337		0.86 (0.76–0.95)	1.58E-03
	Hip and/or knee OA	337		0.92 (0.84–1.00)	3.64E-02
AAM (12)	Overall OA	119	58.00	0.94 (0.86–1.03)	2.09E-01
	Hip OA	119		1.16 (0.97–1.34)	1.23E-01
	Knee OA	119		1.02 (0.85–1.19)	2.31E-01
	Hip and/or knee OA	119		1.02 (0.85–1.19)	6.99E-01
ANM	Overall OA	51	68.14	1.00 (0.97–1.03)	9.05E-01
	Hip OA	51		0.98 (0.92–1.03)	4.43E-01
	Knee OA	51		1.00 (0.96–1.04)	9.46E-01
	Hip and/or knee OA	51		1.00 (0.97–1.04)	7.95E-01
AFB	Overall OA	10	36.49	0.80 (0.68–0.92)	1.80E-04
	Hip OA	10		0.76 (0.51–1.00)	2.65E-02
	Knee OA	10		0.81 (0.63–0.98)	1.57E-02
	Hip and/or knee OA	10		0.79 (0.64–0.93)	1.33E-03
AAM (11) no BMI	Overall OA	285	62.99	0.96 (0.90–1.03)	3.01E-01
	Hip OA	286		1.09 (0.96–1.24)	1.86E-01
	Knee OA	286		1.01 (0.88–1.16)	8.90E-01
	Hip and/or knee OA	286		0.96 (0.86–1.06)	4.15E-01
AAM (12) no BMI	Overall OA	90	58.44	1.01 (0.92–1.10)	9.04E-01
	Hip OA	90		1.23 (1.01–1.49)	4.28E-02
	Knee OA	90		1.06 (0.92–1.22)	4.10E-01
	Hip and/or knee OA	90		1.09 (0.97–1.22)	1.60E-01
AFB no BMI	Overall OA	8	34.02	0.83 (0.72–0.94)	4.91E-03
	Hip OA	8		0.77 (0.59–1.02)	6.85E-02
	Knee OA	8		0.80 (0.65–0.98)	2.83E-02
	Hip and/or knee OA	8		0.78 (0.66–0.92)	3.22E-03

OA: osteoarthritis; AAM: age at menarche; ANM: age at natural menopause; AFB: age at first birth; BMI: body mass index; SNPs: single nucleotide polymorphisms.

datasets or palindromic with ambiguous A/T or G/C. Detailed information for incorporated instrumental SNPs is presented in Supplementary Tables 2–4.

The MR analysis indicated that genetically determined each additional year in AAM (11) was associated with a decreasing risk of knee OA after correcting for multiple testing (IVW: OR = 0.86, 95% CI: 0.76–0.95, $P = 1.58 \times 10^{-3}$). The causality between AAM (11) and overall OA ($P = 5.95 \times 10^{-3}$) and hip and/or knee OA ($P = 3.64 \times 10^{-2}$) was only normally significantly positive, with IVW $OR_{per-SDincrement(95\%CI)}$ of 0.91 (0.85–0.98) and 0.92 (0.84–1.00), respectively. To the best of our knowledge, such causality was not observed in another AAM (12) exposures, with *P* values above the threshold and OR of 0.94, 1.16, 1.02 and 1.02 for OA and three subtypes, respectively. The available evidence also made it difficult to explain the causality of ANM to OA and subtypes. Furthermore, the IVW method indicated that AFB exerted a causal effect on OA and all subtypes in an unfavorable manner, with OR of 0.80 and 0.79 for overall (95% CI: 0.68–0.92, $P = 1.80 \times 10^{-4}$) and hip and/or knee OA (95% CI: 0.64–0.93, $P = 1.33 \times 10^{-3}$), respectively. However, as shown in Table 1 and Figure 1, some nominally significant positive causality correlations with OA did not pass multiple-testing correction for the Bonferroni method (hip OA: OR = 0.76, 95% CI: 0.51–1.00, $P = 2.65 \times 10^{-2}$; knee OA: OR = 0.81, 95% CI: 0.63–0.98, $P = 1.57 \times 10^{-2}$). Additional methods, including weighted median, weighted mode and MR-Egger section, validate the uniformity conclusion (Supplementary Table 5).

To effectively control pleiotropy, we next investigated the MR-PRESSO and *p* value for the MR-Egger intercept test. The sensitivity analysis revealed the absence of outliers (Supplementary Table 7) and horizontal pleiotropy (Supplementary Figure 2). As shown in Supplementary Figure 2, symmetry in funnel plots did not show any evidence of publication bias. In addition, the leave-one-out analysis

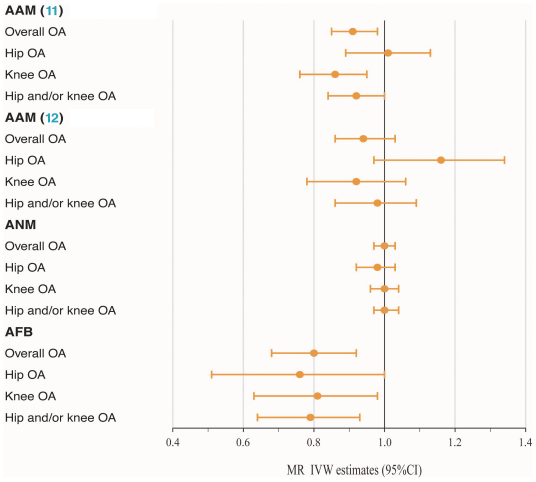

FIGURE 1
Forest plot of hormonal reproductive factors on the risk of OA and subtypes in IVW estimates.

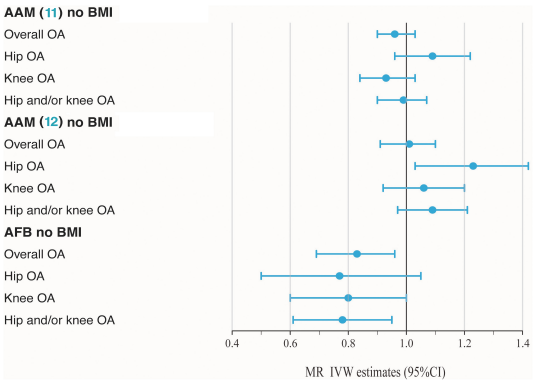

FIGURE 2
Forest plot of hormonal reproductive factors on risk of OA and subtypes in IVW estimates adjusting for BMI.

showed that none of the individual genetic markers drove the majority of the association signal. The scatter plots for effect sizes of SNPs for three hormonal related exposures and those for OA and subtypes are shown in Supplementary Figure 1.

BMI is a known modifiable risk factor that plays an important role in the etiology of OA and shapes reproductive exposures. A total of 82 SNPs were found to be associated with BMI ($P < 5 \times 10^{-8}$), including 51 in AAM (11), 29 in AAM (12) and 2 in AFB. SNPs associated with BMI have been annotated with * in the Supplementary Tables 2–4. As shown in Table 1 and Figure 2, no casualty between AAM (11) no BMI and keen OA were found when we performed MR again (IVW: OR = 1.01, 95% CI: 0.88–1.16, P = 0.89), which indicates that BMI-associated SNPs confounded causality in our initial calculation. The negative result was confirmed in AAM (12) no BMI. Similarly, after deleting two BMI-associated SNPs, there was not enough evidence to indicate the causal relationship between AFB no BMI and overall OA (IVW: OR = 0.83, 95% CI: 0.72–0.94, $P = 4.91 \times 10^{-3}$). However, a similar causal effect association between AFB and hip and/or knee OA could still be measured (IVW: OR = 0.78, 95% CI: 0.66–0.92, $P = 3.22 \times 10^{-3}$). Additional methods section validate the uniformity conclusion (Supplementary Table 6).

Discussion

In the current study, we performed two-sample MR to investigate the causality between three hormonal reproductive factors and the risk of OA. The concern was that 1 year later in AFB was associated with a reduced risk of OA. These findings support the hypothesis that AFB may play a causal role in the pathway of developing OA. The adverse causal effects were robust in our MR after pruning potential confounder BMI.

Several observational studies confirm the causal association of hormonal reproductive factors with OA, while current results on this topic from conventional epidemiological studies remain controversial. A large cohort study with 30.727 cases is consistent with our conclusions, affirming the role of AFB in OA etiology (15). Other studies also point toward some positive association, and a prospective cohort study containing over 30,000 women found that older age at menarche was associated with a decreased risk of total knee replacement (TKR) due to primary OA (28). Researchers proposed that one possible explanation could be that lower AAM may be a marker of other factors, such as higher BMI when young (6). High BMI is known as a risk factor for OA, but it is unlikely that BMI would explain the casualty found here, as we adjusted our analyses for BMI, and our findings were consistently observed within subgroups of no BMI. However, the assumed relationship between the female hormonal aspects and OA was not clinically significant in another cohort study (29). Since hormonal reproductive factors are prone to bias due to interference from potential confounders which difficult to be excluded by traditional epidemiology, MR estimates reflect the causality at the genetic level.

Given the complexity of these confounders, the underlying mechanism of hormonal reproductive factors in the development of OA remains to be elucidated. Estrogen is considered to strongly associate with the female hormonal reproductive cycle, in which receptors are found on bone and chondrocyte cells (30). Several studies show evidence of the associations between radiographic changes in OA and high bone density since considering estrogen could prevent bone loss (31, 32). Consequently, greater exposure to estrogen,

while preventing bone loss, may plausibly promote OA. Gao et al. studied estrogen and estrogen metabolites in Chinese women with OA. Compared to the controls (healthy and rheumatoid arthritis women), premenopausal women with OA had a significantly lower concentration of 2-hydroxyestrone and free estrogen in serum. In postmenopausal women, the serum concentration of 2-hydroxyestradiol was increased compared to that in controls, while free and total estrogen were significantly decreased. Apart from estrogen deficiency, rapidly elevated serum levels of 2-hydroxyestrone in the perimenopausal period may correlate with the pathogenesis of OA (33, 34). Furthermore, it has been reported that estrogen may have different effects on the initiation and progression of OA. Hence, it is difficult to ratiocinate the biological mechanisms that underlie this study due to these heterogeneous effects. However, the effects of female hormones on OA can be further explored in animal models and *in vitro* studies.

Our study has several strengths. The large sample size and richness of the data set for reproductive variables of interest led the estimated effects to be close to the truth. In addition, three different reproductive traits (AAM, ANM, and AFB) were incorporated to reflect the length of the reproductive period and complementing each other well. To reduce the interference of potential factors, we examined OA directly rather than proxies of OA, such as hospitalization or joint replacement. Moreover, we were able to adjust for not only hormonal reproductive factors but also reported confounders of OA, such as BMI. Notwithstanding, we must acknowledge several limitations. Firstly, there was no stratification of sex in the existing GWAS data set, while our selection of hormonal reproductive factors was female specific. However, since most cases in the GWAS dataset were from females (63.7% in overall OA), we thought the estimated effects would be close to the truth. Secondly, to diminish population stratification, our samples were restricted to the European population, which leads our findings to be applicable for European populations. Finally, the design of our study precluded us from considering other factors, such as environmental effects and hormone use, in addition to the only confounder BMI regarded in the current study.

In summary, our findings add to a growing body of evidence surrounding the unfavorable effects of early age at first birth on OA risk, suggesting the essential for relevant health problem management in susceptible populations. Further large-scale studies or longitudinal studies are required to validate our findings.

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

Each participating study obtained written informed consent from all participants and received approval from the appropriate local institutional review boards.

Author contributions

ZY was responsible for the conception, design of the study, full access to all the data in the study, took responsibility for the integrity of the data, and the accuracy of the data analysis. BW and JW Performed the statistical analysis and drafted the manuscript. All authors were involved in drafting the article or revising it critically for important intellectual content, and approved the final version to be published.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2022.941067/full#supplementary-material

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Identification of biomarkers associated with CD8+ T cells in rheumatoid arthritis and their pan-cancer analysis

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Introduction: Rheumatoid arthritis (RA), a prevailing chronic progressive autoimmune disease, seriously affects the patient's quality of life. However, there is still a lack of precise treatment and management methods in clinical practice. Previous studies showed that CD8+ T cells take a lead in the progression of RA.

Methods: Genes closely related to CD8+T cells in RA were identified through multiple RA datasets, CIBERSORT, and WGCNA algorithms. Further machine learning analysis were performed to identify CD8+T cell-related genes most closely related to RA. In addition, the relationship between these three key genes and 33 cancer species was also explored in this study.

Results: In this study, 10 genes were identified to be closely related to CD8+T cells in RA. Machine learning analysis identified 3 CD8+T cell-related genes most closely related to RA: CD8A, GZMA, and PRF1.

Discussion: Our research aims to provide new ideas for the clinical treatment of RA.

KEYWORDS

CD8+ T Cells$_1$, rheumatoid arthritis$_2$, WGCNA$_3$, Pan-Cancer$_4$, ssGSEA$_5$

Introduction

Rheumatoid arthritis (RA) is a common chronic autoimmune disease associated with systemic inflammatory processes. This chronic damage first affects the patient's bones and joints (1). The progression of the disease will affect the patient's quality of life, bringing a huge economic burden to individuals and society. Another prominent

manifestation of RA is systemic inflammatory lesions outside the joints, in particular, the digestive system, nervous system, cardiovascular system, etc (2). Furthermore, because of the significant variability of RA disease, it is challenging to distinguish it clinically from other autoimmune disorders (such as systemic lupus erythematosus, Sjögren's syndrome, etc.) (3) and face challenges in the administration of chemotherapy (4). In the developed world, RA affects between 0.5 and 1% of adults, or about 24.5 million people, with 5 to 50 new cases per 100,000 people every year (5, 6). The aforementioned issues require immediate attention given the significant prevalence of RA in the general population.

CD8+ T cells, also known as cytotoxic T cells, play an important role in the elimination of malignant cells and intracellular inflammation in the body (7). Current research shows that in the face of infection, inflammation, tumor, and other pathological conditions, the metabolic level of CD8+ T cells and the anti-inflammatory phenotype exhibited by the body will undergo various changes (8). Some studies show that its epigenetic modifications also alter (9). The current consensus is that CD8+ T cells promote the progression of RA by releasing pro-inflammatory and cytolytic mediators (10, 11). The study by M Margarida Souto-Carneiro et al. (12) mentions that metabolic demands in hypoxic tissues sustain the continued damage of this cell to the joint. A study by Helena Carvalheiro et al. (10) revealed the full landscape of CD8+ T cells in RA, revealing that CD8+ T cells are characterized by upregulation and secretion of inflammatory mediators throughout RA. The above studies all illustrate the important role of CD8+ T cells in the progression of RA and may become an excellent intervention target for clinical RA treatment.

In this study, based on the results of the CIBERSORT algorithm and weighted gene correlation networks analysis (WGCNA) analysis, we finally identified 10 key CD8+ T cell-related genes. The three genes most closely related to CD8+ T cell infiltration in RA, namely CD8A, GZMA, and PRF1, were found to be associated with a variety of cancers. This points out a new path for the treatment of RA.

Material and methods

Collection and processing of gene expression data

The GSE55235 (13), GSE1919 (14), GSE48780 (15), GSE55457 (13), and GSE55584 (13) datasets were retrieved and downloaded from the Gene Expression Omnibus(GEO, https://www.ncbi.nlm.nih.gov/geo/). Limma package was used to normalize the data and convert between probe ID and gene symbol through platform information, removing probes without gene symbol and taking the average expression value of multiple probes under the same symbol (16). We used variation coefficients to select the most significantly varied genes for analysis.

Analysis of immune cell infiltration

The proportion of 22 immune cell types and the infiltration of immune cells in samples from GSE55235, GSE1919, GSE48780, GSE55457, and GSE55584 datasets was calculated using the CIBERSORT algorithm (17).

Construction of WGCNA co-expression network

"WGCNA" was used to construct a gene co-expression network for genes in the GSE55235 dataset with a variation coefficient greater than 0.1. The top 25% of highly expressed variants were analyzed (18). The reliability of the constructed scale-free network is ensured by removing abnormal samples. For that, they were used to approximate appropriate soft threshold rates and obtain adjacency values between genes whose variances were more significant before applying power functions. The adjacency values were then converted into topological overlap matrices (TOM) and derived the dissimilarity (1-TOM) values. The dynamic tree-cutting method was finally used to identify modules by hierarchical clustering of genes.

Building blocks feature relation

Each module underwent component analysis according to its features, and the correlation between module characteristics and T-cell subtypes was then analyzed using the Pearson test. We considered the module significantly correlated with T cell subtypes when $P<0.05$. The central module was defined as that with the highest correlation coefficient with CD8+T cells.

Hub gene selection and validation

Based on the module connectivity and clinical characteristic relationships of each gene in the central module, candidate central genes were chosen. Pearson's correlation between genes' absolute values is used to describe module connectivity. The overall Pearson's correlation between each gene and each characteristic was used to identify the relationship between clinical traits. For candidate central genes, we set gene significance >0.75. Verify whether there is a significant correlation between the candidate hub genes and CD8+T, to determine the reliable hub genes. GSE1919, GSE48780, GSE55457, and GSE55584 datasets were used to verify the

Spearman correlation between CD8+T cell and central gene expression. Hub genes were defined as candidate hub genes that were significantly associated with CD8+T cells in the GSE55235, GSE1919, GSE48780, GSE55457, and GSE55584. In addition, CIBERSORT, MCPCOUNTER, QUANTISEQ, TIMER, and XCELL algorithms were used to verify the correlation between genes and CD8+T cells in different tumors.

Identification of key genes using LASSO regression and random forest

Critical genes for T cell CD8+ were identified using the LASSO regression and random forest algorithms. Least Absolute Shrinkage and Selection Operator (LASSO) regression is performed using the "glmnet" package. RF is implemented using the "randomForest" package (19). A Venn diagram was used to visualize the results of the two algorithms and obtain the intersection genes.

Functional and pathway enrichment analysis

We examined genes using Gene Ontology (GO) analysis (20), which comprised molecular function (MF), cellular component (CC), and biological process (BP), and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis (21).

Hub gene correlation

Genes GSE55235, GSE1919, GSE48780, GSE55457, and GSE55584 were correlated, and the analysis results were then visualized using "ggplots2". We obtained high-throughput expression data of cancer and normal tissues from the TCGA (22) and GETx databases (http://commonfund.nih.gov/GTEx), respectively, and evaluated the correlation between genes in various tumors and various normal tissues to further illustrate the correlation between central genes.

The association between genes and transcription factors

Transcription factors are involved in various complex biological processes by regulating the transcription process through specific DNA sequence recognition. RNA-seq data were obtained from TCGA database and we used ChEA3 (https://maayanlab.cloud/chea3/) database to identify the transcription factors of hub gene and demonstrated them using Cytoscape software.

Gene set enrichment analysis

A gene set's significance between two biological states can be determined using gene set enrichment analysis (GSEA) (23). A single gene GSEA analysis was carried out to better investigate the potential mechanism through which Hub genes affect RA. At the same time, h.all.v7.5.1.symbols.gmt in the Molecular Signatures Database (MSigDB: https://www.gsea-msigdb.org/gsea/index.jsp) was selected as the reference gene set, and Spearman rank correlation coefficient was obtained by using the package "corrplot", and adjustment P value <0.05 was used as the screening criterion.

Construction of protein-protein interaction network

GeneMANIA (http://genemania.org/), a website for creating protein-protein interaction (PPI) networks, may be used to make predictions for the functions of genes and find genes with similar effects. Among them, providing physical interaction, co-expression, co-localization, gene enrichment analysis, genetic interaction, and locus prediction are some of the bioinformatics methods used by network integration algorithms.

Results

RA gene expression data acquisition and evaluation of immune cells infiltration

Microarray expression data of rheumatoid arthritis (GSE55235) were downloaded from the GEO database, and genes with a variation coefficient greater than 0.1 were selected for subsequent analysis. We next used the R package "CIBERSORT" to analyze the corresponding expression data in the dataset GSE55235 to ascertain the percentage of various immune cell subtypes in each sample from the dataset. The proportion of each block's seven T cell subtypes was then chosen as the trait data for the WGCNA.

Construction of WGCNA co-expression network

Based on genes with a variation coefficient greater than 0.1 from the GSE55235 dataset, the R package "WGCNA" was used to construct a gene co-expression network. The average association coefficient and Pearson correlation coefficient were then calculated, and cluster analysis on all samples in the dataset was performed. A scale-first network was built using β=7 as the soft threshold rate (Figures 1A, B). To create hierarchical

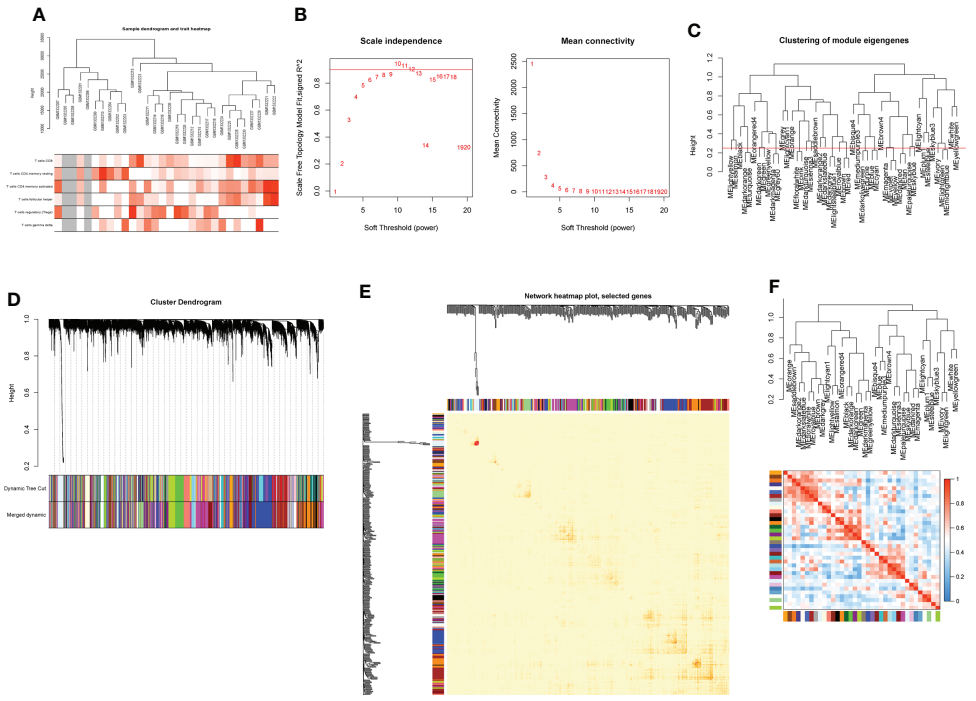

FIGURE 1
Construction of WGCNA co-expression network. **(A)** Sample clustering dendrogram with tree leaves corresponding to individual samples. **(B)** Soft threshold β = 7 and scale-free topological fit index (R2). **(C)** Clustered dendrograms were cut at a height of 0.25 to detect and combine similar modules. **(D)** Shows the original and combined modules under the clustering tree. **(E)** Collinear heat map of module feature genes. Red color indicates a high correlation, blue color indicates the opposite results. **(F)** Clustering dendrogram of module feature genes.

clustering trees, the dynamic hybrid cutting technique was employed. Each branch is a module that combines all genes with similar expression levels, and each leaf represents a gene (Figures 1C, D). Functionally equivalent modules were then combined into one large module, resulting in 37 modules (Figures 1E, F, 2A).

Hub module identification and function enrichment analysis

Among the 37 modules, the blue module had the most significant relationship with CD8+T cell (R^2 = 0.69, P=2E-05). We designate the blue module as the hub module as a result. In this module, potentially important genes that are most closely related to CD8+ T cells were shown to have a stronger correlation. Gene with gene significance >0.75 were selected as candidate central genes, and a total of 10 genes (NKG7, CD8A, DHRS9, CCL5, IL2RG, TNS3, PARP12, GZMA, PRF1, CYTH4) were selected (Figure 2B).

The genes in the blue module were then subjected to GO/KEGG analysis. The findings demonstrated that genes for immune response-activating cell surface receptor signaling pathway, antigen receptor-mediated signaling pathway, and T cell activation in BP were highly enriched in the blue module. The external side of the plasma membrane, MHC protein complex, and secretory membrane were significantly enriched in CC. While cytokine receptor activity, MHC protein complex binding, and SH2 domain binding were significantly enriched in MF (Figure 2C). Module genes were significantly enriched in Epstein-Barr virus infection, Chemokine signaling pathway, Antigen processing, and presentation in KEGG (Figure 2D).

Screening and identification of hub genes

We performed LASSO and random forest analysis on the above-mentioned 10 genes, and a total of 6 genes (NKG7, CD8A, TNS3, PARP12, GZMA, PRF1) were obtained by LASSO analysis (Figure 3A). Genes of Importance >0.6 were selected from RF analysis results for analysis (CD8A, DHRS9, CCL5, IL2RG, TNS3, GZMA, PRF1)(Figure 3B). Next, a Venn diagram was used to visualize the intersection genes of LASSO and RF, and a total of 4 genes (CD8A, TNS3, GZMA, PRF1) were found (Figure 3C). GSE55235 (Figures 4A–C), GSE55457 (Figures 4D–F), GSE55584 (Figures 4G–I), GSE1919 and GSE48780 (Figures 4J–L) were used to identify and validate the correlation between the levels of

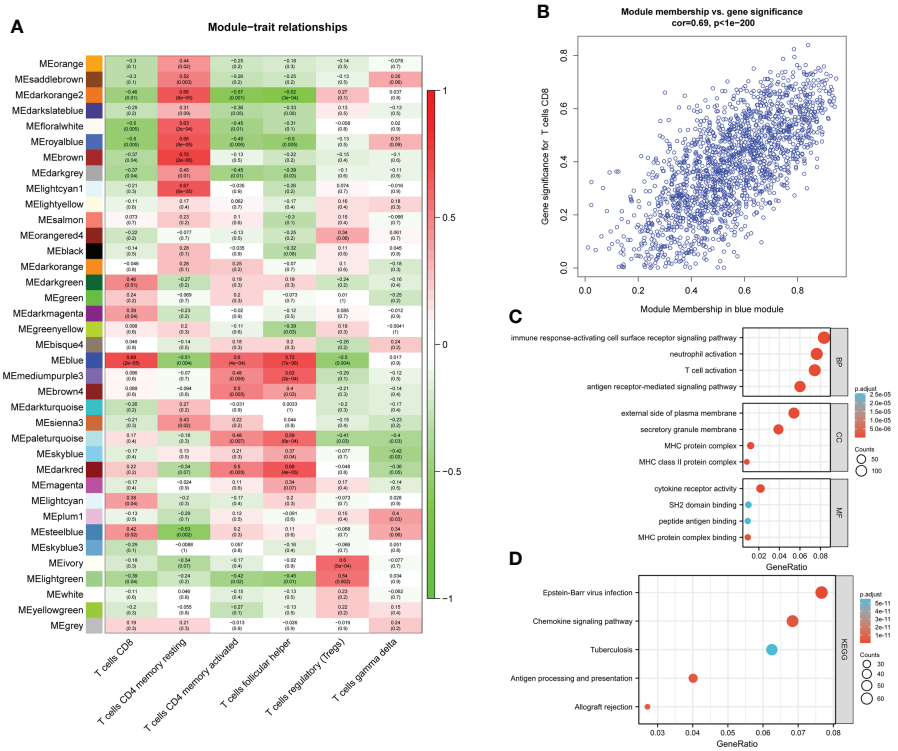

FIGURE 2
module−trait correlations and Functional enrichment analysis **(A)** Heat map of module−trait correlations. Red represents positive correlations and blue represents negative correlations. **(B)** Module Membership in the blue module. **(C)** GO analysis. **(D)** KEGG analysis.

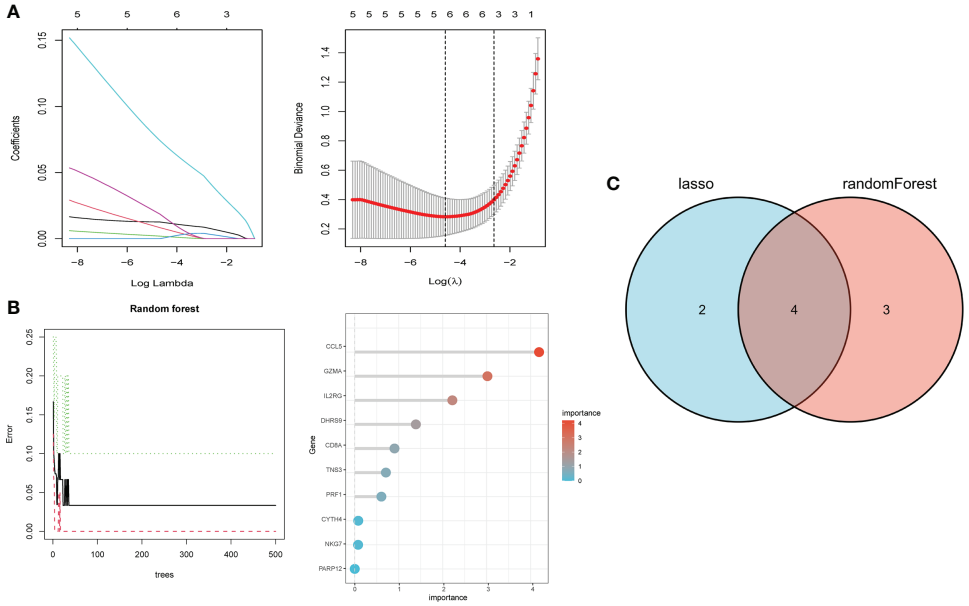

FIGURE 3
Hub gene selection. **(A)** Adjustment of feature selection in the minimum absolute shrinkage and selection operator model (lasso). **(B)** randomForest error rate versus the number of classification trees, the top 20 relatively important genes. **(C)** Three algorithmic Venn diagram screening genes.

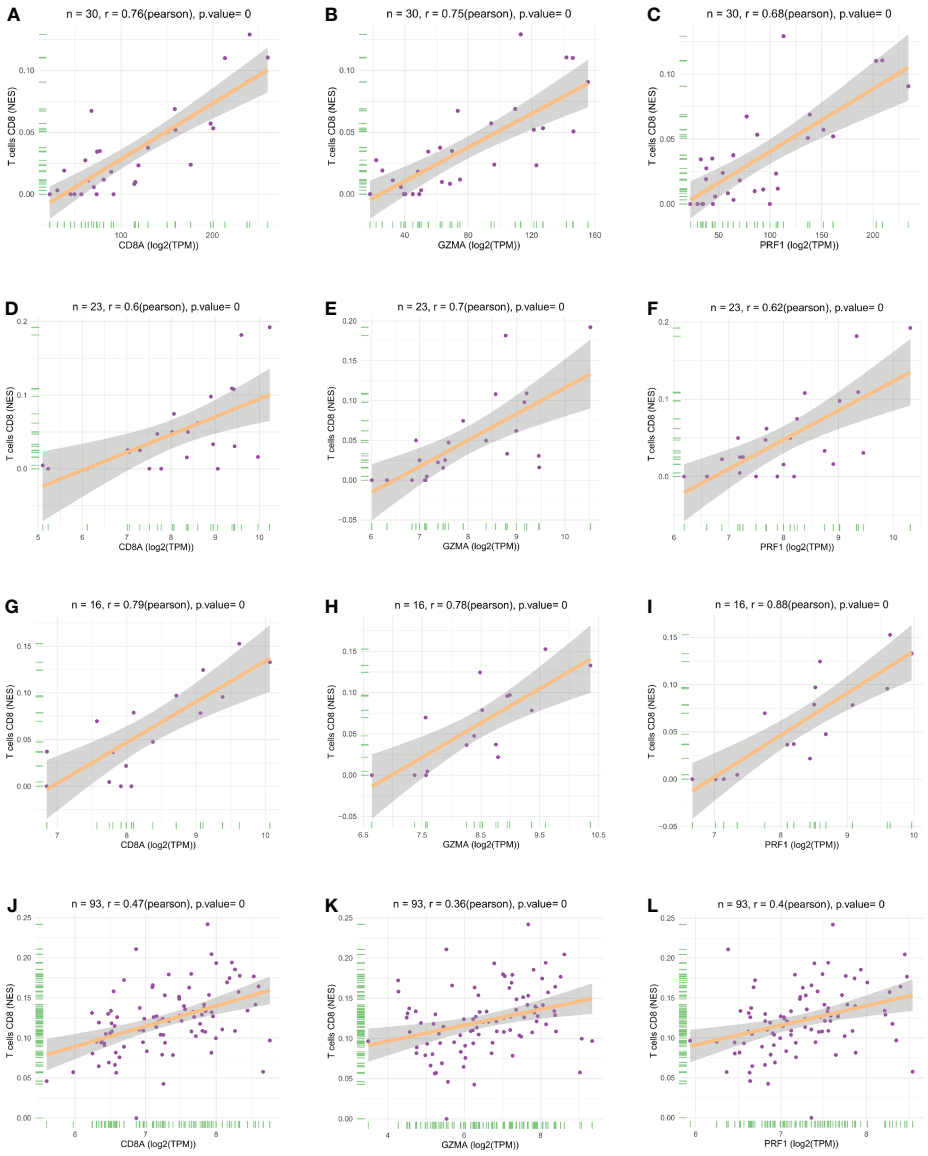

FIGURE 4
Scatter plots of hub gene expression versus CD8+ T cell infiltration levels in three different datasets: **(A-C)** GSE55235. **(D-F)** GSE55457. **(G-I)** GSE55584. **(J-L)** GSE1919 and GSE48780.

CD8A, TNS3, GZMA, PRF1, and CD8+ T-cell infiltration. CD8A, GZMA, and PRF1 were identified as reliable Hub genes. The results of the analysis revealed that three genes were significantly positively correlated with the degree of CD8+ T-cell infiltration in these data sets. At the same time, we selected different cancers for analysis (Figure 4). CIBERSORT, McP-counter, QUANTISEQ, TIMER, and XCELL were employed to explore the expression values of genes related to CD8+T cells in different cancers and utilized the "ggPlot" R package for visualization. It was discovered that CD8A (Figure 5), GZMA (Supplementary Figure 1), and PRF1 (Supplementary Figure 2) were positively correlated with CD8+T cell infiltration in various malignancies. These analyses verified that the identified hub genes play a significant role in the tumor immune microenvironment and are highly correlated with the degree of CD8+ T-cell infiltration.

Hub gene correlation

We verified the correlation between hub genes in different data sets and found that CD8A, GZMA, and PRF1 were significantly positively correlated with each other. The

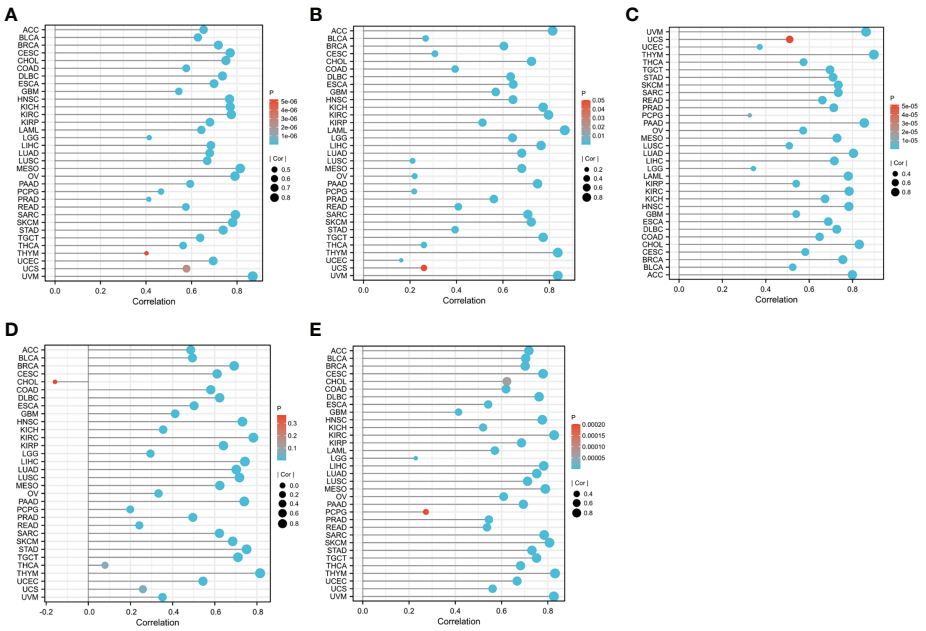

FIGURE 5
The relationship between CD8A expression levels and the degree of CD8+ T cell infiltration in different cancers was statistically significant at P<0.05: **(A)** CIBERSORT. **(B)** MCP-counter. **(C)** QUANTISEQ. **(D)** TIMER. **(E)** XCELL.

relationship between CD8A, GZMA, and PRF1 in various malignancies was then examined (Figure 6). Except for THYM, CD8A, and GZMA being positively correlated in all tumors, CD8A and PRF1 were positively correlated in 33 tumors, and GZMA and PRF1 were positively correlated in 33 tumors (Figures 7A–C). The results of normal tissue analysis showed that CD8A and GZMA were positively correlated in various normal tissues, GZMA and PRF1 were positively correlated in various normal tissues, CD8A and PRF1 were negatively correlated in Bone Marrow, and the rest were positively correlated (Figures 7D–F).

Transcription factor analysis

We identified 8 common transcription factors (EOMES, TBX21, STAT4, ZNF80, GFI1, SCML4, ZNF831, RUNX3) associated with CD8A, GZMA, and PRF1 from the CHEA3 database (Figure 8).

Gene enrichment

To explore the function of the hub gene, we carried out a single gene GSEA analysis. It was found that CD8A was mainly enriched in Rheumatoid arthritis, Asthma, Cholesterol metabolism, etc (Figure 9A). GZEA is mainly enriched in cholesterol metabolism, steroid biosynthesis, mineral absorption, etc (Figure 9B). PRF1 was mainly enriched in Coronavirus disease COVID-19, the Pentose phosphate pathway, and Linoleic acid metabolism (Figure 9C). We then used h.all.v7.5.1.symbols. gmt as a reference gene set to analyze the correlation between genes and genomes. CD8A, GZMA, and PRF1 were found to be positively correlated with interferon gamma response, interferon alpha response, inflammatory response, il6 jak stat3 signaling, complement, and allograft rejection, and negatively with uv response dn, pancreas beta cells, myogensis, cholesterol homeostasis, androgen response, and adipogenesis.

Analysis of hub gene interaction

GeneMANIA database was employed to create PPI networks for Hub genes. To further investigate the function of hub genes, we constructed a 20-gene interaction network (Figure 10A). At the same time, 20 genes were investigated for functional enrichment. Results showed that, in BP, Genes were mainly enriched in T cell-mediated immunity, modification of morphology, or physiology of other organisms. Genes were

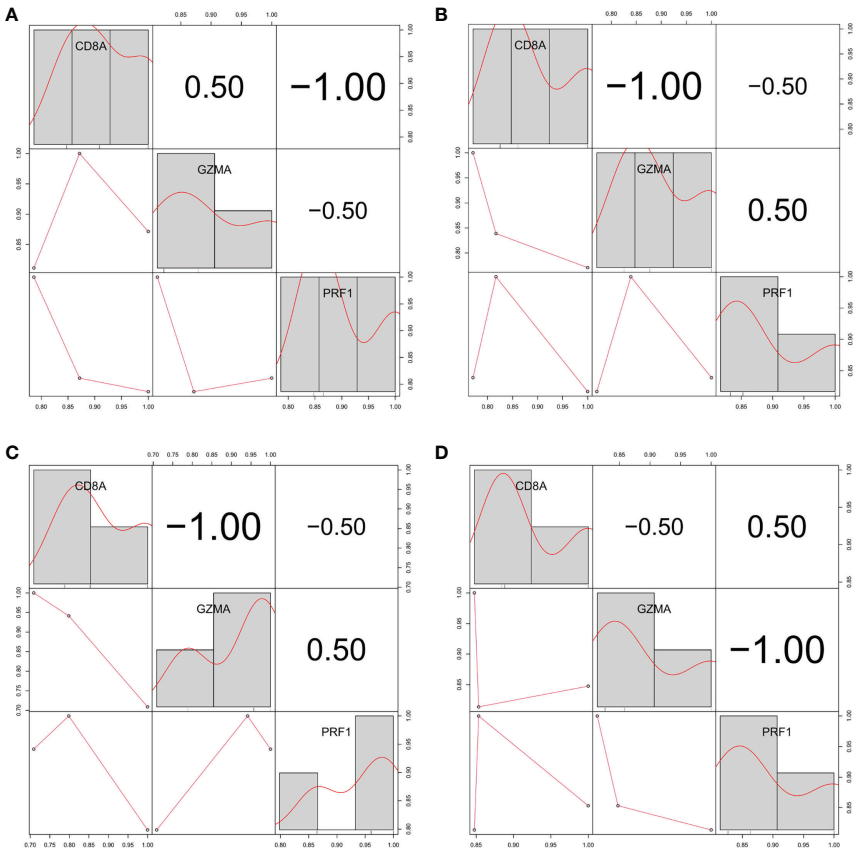

FIGURE 6
Relationship between the expression levels of three hub genes in three different gene sets **(A)** GSE55235. **(B)** GSE55457. **(C)** GSE55584. **(D)** GSE1919 and GSE48780.

mainly enriched in immunological synapses and the external side of the plasma membrane under CC. In MF, genes were mainly enriched in phospholipase activator activity and lipase activator activity (Figure 10B). Genes in the KEGG pathway may regulate Primary immunodeficiency, Viral protein interaction with cytokine, and cytokine receptor. These findings lead us to hypothesize those hub genes are crucial for the immune system (Figure 10C).

Discussion

RA is a chronic progressive autoimmune disease with an increasing incidence that cause great damage to patients' exercise and labor ability. But unfortunately, there are still no effective intervention measures in clinical practice, and there is also a lack of precise diagnosis and treatment methods. A large number of previous studies have shown that CD8+ T cells play an extremely important role in the progression of RA and the clinical outcome of patients (10, 24). Based on this, genes in the GSE55235 dataset were combined with WGCNA and a variety of machinery. The learning algorithm finally obtained three core genes closely related to CD8+ T cells, namely CD8A, GZMA, and PRF1. We further performed validation and analysis on these three genes.

Interestingly, we found that these three core genes were mentioned in previous studies in RA, among which GZMA has the most relevant studies. GZMA is a member of the serine protease family, mainly derived from NK cells and T cells, and plays an important regulatory role in cell death and the release of inflammatory mediators (25). As early as a clinical study in 1999 (26), GZMA was found to be highly expressed in the serum and synovial tissue of RA patients, and in 2017, Llipsy Santiago et al. found in mice that knocking out GZMA can reduce osteoclasts.

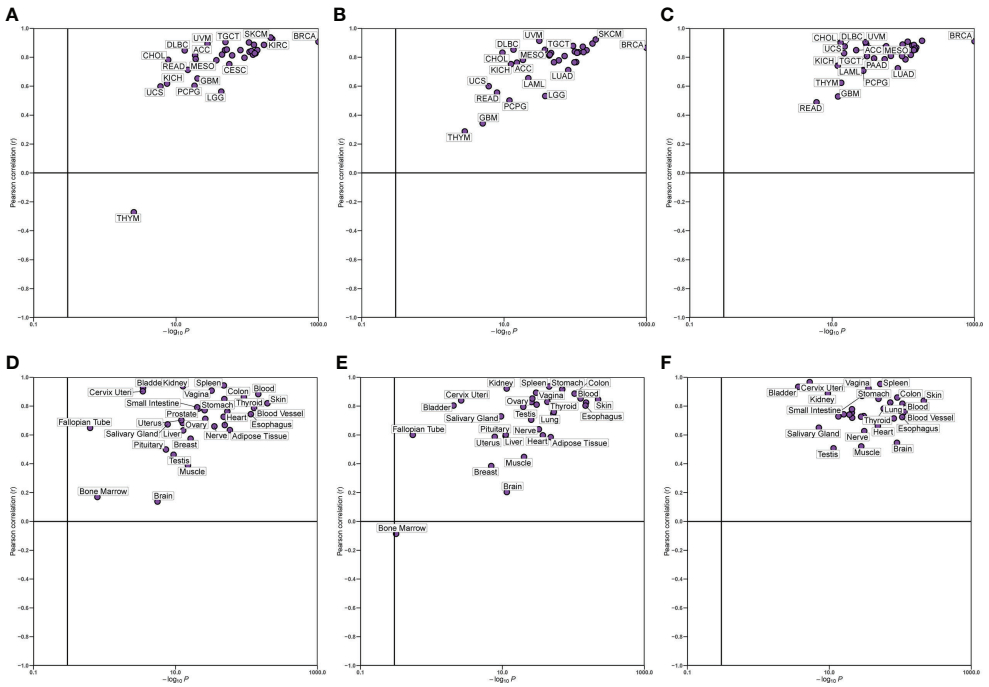

FIGURE 7
Relationship between the expression levels of three hub genes. **(A-C)** In different cancer types. **(D-F)** In normal tissues.

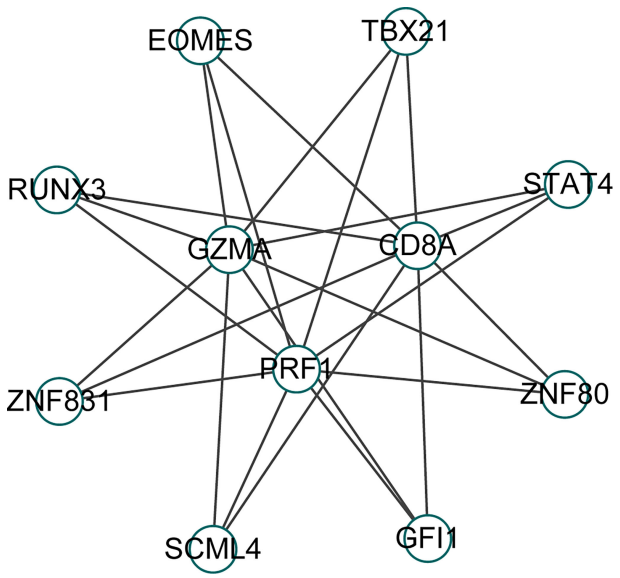

FIGURE 8
Prediction of transcription factors of Hub gene in CHEA3 database.

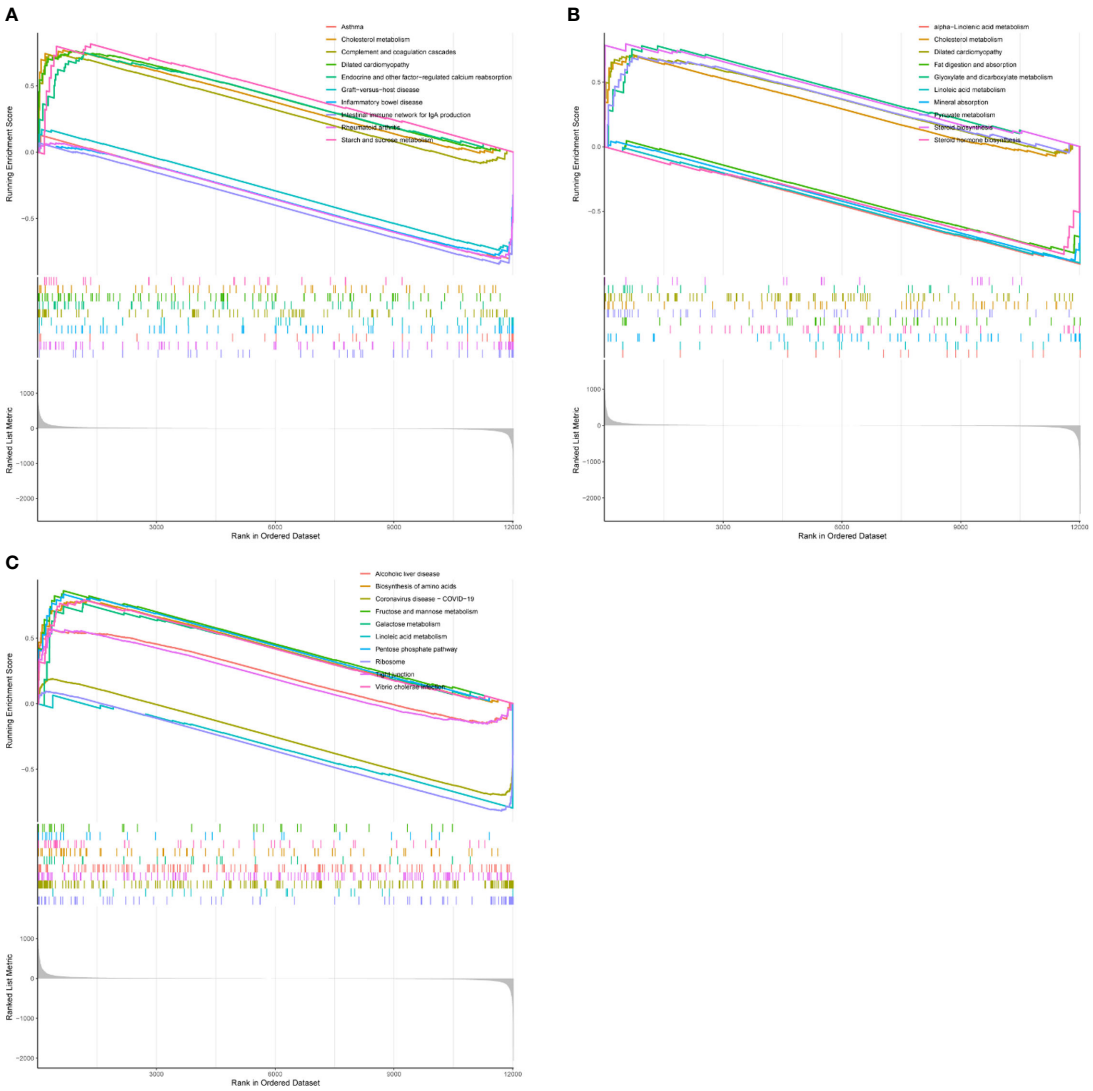

FIGURE 9
GSEA analysis of hub gene. **(A)** CD8A. **(B)** GZMB. **(C)** PRF1.

Active and efficient tissue production of collagen-induced arthritis in mice (a mouse model of RA) (27). More interestingly, the role of GZMA is not limited to RA. Abnormal expression of GZMA has been found in autoimmune diseases such as SLE and Sjögren's syndrome, which shows that the gene has a very objective intervention value (28, 29). For CD8A, this gene is currently considered to be one of the key genes in the differential diagnosis and prognosis prediction of RA in the bioinformatics analysis conducted by several research teams (30, 31), while the study by Cai-Yue Gao et al. found that knocking out the CD8A gene can promote the damage of salivary glands in Sjögren's syndrome mice, which may also be an important mechanism for resident CD8+ T cells to induce joint synovial damage in RA (32). PRF1 belongs to the perforin family of genes. The study by Lan Wang et al. showed that PRF1 is important for rheumatoid disease, while the study by Zoya Qaiyum et al. found the abnormal expression of this gene in the gene map of ankylosing spondylitis (33).

In addition, the relationship between these three key genes and 33 cancer species was also explored in this study. We

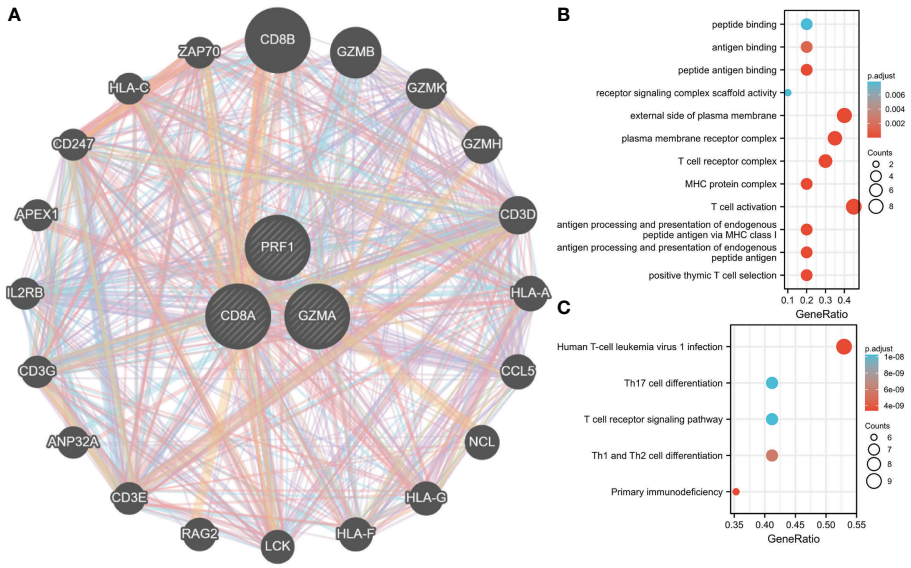

FIGURE 10
Interaction analysis of Hub genes. **(A)** Characterized gene co-expression network. **(B)** GO analysis of co-expressed genes. **(C)** Co–expressed gene KEGG analysis.

found that the CD8A gene is positively associated with a variety of cancers, a result also confirmed by other research groups. For example, Chirag Krishna et al. found that in clear cell renal cell carcinoma, patients with high CD8A expression had more severe ICB resistance symptoms and tumor-associated macrophage infiltration (34); while Bruno Sangro et al. The CD8A gene is a good prognostic predictor in nivolumab-treated patients with advanced hepatocellular carcinoma (35). Similarly, Zhiwei Zhou et al. showed that GZMA can cleave GSDMB and induce tumor cell pyroptosis in an IFN-γ-dependent manner (36). Moreover, PRF1 has also been shown to play a role in the prognosis and progression of various cancers including breast and colon cancer (37, 38). These studies illustrate the important roles of these three genes in human diseases from another dimension.

Since our research is based on bioinformatics methods, it may be subject to the bias of the analysis results due to the quality of the samples in the database. Therefore, more *in vivo* or *in vitro* experiments are needed to further verify the results.

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 authors.

Author contributions

ZZ and JR planned the research concept and designed it, made provisions for study material, collected data and analyzed them, and wrote and approved the manuscript. SX searched for data and wrote programming code. LZ and QZ collected pictures and graphs as well as wrote response letters. SZ and DZ collected data and analyzed them, wrote and approved, and helped correct the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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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.2022.1044909/full#supplementary-material

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Bidirectional two-sample Mendelian randomization study of causality between rheumatoid arthritis and myocardial infarction

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Background: Epidemiological evidence suggests an association between rheumatoid arthritis (RA) and myocardial infarction (MI). However, causality remains uncertain. Therefore, this study aimed to explore the causal association between RA and MI.

Methods: Using publicly available genome-wide association study summary datasets, bidirectional two-sample Mendelian randomization (TSMR) was performed using inverse-variance weighted (IVW), weighted median, MR-Egger regression, simple mode, and weighted mode methods.

Results: The MR results for the causal effect of RA on MI (IVW, odds ratio [OR] = 1.041, 95% confidence interval [CI]: 1.007–1.076, $P = 0.017$; weighted median, OR = 1.027, 95% CI: 1.006–1.049, $P = 0.012$) supported a causal association between genetic susceptibility to RA and an increased risk of MI. MR results for the causal effect of MI on RA (IVW, OR = 1.012, 95% CI: 0.807–1.268, $P = 0.921$; weighted median, OR = 1.069, 95% CI: 0.855–1.338, $P = 0.556$) indicated that there was no causal association between genetic susceptibility to MI and an increased risk of RA.

Conclusion: Bidirectional TSMR analysis supports a causal association between genetic susceptibility to RA and an increased risk of MI but does not support a causal association between genetic susceptibility to MI and an increased risk of RA.

KEYWORDS

rheumatoid arthritis, myocardial infarction, bidirectional, two-sample Mendelian randomization study, causal association

1 Introduction

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease that affects several tissues and organs and causes chronic synovial inflammation, eventually leading to joint destruction, chronic disability, and reduced life expectancy (1). RA results from the interaction of genetic susceptibility, environmental factors, and immune factors,

among which genetic factors determine 50–60% of the risk of RA (2, 3). Myocardial infarction (MI) is a cardiovascular disease in which the formation of plaques on the inner walls of the arteries leads to a decrease in blood flow to the heart, and long-term ischemia and hypoxia result in the death of myocardial cells (4, 5). Some observational studies have shown a close relationship between RA and MI. A cross-sectional study by Dougados et al. (6) of 4,586 RA patients enrolled in 17 countries showed a high prevalence of comorbidities among RA patients, with 6% (95% confidence interval [CI] 5.3%–6.8%) having a history of MI or stroke. In a 10-year cohort study, Lindhardsen et al. (7) found that patients with RA had an increased risk of MI of approximately 70% compared to the general population after adjusting for factors such as sex, age, and socioeconomic status. Among post-MI patients, those with RA have a poor prognosis and an increased risk of death, which is positively correlated with RA duration and steroid dosage (8). A systematic review and meta-analysis showed (9) that the risk of MI increased significantly in patients with RA (relative risk: 1.69, 95% CI 1.50–1.90). However, a cohort study by Rostami et al. (10) found that the weighted genetic risk score of RA had contributed little to the morbidity risk of MI.

Causal inferences from observational studies are susceptible to bias owing to reverse causality and potential confounders (11), which weakens our understanding of the causal association between RA and MI. Randomized controlled trials (RCTs) are the gold standard for causal inferences in epidemiological studies. Some RCTs are difficult to perform owing to medical ethics, subject selection, and extrapolation of results. Mendelian randomization (MR) is a technique that uses genetic variation as an instrumental variable (IV) to assess whether an observational association between exposure factors and outcomes is consistent with a causal effect (12). Genetic variation is not affected by the external environment, social behavior, or other factors, and it is a long-term and stable exposure factor. MR can avoid the effect of confounding factors and reverse causal association on the correlation effects in observational studies, and minimize bias. Published data were collected in this study and bidirectional TSMR analysis was used to determine whether there was a bidirectional causal association between RA and MI.

2 Materials and methods

2.1 Data sources

Relevant genome-wide association study (GWAS) datasets were obtained from the IEU OpenGWAS project (https://gwas.mrcieu.ac.uk). The GWAS dataset for RA was derived from GWAS analysis and included 13,838 cases and 33,742 controls of European ancestry (13). The GWAS dataset for MI was derived from another GWAS analysis and included 14,825 cases and 44,000 controls of European ancestry (14) (Supplementary Table 1).

2.2 Screening of IVs

Single nucleotide polymorphisms (SNPs) were used as IVs. P-value ($P<5.0\times10^{-8}$) was set. To avoid linkage disequilibrium (LD) bias, LD with significant SNPs associated with exposure factors must meet the following conditions: $r^2<0.001$, and genetic distance of 10000 kb. Significant SNPs associated with exposure factors were extracted in the GWAS dataset of outcome variables, and the resulting IVs were recorded with information on the effect allele, allele effect sizes (beta), standard error, and p-value. The F-statistic was used to test the strength of each IV and was calculated using the following formula: $F = R^2(N-2)/(1-R^2)$, where R^2 is the proportion of the exposure factor variation explained by each IV, and N is the sample size of the expourse dataset (15). When F>10, there is no weak IVs bias (16).

2.3 Research design

To better estimate the causal effect, three key assumptions should be met when SNPs are used as IVs in the TSMR analysis (17) (1): IVs must be closely related to exposure factors; (2) IVs are independent of confounding factors; and (3) IVs can only influence the outcome through exposure and not through other pathways.

2.4 Statistical analysis

Summary statistics for the exposure and outcome datasets were harmonized such that the effect of SNPs on exposure and the effect of SNPs on outcome corresponded to the same alleles. TSMR analyses using inverse-variance weighted (IVW), weighted median, MR-Egger regression, simple mode, and weighted mode methods were performed to infer causal associations. We used the IVW as the primary method for MR. When each genetic variation met the IV hypothesis, the IVW method combined the Wald ratio estimates of the causal effects of different SNPs and provided a consistent estimate of the causal effect of exposure on the outcome (18). The results of the IVW method were most reliable when there was no horizontal pleiotropy of the IVs (19). When at least half of the SNPs are effective IVs, the weighted median can provide a consistent estimate of the causal effect (20). MR-Egger regression is used to confirm whether horizontal pleiotropy of IVs exists, and its intercept represents the effect estimate of horizontal pleiotropy (21). When the IVs have horizontal pleiotropy, the MR-Egger regression can still obtain an unbiased estimation of causal association. The weighted median method improves the accuracy of the results compared to the MR-Egger method (22). Simple mode and weighted mode were performed as complementary analyses (23). The Mendelian randomization pleiotropy residual sum and outlier (MR-PRESSO) test was used to detect and correct horizontal pleiotropy by removing the outliers (24). MR power analysis was performed using an online tool (http://cnsgenomics.com/shiny/

mRnd/) (25). Statistical analysis was performed using R (version 4.1.0) and R packages (TwoSampleMR and MR-PRESSO). The test level α was 0.05 ($P < 0.05$), and the difference was statistically significant.

3 Results

3.1 Causal effects of RA on MI

3.1.1 SNPs: Basic information

RA was the exposure factor, and MI was the outcome variable. In total, 15 SNPs were screened and identified as IVs, with F values greater than 10. The variance explained by these IVs was 44% for RA (Supplementary Table 2). The intercept of the MR-Egger regression can be used as an indicator to test whether horizontal pleiotropy of the IVs influences the results of TSMR analysis. The intercept was close to 0 (Egger intercept = 0.006, $P = 0.384$) (Table 1), indicating that there was no horizontal pleiotropy of the IVs, and it was unlikely to influence the results of the TSMR analysis (Figure 1B).

3.1.2 Two-sample Mendelian randomization analysis

The MR results supported a causal association between genetic susceptibility to RA and an increased risk of MI. The MR analysis had 81% statistical power. In the absence of horizontal pleiotropy of IVs, IVW was used as the primary method to estimate the causal association between genetic susceptibility to RA and an increased risk of MI (IVW result: OR = 1.041, 95% CI: 1.007–1.076, $P = 0.017$). Results of the other methods included: MR-Egger, OR = 1.023, 95% CI: 0.973–1.076, $P = 0.389$; weighted median, OR = 1.027, 95% CI: 1.006–1.049, $P = 0.012$; simple mode, OR = 1.016,

TABLE 1 Heterogeneity test and Horizontal pleiotropy test.

Exposure	Outcome	Heterogeneity test (MR-Egger)			Heterogeneity test (IVW)			Horizontal pleiotropy test (MR-Egger)	
		Cochran's Q	Q_df	*P*	Cochran's Q	Q_df	*P*	Intercept	*P*
RA	MI	51.36	13	1.74E-06	54.57	14	1.03E-06	0.006	0.384
MI	RA	14.62	7	0.041	15.18	8	0.056	-0.01	0.621

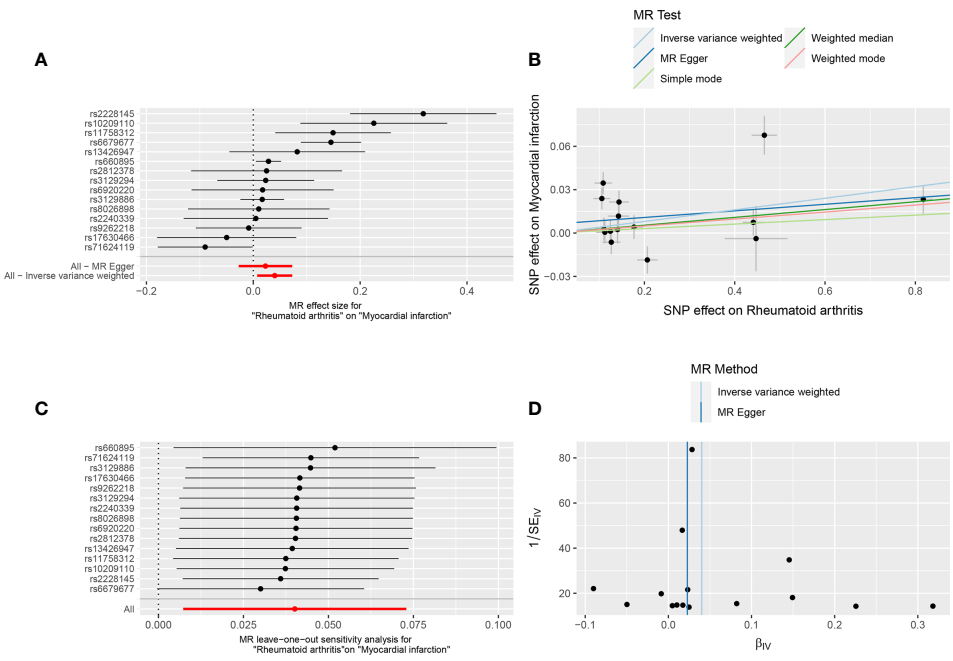

FIGURE 1
Forest plot **(A)**, scatter plot **(B)**, sensitivity analysis **(C)**, and funnel plot **(D)** of the effect of RA on MI.

95% CI: 0.968–1.065, P = 0.534; and weighted mode, OR = 1.024, 95% CI: 1.003–1.046, P = 0.042 (Table 2, Figures 1A, B).

3.1.3 Heterogeneity test and sensitivity analysis

IVW and MR-Egger regression analyses were used to detect heterogeneity between IVs. Heterogeneity was quantified using Cochran's Q test. $P < 0.05$ indicated significant heterogeneity. If there was heterogeneity between the IVs, the random-effects IVW model was used to estimate causal effects (26). MR-Egger regression (Cochran's Q = 51.36, P = 1.74E-06) and IVW (Cochran's Q = 54.57, P = 1.03E-06) (Table 1, Figure 1D) indicated that there was heterogeneity between the IVs, and the random-effects IVW model was used to estimate the causal effect (P = 0.017). The MR-PRESSO test was used to remove the outlier SNPs (rs2228145, rs6679677, and rs71624119) and to estimate the causal effect of TSMR after correction for outliers (P =0.019) (Supplementary Table 3).

The sensitivity analysis used the leave-one-out method to remove SNPs one by one, and the causal effects of the remaining SNPs were compared with the TSMR analysis results of all SNPs to determine whether the causal association was due to a single IV, indicating that the TSMR analysis results were robust (Figure 1C).

3.2 Reverse TSMR analysis

In reverse TSMR, MI was the exposure factor, and RA was the outcome variable. In total, 9 SNPs were screened and identified as IVs, with F values greater than 10. The variance explained by these IVs was 2.6% for MI (Supplementary Table 4). The horizontal pleiotropy test (Egger intercept = −0.01, P = 0.621) (Table 1) indicated that there was no horizontal pleiotropy for the IVs. MR results did not support a causal association between genetic susceptibility to MI and an increased risk of RA (IVW, OR = 1.012, 95% CI: 0.807 – 1.268, P = 0.921). Results of other methods included: MR-Egger, OR = 1.112, 95% CI: 0.723–1.710, P = 0.643; weighted median, OR = 1.069, 95% CI: 0.855–1.338, P = 0.556; simple mode, OR = 1.161, 95% CI: 0.763–1.765, P = 0.505; and weighted mode, OR = 1.095, 95% CI: 0.871–1.377, P = 0.458 (Table 3, Figures 2A, B). Among the heterogeneity test results, MR-Egger regression showed relatively small heterogeneity (Cochran's Q =14.62, P = 0.041), whereas IVW (Cochran's Q = 15.18, P = 0.056) did not find heterogeneity between IVs (Table 1, Figure 2D). MR-PRESSO indicated that there was no horizontal pleiotropy for the global test (RSS_{obs} = 18.31, P = 0.115) (Supplementary Table 3), and no outliers were observed. The leave-one-out method was used for sensitivity analysis, and the results of the TSMR analysis were reliable (Figure 2C).

4 Discussion

This study used the bidirectional TSMR method to analyze published GWAS datasets and determine whether a bidirectional causal association exists between RA and MI in the European population. Our results supported a causal association between genetic susceptibility to RA and an increased risk of MI (IVW, OR = 1.041, 95% CI: 1.007 – 1.076, P = 0.017). However, our results did not support a causal association between genetic susceptibility to MI and an increased risk of RA (IVW, OR =1.012, 95% CI: 0.807 – 1.268, P = 0.921). In the sensitivity analysis, the MR results were robust and reliable.

TABLE 2 Mendelian randomization analysis of causal association between RA and the risk of MI.

Methods	SNPs	Beta	SE	OR (95%CI)	*P*
MR-Egger	15	0.023	0.026	1.023 (0.973,1.076)	0.389
weighted median	15	0.027	0.011	1.027 (1.006,1.049)	0.012
IVW	15	0.040	0.017	1.041 (1.007,1.076)	0.017
Simple mode	15	0.015	0.024	1.016 (0.968,1.065)	0.534
Weighted mode	15	0.024	0.011	1.024 (1.003,1.046)	0.042

TABLE 3 Mendelian randomization analysis of causal association between MI and the risk of RA.

Methods	SNPs	Beta	SE	OR (95%CI)	*P*
MR-Egger	9	0.106	0.219	1.112 (0.723,1.710)	0.643
weighted median	9	0.067	0.114	1.069 (0.855,1.338)	0.556
IVW	9	0.011	0.115	1.012 (0.807,1.268)	0.921
Simple mode	9	0.149	0.214	1.161 (0.763,1.765)	0.505
Weighted mode	9	0.091	0.117	1.095 (0.871,1.377)	0.458

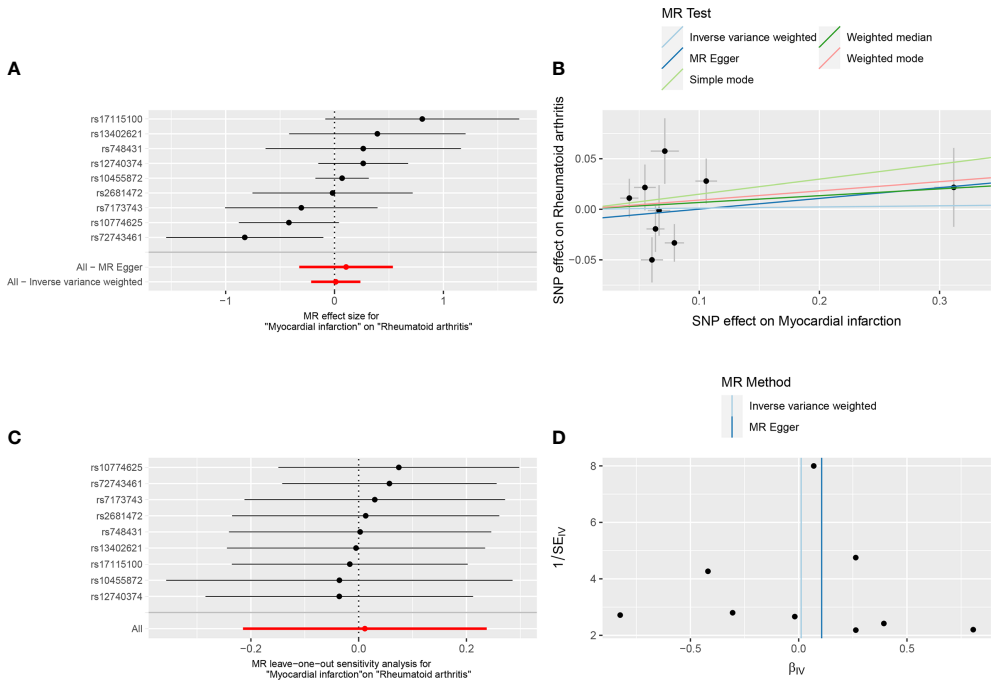

FIGURE 2
Forest plot **(A)**, scatter plot **(B)**, sensitivity analysis **(C)**, and funnel plot **(D)** of the effect of MI on RA.

Several possible reasons have been proposed to explain the association between RA and MI in observational studies. First, MI in RA may be associated with RA-related inflammatory reactions (27). Inflammation can promote the development of atherosclerotic plaques, increase vulnerability, and promote thrombosis (28). Acute phase reactants in patients with RA cause synovitis, and elevated levels of inflammatory cytokines such as tumor necrosis factor α (TNF-α) and interleukin 6 (IL-6) induce atherosclerotic changes and endothelial function damage, leading to an increased risk of MI (29). The TNF-α-induced signaling pathway plays an important role in cellular responses to inflammation and injury, leading to vascular dysfunction and adverse reactions to cardiac remodeling after MI (30). A case-control study showed (31) that the plasma concentrations of IL-6 and IL-6 binary complexes could predict the risk of MI. Early active RA treatment can better control the inflammation. Glucocorticoids (GC) are commonly used as immunosuppressive agents for the treatment of RA. Their anti-inflammatory and immunosuppressive effects can reduce the damage to blood vessels caused by inflammation. However, the long-term use of GC leads to a series of adverse reactions, including cardiovascular diseases (32, 33). A case-control study by Wilson et al. (34) showed that an increase in the cumulative and average daily GC doses was associated with an increased risk of MI. A cohort study by Pujase-Rodriguez et al. (33) showed that even at a lower dose of GC (< 5 mg), the risk of MI increased. Second, there is an increased risk of cardiovascular risk factors (hypertension, dyslipidemia, and diabetes) in RA patients, which, together with inflammation, can promote the formation of atherosclerosis in RA patients and may further contribute to the development of MI (35). Third, genetic factors may also play a role in the association between RA and MI. Vascular endothelial growth factor, a promoter of normal and abnormal angiogenesis, plays an important role in RA pathogenesis (36). Studies by Chen et al. (37) have shown that SNPs in the vascular endothelial growth factor A promoter regions (-2578 and -460) are associated with an increased risk of MI in patients with RA. Palomino-Morales et al. (38) found that the IL6 -174 gene polymorphism is associated with subclinical atherosclerosis, and RA patients with the IL6 -174GG genotype have severe endothelial dysfunction. Methylene tetrahydrofolate reductase 1298 A>C gene polymorphism increases the risk of atherosclerosis in patients with RA (39). Our MR results did not reveal a causal association between genetic susceptibility to MI and an increased risk of RA, and more studies should be conducted in the future.

MR uses genetic variation to estimate the health consequences of the phenotypes affected by these genetic variations (40). This is a relatively novel epidemiological approach that uses genetic variation to infer the causal association between exposure factors and outcome variables. MR provides a way to investigate associations without the typical

biases inherent in observational epidemiological studies, such as reverse causal association and potential confounders. Our results differ from the MR results of Fokina et al. (41), probably because of the different GWAS datasets selected, and newer, larger GWAS studies will be necessary in the future.

This study had some limitations. First, the MR results were based on the European population, and extrapolation of the results is limited. Whether a causal relationship exists in other populations needs to be confirmed by further research. Second, SNPs used for analysis may be correlated with other traits due to genetic polymorphisms and generate confounding bias, which may affect causal inference. Third, the strength of the IV depends on the sample size of the GWAS, and a larger scale GWAS is required to determine more genetic variation for MR.

5 Conclusion

In summary, bidirectional TSMR analysis supports a causal association between genetic susceptibility to RA and an increased risk of MI, but does not support a causal association between genetic susceptibility to MI and an increased risk of RA. However, due to the limitations of the study, further research is necessary.

Data availability statement

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

Author contributions

H-YG: conceptualization, investigation, data curation, writing - original draft. WW: conceptualization, investigation, writing - original draft. HP: investigation, writing - review and editing. HY: conceptualization, writing - review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1017444/full#supplementary-material

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Causal association between systemic lupus erythematosus and the risk of dementia: A Mendelian randomization study

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Introduction: It is well-documented that systemic lupus erythematosus (SLE) is associated with dementia. However, the genetic causality of this association remains unclear. Mendelian randomization (MR) was used to investigate the potential causal relationship between SLE and dementia risk in the current study.

Methods: We selected 45 single nucleotide polymorphisms (SNPs) associated with SLE from publicly available genome-wide association studies (GWAS). Summary level statistics were obtained from the dementia GWAS database. MR estimates were performed using the inverse variance weighted (IVW) method, MR-Egger method and weighted median (WM) method. Cochran's Q test, the intercept of MR-Egger, MR-Pleiotropy Residual Sum and Outlier method, leave-one-out analysis and funnel plot were applied for sensitivity analyses.

Results: No significant causal association was found between SLE and any type of dementia, including Alzheimer's disease, vascular dementia, frontotemporal dementia, and dementia with Lewy bodies. These findings were robust across several sensitivity analyses.

Conclusion: Overall, our findings do not support a causal association between SLE and dementia risk.

KEYWORDS

systemic lupus erythematosus, dementia, causality, Mendelian randomization, risk

Introduction

Dementia is a common neurodegenerative disease with clinical manifestations as a severe decline in cognitive function leading to disruptions in family, occupational and daily life (1). The worldwide prevalence of dementia is estimated to be as high as 7% in the population over age 65 (2). This undoubtedly imposes an immense financial and healthcare burden on individuals, families, medical institutions and society. Alzheimer's disease (AD) is the most common type of dementia, which accounts for approximately 50%-70% of dementia cases. Other common types of dementia include dementia with Lewy bodies (DLB), vascular dementia (VaD), frontotemporal dementia (FTD), and mixed dementia (3, 4). It is well-accepted that the interaction of advanced age, genetic factors, environmental triggers, and metabolic disorders contribute to the initiation and development of dementia (5, 6).

Systemic lupus erythematosus (SLE) is a chronic, systemic autoimmune disease characterized by autoantibody production and multisystem inflammation, predominantly affecting women of childbearing age (7). In recent decades, the prevalence ranges from 20 to 150 cases per 100,000 population and has been increasing yearly (8). SLE has wide clinical heterogeneity and is defined as neuropsychiatric lupus (NPSLE) when it is associated with neurological and psychiatric symptoms (9). The American College of Rheumatology (ACR) defined nineteen NPSLE syndromes in the late 20^{th} century, such as seizures, cerebrovascular disease, anxiety disorders, movement disorders and cognitive dysfunction. Of these, cognitive impairment is the most common which comprises one or more clinical manifestations, such as decreased attention, memory loss, and word-finding difficulties (10–12). This is similar to the American Psychiatric Association (APA) definition of dementia (13). A meta-analysis involving 11 observational studies reported a significantly increased risk of dementia in SLE patients (14). However, owing to the potential biases from residual confounding and the possibility of reverse causality, the genetic causality of this association remains unclear.

Indeed, previous epidemiological studies have shown powerful associations between a variety of risk factors and disease, whereas subsequent studies have demonstrated that these associations are due to interference from residual confounding factors rather than causal associations. Some typical examples include associations between vitamin E and atherosclerotic cardiovascular disease (15, 16), and β-carotene and lung cancer (17, 18). With the recent increased availability of genome-wide association studies (GWAS) databases, mendelian randomization (MR) research has received much attention. The evidence level of the MR studies sits at the interface of randomized controlled trials (RCTs) and observational studies (19), it can mimic an RCT and promise to be a robust statistical approach using instrumental variables (IVs) to clarify the causal association between exposure factors and disease (20). Causality in conventional observational studies is susceptible to interference by potential confounding factors and reverse causality. In MR analysis, alleles are randomly assigned from parents to offspring based on Mendel's law of inheritance (21). Therefore, offspring genotypes are hardly associated with confounding factors. Additionally, MR analysis was able to avoid the problem of reverse causality since genotypes precede exposure in time (22, 23).

In the present study, we performed a two-sample MR analysis using the GWAS database to examine the genetic causality between SLE and common types of dementia risk.

Methods

Study design

We used the publicly available GWAS catalog to conduct a two-sample MR study. No additional ethical approval was required due to the re-analysis of previously summary-level data. Two-sample MR (version 0.5.5) and R (version 4.2.1) were used for MR analysis.

The MR analysis is based on the following three core hypotheses: 1) The selected IVs must be significantly associated with exposure (SLE) (24). We calculate the F-statistic to assess the strength of each genetic instrument. The following formula determines the F-statistic: $F=R^2\times(N-2)/(1-R^2)$; $R^2 = 2\times EAF\times(1-EAF)\times\beta^2$ (25). In this formula, R^2 refers to the cumulative explained variance of the selected IVs on SLE and EAF refers to the effect allele frequency, β refers to the estimated effect of SNP, and N refers to the sample size of the GWAS. If the F-statistic is greater than 10, the IV has a strong potential to predict dementia. 2) The selected IVs are not allowed to affect the outcome (dementia) through other pathways, only through specified exposure (SLE) (26). 3) Confounding factors are not associated with the selected IVs. The overview of the research design is shown in Figure 1.

GWAS data for systemic lupus erythematosus

We extracted single nucleotide polymorphisms (SNPs) from the GWAS database as genetic IVs (24). The significant SNPs associated with SLE ($P<5\times10^{-8}$) were obtained from the latest and most extensive GWAS database, including 14,267 individuals of European ancestry (5,201 cases and 9,066 controls) (27) (Table 1). In order to avoid the potential bias caused by strong linkage disequilibrium (LD), we selected SNPs with $LDr^2 < 0.001$.

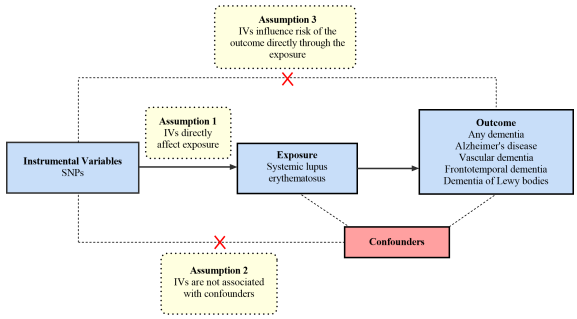

FIGURE 1
An overview of the study design. SNP, single nucleotide polymorphisms.

GWAS data for dementia

GWAS summary data for AD were obtained from an MR study with 954 cases and 487,331 control from the population of European ancestry (28). Summary-level GWAS data with VaD were extracted from the Finn consortium, including 212,389 participants of European ancestry (881 cases and 211,508 controls). Summary statistics for FTD from an international multicenter study comprising 515 cases and 2,509 controls of European ancestry (29). The GWAS data for DLB were derived from another independent GWAS multicenter study with a total of 2,591 cases and 4,027 controls (30). The GWAS summary data in our study are detailed in Table 1.

Statistical analysis

MR estimates of SLE for the risk of dementia were calculated using the inverse variance weighting (IVW) method, weighted median (WM) method and MR-Egger method. The IVW method is the major MR analysis in our study, and it applies a meta-analysis method to integrate the Wald ratio of individual SNPs, which can be assumed that IVs can only influence outcomes through specified exposure. If there is no horizontal pleiotropy, the IVW method is able to achieve unbiased causal estimates (31). Therefore, the IVW method provides the most accurate assessment (32). The WM method and MR-Egger method were applied to the complement of analysis to investigate the bias due to ineffective IV and horizontal pleiotropy effects (33). The estimates of the MR-Egger method are probably inaccurate due to the influence of outlying genetic variants (34). The WM method has a relatively small bias, while its precision is lower, particularly the percentage of IVs with horizontal pleiotropy < 50% (35).

Sensitivity analysis is essential to evaluate potential heterogeneity and horizontal pleiotropy. Cochran's Q test was performed to assess the heterogeneity of effect sizes for selected genetic IVs. The MR-Pleiotropy Residual Sum and Outlier method (MR-PRESSO) analysis was also applied to exclude outliers and moderate horizontal pleiotropy (35). The intercept derived from MR-Egger regression was employed to evaluate vertical pleiotropy (36). Leave-one-out analysis was conducted to explore the effect of removing one of the selected individual SNPs on the overall results (37).

Process of MR analysis

Our MR research was conducted according to the guideline of the STROBE-MR Statement (38). The flow chart of the MR process is shown in Figure 2.

TABLE 1 Details of the GWAS included in the Mendelian randomization.

Year	Trait	Population	Cases	Controls	Samplesize	Websource
2015	Systemic lupus erythematosus	European	5,201	9,066	14,267	DOI: 10.1038/ng.3434
2021	Any dementia	European	7,284	209,487	216,771	www.finngen.fi/en
2022	Alzheimer's disease	European	954	487,331	488,285	DOI: 10.3390/nu14091697
2021	Vascular dementia	European	881	211,508	212,389	www.finngen.fi/en
2010	Frontotemporal dementia	European	515	2,509	3,024	DOI: 10.1038/ng.536
2021	Dementia with Lewy bodies	European	2,591	4,027	6,618	DOI: 10.1038/s41588-021-00785-3

GWAS, Genome-Wide Association Studies.

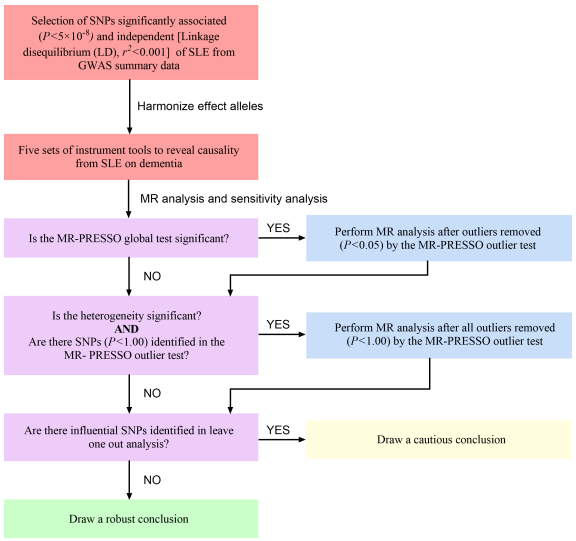

FIGURE 2
The flow chart of the MR process. SLE, systemic lupus erythematosus; SNP, single nucleotide polymorphisms; MR-PRESSO, MR-Pleiotropy Residual Sum and Outlier.

We first harmonized the above-selected SNPs with effect allele in the database of dementia (all dementia, AD, VaD, FTD and DLB). Five sets of genetic instruments were finally extracted to clarify the genetic causality between SLE and dementia. Subsequently, we conduct the MR-PRESSO analysis to moderate horizontal pleiotropy. If the global test *P* value <0.05, which suggests significant horizontal pleiotropy in MR analysis, we will remove SNPs with *P* value <0.05 in the MR-PRESSO outlier test and re-perform the MR analysis. If the heterogeneity remains significant, we will remove all the outliers (*P*<1.00). Finally, we can draw a solid conclusion if the leave-one-out analysis fails to detect SNPs that potentially affect the stability of the outcomes (37).

Results

Genetic instruments for systemic lupus erythematosus

We finally included 45 significant ($P<5\times10^{-8}$) and independent ($LDr^2<0.001$) SNPs as genetic instrumental variables, all of which had an F-statistic > 80, indicating no weak instrumental bias. The detailed information on 45 SNPs is illustrated in Table S1. Finally, the summary information of SNPs for SLE and dementia is presented in Tables S2.1 -S2.5.

Causal effect from systemic lupus erythematosus to dementia

The results of the MR analysis are shown in Figure 3.

For any dementia, no significant causal relationship was found for SLE and risk of any dementia [odds ratio (OR)=0.9884, 95% confidence interval (CI): 0.9627-1.0066, *P*=0.1667], this finding was similar to MR-Egger (OR=0.9657, 95% CI: 0.9201-1.0135, *P*=0.1649) and WM (OR=0.9875, 95% CI: 0.9576-1.0183, *P*=0.4236) (Figure 4A). No significant heterogeneity (Cochran's Q *P*=0.6567) and horizontal pleiotropy (*P* for intercept=0.3859 and global test *P*=0.1520) were found in this MR analysis (Tables S3, S4), the leave-one-out analysis suggests that the results were s robust (*P*=0.1666) (Figure S1A).

For AD, there was no evidence of a potential causal association between SLE and AD risk (OR=1.0000, 95% CI: 0.9999-1.0001, *P*=0.7264). The findings of MR-Egger (OR=1.0000, 95% CI: 0.9998-1.0002, *P*=0.8934) and WM (OR=1.0000, 95% CI: 0.9998-1.0001, *P*=0.6230) were consistent (Figure 4B). In addition, Cochran's Q test suggested no significant heterogeneity (*P*=0.1756) (Tables S3, S4). MR-Egger regression (*P* for intercept = 0.9628) and MR-PRESSO (global test *P*=0.2008) also did not find significant horizontal pleiotropy. Moreover, the leave-one-out test indicates that our results were stable (*P*=0.7264) (Figure S1B).

For VaD, we found that the three methods also reached different conclusions. IVW method had weak evidence of borderline significance for the causal genetic association between SLE and VaD risk (OR=0.9365, 95% CI: 0.8769-1.0002, *P*=0.0506). No such association was found using the MR-Egger method (OR=0.9292, 95% CI: 0.8043-1.0735, *P*=0.3246) (Figure 4C). However, the WM method revealed a significant genetic correlation between SLE and VaD risk (OR=0.8996, 95% CI: 0.8297-0.9754, *P*=0.0103). Since there was no significant heterogeneity (*P*=0.0850) or horizontal pleiotropy (*P* for intercept = 0.9048 and global test *P*=0.0966), we considered the result of IVW more credible (Tables S3, S4). The stability of the MR estimates was also verified by the leave-one-out test (Figure S1C).

For FTD, we did not find a genetic association with SLE (OR=1.0467, 95% CI: 0.9074-1.2075, *P*=0.5310). Similar results were shown on MR-Egger (OR=1.0721, 95% CI: 0.7085-1.6221, *P*=0.7487) and WM (OR=1.0969, 95% CI: 0.9126-1.3185, *P*=0.3243) (Figure 4D). The results of Cochran's Q test, MR Egger regression, MR-PRESSO and the leave-one-out test showed that the MR estimates were relatively robust (Tables S3, S4) (Figure S1D).

For DLB, no genetically significant association was found with SLE (OR=1.0308,95% CI: 0.9829-1.0810, *P*=0.2112). MR-Egger (OR=0.9571,95% CI: 0.8650-1.0590, *P*=0.4016) and WM (OR=1.0201, 95% CI: 0.9550-1.0897, *P*=0.5536) revealed

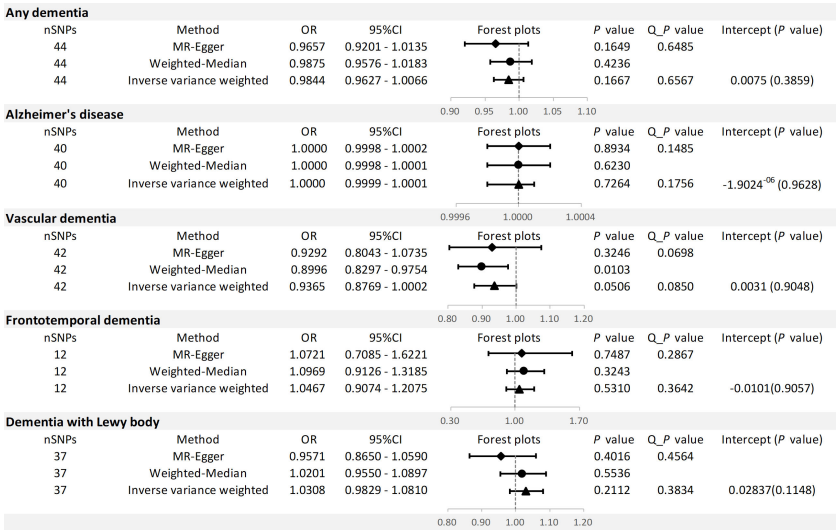

FIGURE 3
MR results and sensitivity analysis for association of SLE and dementia risk.

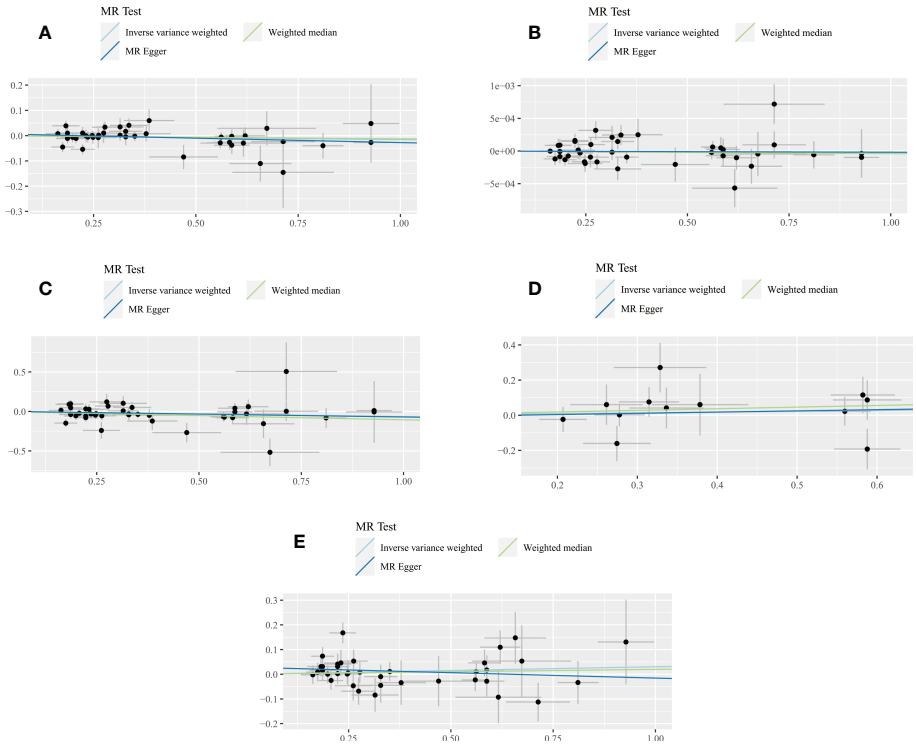

FIGURE 4
Scatter plot of the association between SLE and all dementia **(A)**, Alzheimer's disease **(B)**, vascular dementia **(C)**, frontotemporal dementia **(D)**, dementia with lewy body **(E)**. Three lines reveal the estimated effect sizes by MR methods (inverse-variance weighted, MR-Egger and weighted median).

consistent conclusions (Figure 4E). Sensitivity analysis and heterogeneity test did not indicate potential horizontal pleiotropy and significant heterogeneity (Tables S3, S4). The leave-one-out test demonstrated that the MR estimate was stable when individual SNP was removed (Figure S1E). Finally, the funnel plots on SLE and dementia are presented in Figure S2.

Discussion

In the present two-sample MR study, no genetic causal association was found between SLE and the risk of dementia.

Although cognitive impairment is one of the frequent clinical manifestations of NPSLE patients, progression to dementia is rare (39). A 5-year cohort study found that standardized neuropsychological test scores among patients with SLE were relatively stable and even found signs of improvement during the observation period (40). It revealed that cognitive impairment is stable and reversible in SLE patients. In addition, lupus activity did not appear to have a significant association with cognitive impairment (41).

In recent years, there has been increasing attention to the relationship between autoimmune diseases, especially SLE, and dementia. Several epidemiological studies have shown a potential association between SLE and dementia. Two nationwide population-based cohort studies found that SLE was associated with a higher risk of dementia (42, 43). Another large data analysis that included more than four thousand SLE patients and twenty-four thousand age and gender matched non-SLE controls found an increased risk of dementia in SLE patients (44). Recently, a meta-analysis by Zhao et al. integrating eleven relevant observational studies demonstrated that SLE adversely affects cognition and significantly increases dementia risk (14). In this study, only three relevant studies on the association between SLE and the risk of dementia were included and the variability among epidemiological studies regarding study design, methodology and quality, has made the association between SLE and dementia challenging to ascertain. Moreover, it is noteworthy that most of the current studies were observational. The evidence from observational studies should be interpreted with caution as it is unable to reveal causality and completely exclude the effects of confounding factors.

Our study found no direct genetic causality between SLE and dementia. The higher prevalence of dementia among SLE patients compared to the general population in the observational studies may be attributed to the following reasons. Firstly, pharmacological treatments might influence dementia risk in SLE patients to a certain extent. Glucocorticoids (GCs) play an essential role in the treatment of chronic inflammation (7), with their use in up to 80% of SLE patients, primarily for long courses of treatment. It has been well-established that GCs have neurotoxic effects (45–47). A cohort study that included 123 SLE patients with at least 3 years of follow-up found that long-term use of GCs was a predictor of cognitive impairment (45). The specific mechanism may be that long-term GCs use reduces the hippocampus volume, a crucial brain region in charge of learning and memory. In addition, high plasma levels of GCs and suppression of microglia glucocorticoid receptors (GRs) cause changes in microglia morphology and branching in the hippocampal region. These changes play an important role in the onset and progression of dementia (48, 49). Currently, though disease-modifying antirheumatic drugs (DMARDs) therapy may control rheumatic disease activity effectively, there is conflicting evidence for its effects on cognitive dysfunction. A case-control study that included 957 patients showed that conventional synthetic DMARDs (csDMARDs) (hydroxychloroquine, methotrexate, and sulfasalazine) commonly used in SLE were significantly associated with an increased dementia risk, whereas biological DMARDs (bDMARDs) were not (50). Another study found that bDMARDs, specifical etanercept, were shown to reduce the dementia risk significantly (51). Secondly, approximately 40% of patients with SLE are confirmed positive for anticardiolipin antibodies (aPL) and 50%-70% of these progress to secondary antiphospholipid syndrome (APS). Previous studies have observed a higher risk of dementia in SLE patients with secondary APS, which may be due to the hypercoagulable state and microembolism (52, 53). Our study did not find significant horizontal pleiotropy, therefore SLE patients with secondary APS may have been excluded. Thirdly, SLE is a multisystem autoimmune connective tissue disease with multiple co-morbidities. Approximately 51% of patients have three or more co-morbidities, such as hypertension, obesity, dyslipidemia, and depression. The presence of these diseases has been proven to be independent risk factors for dementia which may lead to an overestimation of the association between SLE and dementia risk (54, 55). Finally, SLE is a remarkably heterogeneous autoimmune disease and may exist different disease groups (56–58). Up to now, several studies have observed significant differences in pathogenesis, clinical manifestations, and genetic susceptibility among patients with SLE from different ancestral backgrounds (59–61). GWAS have attempted to partially explain the complex genetic structure of SLE. However, some alleles have not been sequenced in diverse ancestral backgrounds. Thus, the possibility remains that important causative genes may be buried.

To our knowledge, this is the first MR study to investigate the causal association between SLE and dementia risk. Our research has several strengths. Firstly, the main advantage is the MR design, which can avoid interference from confounding factors and reverse causal association. Secondly, we strictly screened SNPs using plink clumping to ensure the independence of IVs. Thirdly, the F-statistics of the included SNPs were all over 80, so the included genetic instruments were relatively powerful.

However, several limitations are worth mentioning. First, the sample size of the study is relatively small compared to population-based observational studies, although we use the largest and most recent GWAS database. Second, epigenetic

issues such as DNA methylation, RNA editing and transposons inactive are the unavoidable shortcoming of MR analysis. Third, there might be an ethnic bias in our study due to all the selected GWAS database populations being of European ancestry. Forth, detailed demographic and clinical data on participants were not available, so the subgroup analysis was not performed.

Conclusion

In summary, our findings do not support a causal association between SLE and dementia risk, which was inconsistent with previous observational studies. In the future, whole genome sequencing is needed for NPSLE patients to better explain genetic variation. Updated MR studies will be warranted to validate our results when more efficient methods are available to produce less biased MR estimates or when more extensive GWAS summary data are accessible. Meanwhile, further multicenter, large-sample, and follow-up studies should be conducted to longitudinal assess the patient's cognitive function, dynamic monitor laboratory indicators and imaging changes to identify predictive and prognostic factors in the real world.

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 authors.

Ethics statement

Ethical approval was not provided for this study on human participants because we used the publicly available GWAS catalog to conduct a two-sample MR study. No additional ethical approval was required due to the re-analysis of previously summary-level data. The patients/participants provided their written informed consent to participate in this study.

Author contributions

TJ: present idea, perform MR analysis and manuscript writing. WH: evaluate the quality of MR and manuscript writing. FC: Search of the database and quality assessment. XY: figure and table drawing. SG: assisted funding. ZY and CX: study supervision and final approvement. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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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.2022.1063110/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Leave-one-out plots of SLE and all dementia **(A)**, Alzheimer's disease **(B)**, vascular dementia **(C)**, frontotemporal dementia **(D)**, dementia with lewy body **(E)**. The leave-one-out plot visualizes how the causal estimates (point with horizontal line) for the effect of SLE on dementia are influenced by the exclusion of individual SNPs. The leave-one-out analysis suggests no individual SNP significantly affect the risk of SLE on dementia, which indicates that the results are reliable.

SUPPLEMENTARY FIGURE 2
Funnel plot on SLE and all dementia **(A)**, Alzheimer's disease **(B)**, vascular dementia **(C)**, frontotemporal dementia **(D)**, dementia with lewy body **(E)**. The funnel plots are symmetric, which shows that the absence of polymorphism.

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Diagnostic gene signatures and aberrant pathway activation based on m6A methylation regulators in rheumatoid arthritis

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Purpose: Rheumatoid arthritis (RA) is a chronic autoimmune disease (AD) characterized by persistent synovial inflammation, bone erosion and progressive joint destruction. This research aimed to elucidate the potential roles and molecular mechanisms of N6-methyladenosine (m6A) methylation regulators in RA.

Methods: An array of tissues from 233 RA and 126 control samples was profiled and integrated for mRNA expression analysis. Following quality control and normalization, the cohort was split into training and validation sets. Five distinct machine learning feature selection methods were applied to the training set and validated in validation sets.

Results: Among the six models, the LASSO_λ-1se model not only performed better in the validation sets but also exhibited more stringent performance. Two m6A methylation regulators were identified as significant biomarkers by consensus feature selection from all four methods. IGF2BP3 and YTHDC2, which are differentially expressed in patients with RA and controls, were used to predict RA diagnosis with high accuracy. In addition, IGF2BP3 showed higher importance, which can regulate the G2/M transition to promote RA-FLS proliferation and affect M1 macrophage polarization.

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Conclusion: This consensus of multiple machine learning approaches identified two m6A methylation regulators that could distinguish patients with RA from controls. These m6A methylation regulators and their target genes may provide insight into RA pathogenesis and reveal novel disease regulators and putative drug targets.

KEYWORDS

rheumatoid arthritis, N6-methyladenosine, IGF2BP3, cell cycle, M1 macrophages

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease (AD) characterized by tumour-like hyperplasia of synovial tissue, persistent synovial inflammation, bone erosion and progressive joint destruction (1). RA usually occurs in middle-aged women. Currently, we attribute the development of RA to genetic and environmental factors, such as smoking, obesity, stress, neurodepression, and female hormones. Patients with RA have a higher risk of developing malignancies than the general population (2). Recently, the management of clinical symptoms and complications in RA patients has received increasing attention from medical workers (3, 4). An in-depth understanding of the mechanisms underlying RA occurrence and development can help to detect RA and its complications earlier so that measures can be taken to control the development and reduce the activity of the disease.

Previous studies have shown that T/B lymphocytes, macrophages, fibroblast-like synoviocytes (FLSs) and other cells are involved in the pathogenesis of RA (5). Activated FLSs in synovial tissue exacerbate the inflammatory response by secreting proinflammatory factors, chemokines and cell adhesion molecules, which can recruit additional immune cells to synovial tissue (6). Although the pathogenesis of RA remains incompletely elucidated, immune cells and FLSs undoubtedly play a crucial role in the progressive joint destruction and inflammatory response (7). Therefore, studying strategies to inhibit the proliferation and migration of FLSs and the inflammatory response in RA is highly important for elucidating the disease mechanism and developing treatments.

The study of epigenetics, especially RNA modifications, is a hotspot in life science research. Recently, with the development of the first RNA N6-methyladenosine (m6A) map by Cornell University and the discovery of its ubiquity in mRNA, transcriptional modification has gradually become the focus of the biomedical community (8). Among RNA modifications, m6A accounts for the largest proportion of base modifications in mRNAs and functions to regulate RNA stability, protein synthesis and translation; stem cell stress responses, cytotoxic stress responses; and mRNA export (9, 10). Currently, the known m6A methylation regulators consist of eight writers (METTL3, METTL14, WTAP, KIAA1429, RBM15, RBM15B, CBLL1 and ZC3H13), two readers (FTO and ALKBH5) and thirteen erasers (YTHDF1, YTHDF2, YTHDF3, YTHDC1, YTHDC2, HNRNPC, HNRNPA2B1, IGF2BP1, IGF2BP2, IGF2BP3, FMR1, ELAVL1 and LRPPRC) (11). Previous studies have shown that these regulators are involved in biological processes (BPs) such as cell differentiation and apoptosis and immune regulation, which are closely related to cancers and immune diseases (12–14). However, few studies have addressed the regulatory mechanism of m6A in RA, and more attention is needed.

In this study, we selected 19 m6A methylation regulators with expression data in the GSE12021, GSE55235, GSE55457, GSE55584, GSE77298 and GSE153105 datasets. Based on five distinct supervised machine learning approaches, we assessed the potential of these m6A methylation regulators as diagnostic tools by creating binary predictive classification models and assessing their accuracy. Then, by analysing the target genes and pathways of the m6A methylation regulators, we gained a further understanding of the roles of m6A methylation regulators in the pathogenesis of RA (Figure 1). This study is of great significance for elucidating the potential roles and molecular mechanisms of m6A methylation regulators in RA and for exploring new RA biomarkers.

Materials and methods

Dataset collection and processing

Data for 384 samples were accessed *via* the Gene Expression Omnibus (GEO) repository (Supplementary Table 1). The data from GSE12021, GSE55235, GSE55457 and GSE55584 were retrieved from the Affymetrix® GPL96 platform (Human Genome U133A Array), and the data from GSE77298 and GSE153105 were retrieved from the Affymetrix® GPL570 platform (Human Genome U133 Plus 2.0 Array). The raw

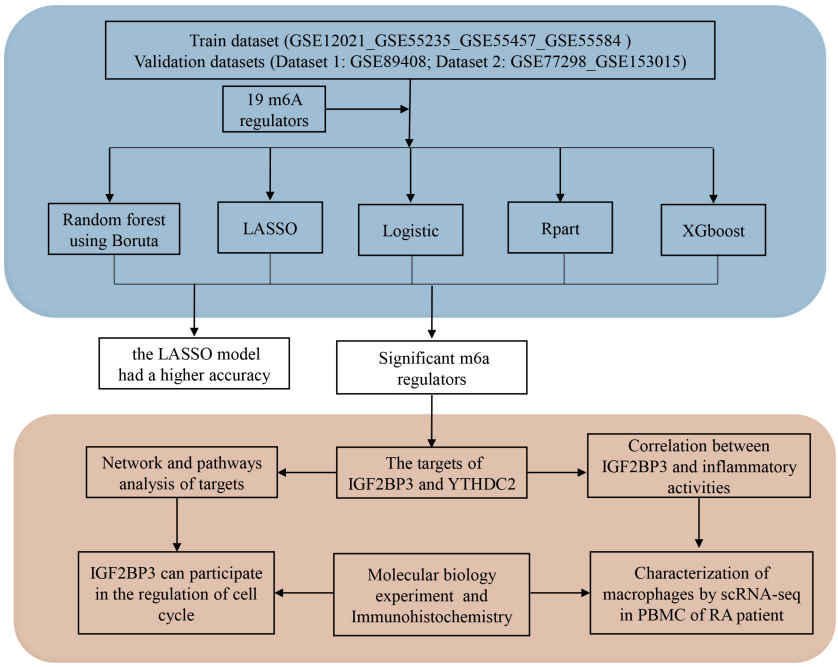

FIGURE 1
Diagram of the study.

data from the Affymetrix® platforms were processed *via* the robust multiarray averaging (RMA) algorithm implemented in the Affy package. After removal of batch effects with the ComBat algorithm, the training dataset was generated by combining the GEO datasets from the Affymetrix® GPL96 platform. Validation dataset 1 was generated by combining the GEO datasets from the Affymetrix® GPL570 platform. GSE89408 (platform: GPL1154) was considered validation dataset 2. In this research, for comparison with the RA group, we defined healthy individuals and patients with osteoarthritis (OA) as the control group.

The samples in GSE12021, GSE55235, GSE55457, GSE55584, GSE77298 and GSE153105 were extracted from synovial tissues. The samples in GSE90081 were taken from peripheral blood mononuclear cells (PBMCs). To investigate the relationship between IGF2BP3 expression and M1 macrophages, single-cell RNA sequencing (scRNA-seq) data from the GSE159117 dataset were analysed.

Cell lines and cell transfection

RA-FLSs were isolated from RA synovium. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 15% foetal bovine serum (FBS) (Thermo, USA) and cultured at 37°C in 5% CO_2 and saturated humidity. The ethics committee of China-Japan Friendship Hospital approved the research (approval number 2021-153-K111).

To silence the expression of IGF2BP3, an IGF2BP3 siRNA (siIGF2BP3) and a control siRNA (siNC) were chemically synthesized by Tsingke Biotechnology Co., Ltd (Beijing, China) and transfected into RA-FLSs and RAW 264.7 cells. The siIGF2BP3 target sequences are shown below: human si-IGF2BP3, 5'- GCAAAGGATT CGGAAACTT -3'; mouse si-Igf2bp3, 5'- GGAGGUGCUGGAUAGUUUACU -3'. JetPRIME® Transfection Reagent was used for cell transfection (Polyplus Transfection, USA).

Random forest optimization using boruta

Boruta has high feature variable selection accuracy in biological data. We used the default settings in the Boruta package (v7.0.0) to evaluate variable importance with 300 iterations (15). After 300 iterations, the confirmed variables were identified. Then, these confirmed variables selected by Boruta were used to construct a random forest model by using the caret package (v.6.0-92). After tuning and modelling, the final selected model was obtained and used to determine whether the subjects were RA patients or non-RA patients.

Regression partition tree

Rpart is a commonly used decision tree modelling method with a good visualization effect and straightforward results. We used the Rpart (v4.1-15) package to build a classification tree model. To avoid overfitting, some rules with weak classification and descriptive abilities were removed to improve the prediction accuracy. The classification tree model was optimized based on the minimum Xerror value, and the optimal classification tree model was used to determine whether the subjects were RA patients or non-RA patients.

Least absolute shrinkage and selection operator

LASSO has the advantage of preserving subset shrinkage and is a biased estimator for dealing with data with complex collinearity. Lasso allows a more refined model to be obtained by constructing a penalty function such that some coefficients are compressed and some coefficients are set to zero (16). LASSO-penalized logistic regression was performed with the glmnet package (version 4.1-4), which then calculated two automatic λ values—one that minimizes the binomial deviance and one representing the largest λ that is still within 1 standard error of the minimum binomial deviance. Both λ values (λ-min=0.02395, λ-1se=0.09203) were selected and used to refit the model, which resulted in a stricter penalty that allowed us to reduce the number of covariates even further than with the former λ. A probability threshold of > 0.5 was used to determine whether the subjects were RA patients or non-RA patients.

Extreme gradient boosting

XGBoost is an extreme gradient boosting algorithm that ranks features from most important to least important and has been used very effectively in diverse classification problems. Based on the default parameters, we used the XGBoost package (version 1.6.0.1) to build the final model for disease diagnosis and rank the features by importance. Features contributing to more than a 5% improvement in accuracy to their branches were selected as 'important' (17). The trained model was used to determine whether the subjects were RA patients or non-RA patients.

Logistic regression

Logistic regression is a machine learning method used to solve binary classification problems to estimate the likelihood of an event. The glmnet package (version 4.1-4) was used to build the final model for disease diagnosis, which was used to determine whether the subjects were RA patients or non-RA patients.

Pathway analysis

M6A2Target (http://m6a2target.canceromics.org/) is a comprehensive database for determining the target genes of writers, erasers and readers (WERs) of m6A modification. It integrates highly confidential targets validated by low-throughput experiments and potential targets with binding evidence indicated by high-throughput sequencing or inferred from m6A WER perturbation followed by high-throughput sequencing. The gene targets of the more important m6A regulators in disease diagnosis were inferred using m6A2Target (18). Then, ClueGO (version 3.0.3) was used for BP functional annotation analysis of the gene targets (19). The clusterProfiler package (version 4.2.2), a universal enrichment tool for interpreting omics data, was used for functional enrichment analysis.

scRNA-seq analysis

First, we imported the H5 file and converted the data to a Seurat object. Then, with the Seurat (version 4.1.1) package, data quality control and clustering were performed on the PBMC population. Each cell subset was annotated based on the celldex package (version 1.4.0).

Real-time qPCR analysis and western blot analysis

RNA isolation and RT–qPCR analysis were carried out according to previous studies (20). β-actin served as an internal control. The sequences of the primers used in the experiment are as follows. Human IGF2BP3: 5′- TCGAGG CGCTTTCAGGTAAA-3′ (forward), 5′- AAACTATCCAGCA CCTCCCAC-3′ (reverse). Mouse Igf2bp3: 5′- CCTGGTGA AGACGGGCTAC-3′ (forward), 5′- TCAACTTCCATCGGTT TCCCA-3′ (reverse).

Protein extraction and Western blot analysis were carried out according to previous studies (20). The primary antibodies included rabbit anti-IGF2BP3 (1:1000, Proteintech, Chicago, USA), anti-CCNB1 (1:1000, Shanghai, China) and anti-C-Myc (1:2,000, Cell Signaling Technology, Beverly, MA, USA). Band densities on autoradiograms were densitometrically quantified (Quantity One software; Bio-Rad), with GAPDH serving as the internal control.

Cell viability assay and cell cycle analysis

The cell viability assay was performed 24 h after transfection of siNC and siIGF2BP3 with a CCK-8 kit from Beyotime (Beijing, China). After transfection, cells were plated in 96-well dishes at a concentration of 5×10^3 cells/well and cultured in DMEM containing 15% FBS for cell attachment. Cell viability was measured with CCK-8 reagent following the manufacturer's protocol at the indicated time points (24, 48 and 72 h).

Cell cycle analysis was performed 48 h after transfection of siNC and siIGF2BP3. Cells were washed twice with ice-cold PBS, harvested, and fixed with 70% ethanol at 4°C overnight. Then, the cells were stained with a Cell Cycle and Apoptosis Analysis Kit (Beyotime, Beijing, China) at 37°C for 30 minutes and detected by flow cytometry (Becton-Dickinson, San Jose, CA, USA). Cell cycle distributions were analysed with ModFit LT 3.1 software (verity Software House, Inc., Topsham, ME, USA).

Flow cytometric analysis and enzyme linked immunosorbent assay

Analysis was performed 48 h after transfection of siNC and iIGF2BP3. After 6h of LPS (100ng/ml) stimulation, cells were collected and washed with PBS. Subsequently, the cells were directly surface stained using anti-CD86 antibodies (Biolegend, California, USA) for 20 min at 4°C. Signals were detected by flow cytometry (Becton-Dickinson, San Jose, CA, USA). Data analysis was conducted with FlowJo software version 10.0 (Tree Star, Inc., Ashland, OR, USA).

After transfection and stimulation, the cell supernatant was collected. According to the protocol of Mouse TNF-alpha ELISA Kit (ABclonal, Wuhan, China), the content of TNF-α in cell supernatant was detected.

Immunohistochemistry

The synovium tissues of six RA patients and six OA patients are obtained from China-Japan Friendship Hospital. Sample processing and data analysis were performed as previously described (20). The ethics committee of China-Japan Friendship Hospital approved the research (approval number 2021-153-K111).

Statistical analyses

Statistical analyses were performed using GraphPad Prism Software (GraphPad Software, San Diego, CA) and R version 4.0.4 software (Institute for Statistics and Mathematics, Vienna, Austria; https://www.r-project.org). We used a leave-one-out (LOO) cross-validation approach to evaluate the performance of the classifiers in the training set. Student's t test was used for comparisons between groups. Measurement data are expressed as the means ± standard deviations, and $P< 0.05$ indicates statistical significance.

Results

Performance of RA classification approaches using the m6A regulators

Considering the important role of m6A methylation regulators in tumour and immune disease progression, we used a public dataset to comprehensively explore the importance of 19 m6A methylation regulators for RA diagnosis. Based on the expression levels of these 19 m6A methylation regulators, a disease diagnosis model (RA *vs.* non-RA) was constructed using five different machine learning methods: random forest optimization using Boruta, Rpart, LASSO, XGBoost and logistic regression. The cross-validation performance in the training set is presented in Supplementary Table 2. The accuracy and AUC of all models except for the Rpart model were greater than 0.8. To compare the performance of each machine learning method, we observed the performance of each model as a classifier in the validation sets. The performance of each machine learning method in the validation sets was also variable (Tables 1, 2; Figures 2A-F). In validation dataset 1, the logistic regression model and LASSO_λ-min model had the highest AUC (0.90), but the LASSO_λ-min model had a higher accuracy (0.901). The Rpart model had the lowest AUC (0.8). In validation dataset 2, the LASSO_λ-min model and LASSO_λ-1se model had the highest accuracy (0.89) and AUC (0.88). Among the models, the Rpart model had the poorest performance. In addition, the number of m6A methylation regulators selected by each machine learning method differed, with Boruta selecting the most (14 regulators) and the Rpart model selecting just one regulator. Considering the performance of each machine learning method in the validation sets and the number of regulators that it selects in the models, the LASSO_λ-1se model not only performed better in the validation sets but also exhibited more stringent in variable screening. These results indicate that the LASSO_λ-1se model has good clinical application value and practicality. Therefore, we further compared the performance of the LASSO_λ-1se model in whole blood samples and calculated an AUC value of 0.83 (Figure 2G), further suggesting that the LASSO_λ-1se model has clinical application prospects in blood-based diagnosis of RA.

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TABLE 1 Model performance of the six classifiers in validation set 1: A random forest wrapper (Boruta), LASSO_λ-min, LASSO_λ-1se, logistic regression, regression partition trees (Rpart) and extreme gradient boosting (XGBoost).

	Random forest	LASSO_min	LASSO_1se	Logistic	Rpart	XGBoost
Regulators selected by model, n	14	11	4	13	1	5
Best threshold	0.481 (0.22,0.829)	0.520 (0.28,0.961)	1.280 (0.26,0.895)	-293.891 (0.3,0.934)	0.5014 (0.24,0.829)	0.903 (0.22,0.809)
Sensitivity	0.78	0.72	0.74	0.7	0.76	0.78
Specificity	0.8289	0.961	0.8947	0.9342	0.8289	0.8092
Positive predictive value	0.6	0.8571	0.6981	0.7778	0.5938	0.5735
Negative predictive value	0.9197	0.9125	0.9128	0.9045	0.913	0.9179
Acuracy (95%)	0.8168 (0.7565~0.8676)	0.901 (0.8512-0.9385)	0.8564 (0.8004-0.9017)	0.8762 (0.8227-0.9183)	0.812 (0.7511-0.8633)	0.802 (0.7403-0.8546)
AUC (95%)	0.811 (0.735-0.888)	0.895 (0.841-0.948)	0.89 (0.830-0.944)	0.899 (0.847-0.95)	0.794 (0.728-0.861)	0.853 (0.792-0.914)

The more important m6A methylation regulators in the RA classification

Different candidate biomarkers were selected by these multivariable machine learning methods. However, biomarkers often have equal accuracy and importance (17). Considering the poorest performance of the Rpart model, we focused on the overlapping m6A methylation regulators selected by the different machine learning methods, including of random forest optimization using Boruta, LASSO, XGBoost and logistic regression (Figure 3A; Supplementary Table 3). Two of the overlapping m6A methylation regulators were selected by every model: IGF2BP3 and YTHDC2. The expression levels of the 19 m6A methylation regulators were further compared in the training dataset. The expression levels of IGF2BP3 and YTHDC2 were significantly different in RA and non-RA patients (Figure 3B). More importantly, based on transcript levels, IGF2BP3 and YTHDC2 also performed well in the diagnosis of RA in the training set (Figure 3C), with AUC values of 0.85 and 0.75, respectively. In addition, when the Boruta (Figure 3D), Rpart (Figure 3E) and XGBoost (Figure 3F) algorithms were used to calculate the importance of the 19 m6A methylation regulators, IGF2BP3 and YTHDC2 were ranked high; and IGF2BP3 has the highest importance.

Pathway and network analysis of the IGF2BP3 and YTHDC2 targets

To investigate the novel roles that these m6A methylation regulators play in RA and examine the related pathways, we predicted their target genes using m6A2Target. IGF2BP3 and YTHDC2 had 287 predicted gene targets in total (Supplementary Table 4); IGF2BP3 had 16 verified targets and 190 predicted targets, and YTHDC2 had 9 verified targets and 77 predicted targets. Based on the predicted gene targets, KEGG pathway enrichment analysis was performed using the ClusterProfiler package (version 4.2.2) to analyse the signalling pathways in which IGF2BP3 and YTHDC2 participate. These predicted gene targets were highly enriched in the following functions and pathways: MYC_TARGETS_V1, E2F_TARGETS, G2M_CHECKPOINT, MITOTIC_SPINDLE, ESTROGEN_RESPONSE_LATE, ALLOGRAFT_REJECTION, OXIDATIVE_PHOSPHORYLATION, DNA_REPAIR, UNFOLDED_PROTEIN_RESPONSE, MYC_TARGETS_V2, and

TABLE 2 Model performance of the six classifiers in validation set 2: A random forest wrapper (Boruta), LASSO_λ-min, LASSO_λ-1se, logistic regression, regression partition trees (Rpart) and extreme gradient boosting (XGBoost).

	Random forest	LASSO_min	LASSO_1se	Logistic	Rpart	XGBoost
Regulators selected by model, n	14	11	4	13	1	5
Best threshold	0.693 (0.273,0.778)	-2.229 (0.273,0.944)	-1.588 (0.273,0.944)	-7331.730 (0.091,0.472)	NA	0.007 (0.273,0.556)
Sensitivity	0.7273	0.7273	0.7273	0.9091	1	0.7273
Specificity	0.7778	0.9444	0.9444	0.4722	0	0.5556
Positive predictive value	0.5	0.8	0.8	0.3448	0.234	0.3333
Negative predictive value	0.9032	0.9189	0.9189	0.9444	NA	0.8696
Acuracy (95%)	0.766 (0.6197, 0.877)	0.8936 (0.769,0.9645)	0.8936 (0.769,0.9645)	0.5745 (0.4218-0.7174)	0.234 (0.123-0.3803)	0.5957 (0.4427-0.7363)
AUC (95%)	0.782 (0.641-0.923)	0.884 (0.780-0.988)	0.881 (0.778-0.984)	0.707 (0.525-0.889)	0.5	0.667 (0.518-0.816)

NA, Not Applicable.

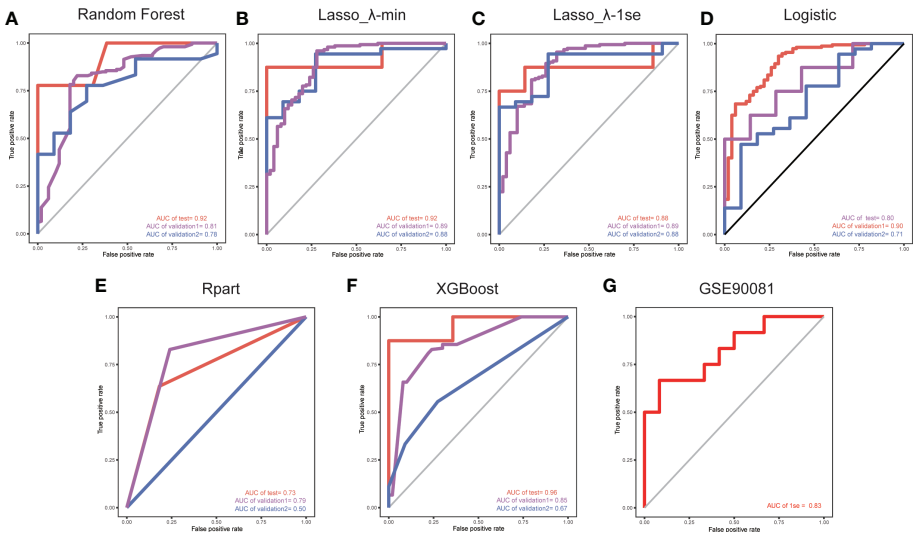

FIGURE 2
ROC curves for validation set 1 and validation set 2, with the model trained on a separate set. The red lines indicate the models trained using a LOO cross-validation approach across the training set. We used five methods to develop models based on the training set: **(A)** a random forest wrapper (Boruta), **(B)** LASSO_λ-min, **(C)** LASSO_λ-1se, **(D)** logistic regression, **(E)** regression partition trees (Rpart) and **(F)** extreme gradient boosting (XGBoost). **(G)** ROC curve of the LASSO_λ-1se model in whole blood samples.

so on (Figure 3G). Interestingly, BP functional enrichment analysis carried out by ClueGO showed that the predicted gene targets participated mainly in processes related to the mitotic cell cycle, translation, cytoplasmic translation and regulation of DNA metabolic processes, which play key roles in the occurrence and development of RA (Figure 3H). To better demonstrate the relationship between IGF2BP3 and YTHDC2, their predicted gene targets and the related pathways, Cytoscape (version 3.9.0) was used to construct a network, which indicated that IGF2BP3 and YTHDC2 can regulate the G2M_CHECKPOINT, MYC_TARGETS_V1 and E2F_TARGETS pathways by acting on CDK1, CDK2, MYC and other targets (Figure 4A).

The importance of IGF2BP3 in the viability and cell cycle of RA-FLSs

Based on the pathway enrichment analysis results, IGF2BP3 and YTHDC2 are closely related to the cell cycle. But, when the Boruta (Figure 3D), Rpart (Figure 3E) and XGBoost (Figure 3F) algorithms were used to calculate the importance of the 19 m6A methylation regulators, IGF2BP3 ranked first, while YTHDC2 ranked lower. In addition, compared with YTHDC2, IGF2BP3 performed better in the diagnosis of RA (Figure 3C). Therefore, we further explored the regulatory effects of IGF2BP3 on the viability and cell cycle of RA-FLSs through molecular biology experiments. To explore the effects of IGF2BP3 on RA-FLSs, siRNAs were transfected into RA-FLSs. The transfection results were confirmed by RT–qPCR and Western blotting and indicated that the siRNA had a good knockdown efficiency (Figures 4B-D). Then, we studied the effect of IGF2BP3 on RA-FLS viability *in vitro*. The CCK-8 cytotoxicity assay revealed that downregulation of IGF2BP3 in RA-FLSs significantly reduced cell viability compared to that of the control cells ($P < 0.05$, Figure 4E). The cell proliferation assay also revealed that downregulation of IGF2BP3 in RA-FLSs significantly inhibited cell proliferation compared to that of the control cells ($P < 0.05$, Figure 4F). In addition, the flow cytometry results showed that low expression of IGF2BP3 had an obvious effect on the G2/M transition. Compared with that in the control group, the proportion of G2/M-phase cells in the siIGF2BP3 group was significantly increased ($P < 0.05$, Figures 4G, H). We also measured the expression of cell cycle-related proteins, showing that siIGF2BP3 reduced CCNB1 and C-MYC expression (Figures 4C, D). In addition, the expression of IGF2BP3 in synovial tissues of patients with OA and RA was detected. We found that IGF2BP3 expression was significantly higher in synovial tissues of RA patients, further affirming the importance of IGF2BP3 in the progression of RA (Figure 4I).

Correlation between IGF2BP3 expression and inflammatory activity

Increasing evidence suggests that m6A modification is an important regulator of immune response regulatory mechanisms and inflammatory regulatory networks (21). To identify the IGF2BP3-associated immune signature in RA, we determined the immune scores and the proportions of immune cells with

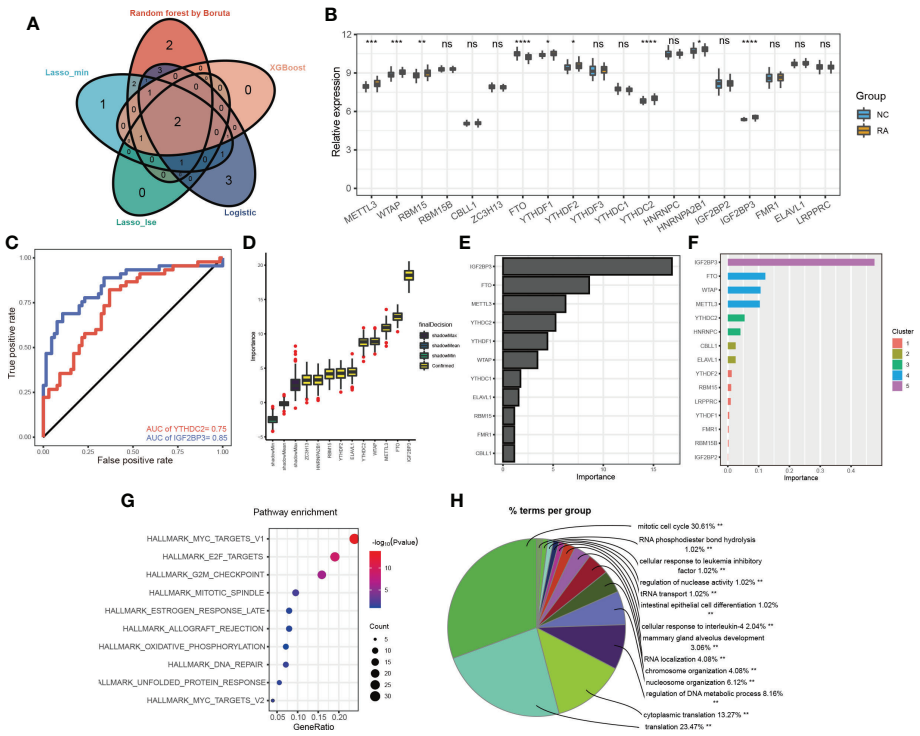

FIGURE 3
The more important m6A methylation regulators in RA classification. **(A)** Venn diagram of the m6A methylation regulators selected by the different machine learning methods; **(B)** the expression levels of 19 m6A methylation regulators in the training dataset; **(C)** the ROC curves for IGF2BP3 and YTHDC2 in the training set; the importance of the 19 m6a methylation regulators calculated by the Boruta **(D)**, Rpart **(E)** and XGBoost **(F)** algorithms; KEGG pathway **(G)** and BP **(H)** enrichment analyses of the gene targets of IGF2BP3 and YTHDC2 *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns (p > 0.05).

xCell (22). First, we found significant differences in the immune score between the two groups, with higher immune scores in the RA patient group than in the NC patient group ($P < 0.001$; Figure 5A). Then, the proportions of immune cells were compared between the two groups. There were significant differences in the proportions of many immune cells, including interdigitating cells (IDCs), natural killer T (NKT) cells, classical dendritic cells (cDCs), macrophages, mast cells, M2 macrophages, Th2 cells, M1 macrophages, and myocytes (Figures 5B, C). Among these cell types, we focused on M1 macrophages because of the closely relationship between M1 macrophages and RA (23). The proportion of M1 macrophages in RA patients was significantly higher than that in control patients. In addition, we investigated the relationship between the proportion of M1 macrophages and the expression level of IGF2BP3 in RA patients and found that they were strongly correlated (Figure 5D). IGF2BP3 expression was also significantly correlated with the expression of M1 macrophage markers, including IL1A, CD86 and TLR2 (Figures 5E-G). Therefore, we thought that IGF2BP3 can participate in the regulation of M1 macrophage polarization.

To further explore the effect of IGF2BP3 on M1 macrophage polarization, we transfected RAW264.7 cells with Igf2bp3-siRNA or NC-siRNA (negative control). RT–qPCR and Western blot analysis were performed to confirm the efficiency of gene silencing and indicated that the siRNA had a good knockdown efficiency (Figures 5H, I). Forty-eight hours after transfection, RAW264.7 cells were treated with 100 ng/ml LPS for 24 h. Then, by measuring the expression of the surface marker (CD86) of M1 macrophages by flow cytometry, we found that the expression level of CD86 in siIgf2bp3 cells was significantly lower than that in siNC cells (Figure 5J). In addition, we further detected the content of TNF-a in the cell supernatant, which indicated that the content of TNF-a in siIgf2bp3 cells was lower than that in siNC cells (Figure 5K). These results further validated the involvement of IGF2BP3 in the regulation of M1 macrophage polarization.

scRNA-seq revealed the relationship between IGF2BP3 expression and M1 macrophage polarization

To further characterize the relationship between IGF2BP3 expression and M1 macrophage polarization, we conducted scRNA-seq in the GSE159117 dataset. Fourteen cell clusters were

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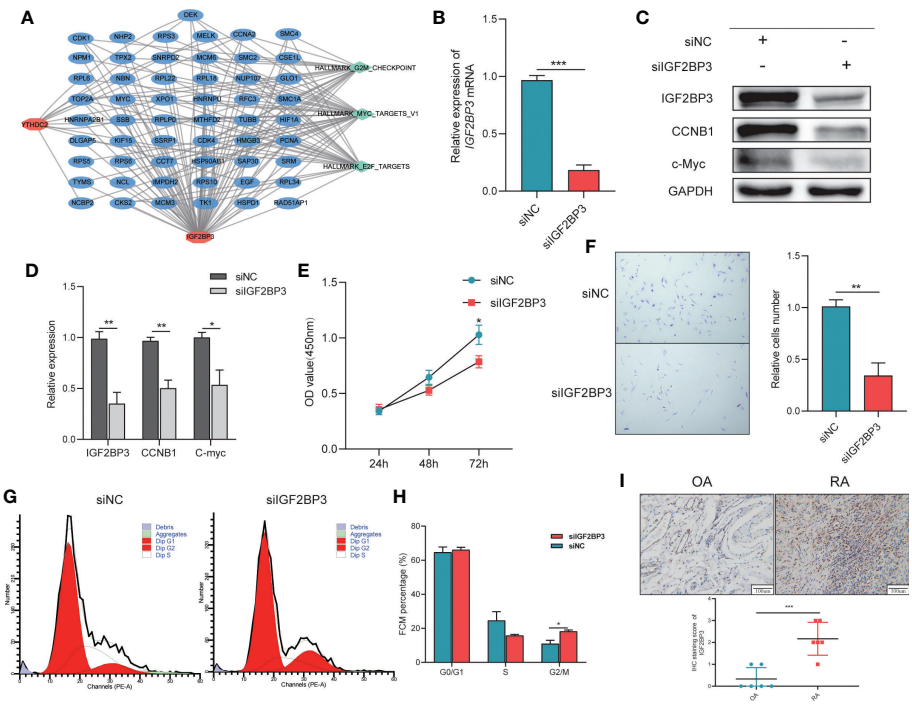

FIGURE 4
The importance of IGF2BP3 in the Viability and Cell Cycle of RA-FLSs. **(A)** The network connecting IGF2BP3 and YTHDC2 pathways and other targets; **(B)** RT–qPCR results showing the efficient depletion of IGF2BP3 expression in RA-FLSs compared with siNC-transfected RA-FLSs; **(C, D)** Expression of IGF2BP3, c-MYC and CCNB1 in RA-FLSs after transfection; **(E)** The proliferative ability of RA-FLSs after transfection was evaluated by a CCK-8 assay; **(F)** Representative images (left) and histograms (right) showing the effect of siFN1 on the cell proliferation of RA-FLSs; **(G, H)** Flow cytometric analysis was used to evaluate the cell cycle distribution of RA-FLSs after transfection; **(I)** Representative IHC staining and IHC staining score of Synovial tissues. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

obtained by a combined uniform manifold approximation and projection (UMAP) analysis (Figure 6A). SingleR (version 1.8.1) was used to identify 7 cell types: B cells, $CD4^+$ T cells, $CD8^+$ T cells, dendritic cells, monocytes, NK cells and T cells (Figure 6B). IGF2BP3 was found to be expressed mainly on monocytes and B cells among the seven cell types (Figure 6C; clusters 4 and 8). Macrophages are the main type of cell derived from monocytes. Therefore, the relationship between CD86 and IGF2BP3 expression was explored in monocytes, and CD86 and IGF2BP3 were found to have a coexpression trend (Figure 6D). Then, we preliminarily investigated the expression of several macrophage markers in monocytes. M1 macrophage markers (including CD86, IL1B, TLR2 and TLR4) were significantly upregulated but M2 macrophage markers (including MSR1, IL10, MMP14 and VEGFA) were downregulated in monocytes (Figure 6E).

Discussion

RA is a systemic autoimmune disorder affecting the synovium of peripheral joints. The average life expectancy of patients with RA is shorter than that of the overall population, and patients with active disease are also prone to develop various diseases, such as cardiovascular disease, pulmonary interstitial disease, and osteoporosis (24, 25). m6A methylation has been shown to be associated with tumours, neurological disorders, metabolic diseases, ADs, viral infections and so on (26). Mutations in the genes encoding m6A methylation regulators are closely associated with inflammation-related diseases, and changes in their expression levels have been observed in RA (21, 27). Therefore, exploring the diagnostic value and mechanism of m6A methylation regulators in RA is highly important for the effective treatment of RA and the improvement of its prognosis.

In this study, based on m6A methylation regulator expression profiles and consensus machine learning approaches, we constructed binary predictive classification models and assessed their accuracy. Among the models, the LASSO_λ-1se model not only performed better in the validation sets but also exhibited more stringent performance. In addition, the LASSO_λ-1se model exhibited better performance in whole blood samples, further suggesting that the LASSO_λ-1se model has application prospects in blood-based diagnosis of RA. Our primary aim in this study was to investigate the relationships between m6A methylation regulators and clinical classification rather than to develop a

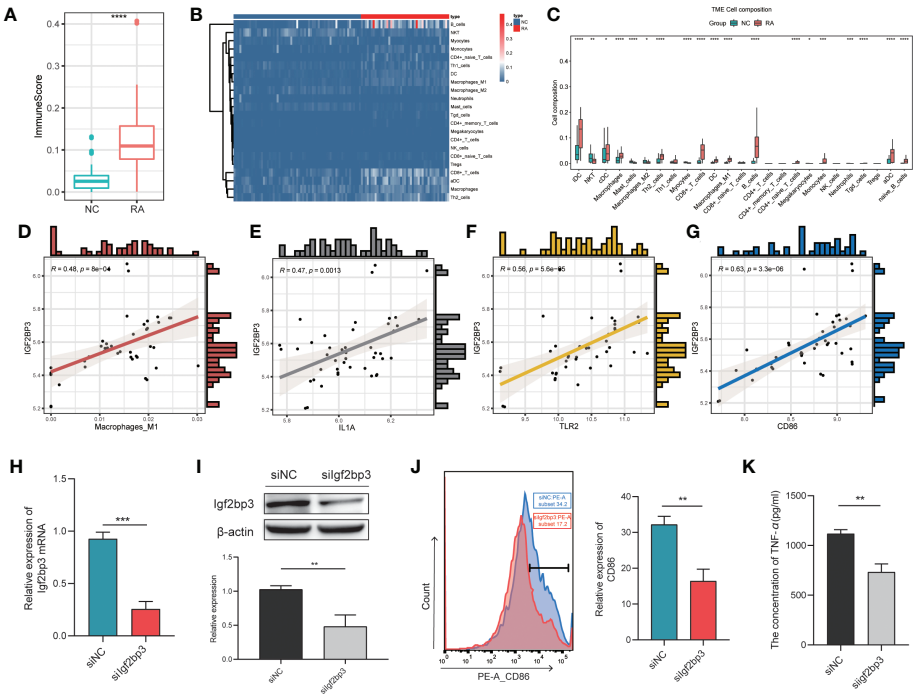

FIGURE 5
Correlation between IGF2BP3 expression and inflammatory activity. Immune scores **(A)** and proportions of immune cells **(B, C)** in the RA and NC groups; **(D)** correlation between the proportion of M1 macrophages and the expression level of IGF2BP3 in RA patients; **(E–G)** correlations between the expression levels of M1 macrophage markers (IL1A, CD86 and TLR2) and IGF2BP3; **(H, I)** RT–qPCR and Western blot analysis confirmed the efficiency of gene silencing; **(J)** expression level of CD86 in RAW264.7 cells after transfection; **(K)** the content of TNF-α in RAW264.7 cells after transfection. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

diagnostic tool. Combined with the comprehensive imaging, haematological and gene expression analyses, a diagnostic model of RA has more clinical diagnostic significance and higher accuracy. This study lays the foundation for the establishment of diagnostic tools by evaluating the accuracy of m6A methylation regulators for clinical classification and affirms the potential diagnostic value of m6A methylation regulators. A limitation of this study is the relatively small sample size used to generate and validate the m6A methylation regulators as classifiers. This may have led to overfitting of some models and thus to overestimation of effect sizes. To alleviate this issue, we validated each model's diagnostic value in different published datasets and validated potentially interesting genes using molecular biology experiments. To develop accurate diagnostic tools, further studies based on larger retrospective and prospective clinical cohorts are warranted.

Machine learning provides an unbiased approach to predict patient status while also offering the potential to identify previously unknown interactions and identify novel biological signatures (17, 28). Our approach of investigating the biomarkers identified through multiple feature selection techniques increases confidence in the generation of reproducible biomarker panels and reduces the number of m6A methylation regulators for potential clinical investigation. The selected m6A methylation regulators (IGF2BP3 and YTHDC2) ranked highly in variable importance. Previous studies have shown that IGF2BP3 and YTHDC2 are closely related to cell proliferation and migration, cell cycle regulation, and immune and inflammatory regulation (29–31). In addition, the study by Fan et al. confirmed that IGF2BP3 not only was significantly overexpressed in RA synovial tissue but also might be a therapeutic target of thymopentin (TP) during RA treatment (32). The above literature reports provide supporting evidence that IGF2BP3 and YTHDC2, identified here as candidate biomarkers, may be associated with disease progression in RA, validating our machine learning approach to identify relevant m6A methylation regulator biomarkers. Pathway enrichment analysis showed that IGF2BP3 and YTHDC2 were involved in the regulation of MYC_TARGETS_V1, E2F_TARGETS, G2M_CHECKPOINT and other pathways, which are closely related to the cell cycle. In particular, IGF2BP3 not only was ranked highest by the Boruta, Rpart and XGBoost methods but also showed better diagnostic value in the training set. We focused on verifying the relationship between IGF2BP3 expression and the cell cycle and further confirmed that IGF2BP3 may affect the proliferation of

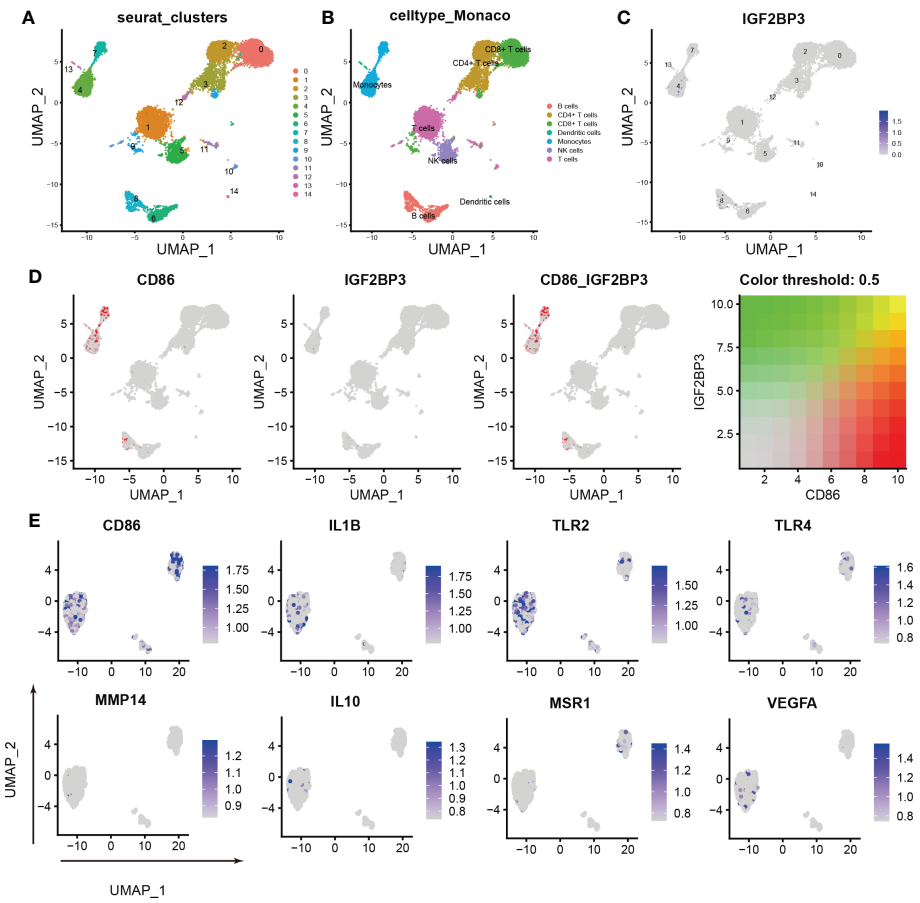

FIGURE 6
Characterization of macrophages by scRNA-seq in PBMCs. **(A)** UMAP plot showing the sources of the collected scRNA-seq cell samples; **(B)** UMAP plot showing 14 cell clusters of 7 cell types in the collected samples; **(C)** UMAP plot showing the IGF2BP3 expression level in the 14 cell clusters; **(D)** scRNA-seq analysis revealed the correlation between IGF2BP3 and CD86 expression; **(E)** UMAP plot showing the expression levels of M1 and M2 macrophage markers.

RA-FLSs by regulating the G2/M transition. Inflammatory cells can secrete a large amount and variety of inflammatory factors and chemokines, leading to the activation of more FLSs and promoting their proliferation and migration, thereby further aggravating the inflammatory response in the disease (33). Among these immune cell types, M1 macrophages attracted our attention for the following three reasons: 1. M1 macrophages, also called classical macrophages, can produce proinflammatory cytokines and thus have potent microbicidal ability but are also prone to cause tissue destruction and exacerbate inflammatory processes that are detrimental to health (34); 2. The synovial lining of RA patients exhibits cell proliferation and a large amount of inflammatory cell infiltration in the interstitium. The degree of inflammatory infiltration determines the severity of the disease (35). 3. Among the inflammatory cells involved in RA, macrophages play a key role. These cells can polarize into different phenotypes and mediate the immune/inflammatory response as well as the repair phase when possible (23). By analysing the relationship between IGF2BP3 expression and M1 macrophage polarization in RA RNA-seq datasets and scRNA-seq datasets, we found that IGF2BP3 plays a crucial role in M1 macrophage polarization. CD86, also known as B7.2, is a T lymphocyte activation antigen with a molecular weight of 80 kD and can be expressed in dendritic cells, monocytes, T lymphocytes and B lymphocytes. Previous studies have shown that CD86 can serve as a marker to elevate the proportion of M1 macrophages (36, 37). By measuring the expression of CD86 by flow cytometry, we found that the expression level of CD86 in siIgf2bp3 RAW264.7 cells was significantly lower than that in siNC RAW264.7 cells. Yang et al. also showed that siIGF2BP3 can reduce MALAT1 expression, thereby impeding p38/mitogen-activated protein kinase phosphorylation and macrophage-mediated inflammation (38). These studies all further verified that IGF2BP3 can regulate macrophage polarization and inflammatory exacerbation during RA progression.

The RA diagnostic model established based on public databases had good performance in multiple validation sets. However, further validation of the diagnostic value of established models in larger independent cohorts is warranted before considering their clinical application. Furthermore, we used five machine learning feature selection algorithms on data from patient synovial tissue to identify two signature m6A methylation regulators in RA, and our findings may provide a new RA marker and reveal novel disease mechanisms. Moreover, this study is the first to confirm the effect of the m6A reader protein IGF2BP3 on the progression of RA and verify its biological function through bioinformatics analysis and molecular biology experiments. This study provides new ideas and strategies for the early diagnosis and targeted therapy of RA and has theoretical innovation prospects. Moreover, it provides theoretical support for the discovery of new markers and drug targets for RA.

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 authors.

Ethics statement

The ethics committee of China-Japan Friendship Hospital approved the research (approval number 2021-153-K111). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

QG and CX designed and wrote the manuscript. The other authors participated in discussions associated with the manuscript and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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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.2022.1041284/full#supplementary-material

SUPPLEMENTARY TABLE 1
Sample information from the different public datasets.

SUPPLEMENTARY TABLE 2
The leave-one-out (LOO) cross-validation performance in the training set a random forest wrapper (Boruta), LASSO_λ-min, LASSO_λ-1se, logistic regression, regression partition trees (Rpart) and extreme gradient boosting (XGBoost).

SUPPLEMENTARY TABLE 3
The selected genes in different machine learning methods.

SUPPLEMENTARY TABLE 4
The gene targets of IGF2BP3 and YTHDC2.

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Exposure to environmental air pollutants as a risk factor for primary Sjögren's syndrome

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Background: Environmental etiology of primary Sjögren's syndrome (pSS), an autoimmune disease, has been proposed. This study determined whether the exposure to air pollutants was an independent risk factor for pSS.

Methods: Participants were enrolled from a population-based cohort registry. Daily average concentrations of air pollutants from 2000 to 2011 were divided into 4 quartiles. Adjusted hazard ratios (aHRs) of pSS for exposure to air pollutants were estimated in a Cox proportional regression model adjusting for age, sex, socioeconomic status, and residential areas. A subgroup analysis stratified by sex was conducted to validate the findings. Windows of susceptibility indicated years of exposure which contributed the most to the observed association. Ingenuity Pathway Analysis was used to identify underlying pathways of air pollutant-associated pSS pathogenesis, using Z-score visualization.

Results: Two hundred patients among 177,307 participants developed pSS, with a mean age of 53.1 years at acumulative incidence of 0.11% from 2000 to 2011. Exposure to carbon monoxide (CO), nitric oxide (NO), and methane (CH4) was associated with a higher risk of pSS. Compared to those exposed to the lowest concentration level, the aHRs for pSS were 2.04 (95%CI=1.29-3.25), 1.86 (95% CI=1.22-2.85), and 2.21 (95%CI=1.47-3.31) for those exposed to high levels of CO, NO, and CH4, respectively. The findings persisted in the subgroup analysis, in which females exposed to high levels of CO, NO, and CH4 and males exposed to high levels of CO were associated with significantly great risk of pSS. The cumulative effect of air pollution on pSS was time-dependent. The underlying

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cellular mechanisms involved chronic inflammatory pathways including the interleukin-6 signaling pathway.

Conclusion: Exposure to CO, NO, and CH4 was associated with a high risk of pSS, which was biologically plausible.

KEYWORDS

air pollution, carbon monoxide, nitric oxide, methane, interleukin-6, Sjögren's syndrome

1 Introduction

Sjögren's syndrome (SS) is an autoimmune disease in which the destruction of the epithelium of the exocrine glands, including the salivary glands and lacrimal glands, leads to the hallmark symptoms of sicca syndrome (1). Patients with SS may also present articular, cutaneous, pulmonary, and muscular complications, with symptoms that may plateau, worsen or go into remission (1). SS affects about 35 million people worldwide, with manifestations of significant fatigue, joint and muscle pain, impairing their quality of life, and the development of extra glandular manifestations, including non-Hodgkin's lymphoma in about 30% to 50% of patients (2). The incidence of primary SS (pSS) is estimated to be 7 cases per 100,000 people per year in the general population with a female-to-male predominance of 9:1 (1). It is significantly higher in middle-aged women than in men as the prevalence rate is 60.82 cases per 100,000 residents with a female-male ratio of 10.72, and a mean onset age of 60.8 ± 15.2 years (3, 4). As with most autoimmune diseases, genetic, environmental, and hormonal factors are incorporated in SS pathogenesis (1). Although the pathogenic pathways underlying SS have yet been completely elucidated (5), studies have described that the etiology and pathogenesis of SS are related to genetic susceptibility to environmental factors, viral infection, vitamin D deficiency, smoking, endocrine alternations and the role of angiogenesis (6).

On the other hand, air pollution has been considered as one consequence of globalization involving industrial development, fossil fuel burning, and climate change. With the rising awareness of air pollution and environmentalism, how urban air pollution could negatively affect human health (7), as a constituent of the ecosystems in the ecotoxicological context, has been brought to the attention of healthcare professionals. Studies have provided evidence that air pollution is associated with enhanced incidence of cardiovascular and respiratory diseases (8). In terms of rheumatic inflammatory diseases, inhalation of air pollutants has been proposed to be an independent risk factor for systemic inflammatory responses, which is associated with the development and progression of autoimmune diseases, such as systemic autoimmune rheumatic diseases (SARD), juvenile idiopathic arthritis (JIA), and rheumatoid arthritis (RA) (9). However, it remains unclear whether the exposure to air pollutants is also a vital factor for the pathogenesis of SS. Therefore, the aim of the present population-based cohort study was to determine whether the exposure to air pollutants could be an environmental risk factor for pSS.

2 Materials and methods

2.1 Data source

With the National Health Insurance Research Database (NHIRD), covering 99% of the population of Taiwan, 25 million people, this population-based cohort study was conducted. The NHIRD consisted of data on reimbursement claims sourced from the National Health Insurance (NHI) program (10–12), a single-payer National Health Insurance (NHI) program which was instituted in 1995 and covered nearly 99% of Taiwan's population by the end of 2008 (13). Outpatient visits, hospital admissions, prescriptions, procedures, and disease diagnostic records were retrieved from NHIRD (14–26).

Air pollution data including concentrations of carbon monoxide (CO), nitrogen monoxide (NO), and methane (CH_4) were obtained from the Taiwan Air Quality-Monitoring Database (TAQMD). This database was released by a governmental agency, Taiwan Air Quality Monitoring Network, which collected daily ambient air pollution data from 78 community-based monitoring stations, available on a real-time basis since 1993. Pollutant data on particulate matter $(PM)_{10}$ ($\mu g/m^3$), $PM_{2.5}$ ($\mu g/m^3$), ozone (O_3) (parts per million, ppm), CO (ppm), CH_4 (ppm), sulfur dioxide (SO_2, parts per billion, ppb), NO_2 (ppb), and NO (ppb), were retrieved for their daily mean concentrations from January 1st, 2000 to December 31th, 2011. The endpoint of the follow-up period of this study was the diagnosis of pSS, or December 2011. The daily average exposed air pollutant concentrations for each participant were estimated using the Inverse Distance Weighting Method (IDW

method). As one means of spatial interpolation, the IDW method was used to give the predicted values of unknown points by weighting the average of known points. In the present study, data on air pollution within two years prior to the diagnosis of pSS were accessed, with the IDW method used to estimate the concentrations of air pollutants based on values of the concentrations measured by monitoring stations surrounding the registered address of each participant, considering the distance between the monitoring station and the registered address of the participant.

2.2 Study population

Populations residing in areas with ambient air quality-monitoring stations were set as the study population. Among them, those with a history of SS before 2000 were excluded. All participants were traced from January 1st, 2000 until the endpoint of the study period. Confounding factors including age, gender, annual individual income, and urbanization levels where the participants lived, were adjusted. The urbanization level was defined following the National Health Research Institutes (NHRI) classification system, in which all residential areas were stratified into 7 urbanization levels considering their population density, resident education level, agricultural activity, and healthcare accessibility (physician number per 100,000 residents) (27). Due to the lack of participants from rural areas of levels 4-7, this study included areas of levels 1-4, with level 1 as the most urbanized and level 4 as the least urbanized. Annual incomes were classified into 4 groups: less than 5,760 USD, 5,760–7,320 USD, 7,320–8,400 USD, and over 8,400 USD.

2.3 Definition of exposure and outcome

The exposure to air pollution was defined using daily average concentrations of each air pollutant from 2000 to the endpoint. The daily average concentrations were classified into four groups using interquartile range (IQR), with three cut-off points (25th, 50th and 75th percentiles) ranging from the lowest concentration level, Quartile (Q) 1, to the highest concentration level, Q4. The American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR) Consensus Group criteria was followed to diagnose pSS as the outcome of this cohort study (28), for which the diagnosis of pSS was made based on anti-SSA (Ro) antibody positivity, focal lymphocytic sialadenitis, abnormal ocular staining, Schirmer's tests, and unstimulated salivary flow rates (28). The diagnosis of pSS had to be confirmed in at least two outpatient visits or at least one hospitalization record within two years, all of which were peer-reviewed by a rheumatologist.

2.4 Statistical analyses

The Chi-squared test was used to determine the differences in age, sex, urbanization level, annual income, season, and the incidence of pSS. A multivariable Cox proportional hazard regression model adjusted for confounding factors was used to derive the association between the exposure to air pollutants and the risk of new-onset pSS. Incidence rates (IRs) and adjusted hazard ratios (aHRs) for new-onset pSS in participants exposed to each concentration level of air pollutants were derived. HRs for quartile levels Q2, Q3, and Q4, were compared to the reference level, Q1. All analyses were conducted with SAS version 9.4 (SAS Institute, Cary, NC, USA). A two-sided *P*-value less than 0.05 was considered statistically significant.

2.5 Bioinformatic analyses

Canonical Pathway Analysis (29–32) was conducted on Ingenuity Pathway Analysis (IPA) software (QIAGEN, Hilden, Germany) using differentially expressed (DE) RNA-seq data of airway epithelial cells exposed to coarse (n=3), fine (n=3), and ultrafine particulate matter (PM) (n=3) versus control group (n=3) obtained from the National Center for Biotechnology Information-Gene Expression Omnibus database (GEO; GSE7010), as well as parotid gland tissue from patients with pSS (n=3) versus healthy adults (n=3) obtained from GEO (GSE40611). Positive and negative z-scores indicated up-regulation and down-regulation of targeted pathways, respectively. Molecular Activation Prediction (MAP) was performed to model the mechanisms by which air pollutants may trigger or regulate the pathogenesis of pSS.

3 Results

177,307 residents with available data for both their exposure to air pollutants and electronic medical records were included in this study. The mean age of patients who developed pSS (N=200) was 53.1 years, with 60.5% over 50 years of age (N= 121) and 85% of patients were females (N=170). Among all included air pollutants, exposure to CO, NO, and CH_4, was significantly associated with higher risks of pSS.

3.1 Baseline characteristics and the incidence of pSS in individuals exposed to CO, NO, and CH_4

Participants exposed to CO in Q1 level were the oldest, with a median age of 41.1 years, and exhibited the least incidence of pSS (0.07%). Participants exposed to Q3 level of CO had a younger median age of 38.7 years and exhibited the highest incidence of pSS (0.18%) (Table 1).

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TABLE 1 Patient information.

Variables	N	%	N	%	N	%	N	%	p-value
CO	Q1 (<0.56 ppm)		Q2 (0.56-0.68 ppm)		Q3 (0.68-0.81 ppm)		Q4 (>0.81 ppm)		
N=177290	N=43942		N=45488		N=40036		N=47824		
Age (mean, SD*)	41.1±16		39.1±15.1		38.7±15.1		39.2±15.2		<0.001
Male sex	19791	45	20018	44	17848	44.6	20728	43.3	<0.001
Annual income (USD) †									<0.001
< 5760	7071	16.1	7790	17.1	6978	17.4	8470	17.7	
5760–7320	12457	28.4	14969	32.9	14357	35.9	16058	33.6	
7320-8400	12914	29.4	10161	22.3	7144	17.8	9664	20.2	
≥ 8400	11500	26.2	12568	27.6	11557	28.9	13632	28.5	
Urbanization level&									<0.001
1 (highest)	9456	21.5	10748	23.6	15509	38.7	24923	52.1	
2	13690	31.2	20404	44.9	9811	24.5	13599	28.4	
3	7616	17.3	6848	15.1	9439	23.6	6374	13.3	
4 (lowest)	13180	30	7488	16.5	5277	13.2	2928	6.12	
New-onset Sjogren's syndrome	29	0.07	42	0.09	71	0.18	58	0.12	<0.001
NO	Q1 (<5.16 ppb)		Q2 (5.16-8.58 ppb)		Q3 (8.58-11.5 ppb)		Q4 (>11.5 ppb)		
N=177307	N=43218		N=45009		N=39988		N=49092		
Age (mean, SD*)	41.0±	16	39.7±	15.5	37.7±	14.7	39.5±	15.2	<0.001
Male sex	19272	44.6	20280	45.1	17486	43.7	21358	43.5	<0.001
Annual income (USD) †									<0.001
< 5760	7061	16.3	7924	17.6	6722	16.8	8606	17.5	
5760–7320	11945	27.6	15079	33.5	14806	37	16016	32.6	
7320-8400	13340	30.9	9550	21.2	7683	19.2	9314	19	
≥ 8400	10872	25.2	12456	27.7	10777	27	15156	30.9	
Urbanization level&									<0.001
1 (highest)	9025	20.9	7924	17.6	6722	16.8	8606	17.5	
2	15053	34.8	15079	33.5	14806	37	16016	32.6	
3	4684	10.8	9550	21.2	7683	19.2	9314	19	
4 (lowest)	14456	33.5	12456	27.7	10777	27	15156	30.9	
New-onset Sjogren's syndrome	35	0.08	45	0.1	53	0.13	67	0.14	0.04
CH_4:	Q1 (<2.00 ppm)		Q2 (2.00-2.04 ppm)		Q3 (2.04-2.10 ppm)		Q4 (>2.10 ppm)		
N=133884	N=35349		N=30071		N=43474		N=24990		
Age (mean, SD*)	38.4±14.2		38.8±14.7		37.9±14.7		41.6±16.6		<0.001
Male sex	15724	44.5	12970	43.1	19132	44	11274	45.1	<0.001
Annual income (USD) †									<0.001
< 5760	5831	16.5	5012	16.7	7436	17.1	4585	18.4	

(Continued)

TABLE 1 Continued

Variables	N	%	N	%	N	%	N	%	p-value
5760–7320	12584	35.6	9808	32.6	15431	35.5	7495	30	
7320-8400	7544	21.3	5937	19.7	9013	20.7	6266	25.1	
≥ 8400	9390	26.6	9314	31	11594	26.7	6644	26.6	
Urbanization level[&]									<0.001
1 (highest)	10340	29.3	11091	36.9	16730	38.5	8745	35	
2	9293	26.3	9736	32.4	15284	35.2	7160	28.7	
3	9085	25.7	5472	18.2	6854	15.8	3881	15.5	
4 (lowest)	6631	18.8	3772	12.5	4606	10.6	5204	20.8	
New-onset Sjogren's syndrome	39	0.11	14	0.05	38	0.09	59	0.24	<0.001

* Respiratory infection (n=6), Epstein-Barr virus infection (2), adenovirus infection (1), cervical lymphadenitis (1), Norovirus enteritis (1), Yersinia enteritis (1), urinary tract infection (1), urticaria (1).
[&]The urbanization level was categorized by the population density of the residential area into 4 levels, with level 1 being the most urbanized and level 4 being the least urbanized area.
[†] The numbers were converted from New Taiwan Dollars to US Dollars. One New Taiwan Dollar equaled 0.03 US Dollar.
HV; Healthy Volunteer, NA; Not applecable, IVIG; Intravenous immunoglobulin, CAAs; Coronary artery abnormalities, ASA; acetylsalicylic acid, PSL; prednisolone, mPSL pulse; intravenous high-dose methylprednisolone pulse therapy, CsA; cyclosporin A, PE; plasma exchange, DEX; dexamethasone, Abs; antibiotics.

Participants exposed to NO in the Q1 level were the oldest, with the median age of 41.0 years; whereas those exposed to the Q3 level were the youngest, with a median age of 37.7 years. Compared with participants exposed to Q1, Q2, and Q3 levels of NO concentration, those exposed to Q4 level exhibited the highest incidence of pSS (0.14%), with the median age of 39.5 years (Table 1). Overall, the exposure to NO was significantly associated with the incidence of pSS (Table 1).

Participants exposed to CH_4 in the Q4 level were the oldest, with a median age of 41.6 years. Participants exposed to Q4 level had the highest incidence of pSS (0.24%), compared with those exposed to lower concentration levels. More participants exposed to Q4 level of CH4 lived in level 1 urbanization areas (35.0%) (Table 1).

3.2 Incidence rates and risks of pSS in individuals exposed to CO, NO, and CH_4

The association between exposed levels of CO, NO, and CH_4 and the incidence of pSS was dose-dependent. Subjects exposed to high concentrations (Q3 and Q4 levels) of CO, NO, and CH_4, presented with significantly higher IRs of pSS, compared to those exposed to Q1 level of these air pollutants (Table 2). Likewise, after adjusting for demographics including age, sex, socioeconomic status, and urbanization levels of residing areas, the risk of pSS in those exposed to Q3 or Q4 levels were significantly higher than that in those exposed to to the Q1 level of each air pollutant (Table 2 and Figure 1). In particular, the aHR was 3.05 (95% CI = 1.96–4.72) for individuals exposed to Q3 level of CO, 1.88 (95% CI = 1.21-2.92) for those exposed to Q3 level of NO, and 2.21 (95% CI = 1.47-3.31) for participants exposed to Q4 level of CH_4 (Table 2 and Figure 1).

These findings persisted in a subgroup analysis stratified by sex, in which the risks of pSS among females exposed to Q3 (aHR =2.83, 95% CI = 1.77-4.52) and Q4 (aHR =1.84, 95% CI = 1.12-3.02) levels of CO, Q3 (aHR =1.64, 95% CI = 1.02-2.65) and Q4 (aHR =1.79, 95% CI = 1.13-2.81) levels of NO, and Q4 level of CH_4 (aHR =2.34, 95% CI = 1.52-3.62), were significantly greater than females exposed to Q1 levels of respective air pollutants; parallelly, males exposed to Q3 (aHR =4.97, 95% CI = 1.37-18.1) and Q4 (aHR =3.99, 95% CI = 1.05-15.1) levels of CO also had significantly higher risks of pSS, compared to males exposed to Q1 level of CO (Table 3 and Figure 2).

Overall, the dose-dependent effect of CO, NO, and CH_4 on the risk of new-onset pSS was observed; on the other hand, the risks of pSS following the exposure to PM_{10}, $PM_{2.5}$, and NO_x, were not consistently increased in individuals exposed to all high concentrations (Supplementary Figure 1 and Supplementary Table 1). Collectively, these findings, suggested an elevated risk of pSS subsequent to the exposure to air pollutants that where small molecules, but not air pollutants of large sizes. Moreover, windows of susceptibility indicated years of exposure that contributed the most to the observed association, in which years between 2007 and 2011 were of high incidence of pSS (9.50%, 16.00%, and 13.00% of all incident cases of pSS happened in 2007, 2009, and 2011, respectively) (Figure 3). This suggested the time-dependent cumulative effect of the exposure to air pollutants on pSS.

TABLE 2 Incidence and risk of pSS in participants exposed to CO, NO, and CH_4.

	Pollutant levels	Event	PY	IR	cHR	95%CI	aHR	95%CI
CO	N=177290							
Q1 (<0.56 ppm)	43942	29	508170	0.57	Ref.		Ref.	
Q2 (0.56-0.68 ppm)	45488	42	529881	0.79	1.39	(0.86, 2.22)	1.5	(0.93, 2.41)
Q3 (0.68-0.81 ppm)	40036	71	460849	1.54	2.7	(1.75, 4.16)***	3.05	(1.96, 4.71)***
Q4 (>0.81 ppb)	47824	58	549247	1.06	1.85	(1.19, 2.89)**	2.04	(1.29, 3.25)**
NO	N=177307							
Q1 (<5.16 ppb)	43218	35	500058	0.7	Ref.		Ref.	
Q2 (5.16-8.58 ppb)	45009	45	520789	0.86	1.24	(0.79, 1.92)	1.32	(0.85, 2.06)
Q3 (8.58-11.5 ppb)	39988	53	463131	1.14	1.63	(1.07, 2.50)*	1.88	(1.21, 2.92)**
Q4 (>11.5 ppb)	49092	67	564216	1.19	1.7	(1.13, 2.56)*	1.86	(1.22, 2.85)**
CH4	N=133884							
Q1 (<2.00 ppm)	35349	39	417257	0.93	Ref.		Ref.	
Q2 (2.00-2.04 ppm)	30071	14	354908	0.39	0.42	(0.23, 0.78)**	0.4	(0.22, 0.75)
Q3 (2.04-2.10 ppm)	43474	38	507527	0.75	0.8	(0.52, 1.26)	0.8	(0.51, 1.25)
Q4 (>2.10 ppm)	24990	59	275499	2.14	2.35	(1.57, 3.53)***	2.21	(1.47, 3.31)***

PY, person-years.
IR, incidence rate, (per 10,000 person-years).
cHR, crude hazard ratio.
aHR, adjusted hazard ratio obtained from a multivariate analysis, after adjusting for age, sex, annual income, and urbanization level.
CI, confidence interval.
Ref., reference group.
*p<0.05; **p<0.01; ***p<0.001.

3.3 Modeled mechanisms of air pollutant-triggered pSS

Canonical pathway analyses indicated multiple air pollutant-associated inflammatory pathways that may trigger pSS, for which their degrees of upregulation were associated with particle sizes, with smaller sizes of air pollutants exacerbating larger extents of inflammation (Figure 4). Among pathways expressed in airway epithelial cells exposed to coarse, fine and ultrafine PM, and in parotid glands of patients with pSS, the highly expressed inflammatory pathways included (1): interleukin (IL)-6 signaling, with Z-scores being 3.272 for pSS,

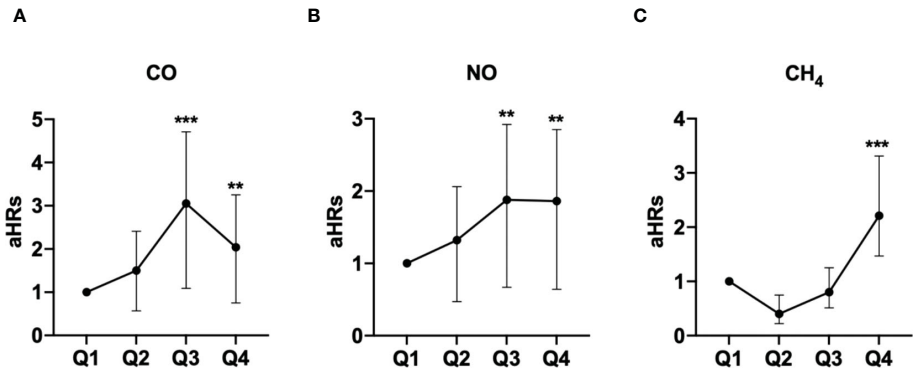

FIGURE 1
Illustrated hazard ratios (HRs) of pSS in individuals exposed to carbon monoxide (CO) **(A)**, nitric oxide (NO) **(B)**, and methane (CH_4) **(C)**. **p<0.01; ***p<0.001.

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TABLE 3 Incidence and risk of pSS in participants exposed to CO, NO, and CH_4, as stratified by sex.

	Pollutant levels	Event	PY	IR	cHR	95%CI	aHR	95%CI
Female								
CO	N=98905							
Q1 (<0.56 ppm)	24151	26	281533	0.92	Ref.		Ref.	
Q2 (0.56-0.68 ppm)	25470	36	298097	1.21	1.31	(0.79, 2.16)	1.41	(0.85, 2.34)
Q3 (0.68-0.81 ppm)	22188	60	257245	2.33	2.53	(1.60, 4.01)***	2.83	(1.77, 4.52)***
Q4 (>0.81 ppm)	27096	48	314121	1.53	1.66	(1.03, 2.67)*	1.84	(1.12, 3.02)*
NO	N=98911							
Q1 (<5.16 ppb)	23946	31	279250	1.11	Ref.		Ref.	
Q2 (5.16-8.58 ppb)	24729	39	288126	1.35	1.22	(0.76, 1.96)	1.29	(0.81, 2.08)
Q3 (8.58-11.5 ppb)	22502	42	262029	1.6	1.44	(0.91, 2.30)	1.64	(1.02, 2.65)*
Q4 (>11.5 ppb)	27734	58	321605	1.8	1.63	(1.63, 2.52)*	1.79	(1.13, 2.81)*
CH4	N=74784							
Q1 (<2.00 ppm)	19625	33	232163	1.42	Ref.		Ref.	
Q2 (2.00-2.04 ppm)	17101	12	202339	0.59	0.42	(0.22, 0.81)**	0.41	(0.21, 0.79)**
Q3 (2.04-2.10 ppm)	24342	30	285615	1.05	0.74	(0.45, 1.22)	0.74	(0.45, 1.22)
Q4 (>2.10 ppm)	13716	53	153862	3.44	2.47	(1.60, 3.82)***	2.34	(1.52, 3.62)***
Male								
CO	N=78385							
Q1 (<0.56 ppm)	19791	3	226637	0.13	Ref.		Ref.	
Q2 (0.56-0.68 ppm)	20018	6	231784	0.26	1.95	(0.49, 7.81)	2.26	(0.56, 9.09)
Q3 (0.68-0.81 ppm)	17848	11	203605	0.54	4.09	(1.14, 14.6)	4.97	(1.37, 18.1)*
Q4 (>0.81 ppm)	20728	10	235126	0.43	3.22	(0.89, 11.7)	3.99	(1.05, 15.1)*
NO	N=78396							
Q1 (<5.16 ppb)	19272	4	220809	0.18	Ref.		Ref.	
Q2 (5.16-8.58 ppb)	20280	6	232663	0.26	1.43	(0.40, 5.05)	1.57	(0.44, 5.58)
Q3 (8.58-11.5 ppb)	17486	11	201103	0.55	3.02	(0.96, 9.50)	3.98	(1.22, 13.0)*
Q4 (>11.5 ppb)	21358	9	242611	0.37	2.05	(0.63, 6.66)	2.44	(0.72, 8.24)
CH4	N=59100							
Q1 (<2.00 ppm)	15724	6	185094	0.32	Ref.		Ref.	
Q2 (2.00-2.04 ppm)	12970	2	152569	0.13	0.41	(0.08, 2.01)	0.38	(0.08, 1.88)
Q3 (2.04-2.10 ppm)	19132	8	221913	0.36	1.12	(0.39, 3.24)	1.12	(0.38, 3.25)
Q4 (>2.10 ppm)	11274	6	121637	0.49	1.58	(0.51, 4.91)	1.45	(0.46, 4.50)

PY, person-years.
IR, Incidence rate, (per 10,000 person-years).
cHR, crude hazard ratio.
aHR, adjusted hazard ratio of a multivariate analysis, after adjustment for age, sex, annual income, and urbanization level.
CI, confidence interval.
Ref., reference group.
*p<0.05; **p<0.01; ***p<0.001.

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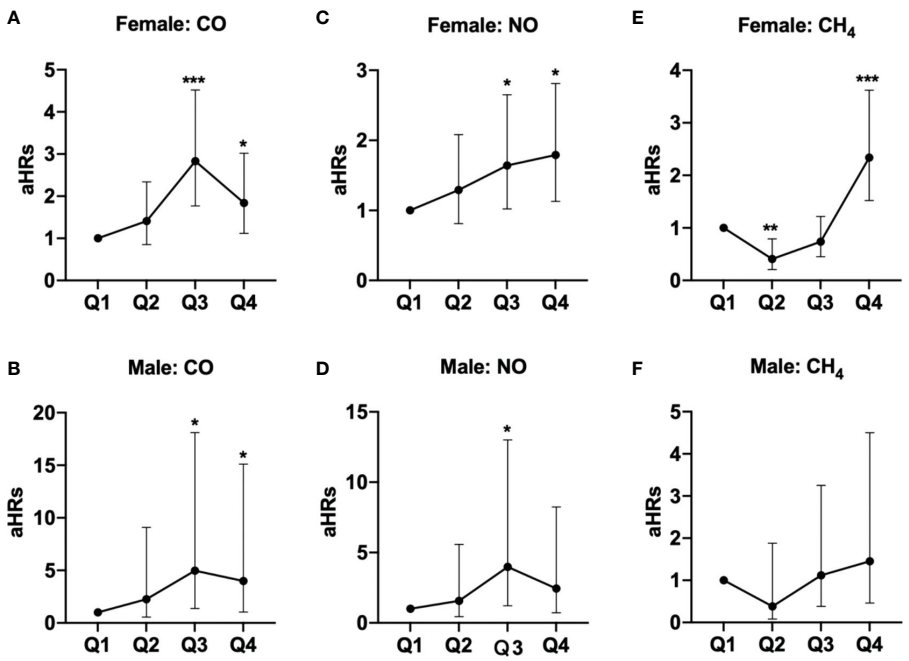

FIGURE 2
Illustrated hazard ratios (HRs) of pSS in females and males exposed to (**A**, female; **B**, male) CO, (**C**, female; **D**, male) NO, and (**E**, female; **F**, male) CH_4. *p<0.05; ***p<0.001.

2.714 for exposure to ultrafine PM, 2.121 for exposure to fine PM, and 1.663 for exposure to coarse PM, (2) Toll-like receptor signaling, with Z-scores being 1.633 for pSS, 2.449 for exposure to ultrafine PM, 2 for exposure to fine PM, and 1.342 for exposure to coarse PM, (3) acute phase response signaling, with Z-scores being 2.921 for pSS, 2.111 for exposure to ultrafine PM, 1.414 for exposure to fine PM, and 0.816 for exposure to coarse PM, (4) adrenomedullin signaling, with Z-scores being 2.401 for pSS, 1.89 for exposure to ultrafine PM, 1.633 for exposure to fine PM, and 1.342 for exposure to coarse PM, (5) NF-κB signaling, with Z-scores being 2.502 for pSS, 1.633 for exposure to ultrafine PM, 0.816 for exposure to fine PM, and 0.707 for exposure to coarse PM, (6) neuroinflammation signaling, with Z-scores being 4.899 for pSS, 1.508 for exposure to ultrafine PM, 0.707 for exposure to fine PM, and -0.378 for exposure to coarse PM, (7) B cell signaling pathway with Z-scores being 4.737 for pSS, 1.508 for exposure to ultrafine PM, 0.707 for exposure to fine PM, and 1.633 for exposure to coarse PM, and (8) osteoarthritis pathway with Z-scores being 3.157 for pSS, 1.387 for exposure to ultrafine PM, 1.387 for exposure to fine PM, and 0.333 for exposure to coarse PM.

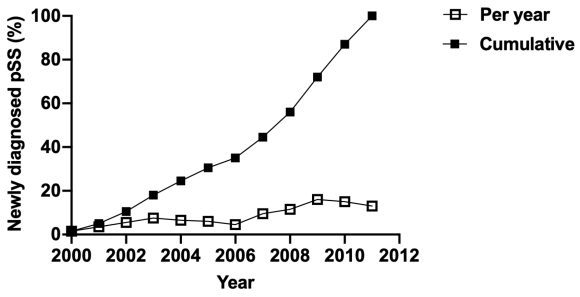

FIGURE 3
Incidence of newly diagnosed pSS per year and cumulative incidence of newly diagnosed pSS.

To further decipher the network that addressed new-onset pSS following the exposure to air pollutants, findings of the MAP analysis (33, 34) supported that IL-6 signaling was activated in both the pathogenesis of pSS (Figure 5A) and the exposure to ultrafine (Figure 5B) or fine PM (Supplementary Figure 1A), in which transcription factor NF-IL6 was activated and subsequently induced the production of multiple inflammatory cytokines, including IL-6 and IL-8, while STAT3 was phosphorylated to transport downstream signaling and the production of acute-phase proteins, such as C-reactive protein (CRP). To the contrary, exposure to coarse PM only activated NF-IL6- but not STAT3-mediated signaling (Supplementary Figure 1B).

Overall, these inflammatory pathways, especially the IL-6 signaling, were upregulated more in response to the exposure to ultrafine particles than that to the exposure to coarse particles

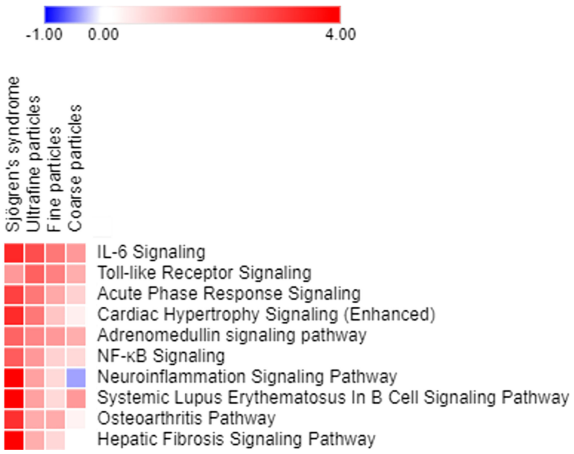

FIGURE 4
Pathways expressed in parotid glands of patients with pSS and primary airway epithelial cells exposed to ultrafine, fine, and coarse PM, ordered based on the sum of z scores.

(Figure 4), which was in line with significant findings in the cohort study on small gaseous molecules.

4 Discussion

In this population-based cohort study, an independent correlation between exposure to small molecule air pollutants and new-onset pSS was observed. Individuals exposed to great concentrations of CH_4, NO, CO, presented with significantly great risks of SS. After adjusting for age, sex, annual income, and urbanization levels of the areas where the participants lived, the association was still notable, for which our findings were not dominated by population density or socioeconomic status. The upregulated pathways in response to the exposure to air pollutants were proinflammatory, and their degrees of upregulation were related to PM sizes. Moreover, in accordance with the above-mentioned findings that small-molecule air pollutants, instead of PM_{10} or $PM_{2.5}$, were associated with increased risks of pSS, the inflammatory pathways were most highly expressed in samples exposed to ultrafine particles, followed by those exposed to fine particles, then those exposed to coarse particles; these scale-dependent findings support the clinical relevance of findings in the present cohort study on significantly great risk of pSS following exposure to small molecule air pollutants (35).

Although previous studies suggested that socioeconomic status may affect the occurrence and development of autoimmune diseases (36), in the present study, the effect of air pollution on pSS was independent of socioeconomic status and where the participants lived. Moreover, results of canonical pathway analyses in the present study illustrated that such an association can be based on significantly triggered inflammatory pathways including the IL-6 signaling pathway that were highly expressed in airway epithelial cells and salivary glands. These findings were similar to previous studies suggesting that inhalation of air pollutants may directly affect the lungs, causing acute and chronic inflammation in the respiratory system (37), and may be associated with non-pulmonary diseases, such as type 2 diabetes, cancer, neurodegenerative diseases, and autoimmune rheumatoid diseases (38, 39). As such, together with previous studies (38, 39), findings of the present study allowed for the conjecture that air pollutants may not only directly induce local pulmonary inflammation but also trigger systemic chronic inflammatory responses, which was to the mechanisms by which air pollutants trigger cardiovascular diseases (8).

As to whether the exposure to air particulates could exacerbate or trigger SS, few studies have addressed the impact of airborne pollutants on SS. Most of these studies were either cross-sectional or animal studies. For instance, one animal study demonstrated that inhaled residual oil fly ash (ROFA) exacerbated lung response in SS mice models, mimicking the harmful effects of airborne pollution on the airway of patients with SS (40). Another cross-sectional study indicated that ocular abnormalities and eye irritation in patients with SS exposed to nitrogen dioxide were significantly more severe than control patients (41). These studies suggested a possible effect of air pollution on SS patients, yet did not provide evidence for whether the exposure to air pollutants was an independent risk factors for pSS, due to the lack of longitudinal and temporal data.

The respiratory immune system as the first-line defense against inhaled agents, when dysregulated by microorganisms, allergens or pollutants, has been known to trigger host immune responses (42) and autoantibody induction (43) that may initiate pSS (44). When these pollutants are transferred from lungs to blood, the onset of systemic inflammatory responses usually involves B cell stimulation, in which autoantibodies could be generated and lead to autoimmunity (38). In the present study, ambient exposure to severe air pollution involving CO, NO, and CH_4 was associated with the risk of pSS, with previous studies suggesting that these three pollutants can trigger the production of excessive inflammatory mediators that may perturb immunity and cause pulmonary toxicity (45, 46). Juxtaposing findings in previous studies and the modeled air pollution-dependent pathogenesis of pSS as demonstrated in the present study, it is recognized that IL-6 plays a role in initiating Th17/Treg imbalance that may contribute to autoimmune responses and chronic inflammation, through driving Th17 immunity and inhibiting the peripheral generation of $Foxp3^+$ regulatory T cells (47); furthermore, these IL-6-dependent autoimmune responses may be triggered by the exposure to air pollutants (34) and have been reported to be associated with the severity of pSS (48), providing the biological plausibility of findings in the present study. Specifically, epidemiological findings in the present study on significantly high risk of pSS following the

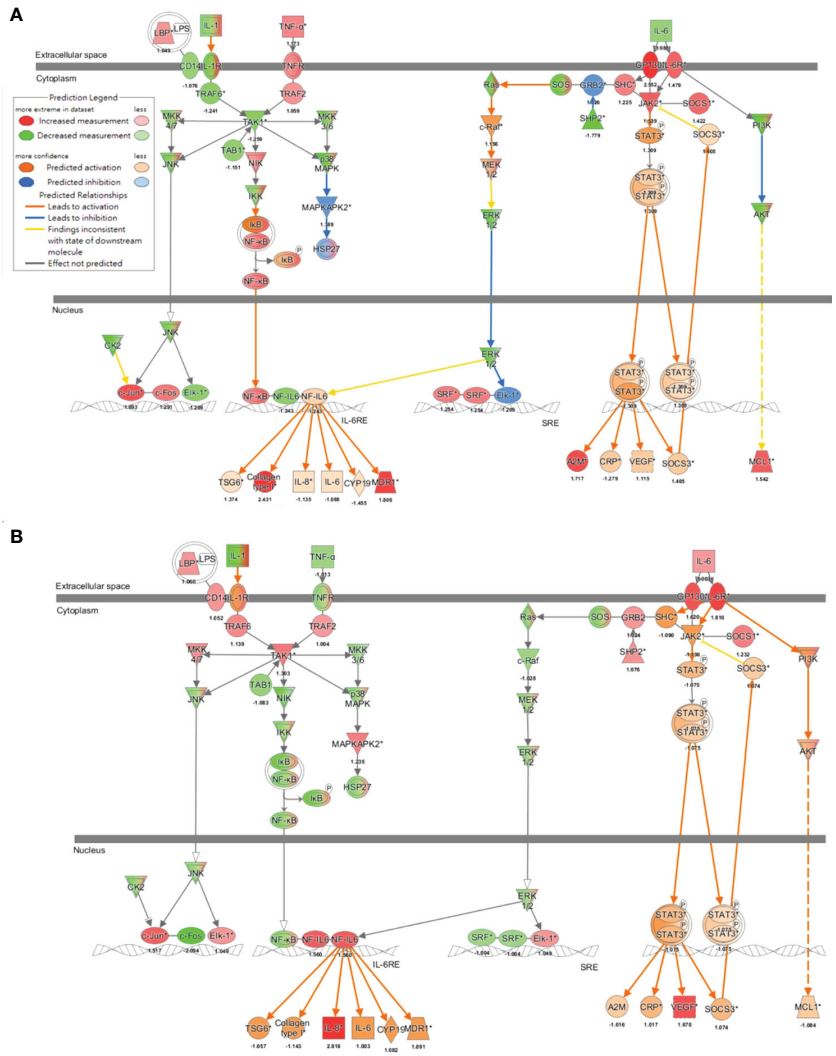

FIGURE 5
Air pollutant-associated IL-6 signaling pathway underlying the pathogenesis of pSS. Molecular Activation Prediction through transcriptomic data from parotid glands of patients with pSS **(A)** and airway epithelial cells exposed to ultrafine PM **(B)**, indicated mechanisms involved in IL-6 signaling.

exposure to air pollutants of smaller molecular sizes, such as CO, NO, and CH_4, instead of PM_{10} and $PM_{2.5}$, were in line with bioinformatic trends that as well indicated the biological effect of these pollutants on the triggered inflammatory pathways in airway epithelium, monocytes (49), and endothelium (50), which demonstrated a link between exposure to air pollutants and subsequent autoimmune responses.

Among all pSS-associated air pollutants that were identified in the present study, the findings on the correlation between pSS and the exposure to NO, echoed with studies suggesting that an increment of NO levels in the human body may disturb immune response, impair organelle function of alveolar macrophages, and elevate cellular levels of IFN-γ and MIP-1α, which can result in disrupted pulmonary immune homeostasis in the alveoli (51). As such, it is also worthwhile to uncover the mechanisms through which NO-mediated damage of alveolar macrophages may contribute to autoimmune pathogenesis underlying pSS, for which studies have suggested that epigenetic events in the immune cells can trigger pSS (6, 45), and that DNA methylation can be induced by exposure to particulate matter (52).

Furthermore, findings in the present study on the great risk of pSS in individuals exposed to high concentrations of air pollutants, were in line with studies implying the impact of environmental pollutants on dry eye disease in patients with SS and in SS models (53), for which increased levels of TNF-α and NF-κB in the cornea were observed (53, 54). Such an ocular involvement was followed by induced apoptosis in corneal superficial/basal epithelium that led to abnormal differentiation

and proliferation of the ocular surface plus a reduced number of goblet cells in the conjunctival fornix (53), which was similar to our findings on air pollution-associated NF-κB upregulation. Additionally, since lung involvement as one of the major systemic complications of pSS (55) has been suggested to manifest as low transfer factors for carbon monoxide (55), our findings indicated that apart from dry eye (56–58) and dry mouth symptoms, the consecutive exposure to air pollutants may exacerbate pSS-related lung pathologies including chronic obstructive pulmonary disease and interstitial lung disease (55).

The strengths of the present cohort study included a large sample size with long-term follow-up, and the reliability of pSS as an outcome, as the diagnosis of pSS in this study was validated by rheumatologists, rather than questionnaires filled in by patients. Results of this study supported the hypothesis that individuals exposed to small molecule air pollutants, as opposed to PM_{10} or $PM_{2.5}$, presented with an elevated risks of SS. The association between exposure to air pollutants and subsequent pSS may involve several chronic inflammatory pathways (59) with existing studies suggesting their relevance to autoimmune diseases such as the B cell signaling pathway (38). The findings on small molecule air pollutant-specific risk of pSS, were in accordance with scale-dependent expression levels of the above-mentioned chronic inflammatory pathways.

Although in the present study, people with previous history of pSS prior to the enrollment were excluded, there could be potential misclassification that resulted in cases who had undiagnosed pSS at baseline that were incorrectly found to develop pSS after the exposure to air pollutants. Moreover, as the exposure to air pollutants has been suggested to be associated with the risk of heart failure, stroke and myocardial infarction (60), it warrants further studies to investigate whether air pollution-associated pSS may be an early sign or concomitant complication of air pollution-associated heart diseases that could lead to hospitalization. Other limitations of this study included the lack of detailed information from electronic medical records involving lab data that may reflect the severity of SS, such as the raw data of SS disease activity index (ESSDAI) (1, 28). In addition, as the community-based monitoring stations of TAQMD may not detect indoor air quality, studies that collect data on indoor air pollutants are required to estimate the effect of indoor air quality; for instance, since NO can result from gas burning and barbecue or cooking (61), further studies that acquire data from NO sensors may validate findings in the present study. All in all, prospective cohort studies with more detailed information are needed to ascertain the observed dose-dependent effect of air pollutants on pSS and other autoimmune diseases, and to provoke patient education on environmental risk factors for pSS (62).

5 Conclusions

In conclusion, the exposure to small molecule air pollutants, especially CO, NO, and CH_4, was an independent risk factor for pSS. Clinical and policy implications of this study include insights to environmental toxicology in a global health context, with more focus on ways to promote public awareness about the importance of environmental health to rheumatic and autoimmune diseases (13).

Data availability statement

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

Author contributions

Conceptualization, KM, L-TW & WC; Methodology, KM, L-TW & C-LL; Writing – Original Draft, KM, HL & L-TW; Writing – Review & Editing, AC & JW; Funding Acquisition, KM, L-TW & WC. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1044462/full#supplementary-material

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Potential diagnostic markers and therapeutic targets for rheumatoid arthritis with comorbid depression based on bioinformatics analysis

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Background: Rheumatoid arthritis (RA) and depression are prevalent diseases that have a negative impact on the quality of life and place a significant economic burden on society. There is increasing evidence that the two diseases are closely related, which could make the disease outcomes worse. In this study, we aimed to identify diagnostic markers and analyzed the therapeutic potential of key genes.

Methods: We assessed the differentially expressed genes (DEGs) specific for RA and Major depressive disorder (MDD) and used weighted gene co-expression network analysis (WGCNA) to identify co-expressed gene modules by obtaining the Gene expression profile data from Gene Expression Omnibus (GEO) database. By using the STRING database, a protein–protein interaction (PPI) network constructed and identified key genes. We also employed two types of machine learning techniques to derive diagnostic markers, which were assessed for their association with immune cells and potential therapeutic effects. Molecular docking and *in vitro* experiments were used to validate these analytical results.

Results: In total, 48 DEGs were identified in RA with comorbid MDD. The PPI network was combined with WGCNA to identify 26 key genes of RA with comorbid MDD. Machine learning-based methods indicated that RA combined with MDD is likely related to six diagnostic markers: *AURKA*, *BTN3A2*, *CXCL10*, *ERAP2*, *MARCO*, and *PLA2G7*. *CXCL10* and *MARCO* are closely associated with diverse immune cells in RA. However, apart from *PLA2G7*, the expression levels of the other five genes were associated with the composition of the majority of immune cells in MDD. Molecular docking and *in vitro* studies have revealed that Aucubin (AU) exerts the therapeutic effect through the downregulation of *CXCL10* and *BTN3A2* gene expression in PC12 cells.

Conclusion: Our study indicates that six diagnostic markers were the basis of the comorbidity mechanism of RA and MDD and may also be potential therapeutic targets. Further mechanistic studies of the pathogenesis and treatment of RA and MDD may be able to identify new targets using these shared pathways.

KEYWORDS

rheumatoid arthritis, depression, bioinformatics, machine learning, molecular docking

Introduction

Rheumatoid arthritis (RA) is a prevalent chronic autoimmune disease, which affects around 0.5–1% of the world's population (1). RA is primarily characterized by joint inflammation and symmetrically active polyarthritis. These conditions affect the metacarpophalangeal joints and result in stiffness, pain, and swelling of the joints (2). It is well known that inflammatory cascades of patients with RA can be initiated or exacerbated by genetic and certain environmental factors (3). Inflammation in patients with RA can lead to systemic responses, including endothelial dysfunction (4). Therefore, RA shares a tight relationship with a number of illnesses, such as diabetes, depression, and myocardial infarction (5, 6). Depression is a common mental illness, affecting 1.5–19.0 in 1,000 adults (7). According to epidemiological research, the proportion of RA patients who also experience comorbid depressive symptoms is 13–20%, which is around three times greater than that of the general populace (8). Another study revealed that the likelihood of developing RA was 1.7 times higher in patients with depression than in controls (9). There is strong evidence that RA and depression are related by mutually influencing each other; RA can lead to MDD and MDD can exacerbate RA. The emergence of a large number of controlled clinical trials of psychotherapy for RA has demonstrated that treating depression is an effective way to improve RA independent of drug treatment (10). Therefore, screening and treatment of depression in patients with RA has important clinical significance.

Similar to many other chronic pain diseases, pain and physical impairment in people with RA as the chronic disease progresses are often cited as the cause of Major depressive disorder (MDD) (11). However, patients with RA experience substantially more symptoms of depression than patients with osteoarthritis, even though pain and dysfunction are similar between the two diseases. This difference may be related to cytokine-related neuroimmunobiological mechanisms (12). Many cytokines, including IL-1, TNF-α, and IL-6, are secreted during the pathological process of RA. These cytokines have been linked to neuroinflammation in the brains of individuals with depression (13–15). Abnormal activation of monoamine neurotransmitters is now recognized as playing a decisive role in the pathogenesis of depression. Cytokines access the brain directly or indirectly, disrupt the metabolism of monoamine neurotransmitters, alter the body's mental and cognitive activities, and lead to depression (16). Proinflammatory cytokines activate serotonin- and tryptophan-degrading enzymes while increasing the creation of glutamate-N-methyl-D-aspartate receptor agonists in the humoral immune system, resulting in serotonin deficiency and glutamate acid overproduction, both of which contribute to depression (17). Furthermore, by reducing the levels of neurotrophic factors in the brain, inflammatory factors may influence neurogenesis and neuroplasticity (18).

RA is highly inheritable, with approximately 60% heritability observed in twin studies (19). Approximately 100 loci that are significantly associated with RA have been identified in the genome. Additionally, a number of RA susceptibility genes have been linked to disease severity (20). Many alleles are weakly associated with RA, but the cumulative effects are observed in the presence of multiple risk alleles (21). It is important to investigate the multi-omics correlation in RA patients with depression, however, there have been no genomic studies on RA associated with depression. It is worth noting that Azathioprine, a Rac1 inhibitor, which is an immunosuppressant commonly used as adjunctive therapy for RA, have been reported to increase the risk of depression (22). Recently, there have been increasing reports of the use of natural products for the treatment of RA combined with depression. *Morinda officinalis* is often used in China because of its anti-osteoporosis, antidepressant, anti-Alzheimer disease, anti-rheumatoid, anti-oxidation, anti-inflammation, and anti-fatigue effects. The crude extracts and pure compounds of this plant are mainly composed of polysaccharides, anthraquinones, iridoid glycosides, and oligosaccharides. More than 100 chemical compounds have been isolated from *M. officinalis* that have been shown to have promising therapeutic effect on depression, osteoporosis, fatigue, and RA (23). Xinfeng Capsule (XFC) is an new effective natural medicine for the treatment of RA (24). In clinical studies, disease activity indexes, number of joint swelling/tenderness, joint morning stiffness duration, and all apoptosis-related indicators were reduced in the XFC group and the leflunomide group after treatment. However, XFC, which is composed of natural products, showed greater improvement on the self-rated depression scale than the leflunomide group (25).

In this study, we explored the common genes between RA and depression to reveal the underlying biological processes in RA combined with depression. This study aimed to explore the common genes of RA and depression to reveal the underlying biological processes in RA combined with depression. Diagnostic markers were identified from common genes to study their association with immune infiltration and their potential as diagnostic biomarkers and therapeutic targets.

Materials and methods

Data processing and analysis

We downloaded the GSE55235 (26), GSE55457 (26), and GSE77298 (25) RA datasets from the Gene Expression Omnibusdatabase (https://www.ncbi.nlm.nih.gov/geo/) using RStudio software (version 4.0.2; URL: https://www.r-project.org/). All dataset processing and analysis were performed in RStudio. The GSE55235 dataset (GPL96 platform), which was uploaded in 2014, contains transcriptome analyses of synovial tissue from 10 RA patients and 8 individuals with healthy joints. The GSE55457 dataset (GPL96 platform) identified 13 synovial membrane samples from patients with RA and 10 normal control synovial membrane samples. The samples in GSE55235, GSE55457 datasets were obtained from patients with RA for more than ten years. The RA dataset GSE77298 (GPL570 platform) contained a total of 23 synovial samples, which were obtained from 16 RA patients and 7 healthy individuals. In this dataset, the synovial samples were obtained from early RA at the Department of Rheumatic. Depression-associated transcriptomes (GSE98793) were obtained from the GEO database. In the GSE98793 dataset (27) (GPL570 platform), whole blood from 128 patients with MDD samples and 64 healthy individuals was collected. MDD was defined as CORE score >=8. According to the different sources of the samples, we categorized the samples into the RA group, depression group, and normal group, respectively. A simplified workflow of the current investigation is presented in Figure 1.

Differentially expressed gene analysis

We used the limma package (version 3.44.0) of R (version 4.0.2) to standardize and correct all gene expression profiling microarray data and annotated the gene names. The SVA package (version 3.36.0) was used to remove batch effects. The RA gene expression profiling dataset, which contained 27 normal control samples and 39 RA samples, was used to analyze the differentially expressed genes (DEGs). The MDD gene expression profiling dataset was preprocessed in the same manner. A conservative threshold ($|\log2FC| > 1.0$, $p < 0.05$) was used to screen for DEGs in patients with RA or MDD. The intersection genes between the DEGs of RA and MDD were generated using an online Venn diagram generator (version 2.1.0; https://bioinfogp.cnb.csic.es/tools/venny).

Construction of co-expressed gene modules

Based on the DEGs of RA and MDD, which were screened using the threshold, we further applied weighted gene co-expression network analysis (WGCNA) to define functional transcriptomic co-expression modules shared by RA and MDD. To identify co-expression modules, the WGCNA package (28) (version 1.71) in R was used to create unsigned co-expression networks. To begin with, the flashClust program in R was used to perform a hierarchical clustering analysis of the samples to discover and eliminate outliers. Second, a "soft" thresholding power (β), generated by the WGCNA's "pickSoftThreshold" algorithm, was utilized to design a physiologically important scale-free network according to the scale-free topology criterion. Third, a dynamic tree-cutting technique was used to create a topological overlap matrix (TOM) based on the adjacency matrix to detect gene modules. Fourth, gene significance (GS) and module membership (MM) were determined for linking modules to clinical features. Finally, we constructed the eigengene network.

Protein–protein interaction network analysis

The STRING database (29) (version 11.0; https://string-db.org/) was used to construct a protein–protein interaction (PPI) network of co-expressed gene modules in RA with comorbid MDD. The parameter settings for the network construction were as follows: organism, Homo sapiens; combined score threshold, 0.7. The PPI network was visualized using the Gephi software (version 0.9.2; https://gephi.org/). Key genes (highly connected genes) were selected using the Cytoscape (30) (version 3.7.1; https://cytoscape.org/) plugin network analyzer (31). The network level (average shortest path length and betweenness centrality) and node level (network degree value and closeness centrality) topological features of the network were calculated. The four network properties reflect the importance of each protein node in the network, we screened out the shared proteins of RA and MDD, which were the top 50 proteins in the PPI network's four network properties. Shared protein-coding genes are the key genes for RA associated with MDD.

Functional enrichment analysis of core genes

The core gene set of RA associated with MDD was composed of DEGs and key genes obtained from PPI network analysis. The primary goals of this research were to identify the comorbidity mechanisms that link RA and MDD as well as to reveal the underlying molecular biological processes of the disease core genes. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were used to identify the characteristic biological and functional attributes (32, 33). GO and KEGG analyses were performed using the clusterProfiler package (34) (version 3.14.3) in R. A p-value of < 0.05 and a q-value of < 0.05 were reserved, and a higher Gene Ratio was considered more significant.

Machine learning methods for the discovery of diagnostic markers

We used a machine learning approach to predict disease-associated genes based on the core genes of RA with comorbid MDD. In this study, two types of machine learning approaches were applied in the process of feature selection and model training: the LASSO regression model and support vector machine (SVM)

FIGURE 1
The complete research workflow.

method. The SVM algorithm was implemented using the caret package (version 6.0-86), kernlab package (version 0.9-29), and e1071 package (version 1.7-9). Ten-fold cross-validation was applied to calculate the misclassification error of our model within the training cohort. To calculate the misclassification error in the training cohort, a ten-fold cross-validation was applied to obtain the accuracy of the model algorithm. We first obtained the diagnostic markers in the RA and MDD datasets, and the overlapping part of the diagnostic markers of the two diseases represented the diagnostic markers in patients with RA and MDD.

Diagnostic core genes and immune cell correlation analysis

The single sample Gene Set Enrichment Analysis (ssGSEA) was applied to explore the relationship between different infiltration degrees of immune cell types and the diagnostic markers of RA with comorbid MDD using the R package "GSVA" (version 1.44.0). By comparing the differences between the groups and the correlation between diagnostic marker expression and immune cell content, we aimed to investigate the link between diagnostic markers and immune cells.

Molecular docking analysis

Eucommia ulmoides Oliver (EUO) has a long history of medicinal use in China. As a medicinal plant used for tonifying kidney, strengthening bones, relieving pain, and enhancing immunity, EUO is also widely used in the treatment of RA, depression, and osteoporosis. The aqueous extract of EUO has been demonstrated to have a cartilage-protecting effect in a rat model of osteoarthritis, potentially by inhibiting chondrocyte apoptosis and improving cartilage metabolism (35). Aucubin (AU), an iridoid glycoside that is an active constituent of EUO, has been extensively studied for the management of neurological diseases (36). However, a comprehensive review of its effects and mechanisms is currently unavailable. Therefore, in this study, we investigated the therapeutic potential of AU. The utilization of molecular docking, a technique commonly employed in virtual screening studies, was carried out to identify potential therapeutic targets for AU (37).

Primarily, the cheminformatics of Aucubin (AU) was obtained from the PubChem database (38) (https://pubchem.ncbi.nlm.nih.gov/), which included chemical name, molecular formula, CAS, PubChem CID, canonical SMILES, and SDF files. The ACD/Labs software (https://www.acdlabs.com/), SwissADME online system (39) (http://www.swissadme.ch/) and ADMETlab 2.0 (https://admetmesh.scbdd.com/) (40)were used to evaluate the pharmacokinetics and safety profile of AU, including absorption, distribution, metabolism, excretion, and toxicity. PyMOL software (version 1.7.0; https://pymol.org/) converted AU's 3D structure, which was downloaded from the PubChem database (41) (http://www.rcsb.org/), from an SDF file to a PDB file while minimizing the energy of small molecules and then saved it as a PDBQT format file. The 3D structures of potential targets were downloaded from the PDB database (http://www.rcsb.org/). PyMOL software removed water molecules and hetero-ions from the PDB file of the target protein. The protein ligands then underwent hydrogenation and the charge was added in AutoDockTools (42) (version 1.5.6) software. Finally, the data were saved as a PDBQT file. The docking box parameters were determined based on the binding region of the protein receptor and original ligand, and the box size was set to 30Å × 30 Å × 30 Å. AutoDock Vina (version 1.1.2; http://vina.scripps.edu/) software performs refined the semi-flexible molecular docking and calculated the affinity (kcal/mol) of all potential key targets for AU. Generally, the lower the affinity value, the stronger the binding of the small molecule to the receptor. Discovery Studio Visualizer (https://www.3ds.com/) was used to visualize the 2D schemes of the AU-target protein interaction.

Cell culture and MTT assay

Rat adrenal pheochromocytoma cells (PC12) and human rheumatoid fibroblast-like synoviocytes (HFLS) were obtained from iCell Bioscience Inc. and JENNIO Biological Technology, respectively. PC12 cells were cultured in 1640 basal medium containing 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin, while HFLS cells were cultured in DMEM basal medium with the same supplements. The cells were incubated at 37°C in an atmosphere containing 5% CO2. PC12 cells were seeded at a density of $2x10^4$ cells/well and incubated for 24 hours before exposure to AU. Different concentrations of AU (ranging from 0 to 160 μM) were then added to the wells and incubated for an additional 24 hours. MTT solution (10 μM) was added to each well and incubated for a further 4 hours. The medium was then removed and DMSO (200 μl) was added to dissolve the formazan crystals formed by the viable cells. The absorbance at 490 nm was measured using a microplate reader. To evaluate the effect of AU on cell proliferation ability, different concentrations of AU ranging from 0 to 5 mM were prepared and tested.

Quantitative real-time PCR

Logarithmically growing cells in a stable state were seeded in six-well plates at a density of 1×10^6 cells per well. HFLS cells were divided into two groups: a control group without treatment and a group treated with 16 μM AU solution. PC12 cells were divided into three groups with different concentrations of AU treatment: 10 μM, 500 μM, and 5 mM, as well as a control group. All groups were incubated for 24 hours. Total RNA was extracted from the cells in each group using TRIzol Reagent (Cwbio, China) and reverse transcribed into cDNA using the SYBR Green Master Mix kit (TransGen Biotech, China). Human GAPDH was used as an endogenous control, and the primer sequences are listed in Table 1. Data were analyzed using the comparative Ct method (2-△△Ct).

Statistical analysis

R version 4.0.2 and GraphPad Prism 8.0 software were used for statistical analysis and visualization. One-way ANOVA was used to compare groups of samples in multiple groups, with the assumption of normality and homogeneity of variances. The significance level was set at $\alpha = 0.05$, and a P value < 0.05 was considered statistically significant.

Results

Identification of DEGs

The RA datasets from the GEO dataset contained 12403 genes in 64 synovium samples from 39 RA patients and 25 healthy individuals. A total of 576 genes were identified as RA-related DEGs in the datasets, of which 201 were downregulated and 375 were upregulated, as shown in a heatmap (Figure 2A). We obtained 1127 MDD-related DEGs in the GSE98793 dataset, of which 477 genes were downregulated and 650 genes were upregulated, as shown in a heatmap (Figure 2B). The 48 common genes between RA- and MDD-related DEGS are indicated by the Venn diagram (Figure 2C).

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TABLE 1 Primer sequence.

Primer	Sequence (5′-3′)
human-AURKA-F	GGGTGGTCAGTACATGCTCC
human-AURKA-R	GGCTCCCTCTGTTACAAAGTCA
RAT-AURKA-F	GCGAATGCTTTGTCCTACTGC
RAT-AURKA-R	CATCCGACCTTCAATCATCTCC
human-BTN3A2-F	GGCAGGTGGTGAACGTGTATG
human-BTN3A2-R	ACTTCGACGTGAAGATTAGAACCC
rat-BTN3A2-F	TAGGCACCAACGGCATTTC
rat-BTN3A2-R	CAACATAGGCCCAATACCCAC
human-CXCL10-F	TAGAACTGTACGCTGTACCTGC
human-CXCL10-R	TGTAGCAATGATCTCAACACG
rat-CXCL10-F	CTGCACCTGCATCGACTTCC
rat-CXCL10-R	CTTCTTTGGCTCACCGCTTTC
human-ERAP2-F	GCTGCTGAACTCTTCTCCC
human-ERAP2-R	TCCTGATGCTTGCTCGTT
human-MARCO-F	GGGACAATTTGCGATGACGA
human-MARCO-R	GGCCCTTCCTTTGGAGTAAC
rat-MARCO-F	GCACGTCCCAAAACACACAT
rat-MARCO-R	ACTTGCTGACGCAGTTGCTC
human-NeuN-F	GCCCGAGTGATGACCAACAAGAAG
human-NeuN-R	GTGGCGCAGCCCGAAATGTA
rat-NeuN-F	CCGTTTGCTTCCAGGGTCG
rat-NeuN-R	GCCGATGGTATGATGGTAGGGAT
human-MAP-2-F	GCCAGGCAGTGATTACTATGA
human-MAP-2-R	GATGGATAACTCTGTGCGAGA
rat-MAP-2-F	CTTGCCTATGTCTTGCCTTGA
rat-MAP-2-R	TCCATCGTTCCGCTAGTGTT
human-βIII -tubulin-F	GCCACGCTGTCCATCCACCA
human-βIII -tubulin-R	CGAAGCCGGGCATGAAGAAGT
rat-βIII -tubulin-F	CATCAGCAAAGTGCGTGAGGAG
rat-βIII -tubulin-R	GACAGGGTGGCGTTGTAGGG
human-GAPDH-F	AATCCCATCACCATCTTCCA
human-GAPDH-R	AAATGAGCCCCAGCCTTCT
rat-GAPDH-F	GGAAAGCTGTGGCGTGAT
rat-GAPDH-R	TCCACAACGGATACATTGGG

Identification of co-expression gene modules

We performed WGCNA to identify co-expressed gene profiles in 39 RA datasets, 128 MDD datasets, and 91 healthy individual datasets. First, we divided the dataset samples from different sources into two groups with no detected outliers according to disease type: disease group (RA or MDD) and healthy control group (HC). Then, 8 and 5 were chosen as the optimal soft-threshold power β for the RA and MDD datasets, respectively, based on the scale independence of R2 greater than 0.9 and the mean connectivity tending to 0 to ensure a biologically meaningful scale-free network (Figures 3A, B).

Genes in the RA dataset were clustered into four modules, and the MDD dataset was clustered into five modules through hierarchical clustering analysis and dynamic branch cut methods for the gene dendrograms (Figures 3C, D). To identify the key modules related to RA and MDD, GS and MM were calculated to relate the modules to clinical traits. MM was defined as the correlation between gene expression values and module eigengene (ME). GS was defined as the correlation between genes and samples, as shown in Figures 3E, F. Figures 3E–G shows four RA modules and five MDD modules obtained using WGCNA. Two MDD-related modules (MDD-MEturquoise and MDD-MEblue) and RA-MEyellow shared 19 and 12 genes, respectively. Most DEGs (65%) found in RA and MDD were concentrated in these modules. Therefore, these modules can be considered as co-expressed gene modules closely related to RA with comorbid MDD.

The PPI network key genes

The interaction data of 943 genes that were composed of all genes in the co-expressed gene modules (RA-MEyellow and MDD-MEturquoise) were obtained from the STRING database and imported into Cytoscape to visualize the PPI network (Figure 4A). Similarly, a total of 644 genes in the co-expressed gene modules (RA-MEyellow and MDD-MEblue) were merged, and the interaction data were imported into Cytoscape to visualize the PPI network (Figure 4B). Based on the four network properties, we removed the relative nodes (network degree value < 5). All remaining nodes were screened to obtain the top 50 important nodes for each network property in the two PPI networks. Finally, we obtained 17 top genes of the co-expressed gene modules (RA-MEyellow and MDD-MEturquoise) and 22 top genes of the co-expressed gene modules (RA-MEyellow and MDD-MEblue) at the intersection of the Venn diagram. Twenty-six top genes as hub genes of RA with comorbid MDD based on PPI network analysis were obtained after the merger, as shown in Figures 4C, D.

GO and KEGG pathway enrichment analysis

Based on the 48 DEGs shared by RA and MDD, we added the 26 key genes obtained from the PPI network analysis and finally obtained 55 genes as the core genes for RA associated with MDD. GO enrichment was analyzed using the clusterProfiler package in R (Figures 5A–C). The results of these analyses showed that 705 GO entries were obtained in this study, including 611 biological process (BP), 54 molecular functions (MF), and 39 cellular components (CC). Regarding BP, the core genes were mainly enriched in

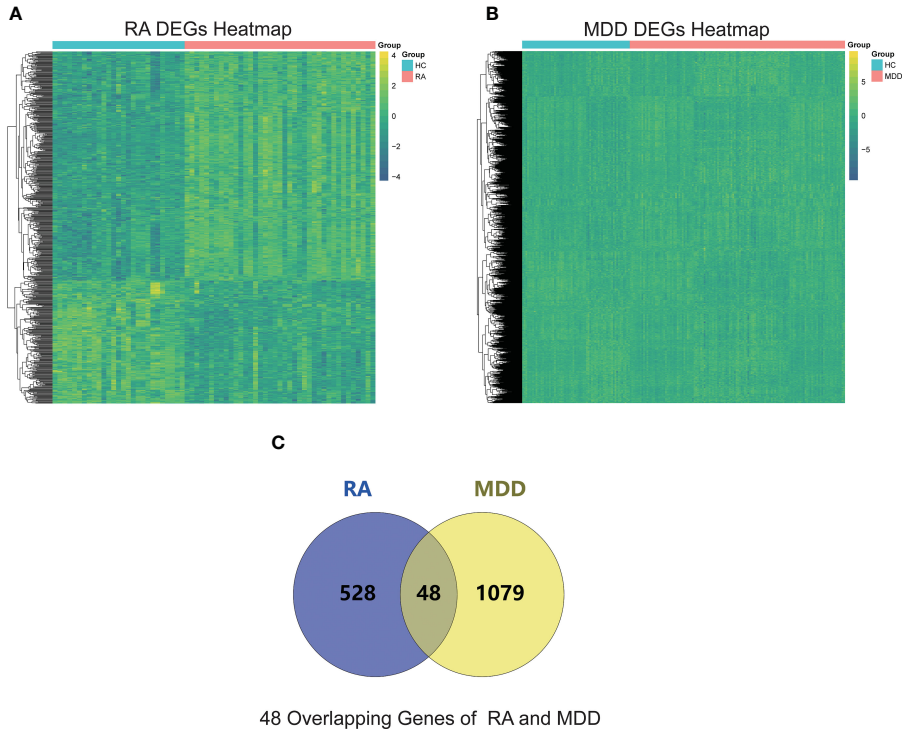

FIGURE 2
RA and MDD DEGs analysis. **(A)** A heat map of RA DEGs analysis results based on the merged GSE55235, GSE55457, and GSE77298 datasets. **(B)** A heat map of MDD DEGs analysis results based on the GSE98793 dataset. **(C)** Identification of 48 overlapping genes between the DEGs of RA and MDD.

lymphocyte differentiation (GO:0030098), leukocyte migration (GO:0050900), T cell activation (GO:0042110), immune response-activating cell surface receptor signaling pathway (GO:0002429), and immune response-activating signal transduction (GO:0002757). As for the MF, the core genes were mainly enriched in cytokine receptor binding (GO:0005126), peptide binding (GO:0042277), amide binding (GO:0033218), phosphatase binding (GO:0019902), G protein-coupled receptor binding (GO:0001664). Finally, regarding CC, the core genes were mainly enriched in the external side of the plasma membrane (GO:0009897), membrane raft (GO:0045121), membrane microdomain (GO:0098857), membrane region (GO:0098589), and plasma membrane signaling receptor complex (GO:0098802).

A KEGG pathway analysis was performed (Figure 5D). The core genes were mainly focused on 89 pathways. The pathways in the KEGG enrichment analysis were related to Epstein-Barr virus (EBV) infection (hsa05169), Th17 cell differentiation (hsa04659), Th1 and Th2 cell differentiation (hsa04658), tuberculosis (hsa05152), PD-L1 expression, and the PD-1 checkpoint pathway in cancer (hsa05235). We found that RA and MDD share many molecular mechanisms.

Receiver operating characteristic curve analysis of diagnostic markers

Based on the 55 core genes of RA associated with MDD, the LASSO regression model and SVM-based method were used to screen diagnostic markers related to disease diagnosis. As shown in Figures 6A–C, following the 10-fold cross-validation procedure, LASSO regression identified 16 diagnostic core genes of RA in the model. The other 44 diagnostic core genes of RA were screened by SVM-based method. As shown in Figures 6D–F, 15 and 27 MDD diagnostic core genes from the core genes were also identified by LASSO regression and SVM, respectively. The common diagnostic core genes of these two diseases are considered diagnostic markers for RA with MDD. As shown in Figure 6G, six diagnostic markers were obtained: *AURKA*, *BTN3A2*, *CXCL10*, *ERAP2*, *MARCO* and *PLA2G7*.

We drew the receiver operating characteristic (ROC) curve of the diagnostic markers in RStudio to determine their diagnostic value. The results showed that most of diagnostic markers (Figure 7) had significant diagnostic value in the disease classification. However, their prediction performance in the RA dataset was much better than in the MDD dataset, which may be attributed to the fact that MDD is a mental disease that rarely leads to organ lesions or inflammation.

Immune cell correlation analysis

The results of ssGSEA showed that in RA, the scores of immune cell content were higher in most RA groups but lower in the control group, and 20 out of 28 immune cells (activated B cells, activated CD4 T cells, activated CD8 T cells, activated dendritic cells, CD56 bright natural killer cells, CD56 dim natural killer cells, Gamma delta T cells, immature B cells, MDSCs, macrophages, monocytes,

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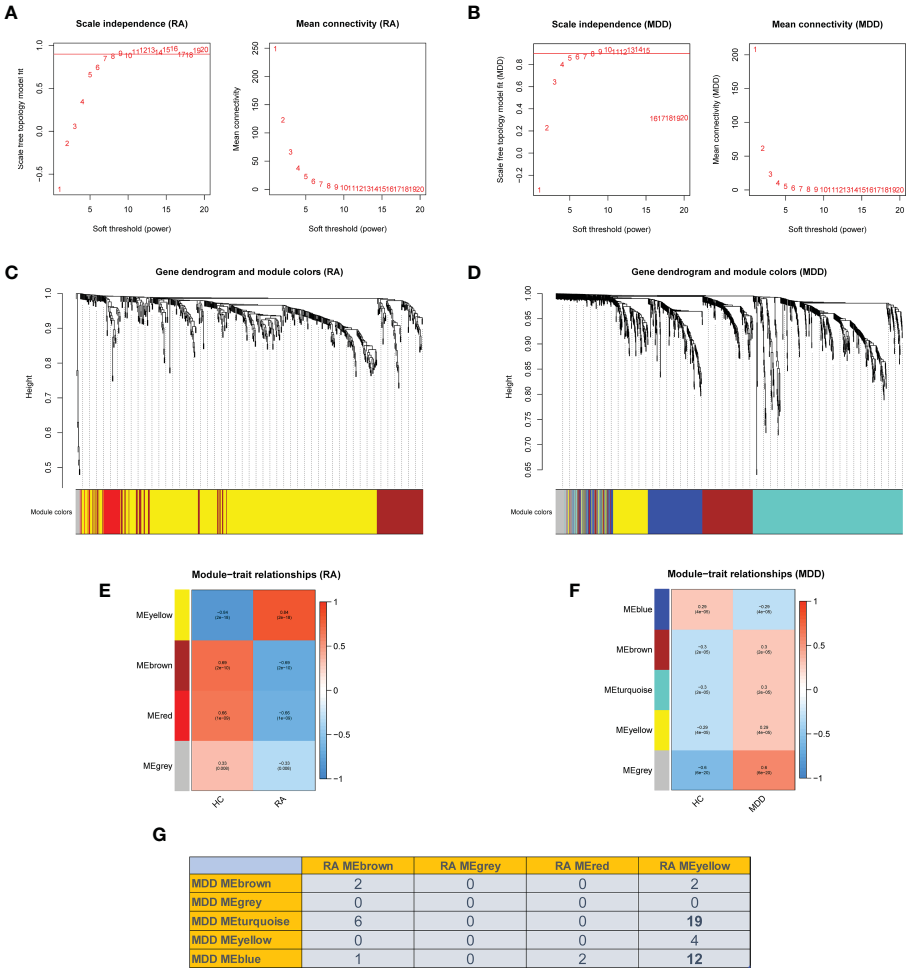

G

	RA MEbrown	RA MEgrey	RA MEred	RA MEyellow
MDD MEbrown	2	0	0	2
MDD MEgrey	0	0	0	0
MDD MEturquoise	6	0	0	19
MDD MEyellow	0	0	0	4
MDD MEblue	1	0	2	12

FIGURE 3
Weighted co-expression network related dataset construction and identification of related key modules in RA and MDD. **(A, B)** Analysis of network topology for various soft thresholds (β). The left panel shows the scale-free fit index (scale independence, y-axis) as a function of the soft threshold power (x-axis); the right panel displays the mean connectivity (degree, y-axis) as a function of the soft threshold power (x-axis). **(C, D)** Gene dendrograms were obtained by average linkage hierarchical clustering. The colored row underneath the dendrogram shows the module assignment determined by the dynamic tree cut method. **(E, F)** Module-trait relationships. Each row in the heatmap corresponds to an ME and each column to a clinical trait. Each cell contains the corresponding correlation and *p*-value. **(G)** Number of intersecting genes in related key modules in RA and MDD.

natural killer T cells, natural killer cells, regulatory T cells, T follicular helper cells, type 1 T helper cells, type 17 T helper cells, effector memory CD4 T cells, memory B cells, and central memory CD4 T cells) were significantly different between the two groups, as shown in Figures 8A, C. However, the immune cell content scores were lower in most MDD groups, except for activated B cells, activated dendritic cells, macrophages, natural killer cells, type 1 T helper cells, central memory CD4 T cells, and central memory CD8 T cells. There was no significant difference in the number of other immune cells between the two groups, as shown in Figures 8B, D.

We found that the levels of six immune cell types (activated B cells, activated dendritic cells, macrophages, natural killer cells, type 1 T helper cells, and central memory CD4 T cells) were significantly different in both RA and MDD, as shown in Figures 8E, F. In RA, *CXCL10* and *MARCO* are closely related to the content of various immune cells, while in MDD, except for *PLA2G7*, the expression levels of the other five diagnostic markers are correlated with the content of most immune cells.

In silico validation of the targets using molecular docking

According to the ADMET evaluation, AU exhibits many of the qualities of an ideal reagent for drug-like qualities (Lipinski), water solubility (solubility), lipophilicity (LogP), and other parameters. However, as shown in Table 2, the intestinal absorption (GI absorption) and oral availability (bioavailability) were low. Thus far, there has been no noted toxicity (hERG, AMES Toxicity, Skin Sensitization).In this study, we selected three target proteins (*AURKA*, *ERAP2*, and *PLA2G7*) for molecular docking analysis to predict their potential therapeutic effect on patients with RA and MDD.

The total kollman charges for *AURKA*, *ERAP2*, and *PLA2G7* were added as -130.535, -451.539, -190.286. The hydrogen atoms and gasteiger charge for AU (0.0002) were added and saved in the pdbqt format. In this paper, molecular docking took the binding sites of the original ligands as the reference binding sites, and the

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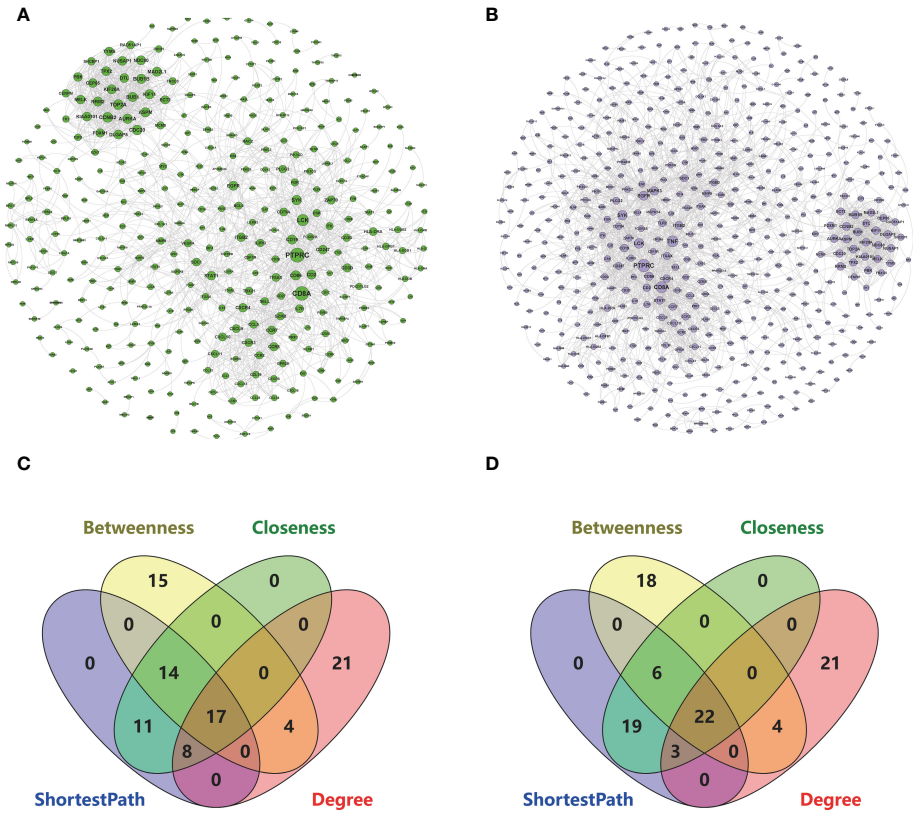

FIGURE 4
Based on the STRING database, 26 key genes were identified based on the two PPI network analysis in RA and MDD. **(A)** The left PPI network A is composed of genes in RA-MEyellow module and MDD-MEturquoise module (degree shown by node size). **(B)** The right PPI network B is composed of genes in RA-MEyellow module and MDD-MEblue module (degree shown by node size). **(C)** Screening of 17 key genes in the left PPI network A based on ShortestPath, Betweenness, Closeness, and Degree. **(D)** Screening of 22 key genes in the right PPI network B based on ShortestPath, Betweenness, Closeness, and Degree.

specific information was shown in Table 3. The docking scores between AU and *AURKA* were -7.7 (kcal/mol). As shown in Figures 9A, B, AU interacted with *AURKA* by forming a hydrogen bond with Lys141, Asp274, Asn261, and interacted with the surrounding residues by forming other bonds. The docking scores of AU and *ERAP2* were -8.4 (kcal/mol). As shown in Figures 9C, D, AU interacted with *ERAP2* by forming a hydrogen bond with Gly334, His370, Glu200, and interacted with surrounding residues by forming other bonds. The docking score between AU and *PLA2G7* was -6.0 (kcal/mol). As shown in Figures 9E, F, AU interacted with *PLA2G7* by forming a hydrogen bond with Trp105, Lys109, Thr113, and interacted with surrounding residues by forming other bonds.

In vitro validation of the targets using MTT assay and qPCR

The results of the MTT assay indicated that the concentration of the test drug had no toxic effect on the cells within the range of 0-160 μM (Figure 10A). Upon adjusting the concentration range from 0-5 mM and reducing the cell density to 1×10^4 cells/well, the MTT assay showed that AU significantly increased the proliferation of PC12 cells (Figure 10B). To further investigate the effect of AU at various concentrations on the gene expression levels of six diagnostic markers and MAP-2, βIII-tubulin, we examined the expression levels of these genes in PC12 and HFLS cells after 24 hours of AU treatment. In HFLS cells, the expression levels of the six diagnostic markers did not change significantly, but the expression level of βIII-tubulin was significantly downregulated (Figure 10C). In PC12 cells, the expression levels of *CXCL10* and *BTN3A2* were significantly downregulated following AU treatment (Figure 10D).

Discussion

Patients with depression have a 14–48% chance of developing RA (43). Depression is the most common comorbidity associated with RA. However, it is frequently neglected and under-treated in clinical practice. Depression has various effects on the progression of RA, including disease activity, other arthritis-related comorbidities, pain levels, quality of life, and mortality, all of which lead to worse clinical outcomes. Furthermore, RA and depression initiate a vicious cycle that exacerbates the other symptoms. This strong association between depression and RA is

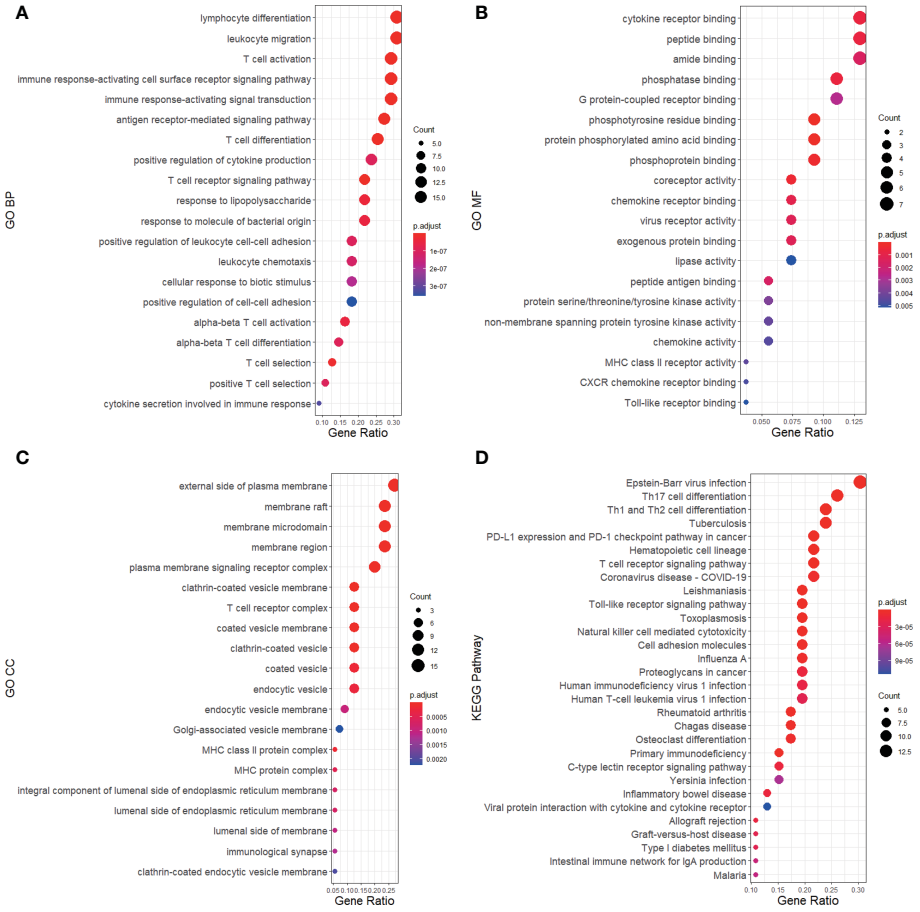

FIGURE 5
GO and KEGG pathway annotation for RA and MDD 55 core genes merged from 48 DEGs and 26 key genes of PPI network. The size of the ball represents the number of genes, and the color change of the ball corresponds to different *p*-values. **(A)** The first 20 significantly enriched GO annotations of BP. **(B)** The first 20 significantly enriched GO annotations of MF. **(C)** The first 20 significantly enriched GO annotations of CC. **(D)** The first 30 significantly enriched KEGG pathways.

already partly explained by the assumption of a model based on the hypothesis of inflammation and crosstalk between the central, peripheral, and immune systems. The management of individuals with dual diagnoses should be closely monitored to avoid undue distress.

Gene expression variations and patterns shed light on the mechanism of RA comorbidity with depression and may aid in the identification of targets for therapeutic intervention. In this study, we used WGCNA to construct network hierarchical clustering trees and co-expression modules associated with RA and depression. We obtained 26 key genes of RA that were associated with MDD based on PPI network analysis. In functional enrichment analysis, some of these shared molecular mechanisms have been experimentally validated, and some of them, such as tuberculosis, are reported for the first time.

It has been reported that Epstein-Barr virus (EBV) infection promotes autoimmunity, and in many studies, the evidence for whether EBV infection is causal of autoimmunity appears high (44). A study published in 1970 showed that there were quantitative differences in EBV protein antibodies in RA patients, and that the route of EBV infection may be closely related to the occurrence and development of RA and MDD complications (45). EBV is a double-stranded DNA virus belonging to the herpes family. The globally prevalent EVB virus has significant effects on the immune system and is considered an attractive candidate pathogen for RA. EVB can be latent in the B lymphocyte and the salivary gland epithelium for a long time, with a lifetime prevalence of 90%. Evidence of *in vitro* EBV infection was observed in the lymphocytes of RA patients and antibodies to EBV antigens were significantly increased in their serum. As a polyclonal activator of B cells, EVB virus can induce rheumatoid factor and autoantibody production *in vitro* and *in vivo*. These immunopathological events may explain the link between EVB virus and RA disease (46). Children with EBV infection are at high risk of depression in adulthood (47).

Lymphopenic mice demonstrate that an adaptive immune system composed of T cells and B cells can be a potential factor in depression. T helper (Th) cells differentiate into different lineages under the influence of cytokine environment, antigen stimulation, and co-stimulation. A decrease in regulatory T cells and an increase in Th17 cells were observed in patients with depression. The discovery that Th17 cells are involved in depression evolves from the classic theory that Th17 cells produce inflammatory cytokines

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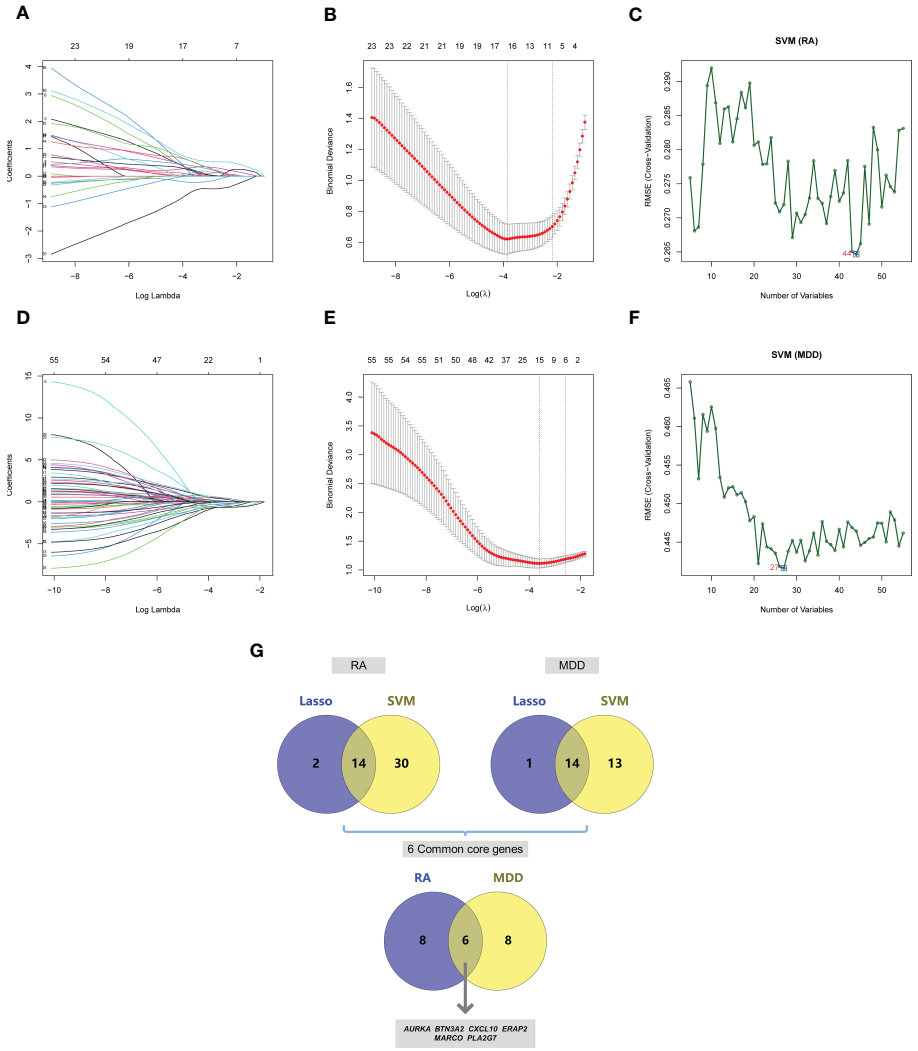

FIGURE 6
Screening of core genes and validation based on machine learning methods in RA and MDD. **(A)** Coefficient profiles of variables in the LASSO regression model in RA. **(B)** Ten-fold cross-validation for turning parameter (λ) selection in the LASSO regression model in RA. **(C)** The optimum root mean squared error (RMSE) of SVM-based method based on 44 characteristic genes in RA. **(D)** Coefficient profiles of variables in the LASSO regression model in MDD. **(E)** Ten-fold cross-validation for turning parameter (λ) selection in the LASSO regression model in MDD. **(F)** The optimum root mean squared error (RMSE) of SVM-based method based on 27 characteristic genes in MDD. **(G)** 14 core genes in RA and 14 core genes in MDD screened by LASSO regression model and SVM-based method, and 6 common core genes were obtained after taking the intersection.

IL-17A and IL-6, which is required for differentiation, and contribute to depression onset and maintenance (48). Increased physiological levels of IL-7 affect joint inflammation, osteoclastogenesis, and neovascularization associated with autoimmune diseases. The increased content of IL-7 in the synovial tissue and fluid of RA allows monocytes to enter the inflamed joints to form macrophages and mature osteoclasts (49).

The chronic immune response in RA may be driven by activated Th1 cells without sufficient Th2 cell differentiation to downregulate inflammation. The combined effect of Th1 cell activation-driven cascades and the inability of Th2 cell differentiation to downregulate the inflammation is the underlying mechanism of chronic immune responses in RA. Th1 cells infiltrating the synovium can secrete abundant proinflammatory cytokines and induce macrophage and neutrophil infiltration (50). PD-1 is an immunosuppressive molecule that inhibits inflammatory responses. It controls the inflammatory activity of T cells by adjusting the immune system's response to human cells, helping to improve immunotolerance. Therefore, impairment of the PD-1/PD-L1 pathway is considered to play an important role in many immune-mediated diseases including RA (51).

To further explore the diagnostic markers of RA complicated by MDD, six diagnostic markers were obtained from 55 core genes based on the two algorithms. *AURKA* encodes a cell cycle-regulated kinase that appears to play a role in microtubule formation and/or spindle pole stabilization during chromosome segregation. *BTN3A2* is the gene most closely connected with treatment response according to a genome-wide methylation analysis of DNA in RA patients receiving anti-rheumatic therapy for the first time (52). *BTN3A2* has also shown a pleiotropic association with MDD (53). TNF stimulates neurons to produce *CCL2*, *CCL7*, and *CXCL10*.

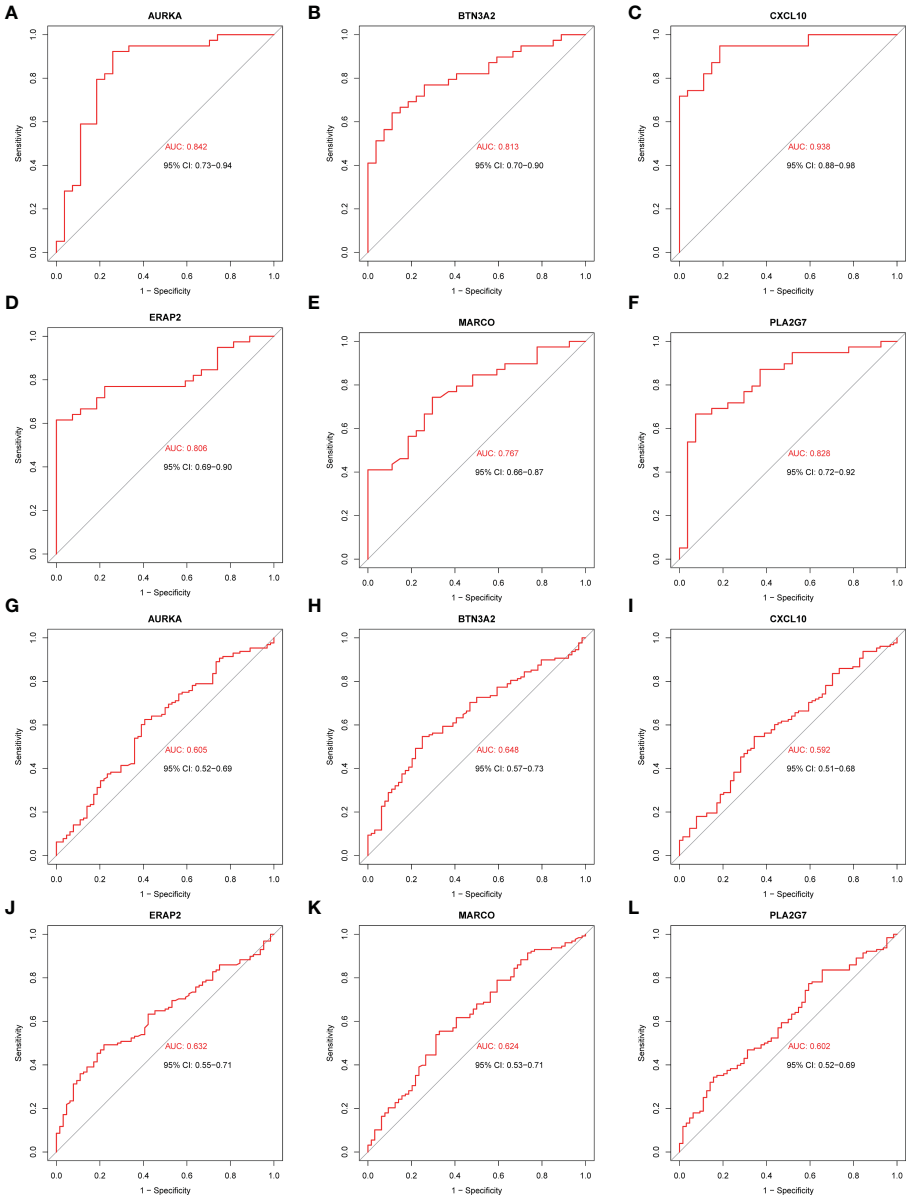

FIGURE 7
ROC curves of the 6 common core genes in RA and MDD. **(A, B, C, D, E, F)** ROC curves of *AURKA*, *BTN3A2*, *CXCL10*, *ERAP2*, *MARCO*, *PLA2G7* in the RA dataset, respectively. **(G, H, I, J, K, L)** ROC curves of *AURKA*, *BTN3A2*, *CXCL10*, *ERAP2*, *MARCO*, *PLA2G7* in the MDD dataset, respectively.

These chemokines are closely related to RA and depression by interfering with the microglial elongation process (54). MDX-1100, an anti-*CXCL10* monoclonal antibody, had demonstrated well tolerated and clinically effective in patients with RA who had an inadequate response to methotrexate (55). This further confirms that *CXCL10* plays a role in the immunopathogenesis of RA. The *ERAP2* gene has been shown to be expressed considerably more in the CD4 + T cells of patients with RA who react to glucocorticoid medication, suggesting that *ERAP2* may be a clinical predictor of response to glucocorticoid therapy in patients with RA (56). *MARCO*, a macrophage receptor with a collagen structure, is involved in the uptake of apoptotic cells, and the ability of macrophages to promptly clear apoptotic cells has been linked to autoimmune diseases (57). Lower levels of the platelet-activating factor acetyl hydrolase, a protein encoded by *PLA2G7*, may result in a loss of anti-inflammatory function, triggering juvenile RA (58). The relationship between the immune cell types and the diagnostic markers of RA were evaluated using ssGSEA which showed that the six candidate diagnostic genes of RA complicated by MDD were correlated with immune cell content to varying degrees.

The long onset time of RA is a serious threat to human health and quality of life. In recent years, there have been many applications of natural product in arthritis. AU with antioxidant, anti-inflammatory, neuroprotective, and osteoprotective properties are high-profile natural small molecules. AU has a wide range of biological effects and is a compound with rich potential sources, a

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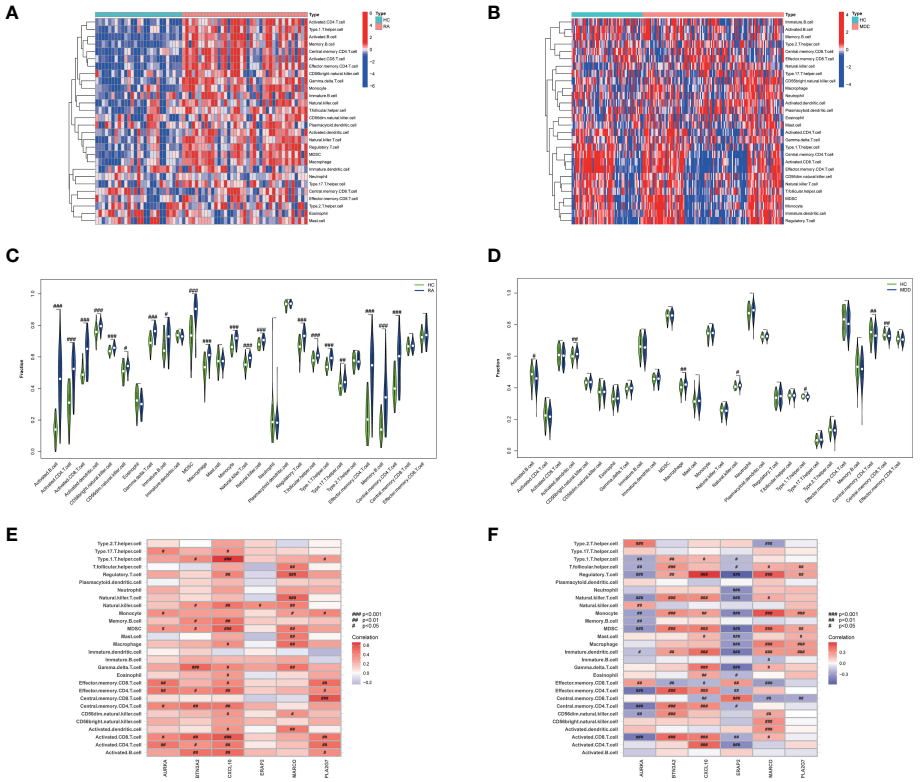

FIGURE 8
The 28 immune cells and their correlation with the 6 common core genes in RA and MDD. **(A)** Heatmap of 28 immune cell expression scores in RA. **(B)** Heatmap of 28 immune cell expression scores in MDD. **(C)** Comparison of 28 immune cells in samples with HC and RA by Fraction. **(D)** Comparison of 28 immune cells in samples with HC and MDD by Fraction. **(E)** Spearman correlation analysis of the 6 common core genes and 28 immune cells in RA. **(F)** Spearman correlation analysis of the 6 common core genes and 28 immune cells in MDD. #$p < 0.05$; ##$p < 0.01$; ###$p < 0.001$.

TABLE 2 ADMET properties of AU by ACD/Labs, SwissADME and ADMETlab 2.0.

Name	*Aucubin* (AU)	Source
PubChem CID	91458	PubChem
Molecular Formula	C15H22O9	
CAS	479-98-1	
MW	346.33	SwissADME
TPSA	149.07	
Lipinski (violations)	1 (NHorOH > 5)	
GI absorption	Low	
Log P	Hydrophilic	ACDLabs
Solubility	Soluble	
BBB permeant	No	
Pgp substrate	Yes	
Bioavailability (%) (Dose, mg = 50.00)	3.17	
hERG	Non-inhibitor	
AMES Toxicity (Probability value)	0.1-0.3	ADMETlab 2.0
Skin Sensitization (Probability value)	0.0-0.1	

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TABLE 3 Summary of molecular docking details.

Targets	Grid dimensions (Å)	Center grid box			Number of poses generated	Affinity (kcal/mol)	
		center x	center y	center z		Aucubin	Original ligand
AURKA	30×30×30	-7.525	26,575	79.368	9	-7.7	-6.8
ERAP2	30×30×30	88.371	9.906	123.592	9	-8.4	-8.2
PLA2G7	30×30×30	25.469	4.174	-2.949	9	-6.0	-7.6

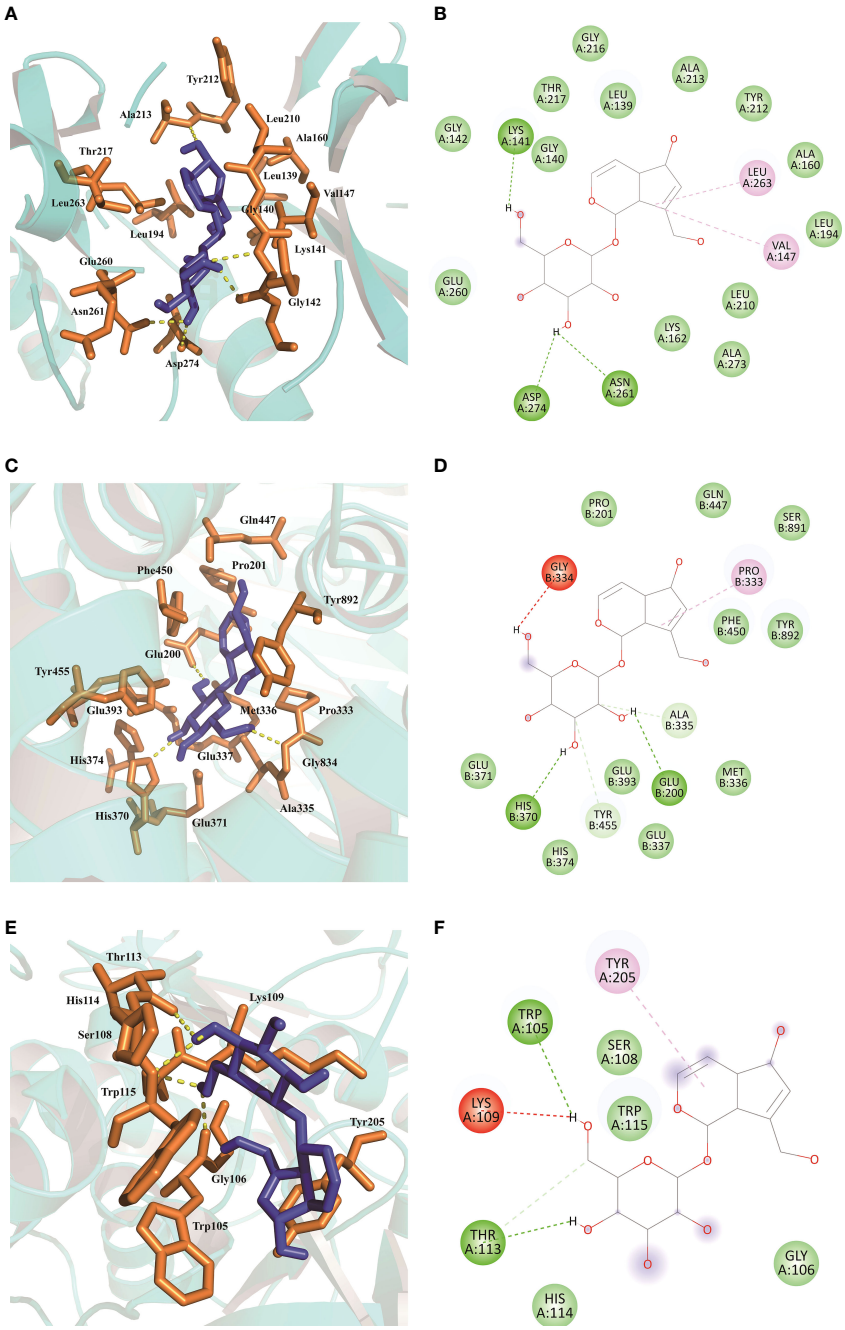

FIGURE 9
Molecular docking results of AU interaction with *AURKA*, *ERAP2* and *PLA2G7*. **(A, B)** Molecular docking conformation of AU interaction with *AURKA*. **(C, D)** Molecular docking conformation of AU interaction with *ERAP2*. **(E, F)** Molecular docking conformation of AU interaction with *PLA2G7*.

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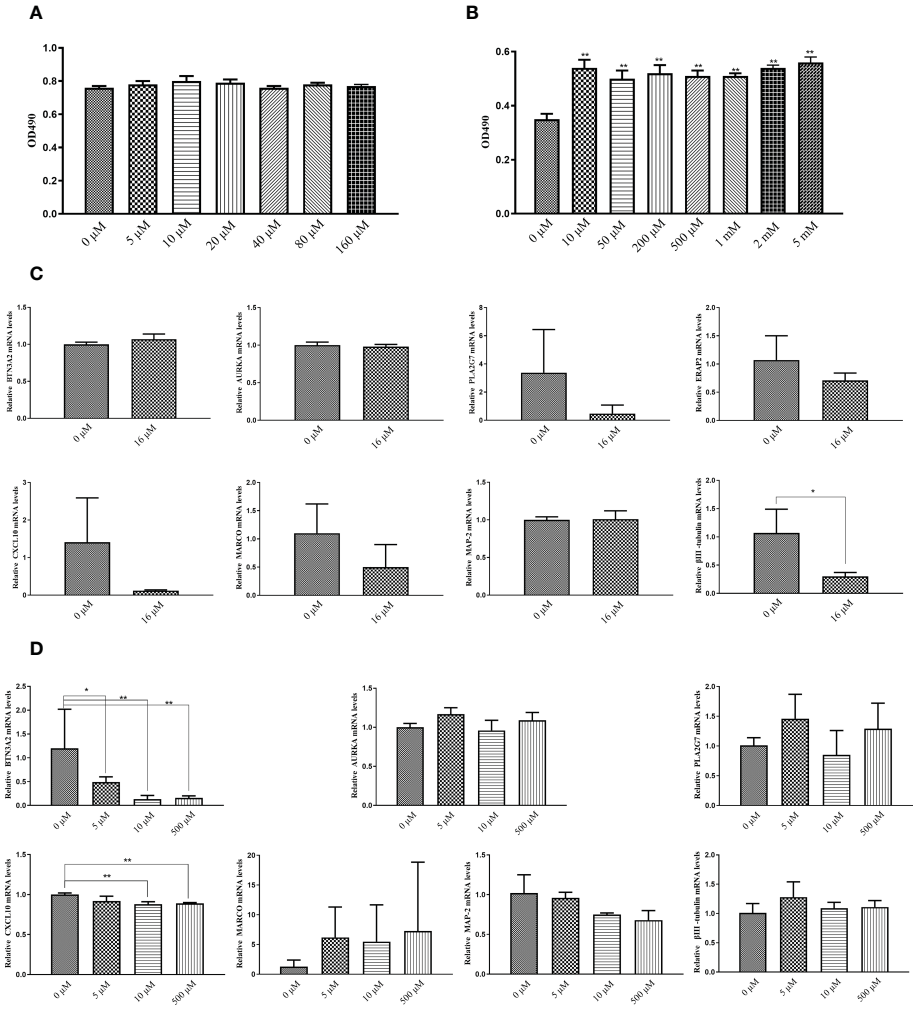

FIGURE 10
In vitro validation of the targets using MTT assay and qPCR. **(A)** MTT assay to measure cell viability in PC12 cells after treatment with AU at 0 to 160 μM concentrations (In comparison with the control group, *P < 0.05, and ** P < 0.01). **(B)** MTT assay to measure PC12 cell proliferation after treatment with AU at 0 to 5 mM concentrations (In comparison with the control group, *P < 0.05, and ** P < 0.01). **(C)** Quantitative analysis of *BTN3A2, AURKA, PLA2G7, ERAP2, CXCL10, MARCO, MAP-2*, and βIII-tubulin gene expression in HFLS cells by real-time PCR (In comparison between two groups, *P < 0.05, and ** P < 0.01). **(D)** Quantitative analysis of *BTN3A2, AURKA, PLA2G7, CXCL10, MARCO, MAP-2*, and βIII-tubulin gene expression in PC12 cells by real-time PCR (In comparison between two groups, *P < 0.05, and ** P < 0.01).

good safety profile, and many beneficial biological activities. It has high application potential in health products and medicines, and can be used to treat RA, depression, hypertension, lower back pain, and other diseases (59). In animal models of neurological diseases, AU inhibits the activation of glial cells, which are responsible for brain inflammation (60, 61). Modern medical research has demonstrated that AU can increase the biomechanical quality of the femur, bone mineral density, and bone microarchitecture to prevent osteoporosis (62). In a molecular docking study, three target proteins (*AURKA*, *ERAP2*, and *PLA2G7*) predicted the potential therapeutic effect of AU on RA with MDD.

BTN3A2 expression has been found to be increased in patients with RA, and inhibiting *BTN3A2* may improve RA symptoms in animal models (63). Elevated levels of *CXCL10* have been associated with impaired cognitive performance in patients with depression (64), and may accelerate disease progression in RA patients (65). *In vitro* studies suggest that AU may exert therapeutic effects by decreasing the expression of *CXCL10* and *BTN3A2*. There is a growing body of evidence supporting the role of adult neurogenesis in the pathology and physiology of brain homeostasis and depression (66). AU's effect on PC12 cell proliferation may be one mechanism by which it improves depression. HFLS cells, found in the synovial lining of joints, may become activated and produce abnormal amounts of pro-inflammatory cytokines and matrix metalloproteinases (MMPs) in RA patients, potentially leading to joint damage and inflammation (67). βIII-tubulin has been shown to positively regulate the activation of HFLS cells (68), and decreased βIII-tubulin gene expression suggests that AU may inhibit activation of HFLS cells in RA patients.

In conclusion, 55 core genes are likely to be involved in the mechanism underlying RA with MDD, which predicts multiple therapeutic pathways closely related to the disease. Six diagnostic markers not only affect immune cells but are also potential therapeutic targets for RA with comorbid MDD.

Data availability statement

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

Author contributions

J-JS was responsible for data collection and sorting, computational modeling, and analysis; T-TZ wrote the research paper; J-YW provided research guidance for writing the research paper; L-DT participated in data analysis; YY provided research guidance on rheumatoid arthritis; LZ provided the design and general guidance of the research framework. J-JS and T-TZ contributed equally to this work and should be considered as co-first authors. J-YW and LZ contributed equally to this work and should be considered as co-corresponding authors. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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Assessing the association of leukocyte telomere length with ankylosing spondylitis and rheumatoid arthritis: A bidirectional Mendelian randomization study

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Background: Telomere length shortening can cause senescence and apoptosis in various immune cells, resulting in immune destabilization and ageing of the organism. In this study, we aimed to systematically assess the causal relationship of leukocyte telomere length (LTL) with ankylosing spondylitis (AS) and rheumatoid arthritis (RA) using a Mendelian randomization study.

Methods: LTL (n=472174) was obtained from the UK Biobank genome-wide association study pooled data. AS (n=229640), RA (n=212472) were obtained from FinnGen database. MR-Egger, inverse variance weighting, and weighted median methods were used to estimate the effects of causes. Cochran's Q test, MR Egger intercept test, MR-PRESSO, leave-one-out analysis, and funnel plots were used to look at sensitivity, heterogeneity, and multiple effects. Forward MR analysis considered LTL as the exposure and AS, RA as the outcome. Reverse MR analysis considered AS, RA as the exposure and LTL as the outcome.

Results: In the forward MR analysis, inverse variance-weighted and weighted median analysis results indicated that longer LTL might be associated with increased risk of AS (IVW: OR = 1.55, 95% CI: 1.14-2.11, p = 0.006). MR Egger regression analysis showed no pleiotropy between instrumental variables (IVs) (Egger intercept= 0.008, p = 0.294). The leave-one-out analysis showed that each single nucleotide polymorphism (SNP) of AS was robust to each outcome. No significant causal effects were found between AS, RA and LTL in the reverse MR analysis.

Conclusion: Longer LTL may be related with an increased risk of developing AS, and these findings provide a foundation for future clinical research on the causal association between LTL and AS.

KEYWORDS

leukocyte telomere length, ankylosing spondylitis, rheumatoid arthritis, Mendelian randomization, single nucleotide polymorphism

1 Introduction

Telomeres are small, highly conserved DNA repetitive sequences (TTAGGG) at the ends of eukaryotic cell linear chromosomes. These DNA components progressively shorten with each cell cycle and have an essential function in maintaining cellular chromosome stability. In most cell types, telomeres shorten with human age, eventually leading to replicative senescence (1, 2). Telomere length is usually referred to as leukocyte telomere length (LTL), which reflects both telomere length in other tissues and the senescence status of immune-related cells within the circulatory immune system (3). Although the relationship between leukocyte telomere length and disease is complex, LTL has been proposed as a marker of biological age and is associated with a high risk of multiple age-related diseases: including cardiovascular disease and cancer (4–6). In recent years, the involvement of telomere length, telomerase and protein complex systems in the pathogenesis of autoimmune diseases has become a hot research topic (7–9).

During the human immune response, immune cells grow exponentially and die when not needed, so they usually have extremely high replication rates. The telomeres within them are under tremendous stress. In addition to reflecting cell replication history, telomere shortening is influenced by factors such as oxidative stress and inflammation (10). As common autoimmune diseases, rheumatoid arthritis and ankylosing spondylitis, clinical and experimental data suggest that immune cells play an essential role in the pathogenesis of the diseases (11, 12). Furthermore, rheumatoid arthritis and ankylosing spondylitis may be related to single nucleotide polymorphisms (SNPs) (13). It was found that T lymphocytes from patients with rheumatoid arthritis and axial spondyloarthritis are susceptible to apoptosis due to mechanisms of abnormal telomere length or lack of upregulation of telomerase activity (11, 14, 15). However, as high-quality observational studies, we lack large samples of RCTs to explore whether a causal effect is associated with LTL and AS, RA.

Mendelian randomization (MR) is an instrumental variable (IV) analysis that uses SNPs as unconfounded proxies for exposure to estimate their effects on outcomes of interest. This reduces bias in observational epidemiological studies (16, 17). In MR analysis, according to Mendelian inheritance laws, SNPs are assumed to be randomly distributed in the general population, simulating the randomization process (18). Conceptually, MR is similar to RCT in that randomization occurs during meiosis and can be an essential strategy to strengthen causal inference when RCT is impractical or unethical (16). In the present study, we first performed a forward MR analysis to assess whether there was a causal effect between LTL as an exposure factor and AS, RA. Then, we performed a reverse MR analysis to assess whether there was an association with LTL using AS and RA as exposure factors. In this investigation, genetic variation as IV inferred a causal connection between outcome and exposure. Eliminating confounding factors and reverse causation effectively avoided bias in traditional epidemiological investigations (19). Furthermore, it may be more persuasive than traditional observational studies and bring fresh insights into treating and diagnosing AS and RA.

2 Materials and methods

2.1 Study design and data sources

Using summary data from genome-wide association studies (GWAS), we ran MR analysis to evaluate the bidirectional relationship of LTL with AS and RA. Data for the MR analyses were obtained from 2 GWAS public summary statistics databases containing mainly European ancestry. Pooled GWAS results for LTL were derived using genome-wide pooled data that screened 472,174 well-characterized adults in the UK Biobank (UKB), LTL quantified as telomere repeat copy ratios relative to single gene copy ratios, genetic variation in LTL GWAS adjusted for age and sex (4). The GWAS data for AS and RA were obtained from the FinnGen database, which collects and analyzes genomic and health data from 500,000 Finnish biobank participants. The AS, RA dataset contains 229,640 (2252 cases, 227,388 controls) and 212,472 (9855 cases, 202,617 controls) participants, respectively, and independent variant loci genetically associated with AS and RA were identified by comparison with the healthy population. Since all of the analyses in this paper were based on data that was available to the public, there was no need for an institutional review board to give ethical approval for this study.

2.2 Selection of instrumental variables for MR analysis

We performed stringent filtering steps to control SNP quality in two different GWAS pooled data. First, SNPs that were linked to the right exposure were chosen using genome-wide significance thresholds ($p < 5 \times 10\text{-}8$). Second, SNPs that have a total linkage disequilibrium (LD, $R2 \geq 0.001$ and 10 Mb). Third, to figure out the strength of genetic tools are, we left out SNPs with F-statistics less than 10. Lastly, SNPs that could be pleiotropic were taken out after MR-polynomial residuals and outliers (MR-PRESSO), and MR analysis was run again to see how stable it was. With the above screening criteria, we screened 8 and 14 SNPs as IVs when using the AS and RA dataset as exposure factors, respectively, and 92 SNPs as IVs when using the LTL dataset as instrumental variables. The SNPs utilized as instrumental variables are described in the supplementary file: Tables S1–S3.

2.3 Statistical analysis

In this study, we used the "TwoSampleMR" package in R (version 4.1.2) for the analysis. The odds ratio (OR) and 95% confidence interval (CI) were used to estimate the degree of causality for the binary outcomes. We employed three techniques of MR analysis (inverse variance weighting, weighted median, and MR Egger) to examine in both directions whether LTL is causally related to AS and RA. IVW was used to weigh the random variable measurements, using the inverse of the variance of each random variable, which minimizes the mean variance. Since random effects of IVW allow each SNP to produce different mean effects, we used

inverse variance weighting as the primary method for MR analysis (20). However, as this method only yields accurate estimates when all genetic variants are valid instrumental variables, we complemented the regression method using MR Egger and weighted medians to assess the IVW method's robustness (21, 22). The MR Egger regression intercept and 95% confidence interval (CI) were used to determine the degree of bias in the arbitrary estimates due to directional pleiotropy when 100% of the genetic variance was considered to be null IVs (21, 23). The weighted median, enables a consistent evaluation of causative effects when 50% of genetic variants are valid IVs (22).

There may be heterogeneity in intravenous fluids from different platforms or populations, which can influence the outcome. This study utilized Cochran's Q test and funnelled plots to evaluate SNP heterogeneity. Horizontal pleiotropy is the association of genetic variations with various phenotypes in multiple pathways, which might render MR analysis ineffective (20). In order to assess unknown pleiotropy, we used several analytical approaches: First, sensitivity analyses used "leave one out" to explore the possibility that individual SNPs drive such causal associations. Second, MR Egger intercept tests were used to assess the pleiotropic association of genetic variants with other potential confounders. The regression intercept evaluates the magnitude of pleiotropy, and the closer the intercept is to 0, the less likely the gene is pleiotropic (21). This study used the bonferroni corrected P value ($p< 0.025$) as the significance threshold.

3 Results

3.1 Forward MR analysis: Causal effect of LTL on AS, RA

In the forward MR analysis, we analyzed the causal effect of LTL on AS and RA. The results of the MR analysis are shown in Table 1. The IVW results showed a significant association between LTL and ankylosing spondylitis (OR = 1.55, 95% CI: 1.14-2.11). The scatter plot of SNPs showing the effect of LTL on AS showed that the risk of AS was associated with a longer LTL (Figure 1A). Among the outcome variables in rheumatoid arthritis, the results of the IVW analysis method showed no significant association between LTL and RA (IVW: OR = 0.89, 95% CI: 0.75-1.05, p = 0.173, Table 1).

To further investigate the association of LTL with AS and RA, we performed pleiotropy, heterogeneity and sensitivity analyses. The results showed no significant cross-sectional pleiotropy bias for the effects of LTL on AS and RA (AS: Egger intercept = 0.008, p = 0.294; RA: Egger intercept = -0.002, p = 0.644. Table 2). In addition, there was no significant heterogeneity between IVs for LTL effects on AS (Cochran Q = 117.15, p = 0.029, Table 2). Similarly, in the funnel plots of IVW and MR Egger, no significant heterogeneity between IVs was observed (Figure 1C). Significant heterogeneity was found between IVs for LTL effects on RA (Cochran Q = 142.51, p < 0.001, Table 2), however, as previously described, the random effects IVW approach allows for heterogeneity generated by SNPs. The leave-one-out method of sensitivity analysis showed that removing any of the 92 SNPs of AS did not significantly change the results (all rows were on the same side of 0) (Figure 1B), which shows that the MR analysis results were reliable.

3.2 Reverse MR analysis: Causal effect of AS, RA on LTL

In the reverse MR analysis, we analyzed whether AS and RA as exposure factors had a causal effect on LTL. The IVW results showed no significant association was found between AS, RA and LTL (Table 3). The results of the pleiotropic analysis showed a significant cross-sectional pleiotropic bias of RA on the IVs of LTL (Egger intercept = -0.0011, p = 0.006, Table 4). Therefore, the effect of RA on LTL may be influenced by other confounding factors, and there is a risk of false negatives.

4 Discussion

With the improvement of medical treatment and the advancement of technology, the average life expectancy of human beings is increasing daily, and the aging problem is gradually attracting the medical community's attention. A series of diseases such as aging-related malignancies, cardiovascular diseases, metabolic diseases and neurodegenerative pathologies have become essential research topics for scientists, and aging of the immune system plays a crucial role in them (4). Aging of the immune system can weaken the body's ability to fight pathogenic

TABLE 1 MR Results of LTL Use on Risk of Ankylosing Spondylitis and Rheumatoid Arthritis.

Outcome	MR Methods	N SNPs	OR (95%CI)	Se	*P* value
AS	MR Egger	92	1.19(0.67-2.12)	0.30	0.562
	Weighted median	92	1.55(1.01-2.38)	0.22	0.045
	IVW	92	1.55(1.14-2.11)	0.16	0.006
RA	MR Egger	92	0.95(0.69-1.30)	0.13	0.746
	Weighted median	92	0.88(0.70-1.09)	0.10	0.242
	IVW	92	0.89(0.75-1.05)	0.07	0.173

AS, Ankylosing Spondylitis; RA, Rheumatoid Arthritis; LTL, Leukocyte Telomere Length; MR, mendelian randomization; IVW, inverse variance weighted; N SNPs, number of genetic instruments; OR, odds ratio; SNP, single nucleotide polymorphism.

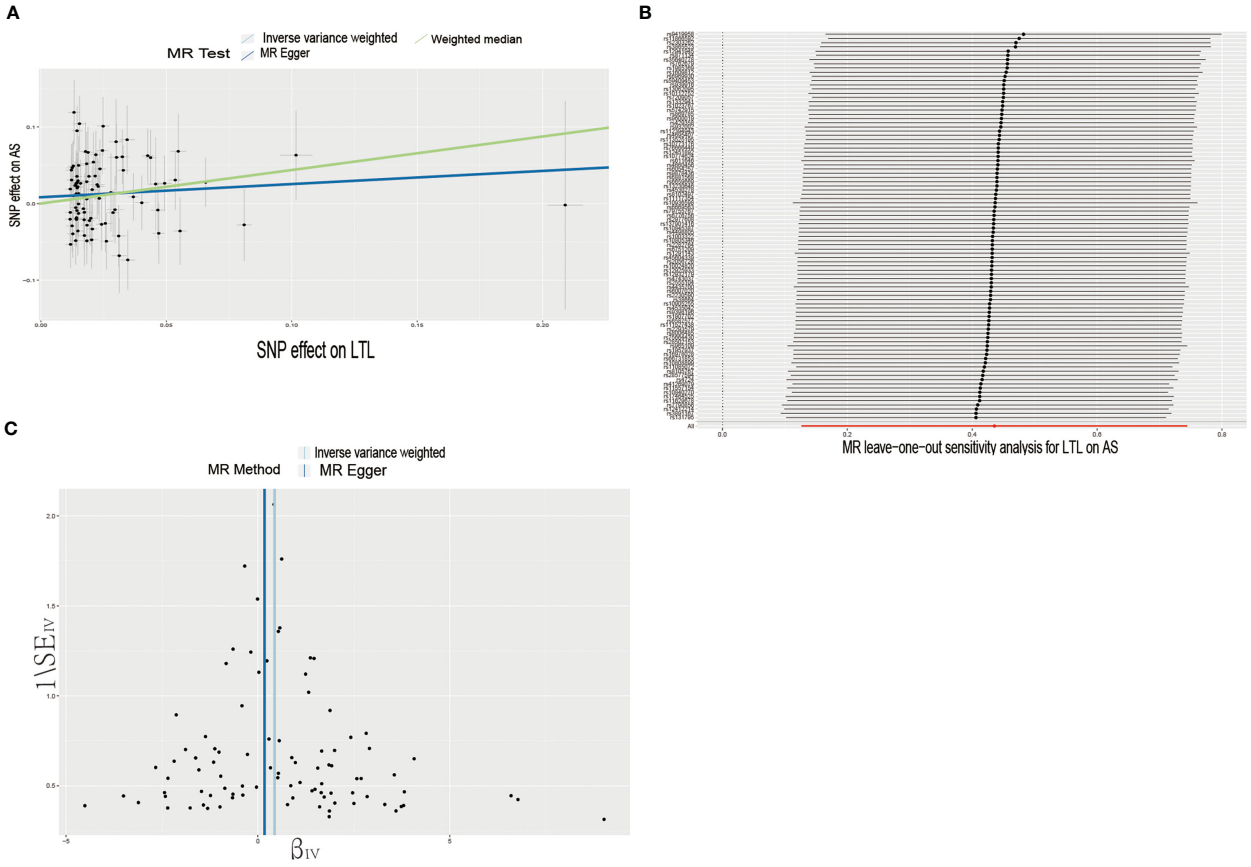

FIGURE 1
Causal effects of LTL on AS. **(A)** Scatter plot of the association between LTL and AS. This study used three methods to calculate the causal relationship between exposure factors and outcomes. The dark blue line represents MR Egger, the green line represents the weighted median, and the light blue line represents IVW. **(B)** Leave-one-out analysis to assess the effect of each SNP in driving causality. **(C)** Application of funnel plots to detect whether the observed associations are significantly heterogeneous. IVW, inverse variance weighting; LTL, leukocyte telomere length; AS, Ankylosing Spondylitis; MR, Mendelian randomization; SNP, single nucleotide polymorphism.

microorganisms and kill tumor cells (24). On the other hand, increase the risk of developing autoimmune diseases, leading to a long-term chronic inflammatory state of the body (25). RA and AS, as chronic autoimmune diseases, both autoimmunity and auto-inflammation are involved in the pathogenesis of the disease (26–29). In this study, we used large-scale GWAS data from UK Biobank and Finnish Biobank participants to assess the possible causal relationship between LTL, AS, and RA by multiple MR methods. According to our research, in a European population, a longer LTL was related with an increased chance of developing AS. Reverse MR analysis revealed that genetically predicted AS and LTL have no causal link.

Ankylosing spondylitis, the most prevalent kind of spondyloarthritis, is a chronic inflammatory disease of the mid-axis spine that can manifest in a variety of clinical manifestations. Chronic back pain and growing rigidity of the spine are the most prevalent symptoms of the disease. AS is viewed as a combination of autoimmunity and autoinflammation. These components of innate immunity promote the onset of the disease, while the adaptive component is responsible for the continuation of the inflammatory process (29). In this investigation, we discovered that longer telomeres were related with an increased risk of AS.

Similarly, Tamayo et al. found in a cross-sectional research that patients with rheumatic illnesses had longer telomeres than controls

TABLE 2 Heterogeneity and pleiotropy analysis in forward MR analysis.

Outcome	MR Methods	Cochran Q statistic	Egger intercept	Heterogeneity *p*-value	Pleiotropy *p*-value
AS	MR Egger	117.15	0.008	0.029	0.294
	IVW	118.60		0.028	
RA	MR Egger	142.51	-0.002	<0.001	0.644
	IVW	142.85		<0.001	

AS, Ankylosing Spondylitis; RA, Rheumatoid Arthritis; MR, mendelian randomization; IVW, inverse variance weighted.

TABLE 3 MR Results of Ankylosing Spondylitis and Rheumatoid Arthritis Used on the Effect of LTL.

Exposure	MR Methods	N SNPs	OR (95%CI)	Se	*P* value
AS	MR Egger	8	1.00(0.99-1.00)	0.004	0.828
	Weighted median	8	1.01(1.00-1.01)	0.003	0.110
	IVW	8	1.00(1.00-1.01)	0.002	0.069
RA	MR Egger	14	1.02(1.01-1.03)	0.005	0.001
	Weighted median	14	1.01(1.00-1.02)	0.004	0.011
	IVW	14	1.01(1.00-1.01)	0.004	0.060

AS, Ankylosing Spondylitis; RA, Rheumatoid Arthritis; LTL, Leukocyte Telomere Length; MR, mendelian randomization; IVW, inverse variance weighted; N SNPs, number of genetic instruments; OR, odds ratio; SNP, single nucleotide polymorphism.

TABLE 4 Heterogeneity and pleiotropy analysis in reverse MR analysis.

Exposure	MR Methods	Cochran Q statistic	Egger intercept	Heterogeneity *p*-value	Pleiotropy *p*-value
AS	MR Egger	3.04	0.002	0.804	0.312
	IVW	4.25		0.750	
RA	MR Egger	11.59	-0.001	0.479	0.006
	IVW	22.49		0.048	

AS, Ankylosing Spondylitis; RA, Rheumatoid Arthritis; MR, mendelian randomization; IVW, inverse variance weighted.

(30). Four years later, the researchers continued their research on spondyloarthritis and discovered that individuals with rheumatic illnesses characterized by persistent systemic inflammation had longer peripheral blood leukocyte telomeres than controls (31). In contrast, this was not found in other rheumatic diseases without chronic systemic inflammation, such as osteoarthritis and osteoporosis (31). The mechanism behind this has not been fully clarified, and it has been suggested that chronic inflammation accompanying rheumatic diseases leads to this relative telomere lengthening effect. This may result from the breakdown of proteins of the sheltered complex or related factors associated with DNA repair or recombination (32). In addition, another study comprising 91 patients with Birdshot uveitis and 150 healthy controls revealed that the Birdshot patients had longer telomeres than the healthy controls, indicating a complicated telomere biology in chronic inflammation (33). Interestingly, like AS, Birdshot uveitis patients are closely associated with HLA class I (HLA-B27) (30, 31).

The ability of activated naive T cells to upregulate telomerase expression is also a possible explanation (34–37), but this ability remains contentious. In highly proliferative cells such as stem cells, germ cells, and the majority of cancer cells, telomerase plays a crucial role in telomere maintenance. In contrast to mature thymocytes, young nave T cells exhibit more telomerase production and activity during proliferation despite being normal somatic cells. They are barely detectable in mature resting naïve T cells (38, 39). Several studies have shown a slight increase in atopic abnormalities in AS patients compared to RA patients (40). Specific immunological pathways have been implicated in GWAS of this genetic disorder. These pathways include the IL-23/17 pathway, regulation of NF-kappaB activation, amino acid trimming of MHC antigen presentation, and genes that regulate CD8 and CD4 T cell populations (41). These findings imply that Th2 cells and chemokines may contribute to the development of AS (42). Interestingly, CD8+ antigen-specific T cells show a stronger inflammatory response in people with longer telomere lengths, and CD4+ antigen-specific T cells have longer telomere lengths than naive cells (43, 44). Lastly, the lengthening or shortening of leukocyte telomeres should not be considered a static event, but rather a dynamic one that occurs throughout time. The length of peripheral blood leukocyte telomeres may serve as a measure of chronic systemic inflammatory activity in AS, requiring subsequent pathological examination.

However, there are some concomitant limitations in our study. First, the data used in this study were primarily conducted on participants of European ancestry, the findings may be biased towards other ethnic groups with different lifestyles and cultural backgrounds. Secondly, we used data measuring TL GWAS in blood leukocytes, and LTL may not be sufficiently representative of telomere length in other cell or tissue subgroups associated with AS. Third, the uncertainty and incompatibility of sample sizes between the two major databases used in this study may simultaneously lead to some bias in our MR analysis. Fourth, MR evaluates inferred causal hypotheses by assigning genetic variants at random, it is challenging to discriminate between mediation and pleiotropy using MR methods alone. Numerous polymorphisms in the human genome may influence one or more phenotypes.

5 Conclusion

In conclusion, the present study found that longer LTL may be associated with an increased risk of AS. Our results suggest that LTL may be involved in the pathogenesis of AS. In the future, further

studies are worthwhile to explore the correlation between LTL and AS in the pathogenesis and treatment strategies of AS.

Data availability statement

Data from LTL are available in the UK Biobank (https://figshare.com/s/caa99dc0f76d62990195). Data for Ankylosing Spondylitis and Rheumatoid Arthritis were obtained from the FinnGen database (https://storage.googleapis.com/finngen-public-data-r7/summary_stats/finngen_R7_M13_ANKYLOSPON.gz and https://storage.googleapis.com/finngen-public-data-r7/summary_stats/finngen_R7_M13_RHEUMA.gz).

Ethics statement

All of the analyses in this research are based on publicly accessible summary data, institutional review board approval was not necessary for this study.

Author contributions

The final manuscript was read and approved by all writers. WD and JY designed the study, gathered and analyzed the data, and prepared and revised the final publication. WH and CJ collected and analyzed the data. SK and ZJ conceived the study and revised the text.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1023991/full#supplementary-material

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Circulating miRNA-19b as a biomarker of disease progression and treatment response to baricitinib in rheumatoid arthritis patients through miRNA profiling of monocytes

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Introduction: A number of studies have demonstrated a key role of miRNA isolated from cells, tissue or body fluids as disease-specific biomarkers of autoimmune rheumatic diseases including rheumatoid arthritis (RA) and systemic sclerosis (SSc). Also, the expression level of miRNA is changing during disease development, therefore miRNA can be used as biomarkers monitoring RA progression and treatment response. In this study we have investigated the monocytes-specific miRNA that could serve as potential biomarkers of disease progression observed in sera and synovial fluids (SF) in early (eRA) and advanced (aRA) RA and in RA patients before and 3 months after selective JAK inhibitor (JAKi) -baricitinib treatment.

Methods: Samples from healthy control (HC) (n=37), RA (n=44) and SSc (n=10) patients were used. MiRNA-seq of HC, RA, and SSc monocytes was performed to find versatile miRNA present in different rheumatic diseases. Selected miRNAs were validated in body fluids in eRA (<2 years disease onset) and aRA (>2 years disease onset) and RA patients receiving baricitinib.

Results: Using miRNA-seq, we selected top 6 miRNA out of 95 that were significantly changed in both RA and SSc monocytes compared to HC. To identify circulating miRNA predicting RA progression, these 6 miRNA were measured in eRA and aRA sera and SF. Interestingly, miRNA (-19b-3p, -374a-5p, -3614-5p) were significantly increased in eRA sera vs HC and even further upregulated in SF vs aRA sera. In contrast, miRNA-29c-5p was significantly reduced in eRA sera vs HC and even further decreased in SF vs aRA sera. Kegg pathway analysis predicted that miRNA were involved in inflammatory-mediated pathways. ROC analysis demonstrated that miRNA-19b-3p (AUC=0.85, p=0.04) can be used as biomarker predicting JAKi response.

Discussion: In conclusion, we identified and validated miRNA candidates which were present simultaneously in monocytes, sera, SF and that can be used as biomarkers predicting joint inflammation and monitoring therapy response to JAKi in RA patients.

KEYWORDS

rheumatoid arthritis, miRNA, baricitinib, monocytes, sequencing, synovial fluids, biomarkers, JAKi inhibitors

Highlights

- Global distribution of miRNA profile is similar in both RA and SSc monocytes
- Selected circulating miRNA can be used as no-invasive biomarkers of joint inflammation in RA patients
- MiRNA-19b can help to predict the responders' group for baricitinib in RA patients

Introduction

Autoimmune rheumatic diseases (ARDs) are a group of distinct disorders that share similar clinical, laboratory, and immunological symptoms. Their basic pathobiological finding is: the development of excessive self-reactive and antigen-controlled immune response. Two of the major ARDs are rheumatoid arthritis (RA) and systemic scleroderma (SSc) which, despite the often-different clinical symptoms of the disease, share a similar pathophysiological basis. They can also cause similar complications, including interstitial pneumonia, glomerulonephritis, and serositis (1, 2). Moreover, SSc-related skin involvement and SSc-related arthritis usually respond well to treatment with the drug commonly used to treat RA which is methotrexate (3, 4). This drug belongs to synthetic disease-modifying anti-rheumatic drugs (sDMARD). Monocytes and macrophages play an extremely important role in the pathogenesis and course of both RA and SSc. Indeed, monocytes produce numerous cytokines and chemokines that may be a hallmark of both of these diseases (5, 6). Monocytes are mainly responsible for chronic inflammation and bone erosion in RA, as well as chronic and progressive tissue and organ fibrosis in SSc, which are the main symptoms of these ARDs (7, 8). Thus, modalities produced by monocytes appear to be very good candidates for new markers in both RA and SSc, which can uniquely identify these diseases.

MicroRNA (miRNA) are small (22–24 nt) non-coding RNA sequences that are involved in the negative regulation of gene transcripts and subsequently resulted in their degradation. Therefore, miRNA play an important role in biological processes as post-transcriptional modulators in numerous physiological processes as well as in pathogenesis of ARDs. Indeed, our previous studies revealed that altered expression of specific miRNA (-146b, -26a-3p, -5196, -29b) correlated with clinical parameters including DAS28, mRSS, CRP and ASDAS for RA, SSc, AS (Ankylosing spondylitis). Furthermore, using functional assays we have demonstrated that these miRNA inhibited their target genes including RARA, IFN type I genes, FRA2 and TAB1, respectively in RA and SSc monocytes and SSc fibroblasts (7–10). We have also observed that circulating miRNA-146b was strongly elevated in synovial fluids (SF) of RA patients, suggesting that miRNA-146b can be used as a biomarker of joint inflammation (9). However, a global comparison between miRNA profile of both RA and SSc monocyte population and subsequent validation in sera has not been performed. Thus, the aim of this study was to analyse the global miRNA expression profile of RA and SSc monocytes and to compare their expression pattern to sera-derived RA and SSc miRNA. There is also a great interest in the identification of miRNA that could predict disease progression in order to implement strong therapeutic intervention before joint deformities and functional impairments occur. Indeed, to find a no-invasive biomarker predicting joint inflammation, further qPCR validation of selected miRNA candidates was performed in sera of early and advanced RA and SF. Interestingly, finding unique miRNA monitoring JAK inhibitors (JAKi) therapy response is also of great need to maximize the clinical benefit for the individual RA patient. Relatively modern therapy by JAKi is used as an alternative for biological agents following the failure of the first-line treatment including sDMARD. Therefore, we have measured selected miRNA in RA patients before and 3 months after baricitinib (selective JAK1/2 inhibitor) treatment. Overall, we believe that our study might help clinicians in better patient stratification based on miRNA-driven biomarkers allowing accurate assessments of disease progression and treatment response to JAKi.

Materials and methods

Clinical characteristics of the patients

In this study, we used blood from 54 patients who met the 2010 and 2013 American College of Rheumatology/European League Against Rheumatism classification criteria for RA and SSc, respectively (11, 12). Clinical and laboratory parameters of RA (n=44) and SSc (n=10) patients included in the study are displayed

in Table 1. Healthy controls (HC) (n=37) were characterized as follows: median age was 42 with a range between 22-67 years of age and the ratio of female to male was 29 to 8. This study was approved by the local ethic committee (approval no. KBT-5/3/2019, KBT-6/5/2020) at the National Institute of Geriatrics Rheumatology and Rehabilitation (NIGRiR). The patients' blood and synovial fluids were obtained from NIGRiR and all patients gave written informed consent prior to inclusion.

Design, participants and cell purification

The blood was collected in EDTA-coated tubes and $CD14^+$ monocytes were separated according to the manufacturer's protocol with the $CD14^+$MACS beads isolation kit (Miltenyi-Biotec, The Netherlands) as previously described (9). In experiments measuring cell-free miRNA, we used sera from SSc patients, sera from early RA (eRA) with <2 years disease onset, and advanced RA (aRA) with >2 years disease onset and plasma from RA patients receiving baricitinib before and 3 months after therapy.

TABLE 1 Clinical and laboratory data of RA and SSc patients.

Parameters of RA patients (n = 44)	RA Patients
Age, years, median (range)	52 (23–73)
Sex F/M	38/6
Disease duration, years, median (range)	2,75 (0–30)
Anti-CCP Abs, % (*n*)	79,5% (n=35)
RF, % (*n*)	63,6% (n=28)
CRP, mg/L, median (range)	10 (1-188)
ESR, mm/h, median (range)	24 (3-104)
DAS-ESR, median (range)	5,62 (2,16-8,17)
DAS-CRP, median (range)	5,52 (1,91-7,78)
Treatment	
Methotrexate, % (n)	50% (n=22)
Leflunomide, % (n)	4,5% (n=2)
Sulfasalazine, % (n)	15,9% (n=7)
Hydroxychloroquine, % (n)	13,6% (n=6)
Baricitinib, % (n)	31,8% (n=14)
Methylprednisolone, % (n)	25% (n=11)
Parameters of SSc patients (n = 10)	SSc Patients
Age, years, median (range)	58 (41–86)
Sex F/M	10/0
Disease duration, years, median (range)	4 (0–30)
Modified Rodnan skin score in 17 body surfaces, median (range)	10,5 (4-25)
CRP, mg/L, median (range)	5 (3-12)
Treatment	
Cyclophosphamide % (n)	20% (n=2)
Mycophenolate mofetil % (n)	10% (n=1)
Methotrexate, % (n)	10% (n=1)
Prostanoids, % (n)	30% (n = 3)

Global miRNA profiling of RA and SSc monocytes

MiRNA from monocytes was isolated using a miRNeasy kit (Qiagen, UK) according to the manufacturer's protocol. Global miRNA profiling was performed in 10 HC, 10 SSc and 12 RA monocytes. The results from miRNA-seq were normalized by the mirDeep2 pipeline and subsequently analysed using R programming language. The processed fasta file was mapped to the human genome using the mirAligner software (version 3.5). Following post-processing analysis, miRNA with adjusted p-values < 0.05 and false discovery rate (FDR) < 0.05 were considered significantly differently expressed between the HC and RA/SSc group. In order to increase the power and integrated information of the miRNA-seq analysis, we used miRNet webtool and the Reactome database to identify and highlight the target genes related to inflammatory and immune-related pathways, as well as investigated the miRNA-gene network. We have focused only on pathways that were significantly enriched based on corrected p-values < 0.05 from the whole list of miRNA.

Circulating miRNA validation in RA and SSc sera and RA synovial fluids

The volume of 300 µl of sera or plasma from SSc (n=10), RA (n=44) patients and and HC (n=37) patients was used to isolate circulating miRNA using NucleoSpin® miRNA plasma/serum (Macherey–Nagel, Germany) according to the manufacturer's protocol. Circulating miRNA expression level was validated using TaqMan ® microRNA RT Kit (Thermo Fisher Scientific, USA) and TaqMan® MicroRNA Assays. The IDs of the probes are: hsa-miR-589-3q (479072_mir), hsa-3614-5p (478836_mir), hsa-miR-374a-5p (478238_mir), hsa-miR-29c-3p (479229_mir), hsa-miR-19b-3p (478264_mir), hsa-miR-503-5p (478143_mir), has-miR-16-5p (477860_mir), all from ThermoFisher Scientific (USA). The expression levels of circulating miRNA in SSc and RA patients were relative to the average HC (arbitrarily set at 1) and were calculated using the following equation: 2^(-delta delta CT). All samples were normalized to miRNA-16-5p as an internal control.

KEGG enrichment analysis

KEGG pathways enrichment analysis of selected 6 miRNA was performed using DIANA-mirPath software v.3. This algorithm was used to predict the interaction between 6 miRNA and their mRNA targets based on the coding sequence (CDS). Illustrated interactions

were previously filtered by FDR correction, p-value threshold less than 0.05, and Fisher's Exact Test.

Statistical analysis

To determine the statistical significances, we performed Mann-Whitney test for unpaired samples and Wilcoxon matched-pairs signed-rank test for non-normally distributed data. Error bars were defined as S.E.M. P-values were expressed as follows: ns for not significant; 0.05 > P > 0.01 as *; 0.01 > P > 0.001 as **; P < 0.001 as ***, P <0.0001 as ****. To validate accuracy of miRNA-derived biomarkers, receiver-operating characteristics (ROC) curve was performed. The cut-off value was established based on maximal Youden index. GraphPad Prism version 4.03 software was used.

Results

Identification of miRNA that were common in both RA and SSc monocytes

In order to find common miRNA for both RA and SSc in monocytes compared to HC we have performed global miRNA profiling using miRNA-seq. The heatmap analysis demonstrated top 75 for RA (Figure 1A) and 95 for SSc (Figure 1B) significantly down-regulated and up-regulated miRNA that were changed in these diseases compared to HC. The principal component analysis (PCA) plot demonstrated that the global miRNA expression pattern was distributed similarly between RA and SSc patients, but was separated from HC, suggesting similarities and overlap in miRNA clustering between RA and SSc monocytes (Figure 1C). In addition, Reactome database revealed that miRNA were predicted to regulate similar immune pathways in both RA and SSc monocytes including innate and adaptive immune system regulation, TGF-β signaling, and MHC class II antigen presentation among others (Figures 1D, E). Subsequently, we selected 6 miRNA which had the same expression pattern both in RA and SSc monocytes compared to HC (Figures 1F, G). Indeed, heatmap analysis revealed that 3 miRNA (-19b-3p, -29c-5p, -374a-5p) were downregulated, whereas 3 other miRNA (-503-5p, -589-5p, -3614-5p) were upregulated in both RA and SSc monocytes. We also investigated genes and their signaling pathways that can be directly targeted by selected 6 miRNA. Using the miRNet web tool, we identified and highlighted genes associated with inflammatory and immune-related pathways (Supplementary Figure S1). We assessed only significantly enriched miRNA-gene networks based on corrected p-values < 0.05.

Validation of selected miRNA in RA and SSc sera

We then measured the expression of selected miRNA in RA and SSc sera in order to compare the cell-specific miRNA expression to cell-free miRNA in both ARDs. Samples used to measure cell-specific miRNA and cell-free miRNA expression were isolated from the same SSc patients. In HC and RA cell-free miRNA analysis, we used a larger cohort than the primary cell-specific miRNA used for miRNA-seq. In Figures 2B-F it can be seen that expression of selected miRNA (-29c-5p, -374a-5p, -503-5p, -589-5p, -3614-5p) in RA sera had similar expression pattern as seen in monocytes. In contrary, miRNA-19b-3p was significantly (p= 0.0016) increased in RA sera (Figure 2A) and downregulated in RA

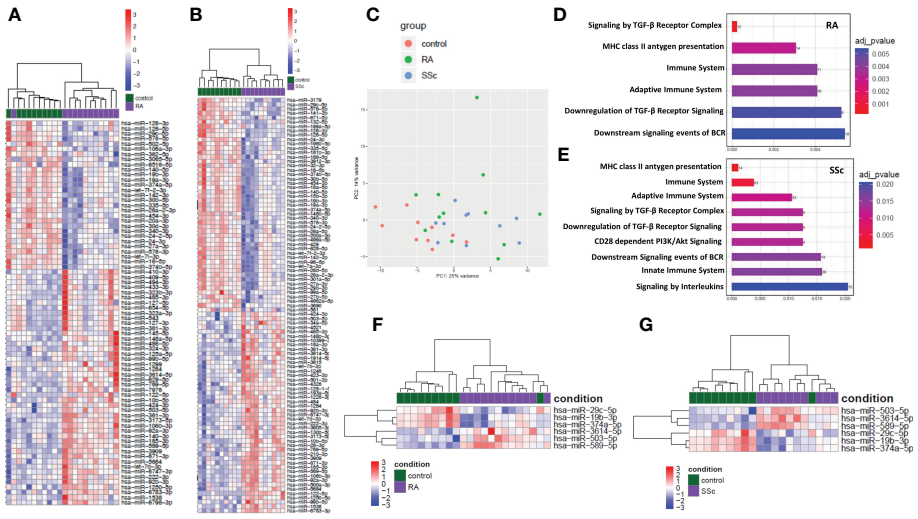

FIGURE 1
Global analysis and distribution of miRNA-seq in RA and SSc monocytes. Heatmap of significantly dysregulated miRNA in RA **(A)** and SSc **(B)** monocytes compared to HC. Principal component analysis (PCA) plot of global miRNA distribution in RA (green), SSc (blue) and HC (red) monocytes **(C)**. Enrichment analysis of abundantly expressed miRNA regulating selected genes involved in immune pathways in RA **(D)** and SSc monocytes **(E)**. Heatmap analysis of selected miRNA in RA **(F)** and SSc **(G)** monocytes compared to HC.

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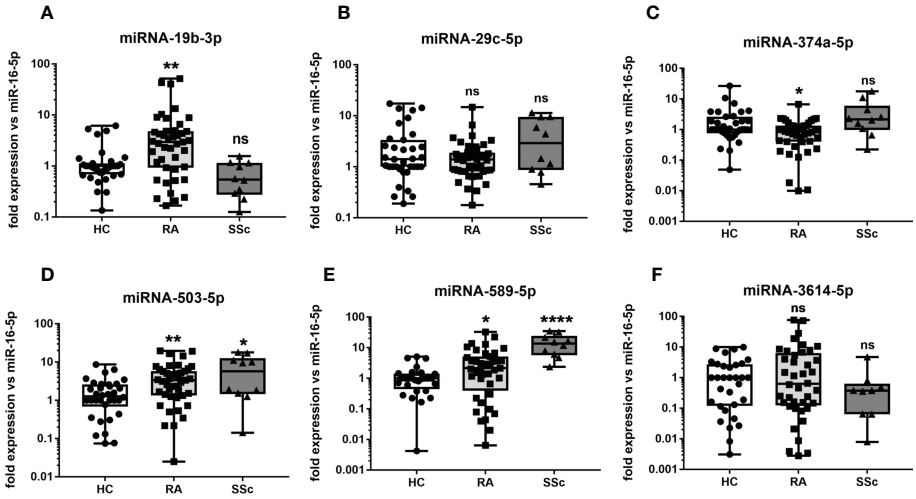

FIGURE 2
The level of circulating miRNA in RA and SSc patients. The level of miRNA-19b-3p **(A)**, miRNA-29c-5p **(B)**, miRNA-374a-5p **(C)**, miRNA-503-5p **(D)**, miRNA-589-5p **(E)**, miRNA-3614-5p **(F)** in sera of RA and SSc patients compared to HC. P-values were expressed as follows: ns for not significant; 0.05 > P > 0.01 as *; 0.01 > P > 0.001 as **; P < 0.001 as ***, P <0.0001 as ****.

monocytes from the miRNA-seq analysis (Figure 1F). In SSc sera, the expression of miRNA-19b-3p and miRNA-589-5p had the same expression pattern as seen in SSc monocytes. The rest of circulating SSc miRNA candidates (miRNA-29c-5p, -374a-5p, -503-5p, -3614-5p) did not differ significantly compared to HC. Overall, these data suggest that 3 cell-free miRNA candidates in RA sera had the same expression pattern as seen in RA monocytes. While in SSc sera only 2 cell-free miRNA had the same expression pattern as seen in SSc monocytes, probably due to the limited sample size of the SSc group. Therefore, we have then performed a deeper analysis only on RA patients in order to find circulating miRNA as biomarkers of disease progression and treatment response.

The expression of circulating miRNA candidates in sera and synovial fluids of RA patients

The next step was to measure selected miRNA in sera of eRA and aRA, but also in SF of aRA. This stage allowed us to identify unique miRNA predicting joint inflammation and ultimately monitoring RA progression based on previously selected cell-specific miRNA. In Figures 3A, D–F, it can be seen that the expression of miRNA (-19b-3p, -503-5p, -589-5p, -3614-5p) were gradually enhanced in eRA sera through high abundance in aRA sera, and the highest expression levels were seen in SF of aRA. On

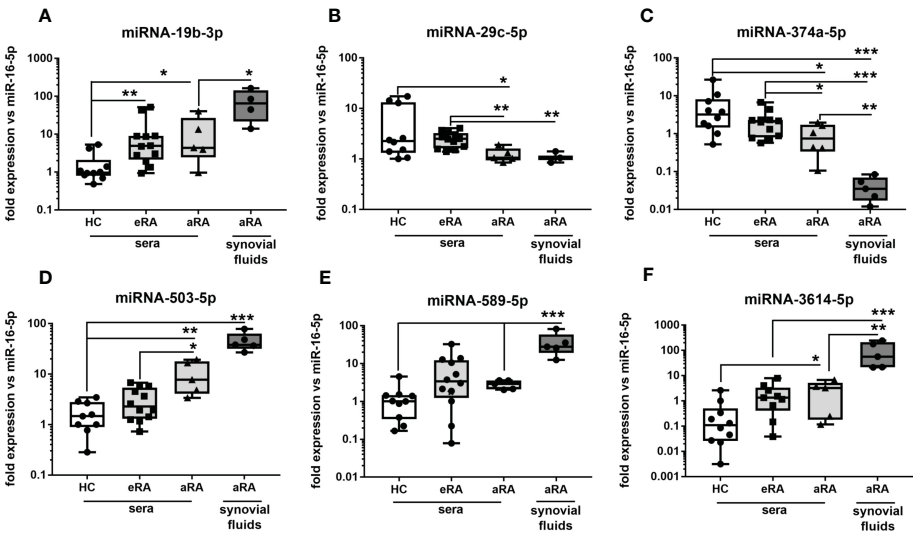

FIGURE 3
The levels of circulating miRNA in body fluids of eRA and aRA patients. The levels of miRNA-19b-3p **(A)**, miRNA-29c-5p **(B)**, miRNA-374a-5p **(C)**, miRNA-503-5p **(D)**, miRNA-589-5p **(E)**, miRNA-3614-5p **(F)** in sera or eRA and aRA and SF of aRA patients compared to HC. P-values were expressed as follows: ns for not significant; 0.05 > P > 0.01 as *; 0.01 > P > 0.001 as **; P < 0.001 as ***, P <0.0001 as ****.

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the other hand, we have observed a gradual reduction of miRNA-29c-5p and miRNA-374a-5p in eRA sera through low abundance in aRA sera and the lowest expression levels were seen in SF of aRA (Figures 3B, C). Overall, these data suggest that cell-free circulating miRNA (-19b-3p, -503-5p, -589-5p, -3614-5p, 29c-5p, -374a-5p) can be used as biomarkers predicting joint inflammation and subsequently cartilage destruction in early-stage of RA.

Functional enrichment analysis of selected miRNA

To further investigate the function of selected 6 miRNA candidates, KEGG pathway analysis was conducted using the online DIANA-mirPath database. Interestingly, 3 out of the top 8 pathways of the enrichment analysis were predicted to be associated with autoimmunity including allograft rejection (hsa05330), autoimmune thyroid disease (hsa05320), and antigen processing and presentation (hsa04612), through significant negative regulation of HLA-DOA and HLA-DQA1 genes by all selected miRNA (Figure 4A). This analysis clearly indicates that selected miRNA candidates were involved in autoimmunity. In addition, RNA-seq analysis confirmed reduced expression of 12 (including HLA-DOA - 1.5 fold reduction and HLA-DQA1 – 1.3 fold reduction) out of 14 detected HLA class II genes in RA monocytes compared to HC (Figure 4B). These results suggest that selected miRNA candidates may negatively regulate HLA class II in RA monocytes. In Supplementary Table S3 and Supplementary Figure S4, there are more detailed analyses of the miRNA-gene network to illustrate the effects of miRNA deregulation in RA and SSc monocytes.

The expression of circulating miRNA candidates in RA patients before and after JAKi therapy

We have also measured selected miRNA in RA patients before and 3 months after JAKi therapy in order to test if these 6 miRNA can be useful as biomarkers for monitoring treatment response. In Figure 5A it can be seen that circulating miRNA-19-3p was significantly (p= 0.0001) reduced in all RA patients after baricitinib treatment reaching a similar level as HC. Also, miRNA-503-5p was significantly (p= 0.0085) downregulated following JAKi therapy in 12 out of 14 RA patients (Figure 5D and Supplementary Figure S2). Simultaneously, the average DAS28 score was significantly reduced (p= 0.0001) from 5.8 to 3.3 in all RA patients upon baricitinib administration (Supplementary Figure S2). The rest miRNA (-29c-5p, -374a-5p, -589-5p, -3614-5p) did not reach statistical significance before and after baricitinib treatment (Figure 5B, C, E, F). Finally, to evaluate the diagnostic potential of miRNA-19b-3p and miRNA-503-5p as biomarkers predicting JAKi treatment response compared to CRP, ROC analysis was introduced. In Figure 5G, it can be seen that changes/delta in miRNA-19b-3p expression (AUC=0.85, p=0.04) before and after drug treatment were more specific to identify the responder group (reduction in DAS28 of more than 2) than changes in miRNA-503-5p (AUC=0.6, p=0.57) or changes in CRP level (AUC=0.58, p=0.62). In addition, it has been demonstrated significant (p=0.027) positive correlation between the expression of delta miRNA-19b-3p and delta DAS28 in baricitinib-treated RA patients (Figure 5H). Overall, these data indicate that miRNA-19b-3p can be used as a biomarker for monitoring both disease progression and JAKi treatment response represented by strong DAS28 reduction in RA patients.

Discussion

In this paper, we have identified unique miRNA candidates expressed in monocytes that were simultaneously present in both ARDs (RA and SSc) using global miRNA-seq. Further qPCR validation of selected miRNA candidates in body fluids of eRA and aRA and RA patients following 3 months JAKi therapy has allowed us to decipher circulating miRNA as universal diagnostic biomarkers of disease progression and JAKi treatment response.

Several studies have demonstrated a key role of miRNA isolated from cells, tissue, or body fluids as disease-specific biomarkers of

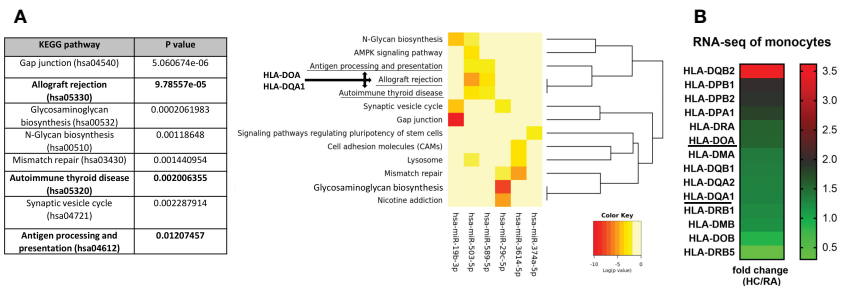

FIGURE 4
KEGG pathway analysis of selected 6 miRNA candidates and their potential target genes. The identification of specific pathways **(A)** and their downstream genes **(B)** including HLA-DOA and HLA-DQA1, which were predicted to be negatively regulated by significantly changed miRNA in RA patients, using the Diana-miPath tool. The fold change analysis of expression level of HLA class II genes between HC and RA monocytes from RNA-seq **(B)** based of FDR < 0.01 and p-values < 0.05.

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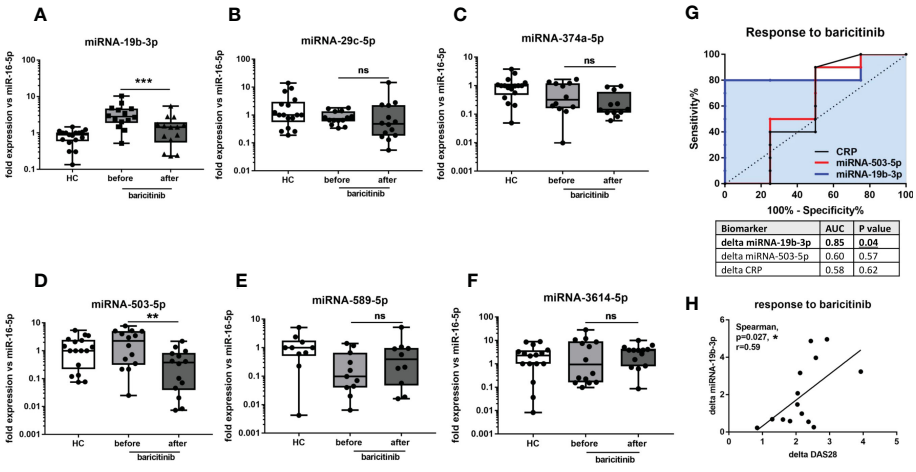

FIGURE 5
The levels of circulating miRNA in HC and RA patients upon baricitinib therapy. The levels of miRNA-19b-3p **(A)**, miRNA-29c-5p **(B)**, miRNA-374a-5p **(C)**, miRNA-503-5p **(D)**, miRNA-589-5p **(E)**, miRNA-3614-5p **(F)** before and 3 months after baricitinib treatment in RA patients compared to HC. ROC analysis of changes/delta in miRNA-19b-3p and miRNA-503-5p expression and CRP level upon JAKi therapy **(G)**. Correlation analysis between the expression of delta miRNA-19b-3p and delta DAS28 in baricitinib-treated RA patients **(H)**. P-values were expressed as follows: ns for not significant; 0.05 > P > 0.01 as *; 0.01 > P > 0.001 as **; P < 0.001 as ***, P <0.0001 as ****.

ARDs including RA and SSc (13–15). In the previous study, we demonstrated that sera-derived miRNA-5196 was increased in SSc, RA and AS patients compared to HC (16). On the other hand, the expression level of miRNA was changing during disease development or therapeutic intervention. Indeed, we have shown that the level of miRNA-5196 was reduced in 80% of RA patients and all AS patients responding to anti-TNF-α therapy (16). In addition, miRNA-5196 was a better biomarker for monitoring TNFi-response than CRP level. The same miRNA-5196 was also investigated in SSc monocytes and was involved in the reduction of profibrotic properties of SSc monocytes (10). In contrast, miRNA-146b was elevated in cell-free fraction (sera and SF) but was reduced in RA monocytes (9). A few other studies also examined the expression of the same miRNA in PBMCs, body fluids, or different tissues. For instance, 49 miRNA candidates were found to be altered in both PBMCs (out of 91) and plasma-derived extracellular vesicles (out of 150) isolated from patients with Chronic Fatigue Syndrome compared to HC. Interestingly, one of the most differentially expressed miRNA in this study published by Almenar-Perez et al., was miRNA-374a (17). Also, the level of miRNA-374a was significantly increased in SF compared to sera isolated from osteoarthritis (OA) patients (18). Similarly, the level of miRNA-29 was significantly increased in both PBMCs and SF of OA patients and correlated positively with age, body mass index (BMI) and Kellgren-Lawrence X-ray classification (19). These miRNA (-374a-5p and -29c-5p) were also selected as candidates in our study (Figures 1F, 2B, C). The other study found that 143 miRNA were present in 61 different tissue biopsies of different organs (20). On the other hand, the results from prostate cancer study demonstrated revers expression of miRNAs (-125b, -126, -141, -143, -221) in tumour tissue compared to plasma (21). Also, expression patter of miRNA-21 and let-7a was opposite between sera and breast cancer tissue (22). Overall, these data suggest that miRNA expression is unique and can be either up- or downregulated across various tissues, cells, or body fluids, which is in line with our results demonstrating that selected monocytes-specific miRNA can be also present in cell-free fractions with similar (miRNA-503-5p, -589-5p, -374a-5p) or opposite expression pattern between sera and monocytes (miRNA-19b-3p).

Circulating miRNA have emerged as minimally-invasive biomarkers that are associated with disease progression and treatment response, thus we have also measured miRNA in RA body fluids to find unique biomarkers that would predict joint inflammation and subsequently joint damage at the early stage. In our paper we have measured selected miRNA. It is known that circulating miRNAs originate as a by-product of dead cells or exosomes (23). Therefore, we decided to measure miRNA in monocytes because these cells are the second population after lymphocytes consisting of even 30 percent of PBMCs. Also macrophages, which differentiate from monocytes, play a role in clearance by phagocytosis which is linked to apoptosis and necrosis (24). We believe this approach allowed us to select versatile miRNA candidate present in both monocyte-derived fractions and in sera-derive fractions in order to increase the power and importance of potential biomarkers. Indeed, based on miRNA-seq of RA and SSc monocytes we have selected 6 candidates, which were present in both diseases. These miRNA candidates (-19b-3p, -29c-5p, -374a-5p, -503-5p, -589-5p, -3614-5p) were subsequently validated in sera of patients with eRA and compared to sera and SF of patients with aRA. This experiment allowed us to test whether selected miRNA candidates present in sera from aRA may reflect the situation observed in the inflamed joint cavity or even predict these changes by measuring miRNA in sera from eRA. In our study, we have demonstrated that expression of miRNA-19b-3p and miRNA-3614-5p was gradually enhanced in eRA sera through high abundance in aRA sera, and the highest expression level was seen

in synovial fluid of aRA. In other studies, it has been indicated that an increased plasma level of miRNA-19b was a good predictor of the risk of OA development and was positively correlated with disease severity (25). On the other hand, TLR2-mediated activation of synovial fibroblasts from RA patients downregulated the level of miRNA-19a/b (26). Whereas, overexpression of miRNA-19a/b reduced MMP-3 and IL-6 secretion. Overall, our and other data suggest that miRNA-19 could be used as a promising biomarker for diagnosis and disease severity of knee inflammation in RA and OA. We have also observed a gradual reduction of miRNA-29c-5p and miRNA-374a-5p in eRA sera through low abundance in aRA sera and the lowest expression level was seen in SF of aRA. A similar pattern of down-regulated expression of the miRNA-29 family was seen in the thymus of the autoimmune disease Myasthenia gravis compared to HC (27). Conversely, miRNA-29c was significantly increased in late-stage OA SF compared to early-stage OA SF (28). This discrepancy in miRNA-29c expression in SF to our finding could be explained by more inflammatory-driven joint damage in RA compared to more mechanical wear and tear on joints observed in OA. We have also revealed that miRNA-503-5p was upregulated in RA and SSc, but did not reach statistical significance in SSc. This observation is in line with the previously published data demonstrating enhanced secretion of miRNA-503 in either extracellular vesicles (EVs), secreted by inflammatory macrophages, or the EVs from peripheral blood mononuclear cells of atherosclerosis patients (29). In addition, miRNA-503 expression was upregulated in SSc skin tissue and promotes angiotensin-II induced cardiac fibrosis (30, 31). We have also measured the level of miRNA candidates before and after baricitinib treatment, in order to identify potentially unique miRNA that are changing during JAKi therapy. Such biomarkers could find broad implications for monitoring response to drug treatment. Interestingly, we noticed that only miRNA-19b-3p and miRNA-503-5p were significantly reduced upon JAKi treatment. However, following ROC analysis only miRNA-19b-3p had a strong potential to be a biomarker predicting reduced disease activity upon 3 months baricitinib treatment. Previously only one study demonstrated that the levels of miRNA-432-5p and miRNA-194-5p were downregulated (out of 61 miRNA candidates) in RA patients achieving clinical remission upon another JAKi - tofacitinib treatment (32). This suggests that only selected miRNA are changing during JAKi therapy. Certainly, the limitation of our study is the number of RA patients before and after baricitinib treatment, which should be justified on larger cohort in order to truly prove that miRNA-19b-3p can be used as biomarker monitoring tsDMARDs-response.

In conclusion, using a global approach, we have selected unique miRNA profiles (miRNA-19b-3p, miRNA-29c-3p, miRNA-374a-5p, miRNA-503-5p, miRNA-589-3q, miRNA-3614-5p) observed not only in monocytes from RA and SSc but also present in sera and SF in RA patients as no-invasive biomarkers of joint inflammation that are predicted to negatively regulate selected HLA class II genes. Further miRNA validation, allowed us, to identify the additional function of miRNA-19b-3p as a new biomarker of disease activity that predicts treatment response to baricitinib in RA patients.

Data availability statement

The datasets presented in this study are deposited in the European Genome-phenome Archive (EGA) repository (EGA-box-1726). However, as the datasets contain sensitive information (DNA and RNA sequences from humans) access to this data is limited/controlled, further inquiries can be directed to the corresponding author/s.

Ethics statement

The studies involving human participants were reviewed and approved by KBT-5/3/2019, KBT-6/5/2020. The patients/participants provided their written informed consent to participate in this study.

Author contributionss

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. MC had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study conception and design, integrity of the paper, project management: MC. Manuscript writing: MC, A-JR, LR. Acquisition of data: MC, TB, AF-G, MM, LR. Analysis and interpretation of data: MC, A-JR.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.980247/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Enrichment analysis of 6 selected miRNA and predicted their target genes which are involved in immune pathways dysfunction both in RA and SSc monocytes.

SUPPLEMENTARY FIGURE 2
The levels of circulating miRNA and CRP in RA patients upon baricitinib therapy. The levels of miRNA-19b-3p **(A)**, miRNA-503-5p **(B)**, CRP **(C)** and DAS28 **(D)** before and 3 months after baricitinib treatment in RA patients.

SUPPLEMENTARY TABLE 3
The list of particular miRNAs (miRNA-19b-3p, miRNA-29c-3p, miRNA-374a-5p, miRNA-503-5p, miRNA-589-3q, miRNA-3614-5p) and the genes that have been dysregulated in RA and SSc.

SUPPLEMENTARY FIGURE 4
MiRNA-gene network analysis illustrating the effects of miRNA deregulation in RA and SSc.

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