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RESEARCH

RNA-seq Based Transcriptome Analysis Reveals Role of Myoglobin in Rheumatoid Arthritis

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Abstract
Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease which manifests as joint destruction and bone erosion, could be caused by both genetic and environmental factors. Currently, the causes of RA are unknown, and targeted therapies are often associated with side effects and contraindications. The detection rate of RA in women is higher than men (3:1), however, there is still a lack of comprehensive understanding of the relationship between sex and RA. We hypothesized gender differences in RA prevalence and their associated mechanisms by performing genome-wide transcriptome analysis of synovial biopsy samples. The results indicated that myoglobin (MB) was differentially expressed between males and females, with higher expression in males than females in healthy populations, while the opposite was observed in RA patients. MB interacted with HLA class II histocompatibility antigen, DM beta (HLA-DMB) and the inflammatory factor interleukin 6 (IL-6) in the human synovial cell line MH7A.

KEY WORDS myoglobin · rheumatoid arthritis · gene expression · synovial tissue · inflammatory cytokines · hypoxia

INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune disease mainly manifests as chronic joint inflammation [1]. RA patients present immune dysfunction characterized by infiltration of inflammatory cells, synovial hyperplasia, and the formation of pannus, leading to bone and cartilage impairment [2]. The global prevalence of RA ranges from 0.5% to 1%, with a male to female prevalence ratio of about 1:3. The pathogenesis of RA is still unclear and the corresponding therapeutic targets are thus also diverse, treatments vary [3], and are often accompanied by different degrees of side effects and contraindications [4]. Most biological therapies target the elevated inflammatory cytokines in RA patients, such as Interleukin-1β(IL-1β), IL-6 or tumor necrosis factor alpha (TNF-α), in only about half of these new RA patients, while significantly increasing the prevalence of infection, tuberculosis, and gastrointestinal side effects [2, 3].

Although the pathogenesis of RA has not been fully resolved, it is generally accepted that both genetic and environmental factors play key roles [5]. Previous reports showed that patients with a family history of RA had a 3- to fivefold increased risk of developing the disease [6]. Genome-wide association analysis using single nucleotide polymorphisms has identified more than 100 gene loci related to the risk of RA [7]. These gene loci are involved in immune mechanisms, some of which are shared with other chronic inflammatory diseases [8]. The pathogenesis of RA may thus be promoted by integrating environmental and genetic effects.

Synovial tissue is mainly composed of fibroblast-like synovial cells (FLS) and a small amount of macrophage-like synovial cells (MLS) [9]. Synovial hyperplasia is the most significant pathological change in RA and may be caused by a

HighlightsThe hypoxic microenvironment and sex affect the susceptibility and prevalence of Rheumatoid arthritis.

Sex-related genes IL-6 and HLA-DMB were related to immunity and inflammation.

Myoglobin interacts with HLA-DMB and IL-6 to regulate Rheumatoid arthritis.

Myoglobin may be a potential drug target for the treatment of Rheumatoid arthritis.

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massive infiltration of synovial tissue cells [10] and the resistance of RA-FLS to apoptosis and autophagy [11]. The infiltration of RA-FLS and MLS are also the main cause of synovial hyperplasia [12], due to the recruitment of inflammatory factors by abnormally activated RA-FLS [13]. Most researchers believe that activation of RA synovial tissue is mainly caused by inflammation and a hypoxic joint environment [14], which plays a key role in activation of the synovial tissue. If the inflammatory stimulus is cancelled, RA-FLS may return to a quiescent state, but epigenetic imprinting due to long-term inflammatory memory remains [15–17]. The activation of RA-FLS by hypoxia has been confirmed [18], and the hypoxic joint environment has thus been identified as an important factor, in addition to the inflammatory joint environment, affecting the activation of RA-FLS [19].

There are currently two recognized theories regarding the origin of RA joint hypoxia. First, the proliferation of synovial tissue and infiltration of inflammatory cells may increase the metabolism of cells in the joint cavity, leading to increased oxygen consumption. Second, there is extensive pannus formation in synovial tissue hyperplasia, and these pannus cannot supply blood, resulting in hypoxia [20]. Collagen-induced arthritis model studies demonstrated hypoxia in synovial tissue in the early stage of the disease. However, these two theories may explain the maintenance of hypoxia but cannot fully explain the source of hypoxia in RA joints [21, 22], and the cause of hypoxia in RA synovial tissue is thus still unknown.

In this study, we performed a bioinformatics analysis of high-throughput sequencing data of synovial tissue from RA and healthy donors. The RA-related differentially expressed genes (DEGs) were identified and mainly enriched in immune and RA-related pathways. Because of the sex bias in the occurrence of RA, we also performed a sex-related differential analysis in both healthy subjects and RA patients, and found that 11 sex-related DEGs were only present in the RA samples. Protein–protein interaction (PPI) network analysis of RA-associated DEGs enriched in the RA pathways and sex-related genes present in the RA-only samples showed that myoglobin (MB) interacted with HLA class II histocompatibility antigen, DM beta (HLA-DMB) and interleukin (IL)-6. These findings were validated by co-immunoprecipitation (IP) experiments. We therefore speculate that MB may mediate the interaction between HLA family members and inflammatory cytokines, thus acting as an intermediate protein regulating RA occurrence. This finding may also be useful in the development of new therapeutic approaches.

METHODS

Sequencing Data Acquisition and Preprocessing

The synovial biopsies RNA-seq data were obtained from Gene Expression Omnibus (GEO) database (GSE89408). In total, 65 rheumatoid arthritis (RA) samples and 13 samples from healthy donors were selected. The samples from healthy donors were derived from 4 females and 9 males, whose average age was 36.1 ± 16.9 y. The RA samples were derived from 48 females and 17 males, whose average age was 54.0 ± 13.3 y.

The quality of raw data was evaluated by FastQC. After being trimmed for adaptors and sequence quality, the reads were aligned to human reference genome GRCh38.96 by utilizing STAR v2.7 aligner [23]. The read counts were calculated using feature Counts [24] and uploaded into R (software version 4.0.5), we screened genes with coefficient of variation (CV) > 5% for subsequent analysis. FPKM of each gene was calculated according to gene length. The number of reads were also mapped to its corresponding gene.

Gene Co-Expression Network Analysis

The co-expression network was established and the key modules were filtered out via a step-by-step network construction function of "WGCNA" package in R [25]. Correlation coefficients between the module Eigengenes and traits were calculated utilizing Pearson's method. Positive or negative correlation modules with correlation coefficients greater than 0.5 [26] were selected as candidates for further research.

Identification and Functional Enrichment Analysis of Differentially Expressed Genes (DEGs)

The DEGs analysis was performed using the R package limma with a threshold of |log2FoldChange|> 1 and *P*-value < 0.05. Then the up-regulated genes or down-regulated genes were defined as robust DEGs. The clusterProfiler package [27] was applied for the Gene Ontology (GO) analysis, which involving cellular components (CCs), biological processes (BPs), molecular functions (MFs), Gene Set Enrichment Analysis (GSEA) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. P values less than 0.05 was considered as statistical significance.

Construction of Protein–Protein Interaction (PPI) Network

The genes with differential expression were uploaded into STRING (https://string-db.org) [28] for the establishment of PPI network with a confidence score more than 0.7. The network was visualized via Cytoscape [29].

Synovial Tissue Sample Collection

Synovial biopsy samples were collected from 8 healthy donors (4 females and 4 males, mean age ± SD 45.8 ± 14.2 years) and 8 RA patients (4 females and 4 males, mean age ± SD 51.2 ± 11.5 years) who participated in sports medicine day surgery for knee pain at Third Affiliated Hospital of Xi'an Jiaotong University. Healthy subjects were defined as those who had no evidence of any form of arthritis on history or examination and had no cartilage damage or synovitis on knee arthroscopy, and no history of chronic diseases, no ongoing medication that could affect the biopsy results. The patients with RA met the American College of Rheumatology criteria for the diagnosis of RA [30]. The study was approved by the Medical Ethics Committee of Xi'an No.5 Hospital (Number: 2023–75). All methods were performed in accordance with the relevant guidelines and regulations and written informed consent was obtained from all participants in this study. The patient and healthy control characteristics are shown in Supplementary Table S1.

Cell Culture and Transient Transfection

Human synovial cell line MH7A was purchased from Riken Cell Bank (Ibaraki, Japan), and it was cultured in RPMI 1640 medium with 10% fetal bovine serum (FBS), under the condition of humidified, 37 °C incubator with 5% CO2 [31, 32]. Lipofectamine 3000 reagent (Invitrogen) was applied for transfection of plasmids into cells, following the instructions provided by manufacturer [33]. The cells were seeded onto dishes 12 h prior to transfection and cultured until reaching approximately 60% confluency. Plasmids were diluted in Opti-MEM culture medium and mixed with P3000 before transfection, Lipofectamine 3000 transfection reagent was diluted in an equal volume of Opti-MEM culture medium and gently mixed with the transfection mixture. The mixture was then incubated at room temperature for 15 min before being added to each corresponding culture plate and gently mixed. Subsequently, the plate was returned to the incubator for further culturing. After 48–72 h of transfection, analyze transfected cells or gather specimens based on the experimental requirements. All the reagents used during cell culture were purchased from Gibco (Life Technologies), unless specially indicated. The inflammation model was established by treating cells with TNF-α (10 ng/ml) for 6 h.

Total RNA Extraction and Quantitative Real-time PCR (qPCR)

Total mRNA was extracted by TRIzol reagent (Invitrogen), and reverse transcripted to cDNA by cDNA synthesis kit (TaKaRa, China) soonly. Real-time quantitative PCR was performed with SYBR green master mix kit (TaKaRa) in a LightCycler instrument (Bio-Rad CFX, U.S.A.). We used GAPDH as reference gene. And real-time quantitative PCR results were analyzed by $2^{-\Delta\Delta Ct}$ method on Bio-Rad CFX Manager Software Version 3.0 (Bio-Rad CFX). The primers used for the rt-qPCR, as well as their sequences were listed in Supplementary Table S2.

Western Blot

Cells were lysed with RIPA Lysis and Extraction Buffer (ThermoFisher, USA) containing Phenylmethanesulfonyl fluoride (PMSF) (ThermoFisher, USA) protease inhibitor. Total protein were collected, and its concentration was detected using Protein Reagent Assay BCA Kit (ThermoFisher, USA). Antibodies used in this research were as follows: anti-IL-6 (1:1000, 21865–1-AP, Proteintech), anti-MB (1:1000, 16048–1-AP, Proteintech), anti-HLA-DMB (1:1000, 21704–1-AP, Proteintech), Major histocompatibility complex, class II, DO alpha (HLA-DOA) (1:1000, Ag10752, Proteintech) and anti-GAPDH (1:2000, AF0006, Beyotime).

Immunofluorescence

The cells were washed 3 times with 1 × PBS and fixed with 4% PFA for 30 min. Second, the cells were washed 3 times with 1 × PBS for 5 min, with 0.25% TritonX-100 for 10 min, 3 times in 1 × PBS for 5 min each time, and then blocked with 1% BSA for 1 h. Third, the first antibody (1:100) was diluted with 1% BSA and was incubated with the samples overnight at 4 °C. PBS was used to wash 3 times for 10 min each. The secondary antibody (1:500) was incubated for 1 h at room temperature in the dark; after washing with 1 × PBS for 3 times, the nuclei were stained with DAPI for 2 min. Finally, the cells were imaged using confocal microscopy (Nikon, Tokyo, Japan) at an emission wavelength of 460 nm. Antibodies used in this research were as follows: anti-IL-6 (1:1000, 21,865–1-AP, Proteintech), anti-MB (1:1000, 16,048–1-AP, Proteintech), anti-HLA-DMB (1:1000, 21,704–1-AP, Proteintech), and HLA-DOA (1:1000, Ag10752, Proteintech).

Immunoprecipitation (IP) and co-IP Assays

Cells were cross-linked by dithiobis (succinimidylpropionate) (Thermo) after washing with phosphate-buffered saline

(PBS), following the protocol suggested by the manufacturer. After lysis of 1×10^7 cells by 1 × IP Lysis Buffer containing complete protease inhibitor mixture, whole cell protein were collected via centrifugation. The extracted protein was incubated with FLAG mAb (Sigma) or immunoglobulin G for control group, then target protein was captured by incubating with Pierce Protein A/G Agarose. After washing twice with the previous mentioned IP Lysis Buffer, proteins complex were eluted in 5 × SDS loading buffer and analyzed by Western blot.

Plasmids Construction

The full-length coding sequence of human *IL-6*, *MB*, *HLA-DMB*, *HLA-DOA*, *Major histocompatibility complex, class II, DR beta 5* (*HLA-DRB5*), *Major histocompatibility complex, class II, DR alpha* (*HLA-DRA*), *TLR2*, *MMP3* and *TNFSF13B* were amplified from synovial tissue cDNA by PCR with specific primers. Then, *IL-6* gene was subcloned into p3xFlag-CMV-10 and pCMV-HA vectors. *MB* gene was subcloned into pCMV-HA vector. *HLA-DMB* gene was subcloned into p3xFlag-CMV-10 vector. *HLA-DOA*, *HLA-DRB5* and *HLA-DRA* were subcloned into pEGFP-C1 vector. *TLR2*, *MMP3* and *TNFSF13B* were subcloned into p3xFlag-CMV-10 vector. The successful construction of these recombinant vectors were validated by sequencing.

Statistical Analysis

Apart from the statistical analysis of RNA-seq data described above, the other statistics were all analyzed by GraphPad Prism version 7 (GraphPad, CA). Unpaired one-tailed or two-tailed Student' s t-test and one-way analysis of variance were used for testing the difference between groups. The post hoc test used Tukey's to confirm the statistical significance. Error bars, P values and statistical test methods are provided in the figure legends.

RESULTS

Identification of RA-Related Gene Modules

To find out the relationship between RA and gene expression disorders, we selected and analyzed RA-related RNA-seq data from GEO database. The datasets contained 78 samples, including 65 from RA tissues and 13 from normal tissues.

The Umap plot shows the cluster of samples in high-dimensional space and the relationship between sample points (Fig. 1A). In accordance with the hierarchical clustering results of the samples (RA_female_54.3 and Normal_female_32), two outliers were removed (Fig. 1B). We then obtained RA-related genes using R package. All gene expression profiles were identified in 20 modules. Correlation analysis between gene modules and traits showed that 4 gene modules were associated with RA and 4 gene modules were associated with non-RA ($R \geq 0.5$; Fig. 1C, D). A total of 6584 RA-related genes and 5724 normal-related genes were identified.

Differential Expression Analysis of RA-Related Genes

The differential expression of RA-related genes were further analyzed. In general, 9.23% (1136/12308) of genes were differentially expressed, comprising 666 up-regulated genes and 470 down-regulated genes (Fig. 2A). These DEGs could distinguish between healthy and RA samples, as shown in the heatmap plot (Fig. 2B). DEGs were distributed throughout the chromosomes: up to 82 up-regulated genes were located on chromosome 1 and at least one on chromosome Y. Conversely, up to 43 down-regulated genes were located on chromosome 11 and at least four on chromosome 21 (Fig. 2C). We investigated the functions of DEGs by GSEA, and pathway enrichment using the Hallmark database and a significant enrichment of these genes were found in adipogenesis, allograft rejection, fatty acid metabolism, k-Ras signaling, and myogenesis. According to reactome database, the DEGs were significantly enriched in pathways of binding and uptake of ligands by scavenger receptors, production of C4 and C2 activators, initial triggering of complement cascade, parasite infection, and scavenging of heme from plasma (Fig. 2D, E). In addition, up- and down-regulated genes were identified, and then pathway enrichment analysis was performed on each group of genes to identify up- and down-regulated pathways, respectively (Fig. 3A, B).

An online enrichment analysis tool were utilized for the pathway enrichment analysis for better biologically characterizing RA-related DEGs. The result demonstrated that these genes were significantly enriched in GO-BP pathways (Additional file 1: Fig. S1A), involving positive regulation of lymphocyte activation, cell activation, leukocyte activation, B cell receptor signaling pathway, immune response-regulating cell surface receptor signaling pathway, immune response-regulating signaling pathway, immunoglobulin production, immune response-activating cell surface receptor signaling pathway, immune response-activating signal transduction, and antigen receptor-mediated signaling pathway. In GO-CC (Additional file 1: Fig. S1B), the genes were significantly enriched in immunoglobulin complex, external side of plasma membrane, immunoglobulin complex, circulating, nucleosome, blood microparticle DNA packaging complex, MHC class II protein complex, protein-DNA complex, contractile fiber, and CENP-A containing nucleosome, and in GO-MF (Additional file 1: Fig. S1C) they were

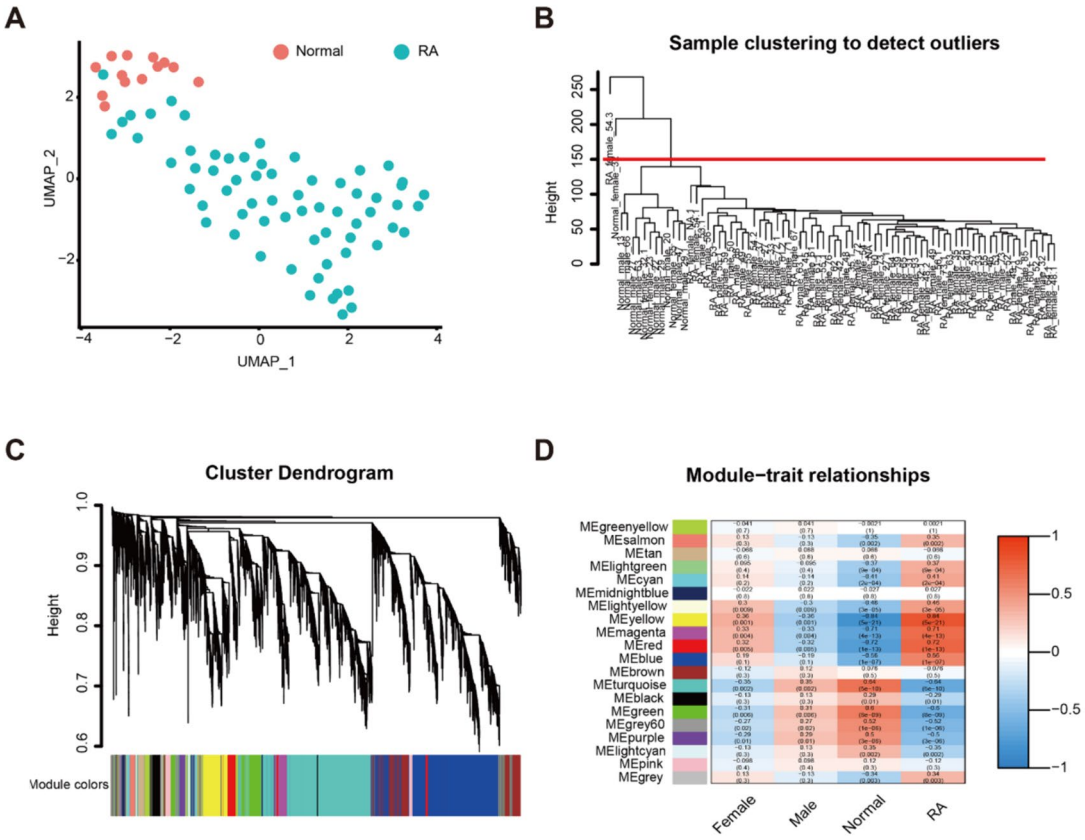

Fig. 1 Identification of RA-related gene modules. **A** Umap plot showing the cluster of samples in high dimensional space and the relationship. **B** Hierarchical clustering showing the clustered samples and deviated samples. Red line to show the cut standard. **C** Gene modules identified by WGCNA. **D** The correlation between gene modules and trait.

significantly enriched in antigen binding, immunoglobulin receptor binding, immune receptor activity, chemokine activity, chemokine receptor binding, MHC protein complex binding, cytokine activity, protein heterodimerization activity, MHC class II protein complex binding, and CXCR chemokine receptor binding. We further examined the enrichment of these genes by KEGG analysis (Additional file 1: Fig. S1D), which showed that they were significantly enriched in systemic lupus erythematosus, viral protein interaction with cytokine and cytokine receptor, hematopoietic cell lineage, neutrophil extracellular trap formation, rheumatoid arthritis, cytokine-cytokine receptor interaction, intestinal immune network for IgA production, graft-versus-host disease, alcoholism, and Th17 cell differentiation.

Furthermore, PPI network of up-regulated and down-regulated RA-related DEGs were established respectively. The result illustrated the PPI relationships of 303 up-regulated DEGs and 208 down-regulated DEGs (Additional file 1: Fig. S2). The PPI network of up-regulated genes is primarily categorized into two components: those related to immune-inflammation and those related to the cell cycle, And the most interacting proteins were CD4 and CDK1. The interaction network of down-regulated genes exhibited increased fragmentation, with one component primarily associated with mitochondrial metabolism.

Identification of Sex-Related Genes

RA is a sex-related disease that is more common in women than men (3:1). To investigate the influence of sex, we analyzed differential gene expression in 13 normal and 65 RA samples and integrated the common sex-related DEGs from the two datasets. In total, thirty-four robust sex-related DEGs were obtained which comprises 32 up-regulated genes and two down-regulated genes (Fig. 4A, B). In addition, 16 sex-related DEGs were only found in the RA sample (RA-Sex-DEGs) (Fig. 4A). The RA-Sex-DEGs were able to distinguish between male and female samples, as shown in dot plots (Fig. 4C), including 14 up-regulated and two down-regulated DEGs (Fig. 4D). The RA-Sex-DEGs were distributed on various chromosomes, with one or two up-regulated genes on most chromosomes, while down-regulated genes were located on chromosome 1 and chromosome 6 (Fig. 4E).

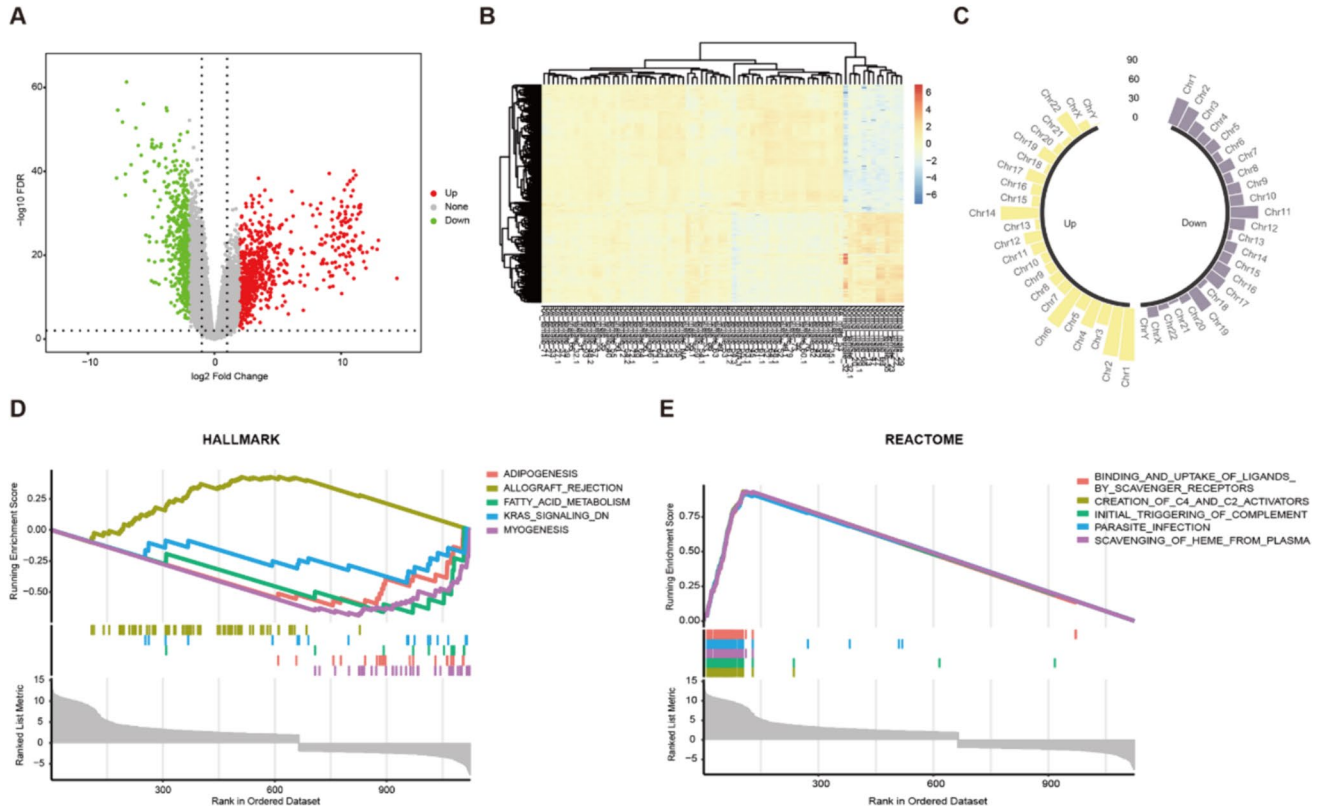

Fig. 2 Differential expression analysis of RA-related genes. **A** The volcano plot showing the differentially expressed RA-related genes. The up-regulated genes presented in red, whereas the down-regulated genes in green. Genes without significant statistic difference are presented in grey. **B** Heatmap showing the differentially expressed RA-related gene expression profile. **C** Circular barplot showing the distribution of differentially expressed RA-related gene in chromosomes. **D** Hallmark geneset enrichment of RA-Related DEGs. **E** Reactome pathway Analysis of RA-Related DEGs.

To clarify the biological characteristics of sex-related DEGs in RA samples, we utilized an online enrichment analysis tool for pathway enrichment analysis. Sex-related DEGs were over-represented in GO-BP pathways involved in muscle-related functions (adaptation, contraction, development) (Fig. 5A), and in GO-CC pathways involved in myofibril, contractile fiber, sarcomere, actin cytoskeleton, striated muscle thin filament, myosin filament, myofilament, myosin complex, stress fiber, and contractile actin filament bundle (Fig. 5B). In GO-MF, these genes were significantly enriched in actin binding, myosin binding, actin monomer binding, microfilament motor activity, structural constituent of muscle, and actin filament binding (Fig. 5C). We further analyzed the enrichment of these genes by KEGG, and found that only eight DEGs were enriched in cardiac muscle contraction, dilated cardiomyopathy, hypertrophic cardiomyopathy, and adrenergic signaling in cardiomyocytes (Fig. 5D), while the enrichment in immune and inflammation pathways was not significant. In addition, we further analyzed the PPIs of RA-Sex-DEGs, and found that 11 DEGs had PPI relationships (Fig. 5E).

We further analyzed the PPI network for these 11 sex-related DEGs in RA and RA-related DEGs which enriched in RA-KEGG. The MB gene had a PPI relationship with IL-6 and HLA-DMB (Fig. 6A). The KEGG pathways including IL-6 and HLA-DMB were related to immunity and inflammation (Fig. 6B). These results suggest that MB may be useful as a novel target for the treatment of RA.

MB Interacts with HLA-DMB and IL-6 to Regulate RA

Our previous bioinformatics analysis showed that HLA-DMB and IL-6 were expressed differently in synovial tissues of normal people and RA patients, while MB was expressed differently between men and women (Fig. 6A). We examined the mRNA and protein expression levels of HLA-DMB, IL-6, and MB in synovial tissues from RA patients and healthy people (normal) by qPCR and western blotting, respectively. The transcriptional levels of IL-6 and

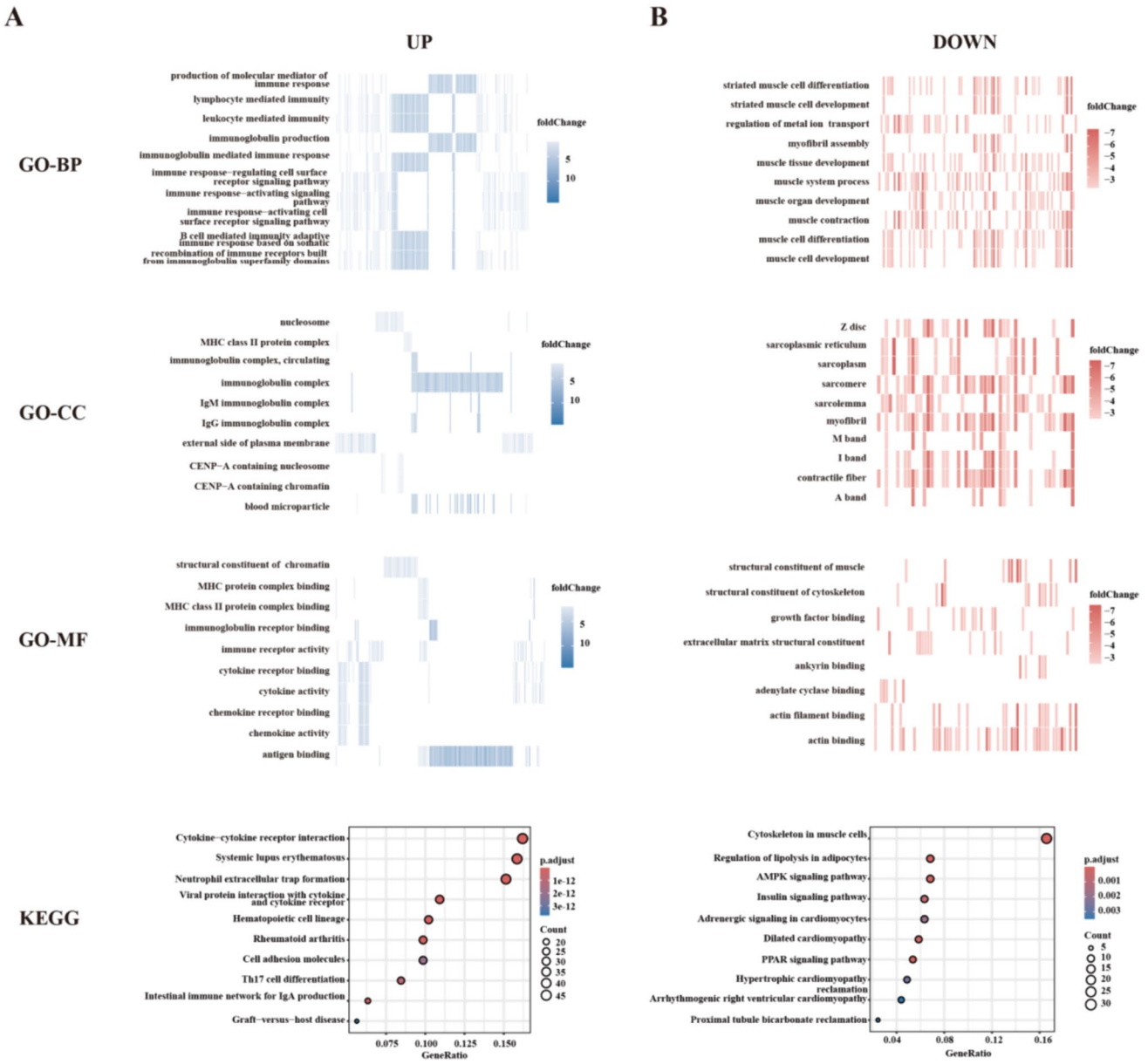

Fig. 3 Enrichment analysis of RA-related DEGs. **A** Gene Ontology and KEGG pathway enrichment analysis of RA-Related upregulated genes. **B** Gene Ontology and KEGG pathway enrichment analysis of RA-Related downregulated genes.

HLA-DMB were significantly overexpressed in synovial tissue of RA patients in contrast with normal people, with no significant sex difference. MB expression was higher in female RA patients than in normal women, while MB expression levels were lower in male RA patients in comparison to normal men (Fig. 7A). In addition, IL-6, MB, and HLA-DMB were all expressed at significantly higher levels in female with RA; MB was expressed at significantly higher levels in normal men. Protein expression levels of MB, HLA-DMB, and IL-6 were basically consistent with the mRNA results (Fig. 7B).

PPI network analysis indicated that MB interacts with HLA-DMB and IL-6, respectively (Fig. 6A). We then co-transfected MH7A human arthritis synovial fibroblast cells with an optimized eukaryotic expression vector expressing Flag-tagged HLA-DMB and HA-tagged MB, and Flag-tagged HLA-DMB and HA-tagged MB, respectively. Co-IP and western blot experiments demonstrated that HLA-DMB and IL-6 interacted with MB protein (Fig. 7C, D). We further explored the role of MB in RA by selecting six proteins interacting with HLA-DMB and IL-6, respectively, in the RA pathway for verification. After constructing expression vectors and co-transfection, co-IP and western blots

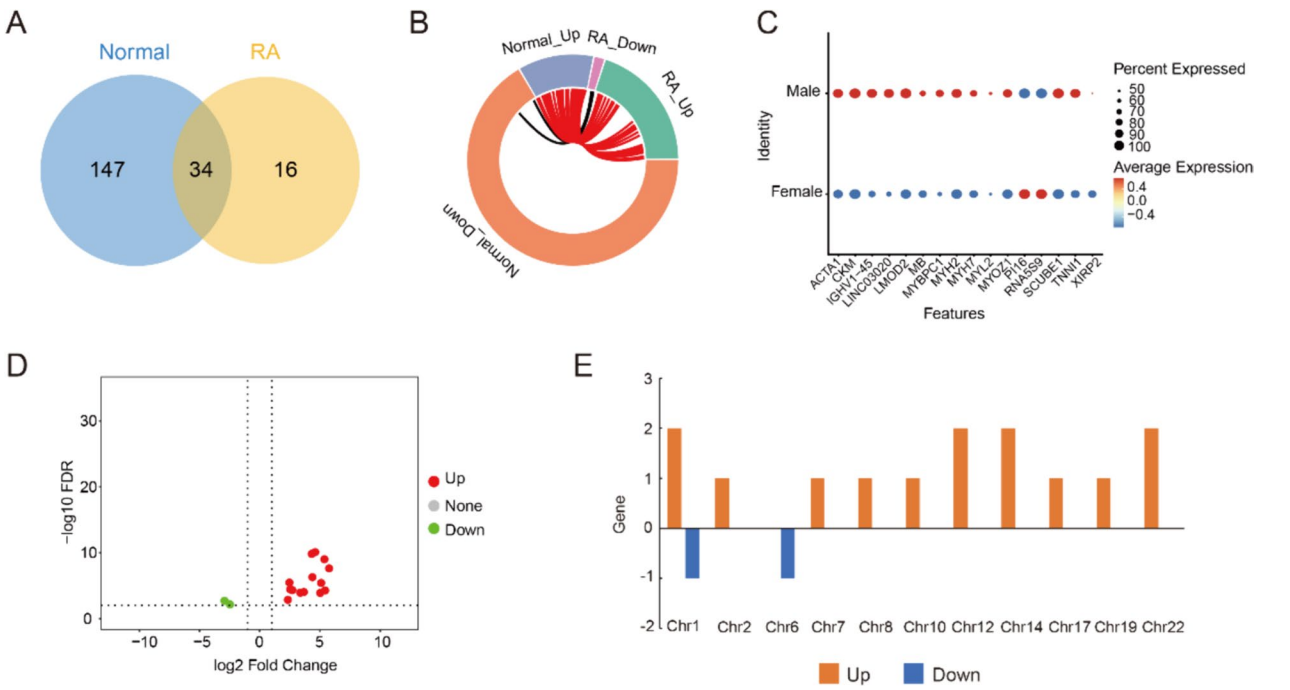

Fig. 4 Differential expression analysis of sex-related genes. **A** and **B** sex-related DEGs in normal and RA samples. **C** Dot plots showing significant differences in sex-related genes. **D** The volcano plot showing the differentially expressed Sex-related genes. The up-regulated genes presented in red, whereas the down-regulated genes in green. Genes without significant statistic difference are presented in grey. **E** Histogram showing the distribution of differentially expressed Sex-related gene in chromosomes.

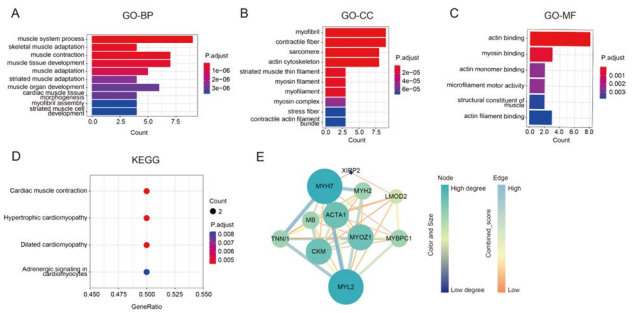

Fig. 5 Enrichment analysis of sex-related DEGs. **A**, **B** and **C** Gene Ontology enrichment analysis of Sex-related DEGs. **D** KEGG pathway enrichment analysis of Sex-related DEGs. **E** PPI network of Sex-related DEGs.

showed that HLA-DMB co-precipitated with the HLA family members HLA class II histocompatibility antigens DO alpha (HLA-DOA), DR-5 beta (HLA-DRB5), and DR alpha (HLA-DRA) (Fig. 8A-C), while IL-6 co-precipitated with Toll-like receptor 2 (TLR2), matrix metallopeptidase 3 (MMP3), and TNF superfamily member 13b (TNFSF13B) (Fig. 8D-F).

To further validate our results, a hypoxia model and an inflammation model were established *in vitro* using MH7A cells as a way to simulate whether hypoxia-induced inflammatory conditions *in vivo* would lead to an increase in the expression of HLA-DMB and MB, as well as recruitment of other HLA proteins that regulate the joint environme. The results showed that the hypoxic environment and TNFα treatment significantly increased the mRNA and protein expression levels of IL-6, IL-8, and IL-1β, resulting in a cellular inflammatory response compared with the control group (Fig. 9A-F). When inflammation occurs, the protein levels of HLA-DMB and MB significantly increase in the cells (Fig. 9G-J). Notably, the results of CO-IP and IF experiments showed that under hypoxic conditions, HLA-DMB and MB proteins interacted, HLA-DMB and IL6 proteins interacted, MB and IL6 proteins interacted, and HLA-DMB and HLA-DOA proteins interacted (Fig. 9K-R). Thus, in conjunction with the previous experiments, it can be shown that a hypoxic environment leads to the development of an inflammatory response that significantly elevates the expression of HLA-DMB, MB, HLA-DMB and IL-6 and binds to each other. These results suggest that under inflammatory conditions, MB interacts with HLA family members, inflammatory factors, and MMP3, which may be involved in the development of RA.

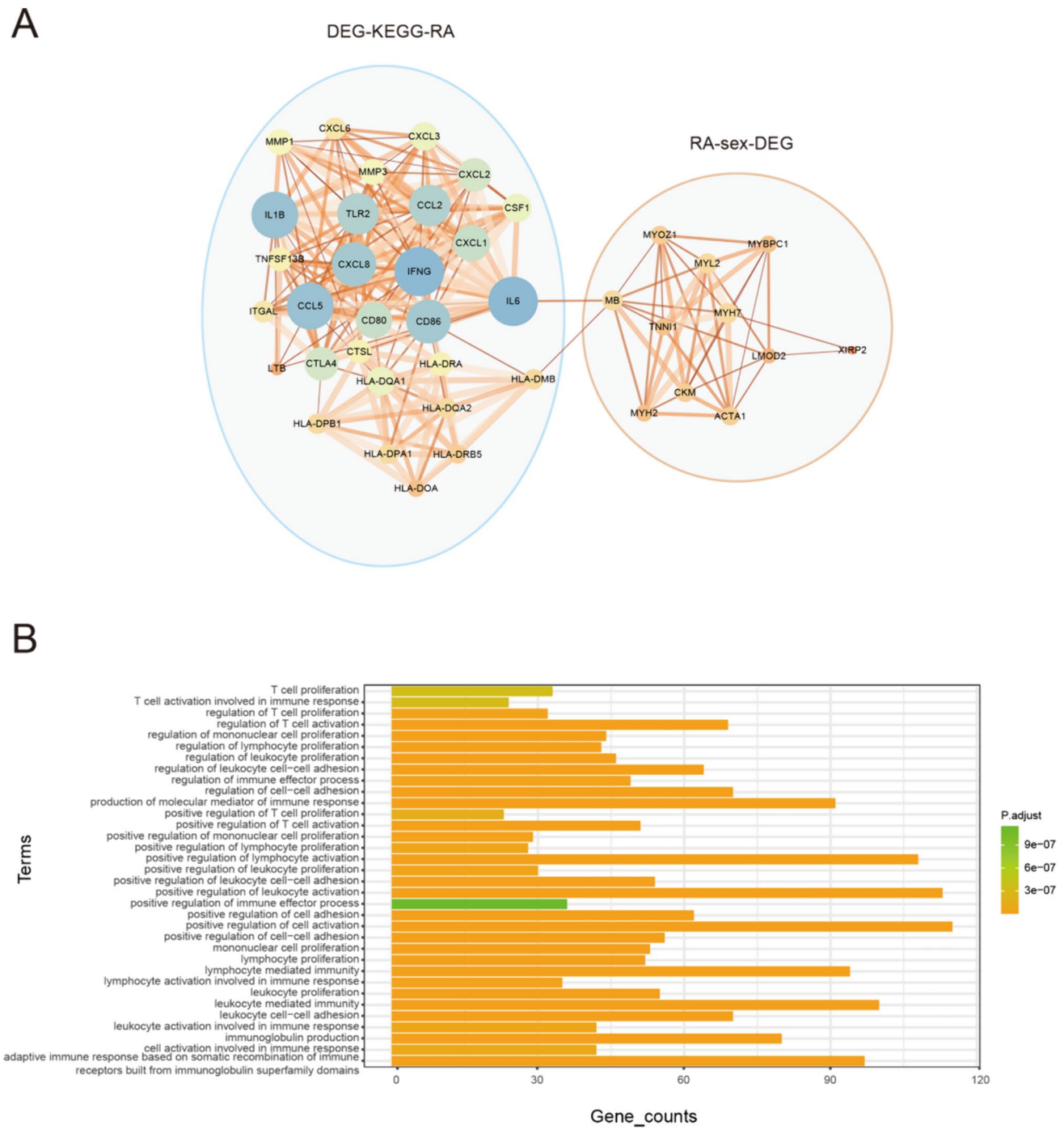

Fig. 6 Enrichment analysis of RA-related hub gene. **A** PPI network of the RA-related DEGs which enriched in RA pathway and Sex-related DEGs. **B** The KEGG pathway including IL6 and HLA-DMB.

DISCUSSION

RA is a chronic inflammatory autoimmune disease influenced by both genetic and environmental factors. The environment has been considered as a major trigger of diseases in genetically predisposed individuals [1]. Hypoxia appears to be a prominent environmental factor affecting RA, and regulates FLS activity through inducing hypoxia-inducible factor 1 α. Interestingly, Yu et al. (2018) found that hypoxia promoted the development of RA by inducing inflammation and angiogenesis, and also by inducing citrullination in human FLS [34]. The clinical immune dysfunctional manifestations of RA patients are as a consequence of close interactions among cells, mediators, autoantibodies, and signal

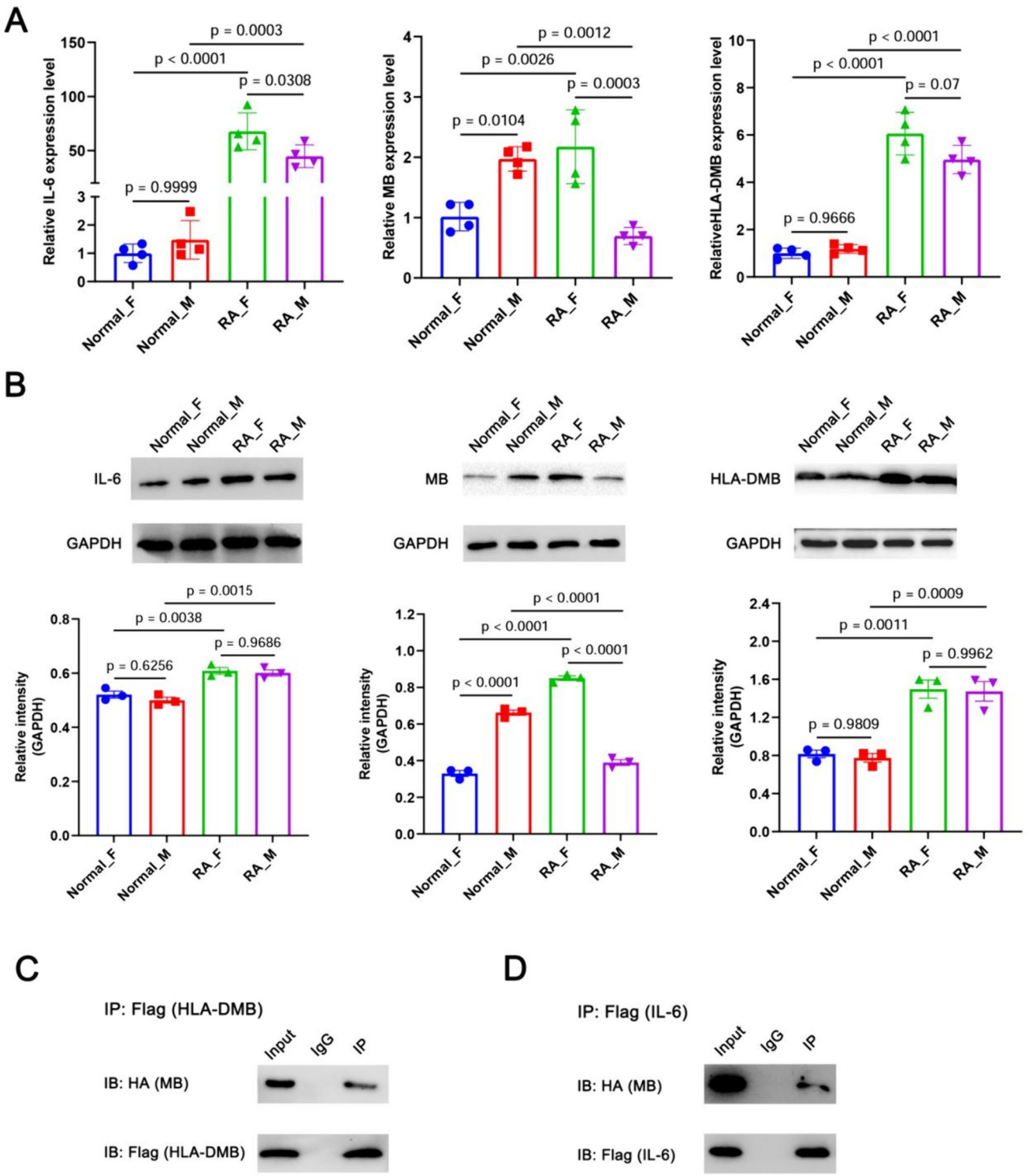

Fig. 7 The Effect of RA on the expression of IL-6, MB and HLA-DMB. **A** The mRNA expression of IL-6, MB and HLA-DMB in RA and normal synovial tissues. ($n=4$, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$, Difference between normal and RA groups; $\#P<0.05$, $\#\#P<0.01$, $\#\#\#P<0.001$, $\#\#\#\#P<0.0001$, Sex difference; NS, no significant difference between Sexs). **B** Representative western blots of IL-6, MB and HLA-DMB in RA and normal synovial tissues. GAPDH was used as a loading control. **C** and **D** Flag-tagged HLA-DMB (**C**) or IL-6 (**D**) and HA-tagged MB were transiently co-expressed in MH7A cells. Flag-tagged HLA-DMB or IL-6 was immunoprecipitated respectively and precipitates were blotted for HA-tagged MB. IgG, immunoglobulin G. Total cell lysate (input) was set as internal reference for co-immunoprecipitation assay and normal mouse IgG served as a negative control.

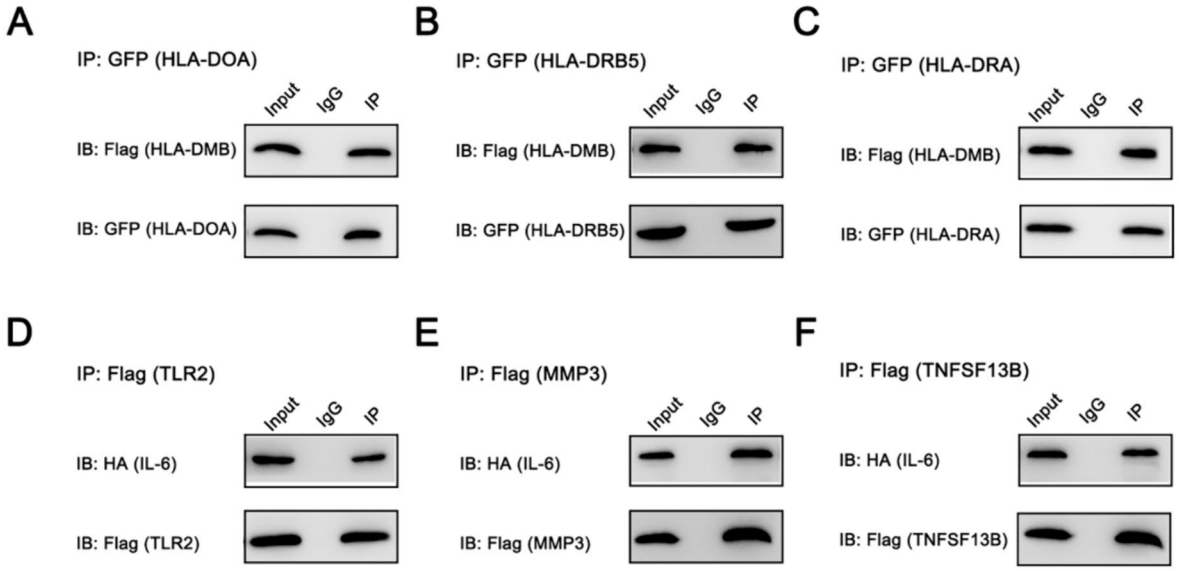

Fig. 8 IL-6 and HLA-DMB interacted with several RA-related genes. **A**, **B** and **C** GFP-tagged HLA-DOA (**A**), HLA-DRB5 (**B**), HLA-DRA (**C**) and Flag-tagged HLA-DMB were transiently co-expressed in MH7A cells. GFP-tagged HLA-DOA, HLA-DRB5, HLA-DRA was immunoprecipitated respectively and precipitates were blotted for Flag-tagged HLA-DMB. **D**, **E** and **F** Flag-tagged TLR2 (**D**), MMP3 (**E**), TNFSF13B (**F**) and HA-tagged IL-6 were transiently co-expressed in MH7A cells. Flag-tagged TLR2, MMP3, TNFSF13B was immunoprecipitated respectively and precipitates were blotted for HA-tagged IL-6. IgG, immunoglobulin G. Total cell lysate (input) was set as internal reference for co-immunoprecipitation assay and normal mouse IgG served as a negative control.

pathways of the innate and adaptive immune systems [35]. Cells of the innate immune system function via specialized receptors, including TLRs, which recognize some microbial epitopes on pathogens and subsequently activate inflammasomes [36]. Proteins encoding TNF superfamily genes also play a key role in disease pathogenesis [37].

This study demonstrated functional links among MB, HLA-DMB, and IL-6 in regulating RA development, and provided evidence for the relevant mechanisms by the analysis and experimental verification of synovial tissue transcriptomic data. HLA-DMB was highly expressed in RA patients and interacted simultaneously with MB and HLA family members. Previous studies showed that HLA family proteins were encoded by genes potentially associated with RA susceptibility [38, 39]. We therefore concluded that after external stimulation or infection, HLA-DMB, which was highly expressed in RA-susceptible individuals, might bind to MB resulting in reduced oxygen partial pressure in the joint and forming a low oxygen environment, or might combine with other HLA family proteins to form a complex regulating MHC class II molecules. Increasing evidence suggests that low oxygen is a key factor in initiating RA [18, 40]. Meanwhile, a low-oxygen environment promotes the accumulation of MB to regulate the joint environment [41]. The current results showed that MB expression was elevated in female RA patients and decreased in male RA patients compared with their normal counterparts, which may be one reason for the higher prevalence of RA in women than in men. In addition, the high expression of MB is closely associated with clinical findings such as DAS28, CRP, ESR, or autoantibodies, and alterations in hormones are also a possible cause [42–44].

Bone destruction is an important marker of disease development in RA patients [45]. Hypoxia also promotes the expression of MMPs and damages the cartilage [46, 47]. Recent data suggested that RA-FLS is the main type of effector cell causing cartilage damage and the main source of MMPs [17, 48]. The most important pathological changes in RA are synovial tissue hyperplasia, invasion of local cartilage, and the infiltration of inflammatory cells [49]. RA-FLS is a key component of the synovial membrane, and RA is mainly caused by the recruitment of inflammatory factors through abnormally activated RA-FLS [13]. Combined with our validation results in a human arthritis synovial fibroblast line, MH7A, these results suggest that MB protein produced in a low-oxygen environment binds to IL-6 and then, with the help of the HLA complex, binds to TLR2 on FLS to promote MMP3 expression and cause cartilage damage. Meanwhile, we also hypothesized that binding of IL-6 and TNFSF13B may lead to inflammatory cell infiltration.

Some limitations of this study should be taken into consideration. First, a relatively small number of samples were included in our study. Second, our research has uncovered an interaction between MB and HLA-DMB as well as IL-6,

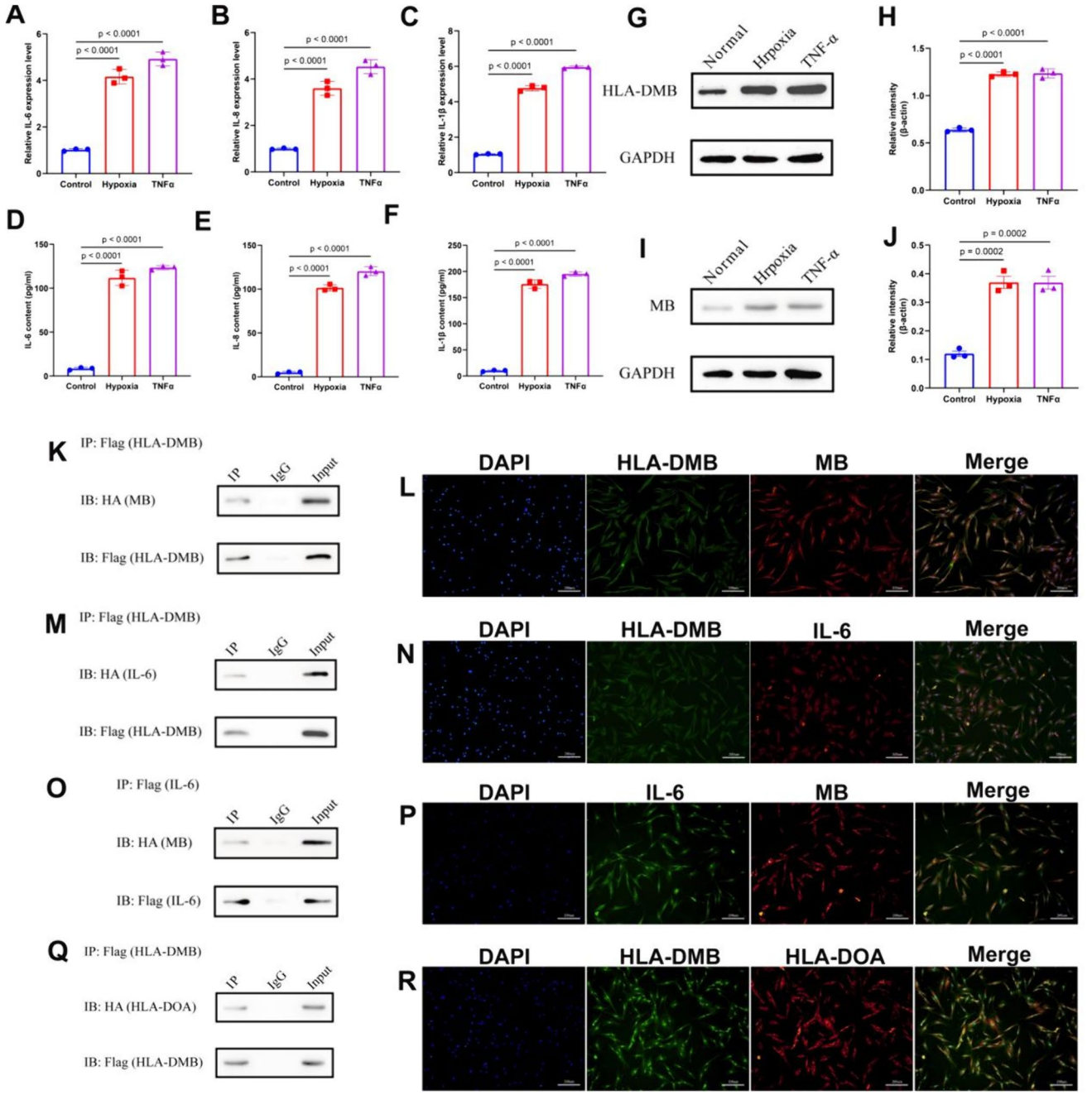

Fig. 9 MB, HLA-DMB and IL-6 have interaction under inflammatory and hypoxic conditions. **A**, **B** and **C** Analysis of mRNA expression levels of IL-6 (**A**), IL-8 (**B**) and IL-1β (**C**). **D**, **E** and **F** Analysis of protein expression levels of IL-6 (**D**), IL-8 (**E**) and IL-1β (**F**). **D**, **E** Analysis of mRNA (**D**) and protein (**E**) expression levels of HLA-DMB. **I**, **J** Analysis of mRNA (**I**) and protein (**J**) expression levels of MB. **K** CO-IP detects the phase between HLA-DMB and MB. **M** CO-IP detects the phase between HLA-DMB and IL-6. **O** CO-IP detects the phase between MB and IL-6. **Q** CO-IP detects the phase between HLA-DMB and HLA-DOA. **L** Under low oxygen condition, the interaction between HLA-DMB and MB was detected by IF. **N** Under low oxygen condition, the interaction between HLA-DMB and IL-6 was detected by IF. **P** Under low oxygen condition, the interaction between MB and IL-6 was detected by IF. **R** Under low oxygen condition, the interaction between HLA-DMB and HLA-DOA was detected by IF.

although the specific regulatory mechanism remains to be elucidated. Further studies that evaluate the role of MB in RA are needed. Despite these limitations,, this study improves our understanding of the mechanism by which a hypoxic microenvironment and sex affect the susceptibility and prevalence of RA, and indicates that MB could be developed as a fresh drug target for the treatment of RA.

Perspectives and Significance

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease affected by both genetic and environmental factors, leading to joint destruction and bone erosion. We found that 11 sex-related DEGs were only present in the RA samples. Protein–protein interaction (PPI) network analysis of RA-associated DEGs enriched in the RA pathways and sex-related genes present in the RA-only samples showed that myoglobin (MB) interacted with HLA class II histocompatibility antigen, DM beta (HLA-DMB) and interleukin (IL)-6. These findings were validated by co-immunoprecipitation (IP) experiments. We therefore speculate that MB may mediate the interaction between HLA family members and inflammatory cytokines, thus acting as an intermediate protein regulating RA occurrence. These results advance our understanding of the sex-related mechanisms affecting the susceptibility and prevalence of RA, and suggest that MB may be a novel potential therapeutic target for RA.

Abbreviations RA: Rheumatoid arthritis; MB: Myoglobin; HLA-DMB: HLA class II histocompatibility antigen, DM beta; IL-6: Inflammatory factor interleukin 6; TNF-α: Tumor necrosis factor alpha; FLS: Fibroblast-like synovial cells; DEGs: differentially expressed genes

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Author Contributions Hb.Wang and Y.W designed the study and wrote the paper. Hb.W, X.T and L.J performed the laboratory experiments. Hb.W and L.S analysed data and mad the figures. All authors reviewed the manuscript and agreed to the published version of the manuscript.

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

Declarations

Ethics Approval and Consent to Participate This study involving human participants were reviewed and approved by the Ethics Committee of the Shaanxi Provincial People's Hospital. The patients/participants provided their written informed consent to participate in this study.

Consent for Publication Not applicable.

Competing Interests The authors declare no competing interests.

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