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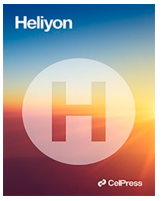

Identification of potential ferroptosis key genes and immune infiltration in rheumatoid arthritis by integrated bioinformatics analysis

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ABSTRACT

Objective: Ferroptosis is of vital importance in the development of Rheumatoid arthritis (RA). The purpose of this project is to clarify the potential ferroptosis-related genes, pathways, and immune infiltration in RA by bioinformatics analysis.
Methods: We acquired ferroptosis-related genes (FRGs) from Ferroptosis database (FerrDb). We obtained the Gene dataset of RA (GSE55235) from the Gene Expression Omnibus (GEO) Database, screened the differentially expressed genes (DEGs) in RA and control samples, and then took the intersection of it and FRGs. Aiming to construct the protein-protein interaction (PPI) networks of the FRGs-DEGs, STRING database and Cytoscape software 3.7.0 would be used. Furthermore, hub genes were identified by CytoNCA, a Cytoscape plug-in. The gene ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment of FRGs-DEGs were performed.
Results: We identified 34 FRGs-DEGs, including 7 upregulated and 27 downregulated genes by taking the intersection of the FRGs and DEGs. PPI analysis identified a total of 3 hub genes (VEGFA, PTGS2, and JUN). GO enrichment analyses and KEGG Pathway enrichment displayed that the FRGs-DEGs are involved in the response to oxidative stress and corticosteroid, heme binding, FoxO-signal pathway. Results of immune infiltration displayed that increased infiltration of T cells, while Macrophages M2 less may be related to the occurrence of RA.
Conclusion: The hub genes involved in ferroptosis in RA may be VEGFA, PTGS2, and JUN, which are mainly involved in FoxO-signal pathway. T cell, Mac, and plasma cells may be involved in the regulation of RA-joints-synovial-inflammation.

1. Introduction

Rheumatoid arthritis (RA) is characterized by the following: it can cause bone inflammation and cartilage destruction in the affected joint and is an autoimmune and inflammatory disease. The basic pathological manifestations of RA are synovitis, pannus formation, and gradual destruction of articular cartilage and bone, finally leading to joint deformity and disability [1,2]. The estimated

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global prevalence rate of RA is 0.46 %, so it is called one of the most common chronic diseases worldwide [3], and the prevalence is expected to increase [4]. The mortality rate of patients with RA remains higher than that of the general population [5,6]. RA affects patients' quality of life and poses a significant social and economic burden on individuals, their families, and the community [7]. The underlying mechanism of RA is not yet fully clarified, therefore, it is important to have an intensive study on the nosogenesis of RA for targeted therapy of RA.

Ferroptosis, proposed by Dixon et al., is a unique iron-dependent form of nonapoptotic cell death that was triggered by erastin or RSL [8]. This type of death is related to reactive oxygen species (ROS) and intracellular iron [9]. The main morphological characteristics of death were abnormal mitochondrial structure, while the biological characteristics were mainly glutathione (GSH) consumption and glutathione peroxidase 4 (GPX 4) inactivation that leads to abnormal accumulation of ROS and iron and induces cell death [10]. A previous study found that erastin (the ferroptosis inducer) leads to increased expression of MMP13 and decreased

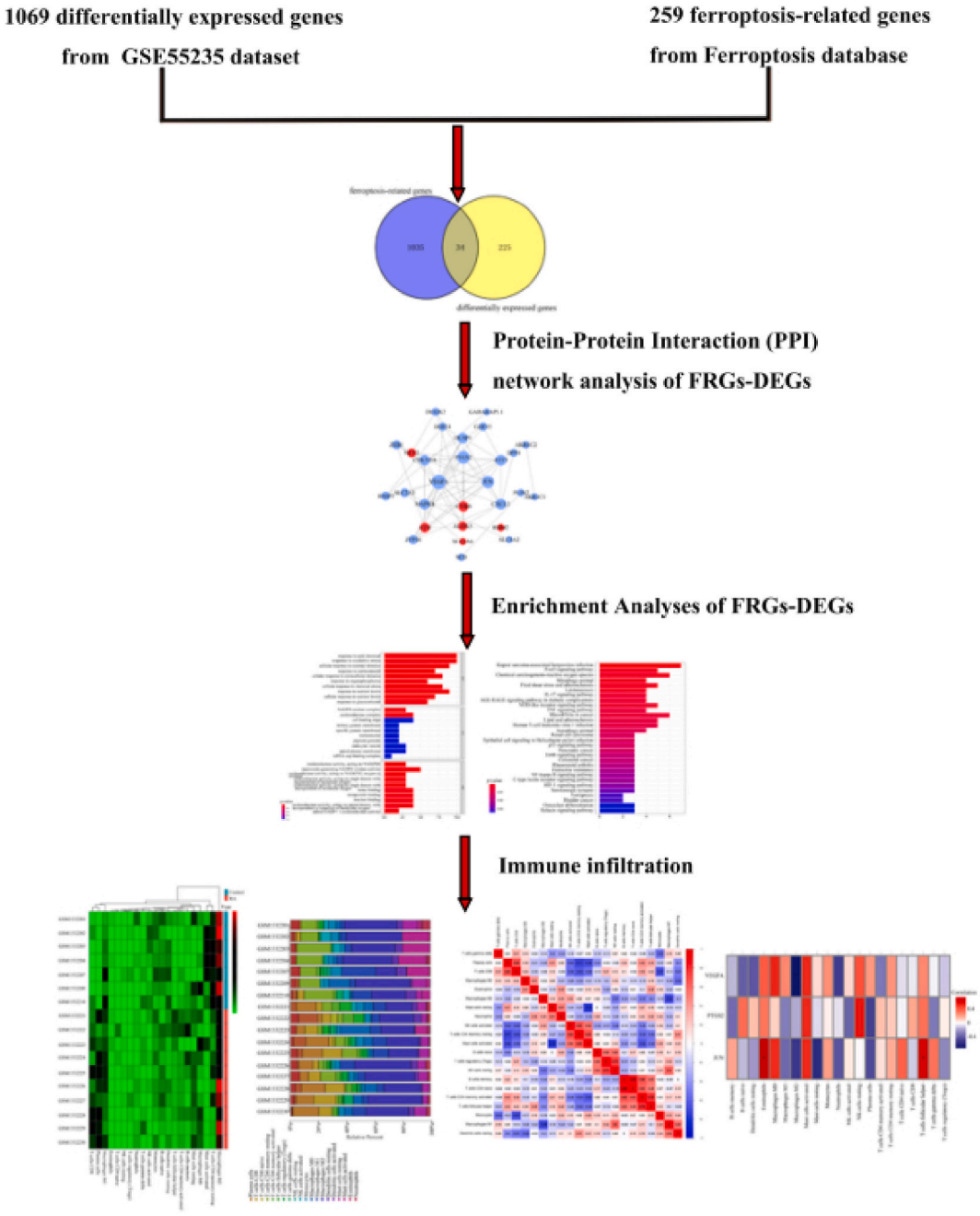

Fig. 1. The flow chart of this study.

expression of collagen II in chondrocytes, which proposed that under inflammation and iron overload conditions the chondrocytes underwent ferroptosis [11]. It has been preliminarily reported that desferrioxamine, which inhibited ferroptosis, could alleviate soft tissue swelling in Wistar rats [12]. An earlier study indicated articular-cartilage injury and chondrocyte-ferroptosis were attenuated by Transient Receptor Potential Melastatin 7 (TRPM7), which provided a hopeful target for the prevention and treatment of RA [13]. Tumor necrosis factor α(TNF-α), interleukin-6(IL-6), and IL-1β are the key proinflammatory cytokines that destroy bone and articular cartilage and play a dominant role in the etiopathology of RA [14]. The GPX pathway of ferroptosis is also associated with RA. The decrease of key inflammatory factors such as TNF-α, IL-1β, and IL-6 has been observed in several anti-RA-drug studies, while the increase in GPX4 expression has been found [15–17]. Hence, investigating deeply the potential role of ferroptosis in RA will help to understand the nosogenesis and would provide a new therapy for RA treatment.

The unclarified nosogenesis of RA prevents its diagnosis and treatment. Furthermore, as far as we know, there has been no bioinformatic-based study targeting the mechanism on the basis of ferroptosis in RA. Aiming to identify the key ferroptosis-related genes in RA and have an intensive study on the potential nosogenesis of ferroptosis in RA, we used data analysis and data mining techniques in the present study. The flow chart of this study is shown in Fig. 1. This study is expected to contribute to the understanding of the ferroptosis mechanism in RA as well as provide new ideas for RA clinical diagnosis and its treatment.

2. Materials and METHODS

2.1. Data sources

The clinical information of RA patients was obtained from Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/). And from that, we obtained the RA Affymetrix microarray dataset GSE55235. This dataset performed genomic expression profiling of 10 RA patients and 10 healthy individuals. In dataset GSE55235, the GPL96 (Affymetrix Human Genome U133A Array) was verified using [18]. As the data were obtained from public databases, there was no need for approval from the local ethics committee.

2.2. Data preprocessing

Probes were converted to gene symbols in accordance with the GPL96 platform-annotation information. A Limma package was used in R software (Version 4.2.1) to identify differentially expressed genes(DEGs) from expression data, using adjusted *P*-value < 0.05 & $|\log2 \text{ (fold change)}| > 1$ as thresholds. Next, a volcano plot and heatmap of DEGs were rendered using the Ggpubr and heatmap packages in R software. We obtained ferroptosis-related genes (FRGs) from Ferroptosis database (FerrDb; http://www.zhounan.org/ferrdb/current/). FerrDb is the world's first manually curated database for regulators and markers of ferroptosis and ferroptosis–disease associations [19]. Afterward, we calculated the intersection of FRGs with DEGs via R software and drew a Venn diagram.

2.3. Protein-protein interaction (PPI) network analysis of FRGs-DEGs

The STRING database is one of several resources for online retrieval of organism-wide protein association networks, and version 11.5 used in this study (https://string-db.org/), used for amalgamating all known and predicted proteins associations (including physical interactions and functional associations) [20]. The PPI network was constructed for further exploration of PPIs by using STRING, with the minimum required interaction score>0.4 [21] and hiding disconnected nodes in the network. And then, Cytoscape software 3.7.0 was used to visualize the PPI network. After that, we calculated and analyzed the topological properties of the following three data for each node in the PPI network using the Cytoscape software plug-in CytoNCA (degree centrality (DC), betweenness centrality (BC), and closeness centrality (CC) [22]. These properties showed the topological significance of each individual node of the entire network. Subsequently, DC $\geq$ 2 $\times$ median DC, BC $\geq$ median BC, and CC $\geq$ median CC were used as the screening criteria to screen putative targets for the hub targets [23].

2.4. Enrichment analyses of FRGs-DEGs

We performed GO(Gene Ontology) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses of FRGs-DEGs by the cluster profiler package of R software to evaluate the biological role and significance of FRGs-DEGs [24,25]. In addition, *P*-value <0.05 was defined as statistically significant.

2.5. Analysis of immune cell infiltration

CIBERSORT deconvolution algorithm is a machine learning method based on linear support vector regression, that can evaluate the infiltration abundance of B cells memory, plasma cells, NK cells, and other 22 sorts of immune cells in tissues [26]. We used R software to analyze the infiltration of 22 sorts of immune cells in RA-tissue-samples and normal-tissue-samples, and the calculation times were set at 100 times for the sake of accuracy [27]. CIBERSORT derives a *p*-value for the deconvolution of each sample, and *P*-value< 0.05 was considered accurate [26]. Therefore, only samples with a CIBERSORT *P*-value< 0.05 were enrolled for further analysis. Then, R software version 3.2 was applied to calculate the related coefficient between different immune-cells in the enrolled sample data and visualize the results, so as to analyze the differences between the RA and the control group as well as the correlation between each immune cell. Additionally, R software was used to conduct and visualize the correlation betwixt hub genes and immune infiltration

[28,29].

3. Results

3.1. FRGs-DEGs

We downloaded the microarray expression profiling dataset GSE55235 from GEO database and gained DEGs by comparing synovial tissues from the healthy and RA groups. We obtained 1069 DEGs by differential expression gene analysis. The volcano plots of the DEGs were shown in Fig. 2. We also identified 259 FRGs that drive, suppress, or mark ferroptosis from the FerrDb. And then, we identified 34 FRGs-DEGs, including 27 downregulated genes and 7 upregulated by taking the intersection of the FRGs and DEGs (Table 1). We displayed the Venn diagram in Fig. 3.

3.2. Protein-protein interaction (PPI) network analysis of FRGs-DEGs

After removing 6 disconnected nodes, the PPI network data (including 28 nodes and 68 edges) were gained from STRING database. The purpose of using the Cytoscape software was to visualize the PPI network of FRGs-DEGs(as shown in Fig. 4). The nodes represented genes and the edges indicated interactions of genes. Red nodes showed genes were upregulated, and blue nodes showed genes were downregulated. The size of the node expresses as the DC value of the node, that was to say, the larger the node was, the greater the DC was, and the more significant the node is in the PPI network. The topological features of every node then were calculated including DC, BC, and CC. The median values of DC, BC, and CC were 4.00, 3.30, and 0.17 respectively. Therefore, we obtained three hub targets namely, VEGFA, PTGS2, and JUN, with DC $>$ 8.00, BC $>$ 8.03 and CC $>$ 0.17.

3.3. Enrichment analyses of FRGs-DEGs

We used GO and KEGG Enrichment Analyses functions of R software to analyze the biological role and significance of 34 FRGs-DEGs. Through the GO enrichment analysis, 489 GO terms with *P*-value$<$ 0.05 in total were obtained. The Biological Process(BP) enrichment analysis confirmed 455 terms. Among them, the top 5 significant enrichment results included response to acid chemical, response to oxidative stress, cellular response to external stimuli, response to corticosteroids, and cellular response to extracellular stimuli. The Cellular Component(CC) enrichment analysis confirmed 2 terms including NADPH oxidase complex and oxidoreductase complex. The Molecular Function (MF) enrichment analysis detected 32 terms. Among the MFs, the most significant were

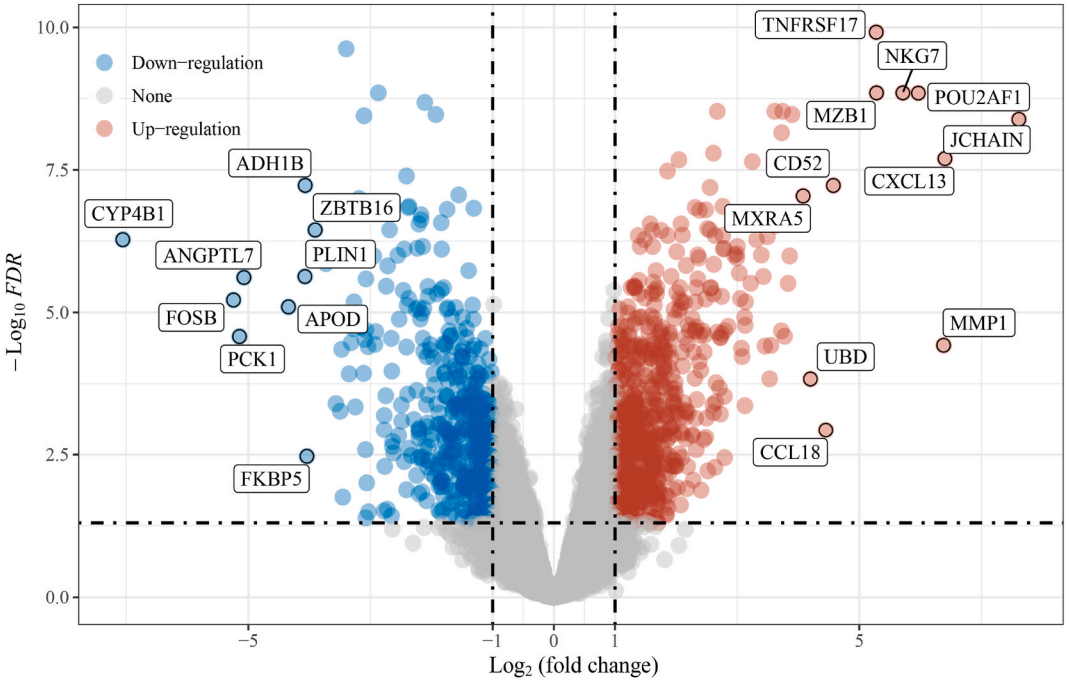

Fig. 2. The volcano plot of DEGs.
Note: (A) Volcano plot: The volcano plot was constructed by the fold-change values and adj.*p*.val. Red dots represented upregulated DEGs (610 genes); blue dots represented downregulated DEGs (459 genes). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
List of FRGs-DEGs.

	Gene symbol	Gene name
Downregulated genes		
1	ANGPTL7	Angiopoietin-related protein 7
2	PTGS2	Prostaglandin endoperoxide synthase 2
3	ATF3	Cyclic AMP-dependent transcription factor ATF-3
4	DUOX2	Dual oxidase 2
5	ZFP36	mRNA decay activator protein ZFP36
6	CDO1	Cysteine dioxygenase type 1
7	GDF15	Growth/differentiation factor 15
8	JUN	Transcription factor Jun
9	CDKN1A	Cyclin-dependent kinase inhibitor 1
10	GABARAPL1	Gamma-aminobutyric acid receptor-associated protein-like 1
11	SCD	Stearoyl-CoA desaturase
12	CXCL2	C-X-C motif chemokine 2
13	MAPK8	Mitogen-activated protein kinase 8
14	DDIT4	DNA damage-inducible transcript 4 protein
15	DPP4	Dipeptidyl peptidase 4
16	DUSP1	Dual specificity protein phosphatase 1
17	ZEB1	Zinc finger E-box-binding homeobox 1
18	SLC2A3	Solute carrier family 2, facilitated glucose transporter member 3
19	CBS	Cystathionine beta-synthase
20	AKR1C1	Aldo-keto reductase family 1 member C1
21	VEGFA	Vascular endothelial growth factor A
22	SLC3A2	4F2 cell-surface antigen heavy chain
23	ARNTL	Aryl hydrocarbon receptor nuclear translocator-like protein 1
24	NNMT	Nicotinamide N-methyltransferase
25	BNIP3	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3
26	AKR1C2	Aldo-keto reductase family 1 member C2
27	PLIN2	Perilipin-2
Upregulated genes		
1	ATM	Serine-protein kinase ATM
2	ALOX5	Polyunsaturated fatty acid 5-lipoxygenase
3	CAPG	Macrophage-capping protein
4	CYBB	Cytochrome *b*-245 heavy chain
5	NCF2	Neutrophil cytosol factor 2
6	RRM2	Ribonucleoside-diphosphate reductase subunit M2
7	SLC2A6	Solute carrier family 2, facilitated glucose transporter member 6

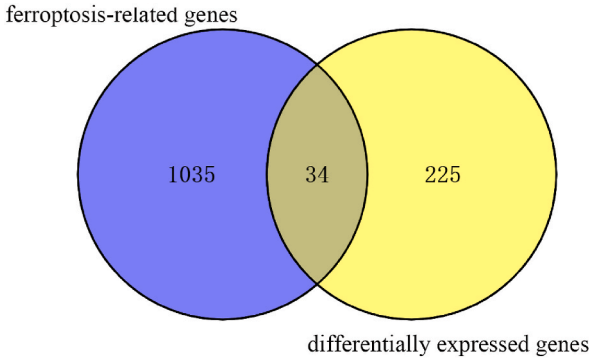

Fig. 3. venn diagram of FRGs and DEGs.

oxidoreductase activity, acting on NAD(P)H, acting on NADPH, iron ion binding, heme binding, and tetrapyrrole binding. The top 10 of the BP, CC, and MF enriched terms were shown in Fig. 5. KEGG pathway enrichment analysis enriched 42 terms with P-value< 0.05. Among them, the highly enriched terms were Kaposi sarcoma-associated herpesvirus infection, FoxO-signal pathway, Chemical carcinogenesis - reactive oxygen species, IL-17 signal pathway, NOD-like receptor signal pathway, TNF-signal pathway, etc. The top 30 enriched pathway terms were shown in Fig. 6.

3.4. Immune infiltration

We finally gained the immune cell content matrix of 10 RA synovial-tissue-samples and 7 normal synovial-tissue samples, by the

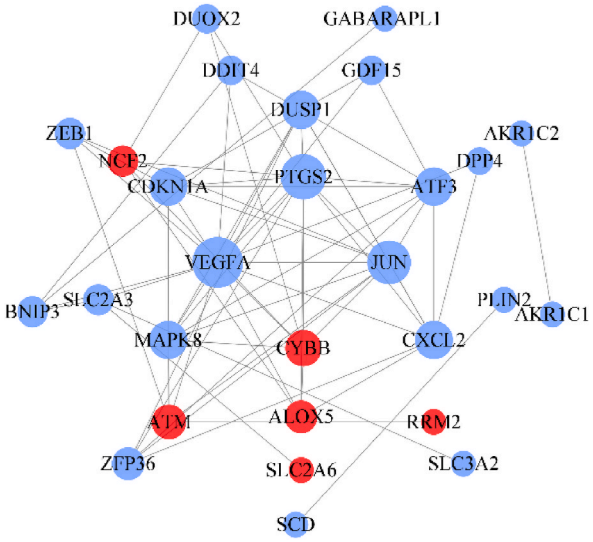

Fig. 4. PPI networks.

CIBOSORT deconvolution algorithm, the samples were screened with $P < 0.05$, as shown in Fig. 7, where the RA group was shown in pink and the control group was shown in blue. We found that the infiltration of immune cells was different between RA and the control group. The results displayed that the contents of T-cell-CD4-memory-restin, Dendritic-cell-restin and Mast-cell-activated were decreased in RA synovial tissues compared to control synovial tissues, while the contents of Plasma-cells as well as T-cell-CD8 increased in the synovium of RA to varying degrees. The percentage of each immune cell infiltration in each sample was presented in Fig. 8. The results showed that T cells and Macrophages(Mac) M2, Plasma cells had a high proportion in the RA group, while the content of Eosinophils was low in both groups. The correlation coefficients between different immune cells in the included samples were calculated and the heat map of correlation coefficients was reported in Fig. 9. There was an apparent direct correlation between T cell CD4 naïve and B cells memory (r = 0.89), T cell CD4 memory activated and T cell CD4 memory activated (r = 0.78), T cells regulatory (Tregs) and NK cells resting (r = 0.73). Plasma cells and T cell CD4 memory resting were negatively correlated (r = −0.71). Mast cells resting had a significant negative correlation with Mast cells activated (r = −0.79). Mac M2 and M1 showed an apparent inverse correlation (r = −0.69). The heatmap of the correlation between hub genes and immune infiltration showed that JUN was directly proportional to Eosinophils(Eos) and T cell follicular helper(Tfh) (as shown in Fig. 10). We also found a negative association between VEGFA and Mac M2.

4. Discussion

Abnormal proliferation of fibroblast-like synoviocytes (FLSs) and the infiltration of immune cells into synovial tissue are two typical features of RA [30,31], and they produce factors that drive inflammation and pannus formation, such as TNF-α, interleukin-6 (IL-6) [32], and prostaglandin (PG) E2, ultimately leading to articular cartilage destruction and bone erosion [33,34]. Recent studies [35,36] have revealed that ferroptosis is important in regulating inflammatory and autoimmune diseases. Lipid peroxidation and abnormal iron metabolism, as critical stimulators for ferroptosis, are involved in the development of RA [37–39]. In the Fenton reaction, Fe II oxidizes H2O2, resulting in the production of hydroxyl radicals (HO*), which oxidize polyunsaturated lipids. glutathione peroxidase 4(GPX4) activity is reduced, and lipid oxides cannot be metabolized by GpX4-catalyzed glutathione reductase, eventually leading to Ferroptosis [40]. More importantly, in the joint cavity of RA patients in large quantities exists a product-oxidative stress called ROS. Clinical trials have confirmed that in RA patients ROS, as a potential marker to judge the disease progression [41,42]. Thus it can be seen that ferroptosis has an important role in developing RA.

With the purpose of constructing the PPI network of FRGs-DEGs, STRING database and Cytoscape software were applied. Then hub genes from the whole network were identified using CytoNCA. The hub genes contained 3 nodes namely, VEGFA, PTGS2, and JUN. VEGFA is one of the most important pro-angiogenic mediators correlated with inflammation-associated synovial angiogenesis in RA [43]. Serum VEGFA levels of RA patients correlate with lesion development in RA. High serum VEGFA levels at an early stage may predict the size of subsequent damage of joints [44]. Both anti-VEGFA and anti-VEGFA-receptor antibodies have been found to suppress pannus formation, delay disease onset and reduce synovial inflammation in the mouse RA model [45]. PTGS2, also known as cyclooxygenase 2 (COX-2), is an essential rate-limiting enzyme in PGs biosynthesis and plays an essential role in the inflammatory response [46,47]. The high expression of COX-2 can increase the content of PGE2, resulting in inflammation, pain, and joint injury [48]. In inflammation, the expression of COX-2 is regulated by various cytokines, such as TNF-α, and IL-1 [49]. The infiltration of mast cells (MCs) in RA patients' synovium exists ahead of joint symptom onset time, and there is a 6-25-fold improvement in the number of mast cells in RA patients in comparison with healthy people [50]. Mishima Shintaro et al. [51] detected that the expression of PTGS2

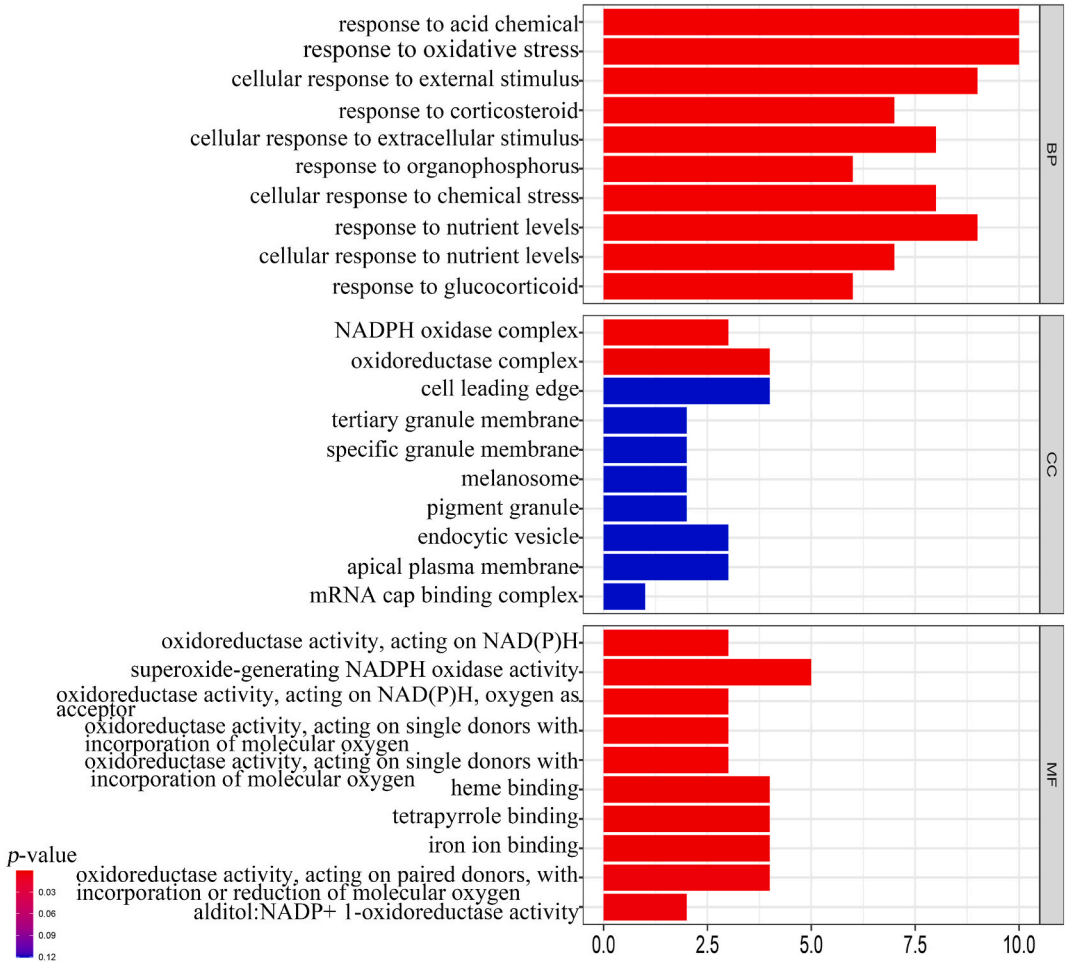

Fig. 5. Barplot of GO enrichment analysis.
Note: The top 10 GO enrichment analysis of 3 components. The enriched terms of GO are expressed by vertical coordinates, and the percentage of genes in each term are expressed by the horizontal coordinates. The color intensity represents the degree of enrichment, and the higher the degree of enrichment from blue to red. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

was apparently higher in synovial MCs of RA patients compared with osteoarthritis patients. Besides, non-steroidal anti-inflammatory painkillers (such as celecoxib) that selectively inhibit cox-2 expression and activity and reduce PGE2 production have become commonly used drugs to reduce the symptoms of joint swelling and pain caused by RA [52]. Jun (C-Jun) belongs to the JUN family proteins including Jun B, C-Jun, and Jun D [53]. JUN can regulate the expression of proinflammatory cytokines and chemokines [54] and induce the expression of the transcription factor Fos-related antigen proteins in the peripheral blood of synovial Mac, thereby regulating the clinical process of RA [55].

GO annotation analysis of the 34 FRGs-DEGs found that most enrichment terms are included in the BP. In enriched BP terms, the response to oxidative stress and response to corticosteroids were closely related to RA. Oxidative stress is a key contributory factor for the etiopathology of RA [56]. These issues have been studied in detail in RA (such as the oxidation of low-thickness lipoproteins, oxidative harm to hyaluronic corrosive and lipoperoxidation outcomes, DNA damage and carbonyl expansion brought about by protein oxidants) [57]. It has been previously shown that ROS can activate different signal pathways having a vital importance in the pathophysiology of RA [58]. Corticosteroids have long been the mainstay of RA therapy owing to their potent immunosuppressive and anti-inflammatory actions [59,60]. However, it is very effective in the short term regarding pain relief, but the long-term effect is not satisfactory and can lead to many side effects [61]. The CC analysis showed that the ferroptosis gene in RA mainly existed in NADPH oxidase complex, and oxidoreductase complex. In terms of MF, it plays a role in oxidoreductase activity, acting on NAD(P)H, acting on NADPH, iron ion binding, heme binding, and tetrapyrrole binding. Excessive stimulation of NAD(P)H is one of the causes of oxidative stress and excessive production of reactive oxygen species [62]. Heme oxygenase-1(HO-1) is an enzyme existing within the human body, which has protective and antioxidant functions. HO-1 levels will be increased because of the stimulation of oxidation and inflammatory cytokines, and thus it exerts anti-inflammatory, antioxidant effects on the body [63,64]. Su et al. [65]. found that calycosin

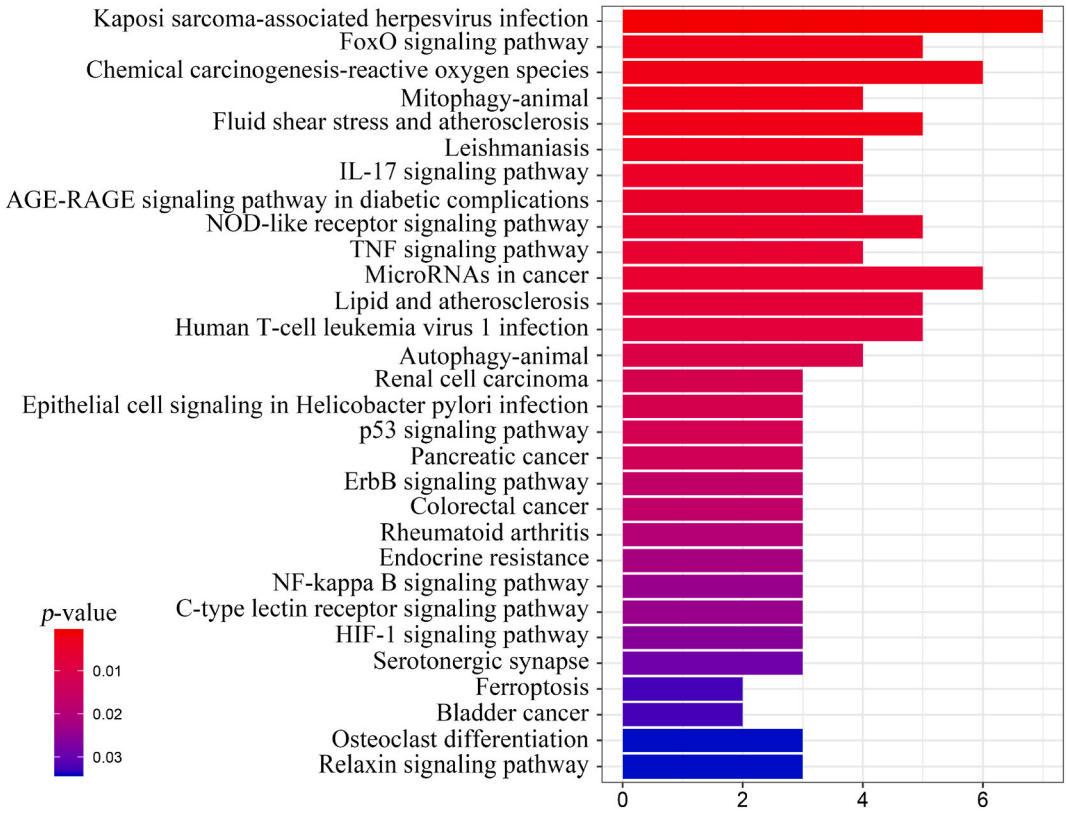

Fig. 6. Barplot of KEGG pathway enrichment analysis.
Note: The top 30 KEGG pathway enrichment analysis. The enriched pathways are in the vertical coordinates, and the percentage of genes in each pathway is expressed in the horizontal coordinates. The color intensity represents the degree of enrichment, and the redder the color, the higher the enrichment. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

could inhibit the expression of proinflammatory factors in synovial fibroblasts of RA patients by increasing HO-1 activity, suggesting the induction of high expression of HO-1 can be used to treat RA. Hence, investigating deeply the mechanism of ferroptosis in RA may be of potential help to improve the clinical efficacy of RA.

KEGG enrichment analysis displayed that ferroptosis-DEGs have main connection with Kaposi sarcoma-associated herpesvirus infection, FoxO-signal pathway, Chemical carcinogenesis-reactive oxygen species, IL-17 signal pathway, NOD-like receptor signal pathway, TNF-signal pathway. Among them, FoxO signal pathway, NOD-like receptor signal pathway, IL-17 signal pathway, and TNF signal pathway were closely related to the occurrence and development of RA, the mediation of inflammation and the destruction of bone and joints. It has been reported that transcriptional FoxO-related factors maintain the survival role of RA synoviocytes in the integration of inflammatory stimuli in RA joint synovial tissue. FOXO3A and FOXO4, members of the FoxO family, protect cells from oxidative stress by transcriptional upregulation of ROS scavenger MnSOD [66]. Cytosolic NOD-like receptors (NLRs) family that are activators of inflammation have been associated with human diseases including infections, autoimmune, and inflammatory disorders [67]. NLRP3, a member of the NLRs family, is a crucial source of IL-1 and IL-18, and plays a part in the nosogenesis of RA [68]. IL-17 inhibition in several animal models of arthritis limits joint erosion and inflammation and uses antibodies to block IL-17 or its receptors, aiming to reduce disease in mice [69]. Initial observations from phase I trials indicate that the signs and symptoms of RA are apparently suppressed after the treatment of anti-IL-17 antibody [70]. TNF-signal pathway is also a significant inflammatory-signal pathway. TNF-α is a crucial player in the occurrence of RA. It can be bound to TNF Receptor-1 on fibroblast synoviocytes, promote the release of inflammatory cytokines such as IL-1, IL-6, and IL-8, and aggravate articular cartilage and bone injury [71]. In addition, it can also induce immune cells in the blood to enter the joint through vascular cell adhesion molecule-1, aggravating joint injury [72]. These signal pathways are significant in ferroptosis intervention in RA.

To further study the role of immune cells in RA-synovium, we used the CIBOSORT deconvolution algorithm to screen the synovium samples of the RA and the control group. The final result showed that T cells, Mac M2, and Plasma cells had a high proportion in the RA group. $CD8^{+}$T cells account for the majority of T cells. In recent years, several studies have confirmed T cell infiltration plays an important role in RA nosogenesis [73,74]. In addition, it has been reported that the pool of $CD8^{+}$T cells tends to expand in the synovial tissues of RA patients [75,76]. Carvalheiro et al. [77]. found that T cell CD8 are abundant in RA and observed the activation status and proinflammatory potential of T cells CD8 subsets in RA patients, suggesting a local and systemic effector cytotoxic role in RA. Tregs are a kind of cells with strong immune suppression, which play an important role in autoimmunity by directly inhibiting and secreting

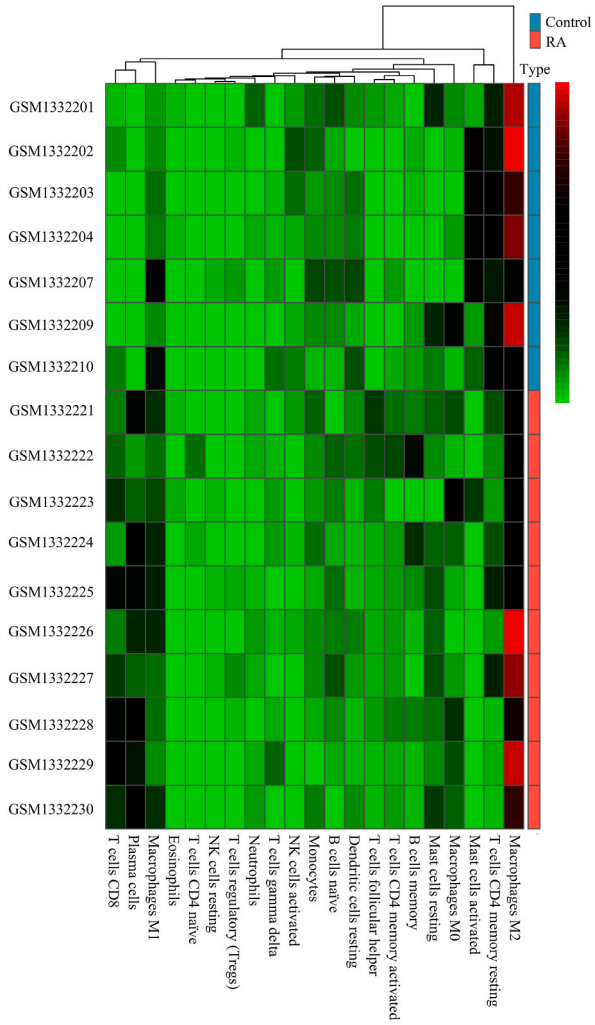

Fig. 7. Heat map of the proportion of immune cell infiltration in RA-synovial-tissue samples versus control-synovial-tissue samples (control in blue, RA in pink). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

anti-inflammatory factors (transforming growth factor-β, IL-10, etc.) to negatively regulate the immune response [78]. These reflect the unique biological behavior of T cells in RA. The above series of studies' conclusions were consistent with the results of this study. Although Mac M2 has a high proportion in RA group, the infiltration level of Mac M2 is significantly reduced in RA. In addition, the correlation analysis showed that Mac M2 and Mac M1 were negatively correlated. Macs are broadly divided into two categories, Mac M1 and Mac M2 [79]. The imbalance between Mac M1 producing proinflammatory factors and Mac M2 producing anti-inflammatory factors is one of the key mechanisms promoting the progression of rheumatoid arthritis, and Mac M2 plays a protective role in RA [80]. There was an apparent direct correlation between T cell CD4 naïve and B cell memory. Theoretically, antigen-presenting cells in RA joints may stimulate synovial $CD4^+$ T cells to differentiate into T helper cells, which in turn activate B cells, some of which are differentiated into plasma cells that produce autoantibodies [81]. The immunocyte correlation analysis shows that most of the immune cells in RA have different degrees of correlation, suggesting that various immune cells may interact to form a highly complex immunosuppressive network. However, the specific effects of these correlations on the development of synovium in RA need to be further studied.

The heatmap of the correlation between hub genes and immune infiltration showed that JUN and VEGFA were closely related to immune cell infiltration. It has been previously shown that the Tfh level was significantly increased in RA [82], which was unanimous to the results of immune infiltration in this study. Tfh is a subset of CD4 T cells, which can mediate the destruction of cartilage and bone in RA patients because it can induce B cell activation, proliferation, and differentiation and produce a large amount of immunoglobulins, such as anticyclic citrullinated peptide (CCP) [83], thus of vital importance in RA nosogenesis. However, the specific mechanism by which Tfh cells promote the nosogenesis of RA remains unclear. We conclude that JUN and Tfh have a synergistic function on the nosogenesis of RA. The proinflammatory effector function of eosinophils is well-known in asthma and likely to contribute to the development of asthma exacerbation [84]. Unexpectedly, Chen et al. [85] proposed that Eos has suppressed

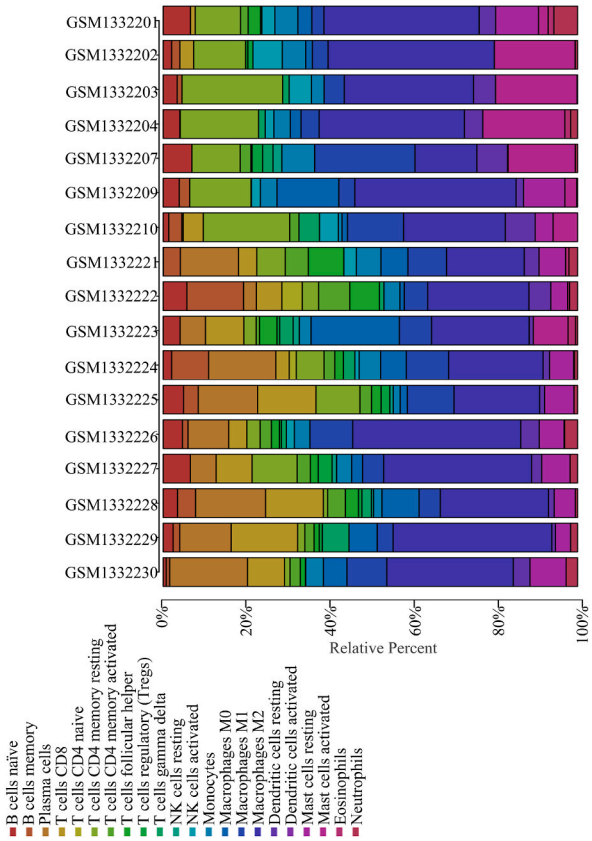

Fig. 8. The bar graph of the percentage of each immune cell infiltration in RA-synovial-tissue samples and control-synovial-tissues samples.
Note: Different colors represent different immune cell types, as shown in the legend, and their lengths represent their relative percent in the enrolled sample. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

inflammatory arthritis. There was a recent study pointing out that there are hitherto undiscovered proresolution features in the Eos subset, which stimulates RA arthritis resolution [86], however, the mechanism by which the double-edged effects of Eos are identified is currently unknown. VEGFA has a negative positive association with Mac M2. VEGFA plays an essential role in the inflammatory response [46,47]. Mac M2 plays a protective role in RA [80]. Therefore, we conclude that PTGS2 and Mac M2 have antagonistic effects in the nosogenesis of RA.

5. Conclusions

In summary, the hub genes involved in ferroptosis in RA may be VEGFA, PTGS2, and JUN, which are mainly involved in FoxO signal pathway and NOD-like receptor signal pathway. T cells, Mac, and plasma cells may be involved in the regulation of RA-joint-synovial inflammation. JUN and Tfh have synergistic effects in the nosogenesis of RA, while PTGS2 and Mac M2 have antagonistic effects. It provides a more detailed molecular mechanism for understanding the influence of ferroptosis on the development of RA. This study speculated that iron death may interfere with RA through abnormal ROS accumulation, regulating oxidative stress, inflammatory response, and corticosteroid response. We know deeply about the underlying molecular nosogenesis by which ferroptosis influences the development of RA through the results of this study, thereby providing a novel detection and targeting of therapeutic modalities by modulating ferroptosis angles.

Data availability statement

Data will be made available on request.

Additional information

No additional information is available for this paper.

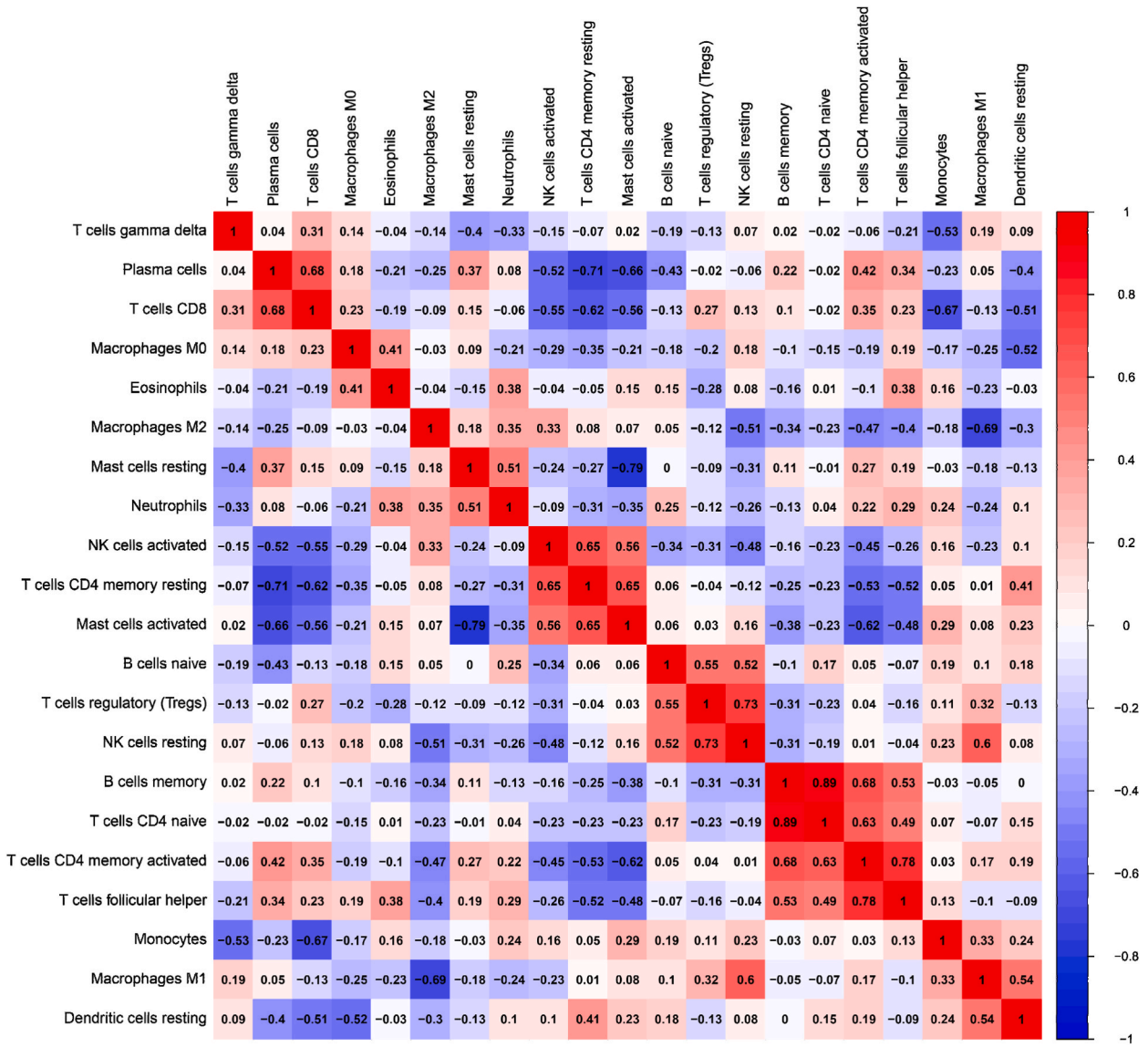

Fig. 9. The heatmap of the correlation between each immune cell infiltration in RA.
Note: red indicates a positive correlation, blue indicates a negative correlation, and the darker color indicates stronger correlation. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

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CRediT authorship contribution statement

Yihua Fan: Conceptualization, Writing – original draft. **Yuan Li:** Conceptualization, Writing – original draft. **Xiaoyan Fu:** Data curation. **Jing Peng:** Data curation. **Yuchi Chen:** Software. **Tao Chen:** Software. **Di Zhang:** Funding acquisition, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to

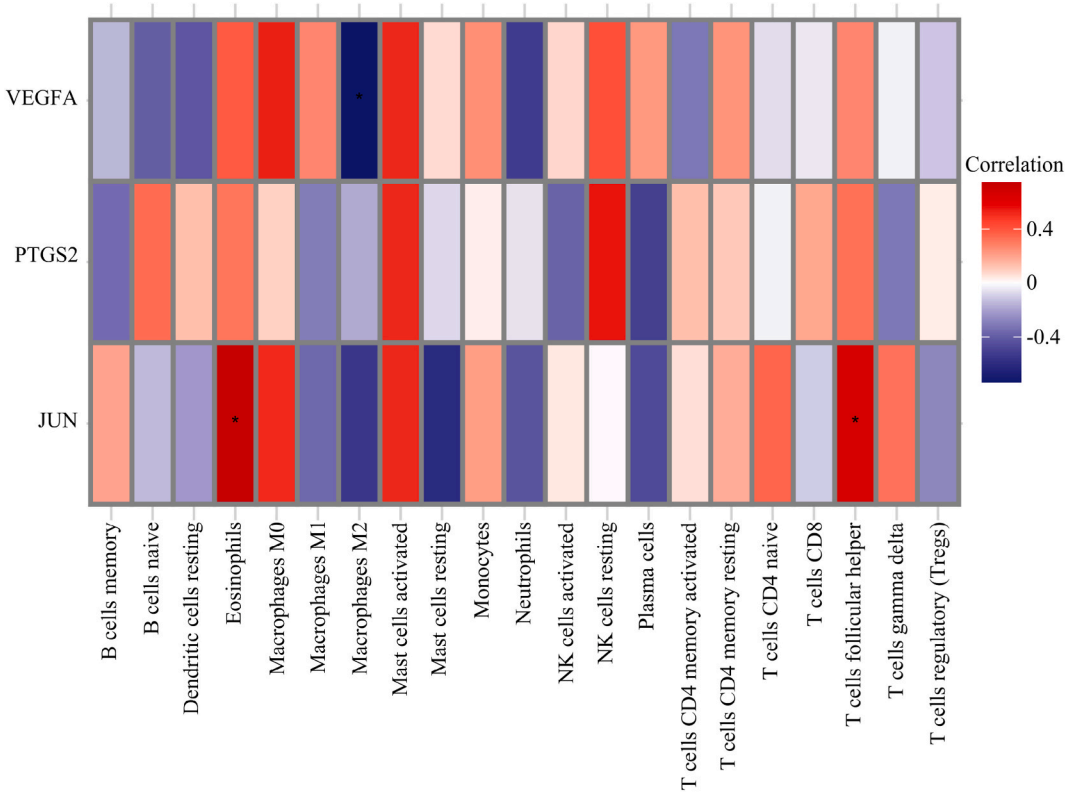

Fig. 10. The heatmap of the correlation between hub genes and immune infiltration.
Note: Red indicates a positive correlation, blue indicates a negative correlation, and the darker the color, the greater the correlation. * appears in the grid, a correlation between the immune cells and hub gene with *P*-value <0.05. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

influence the work reported in this paper.

Acknowledgment

A preprint has previously been published [87].

Abbreviations

RA	Rheumatoid arthritis
FRGs	ferroptosis-related genes
FerrDb	Ferroptosis database
GEO	Gene Expression Omnibus
DEGs	differentially expressed genes
PPI	protein-protein interaction
GO	gene ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
ROS	reactive oxygen species; GSH = glutathione
GPX 4	glutathione peroxidase 4
TRPM7	Transient Receptor Potential Melastatin 7
TNF-α	Tumor necrosis factor-α;
IL-6	interleukin-6
DC	degree centrality
BC	betweenness centrality
CC	closeness centrality
BP	Biological Process
CC	Cellular Component

MF	Molecular Function
Mac	Macrophages
Tregs	T cells regulatory
Eos	Eosinophils
Tfh	T cells follicular helper
FLSs	fibroblast-like synoviocytes
PG	prostaglandin
COX-2	cyclooxygenases 2
MCs	synovial mast cells
HO-1	Heme oxygenase-1
NLRs	NOD-like receptors
CCP	cyclic citrullinated peptide

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