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ORIGINAL RESEARCH

Identification of Shared Biomarkers and Immune Infiltration Signatures between Vitiligo and Hashimoto's Thyroiditis

Jiawei Lu[1], Lebin Song[1], Jiaochen Luan[2], Yifei Feng[1], Yidan Wang[1], Xuechen Cao[1], Yan Lu[1]

[1]Department of Dermatology, The First Affiliated Hospital, Nanjing Medical University, Nanjing, People's Republic of China; [2]Department of Urology, The First Affiliated Hospital, Nanjing Medical University, Nanjing, People's Republic of China

Correspondence: Yan Lu, Department of Dermatology, The First Affiliated Hospital, Nanjing Medical University, 300 Guangzhou Road, Nanjing, Jiangsu, 210029, People's Republic of China, Tel +86 13913967126, Email luyan6289@163.com

Background: Vitiligo and Hashimoto's thyroiditis (HT) are concomitant autoimmune diseases characterized by the destruction of melanocytes or thyrocytes. We aimed to explore the immunological mechanism of this comorbidity and screen their potential biomarkers.

Methods: We downloaded the microarray datasets from the GEO database. Differentially expressed genes (DEGs) and immune-related genes (IRGs) were selected. The immune-related differentially expressed genes (IRDEGs) were obtained by taking the intersection. Candidate biomarkers were elected by Cytoscape software. CIBERSORT was used to depict immune cell infiltration prospects. Correlation analysis was conducted between infiltrating cells and several indicators. The results were validated by real-time quantitative PCR (RT-qPCR).

Results: Three datasets and 60 IRDEGs were obtained in total. Pathway enrichment analysis showed that the T cell receptor signaling pathway, IL-17 signaling pathway, receptor-ligand activity, and signaling receptor activator activity were significantly enriched. We screened out four hub genes, including *IFNG*, *STAT1*, *IL1B*, and *CXCL10*. The ROC curve indicated the highest diagnostic value of *CXCL10* in both vitiligo and HT. Immuno-infiltration analysis revealed significant changes in T cell subsets and macrophage subtypes, which were correlated with four hub genes, melanocyte markers, and thyroid-specific antigens. qPCR validated the hub genes in peripheral blood mononuclear cells from patients with comorbidity.

Conclusion: *IFNG*, *STAT1*, *IL1B*, and *CXCL10*, were the key IRDEGs to vitiligo and HT. These genes may participate in the comorbidity by remodeling the immune cell infiltration pattern, and cross-expressed antigens may mediate the common damage of melanocytes and thyroid tissues.

Keywords: depigmenting skin disorder, autoimmune thyroiditis, immune cell infiltration, bioinformatics analysis

Introduction

Vitiligo is a chronic pigmentary disorder characterized by the development of white macules in the skin or hair. The prevalence of vitiligo is referred to as 0.5–2% around the world and is more common in dark-skinned individuals.[1] Non-segmental vitiligo, the most common clinical subtype, often bilaterally distributes in limbs or scattered over the body. The lesions evolve or progress over time, which is psychologically stigmatizing and brings a lower quality of life.

The loss of melanocytes originates from genetic susceptibility, immunologic abnormalities, oxidative stress, and neuropeptides. Multiple susceptibility loci encoding immunoregulatory proteins have been identified for generalized vitiligo.[2] The endoplasmic reticulum stress-induced unfolded protein response is considered the bridge between oxidative stress and autoimmunity in vitiligo. DAMPs, PAMPs, and innate immune cells further promote the adaptive T-cell response. The infiltrated $CD8^+$ T cells are the culprit to the destruction of melanocytes in vitiligo,[3] which release type 1 cytokines, especially IFN-γ and TNF-α. The recruitment of $CD8^+$ T cells towards the skin is mediated by the combination of CXCR3 and CXCL9/10 which are the chemokines released by keratinocytes dependent on the IFN-γ-activated JAK/

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STAT pathway. IFN-γ-CXCL9/10-CXCR3 makes a positive feedback loop in vitiligo. Th17/ regulatory T cells (Treg) are in a state of imbalance. FoxP3$^+$ Treg cell and its chemokine, CCL22, are down-regulated in the lesions.

Vitiligo is often accompanied by several autoimmune diseases, primarily thyroid diseases. A higher prevalence (12.9–24.1%) of thyroid diseases has been found in vitiligo patients.[4–6]As one of the most prevalent comorbid diseases of vitiligo, HT is recognized as a risk factor for developing papillary thyroid cancer and thyroid lymphoma.[4] HT often occurs in immune-defected individuals with genetic susceptibility or triggers including environmental factors, oxidative stress, trace elements, and infections. Histologically, we can observe diffuse lymphocytic infiltration in lymphoid follicles and germinal centers, which leads to a transient hyperthyroid phase to hypothyroidism of the thyroid gland. HT is characterized by humoral immunity-mediated destruction of the thyroid gland, together with abnormality in innate and cellular immunity. Exogenous and endogenous dangers (PAMPs, DAMPs) may trigger the innate immune response in thyroid cells in the absence of immune cells.[7] As the receptors of PAMPs and DAMPs, Toll-like receptors (TLRs)2/3/9/10 are upregulated in the immune cells in HT.[8] The imbalance of Th17/Treg adds to the development and progression of HT.[9]

Patients with comorbidity have poorer therapeutic responses to the traditional treatment and recurrent clinical courses. A retrospective study reported a higher percentage of the involved body surface area in vitiligo patients with thyroid pathology.[10] Also, patients with vitiligo and autoimmune thyroiditis have a predisposition to developing acral lesions and discolored patches of wrists.[11] In the subgroup analysis, vitiligo had a stronger association with HT in males than females, and they had the strongest association in children and adolescents (aged under 20 years).[12] Genetic factors[13] and autoimmune etiology are primarily involved in the synchronicity of the two diseases, but the exact immunological mechanism of the comorbidity remains unclear.

The rapid development of microarray and high-throughput sequencing technology has brought a valid means of identifying hub genes and potential molecular biomarkers for multiple diseases, such as neoplastic diseases and autoimmune diseases. To our knowledge, this is the first bioinformatics-based study on the immunological mechanism of this comorbidity.

Methods

Access to GEO Datasets

Genes expression profiling of vitiligo and HT were screened using the GEO database (http://www.ncbi.nlm.nih.gov/geo). The following criteria were used to filter the datasets: (1) the gene expression profiling obtained from tissue specimens; (2) expression profiling by array or high-throughput sequencing of mRNA; (3) available raw or processed data for further analysis. Three eligible datasets were enrolled, including GSE75819, GSE138198, and GSE54958. GSE75819 series on the GPL6884 platform (Illumina HumanWG-6 v3.0 expression bead chip) was the dataset of vitiligo. GSE138198 and GSE54958 on the GPL6244 platform (Affymetrix Human Gene 1.0 ST Array) were merged as the dataset of HT.

Data Processing

Raw and series matrix files of the three microarray datasets were downloaded from the GEO database. For all raw data, the probe expression matrix was extracted and normalized with the "normalizebetweenarrays" function of the "limma" package. R package "sva" was utilized to eliminate heterogeneity of the merged dataset (GSE138198 and GSE54958) caused by different experimental batches. Then probe expression matrices were converted into gene expression matrices according to the annotation documents of corresponding platforms.

Identification of Differentially Expressed Genes (DEG) and IRGs

The Immunology Database and Analysis Portal database (ImmPort) was applied to single out the immune-related genes set. The differential analysis was performed by comparing normal tissues with pathological tissues using "limma" package in R computing environment, separately in the vitiligo and HT IRGs sets with conditioned adjusted P-value <0.05. We obtain the qualified immune-related differentially expressed genes (IRDEGs) by taking the intersection of IRDEGs in two datasets.

Function and Pathway Enrichment Analysis

GO function and KEGG pathway enrichment analyses of the co-expressed IRDEGs were performed through R package "ClusterProfiler". Adjusted P-value <0.05 was considered significant.

PPI Network Construction and Module Analysis

Based on the STRING online tool, a biological database designed to construct a protein–protein interaction (PPI) network of targeted genes and analyze the functional interactions between proteins, the PPI network of the IRDEGs was built with a confidence score >0.4. The visualization of the network was processed by Cytoscape software (version 3.8.2). Molecular Complex Detection (MCODE) and cytoHubba are plug-in procedures of Cytoscape software. MCODE was used to explore the significant modules. We selected the hub genes by taking the intersection of the top ten genes ranked by the nine algorithms (MCC, MNC, degree, EPC, Bottleneck, closeness, radiality, betweenness, and stress) in cytoHubba.

Construction of ceRNA Network

We predicted miRNAs of the target gene and then filtered the overlapped mRNA-targeted miRNAs based on four online tools. Similarly, we acquired the target lncRNA of the core miRNA by using StarBase 3.0 (http://starbase.sysu.edu.cn/). The results were displayed after being processed by Cytoscape software.

Immune Infiltration and Correlation Analysis

CIBERSORT is a method for characterizing the immune cell composition of tissues based on their gene expression profiles. The immune cell compositions were predicted via CIBERSORT using the LM22 signature gene file with 1000 permutations, respectively in vitiligo and HT datasets. We explored the correlation of the identified hub genes with the composition of infiltrating immune cells and specific antigens using Spearman's rank correlation analysis in R software. The results were visualized using the "ggplot2" package in R software.

Participants

In the present study, we enrolled six vitiligo patients with concomitant HT identified in the dermatology department of the first affiliated hospital of Nanjing Medical University. We also recruited six age- and sex-matched healthy controls (HCs) for comparison. Ethical approval for this study was obtained from the local Ethics Committee. Written informed consent was signed by all subjects before the study. All experiments were performed according to the Declaration of Helsinki and guidelines for Good Clinical Practice.

Peripheral Blood Mononuclear Cells (PBMC) Isolation

Peripheral venous blood was extracted into sodium-heparin tubes, and PBMC were extracted through a concentration gradient technique (Ficoll-Paque^{TM} Plus, GE Healthcare Life Sciences, Sweden). The cells were washed twice with PBS and centrifuged at 400g for 30 minutes at RT to isolate PBMC.

RNA Isolation and Real-Time Quantitative PCR

Total RNA was isolated from PBMC using Trizol reagent (Invitrogen, Carlsbad, CA, U.S.A.). RNA purity was detected using Quantus Fluorometer. Firstly, total RNA was used to synthesize cDNA with reverse transcription reaction. Secondly, RT-qPCR was conducted on the QuantStudio 7(Thermo Fisher) with SYBR-Green dye (Vazyme). The primers used are shown in Table 1. Each sample was tested in triplicates and underwent a melting curve analysis. Expression levels of target genes were calculated by the $2^{-\Delta\Delta}$Ct method adjusted to the internal control GAPDH.

Statistical Analysis

Statistical analyses were carried out based on R software. Comparisons between the disease and control group that presented normal distribution were performed with the Student's *T*-test. P-value <0.05 was considered significant. ROC analysis was conducted to determine the discrimination value of the genes.

Table 1 Primers

	Forward	Reverse
CXCL10	ACCTCCAGTCTCAGCACCATGA	TGCAGGTACAGCGTACAGTTCT
IFNG	ATTGGAAAGAGGAGAGTGACAGA	TTGGACATTCAAGTCAGTTACCG
IL1B	AGGGACAGGATATGGAGCAACAAG	CATCTTTCAACACGCAGGACAGGT
STAT1	AGGTGGCAGGATGTCTCAGTGG	CAGCGTGCTCCCAGTCTTGC
GAPDH	AAGTATGACAACAGCCTCAAG	TCCACGATACCAAAGTTGTC

Results

Identification of Shared IRDEGs

The flowchart of this research is shown in Figure 1. Three qualified datasets (GSE138198, GSE54958, and GSE75819) were downloaded. After normalization and the removal of the batch effect (Figure 2A–D), immune-related genes were extracted from the vitiligo and HT datasets for differential analysis (Figure 3A and B). We obtained 263 IRDEGs in the vitiligo dataset and 592 IRDEGs in the merged HT dataset. Sixty IRDEGs with consistent trends were acquired by taking the intersection in two datasets (Figure 3C and D). Heatmaps showed evident discrepancies in expression levels of the 60 genes between the diseases group and the healthy control group (Figure 3E and F).

Analysis of the Functional Characteristics of IRDEGs

We further processed IRDEGs for functional enrichment. Enriched KEGG pathways were primarily the T cell receptor signaling pathway, IL-17 signaling pathway, Toll-like receptor signaling pathway, proteasome, and natural killer cell-mediated cytotoxicity (Figure 4A and C). The enriched GO terms were closely related to the metabolism of cytokines

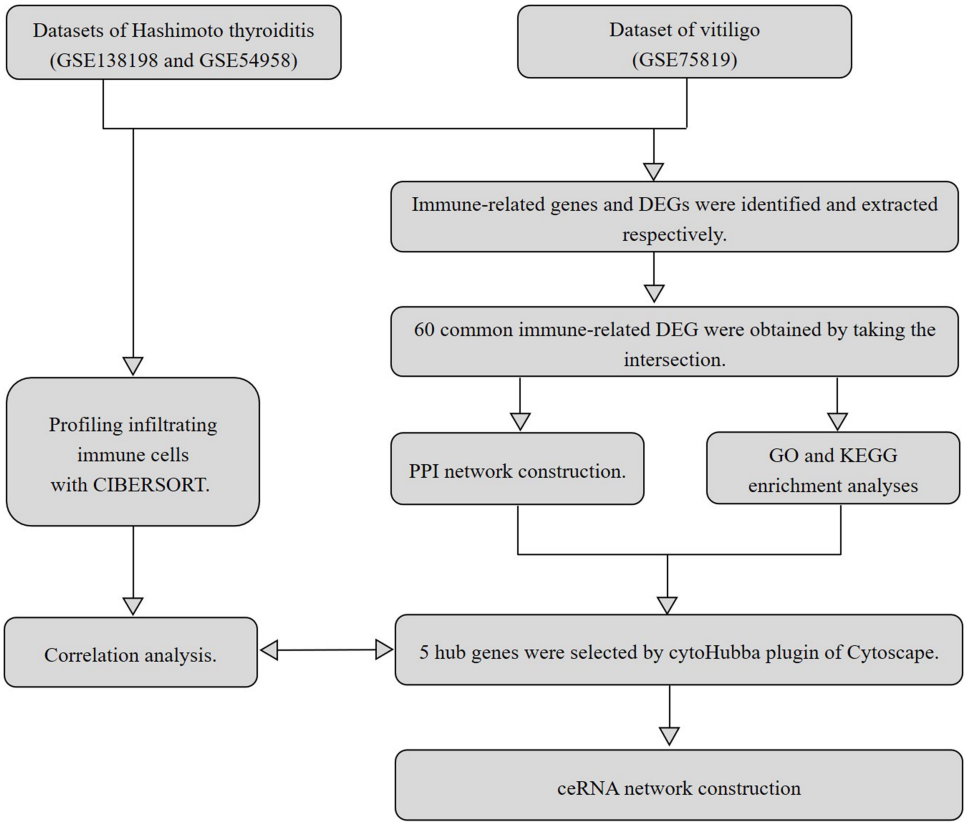

Figure 1 The flow chart of the research.

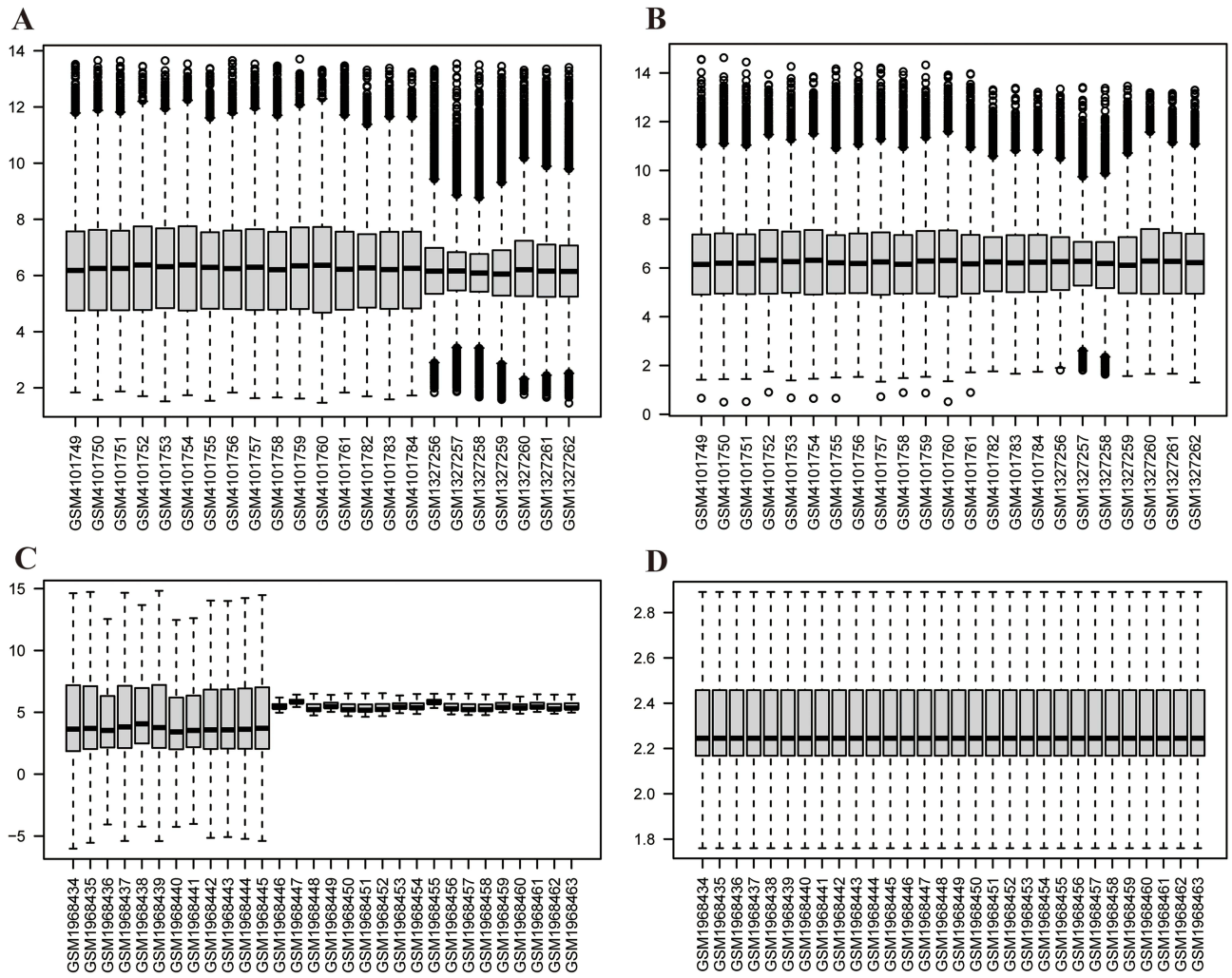

Figure 2 Data processing. Boxplot of the merged Hashimoto's thyroiditis dataset before (**A**) and after (**B**) the removal of the batch effect. Boxplot of the vitiligo dataset before (**C**) and after (**D**) normalization.

(Figure 4B and D). The most prominent clusters are receptor ligand activity, signaling receptor activator activity, positive regulation of cytokine production, cytokine-mediated signaling pathway, cytokine receptor binding, etc.

Construction of PPI Network, Analysis of Modules and Hub Genes

Sixty IRDEGs were predicted by the online database STRING, and visualized on Cytoscape software (Figure 5A). Significant modules of the network were identified by plug-in MCODE, and the top three clusters were ranked based on betweenness algorithm (Figure 5B–5D). We calculated the score of the genes with nine algorithms and selected the top 10 hub genes, respectively (Figure 5E). By taking the intersection of the nine results, we picked out four hub IRDEGs, including *STAT1, CXCL10, IL-1B*, and *IFNG*. The four genes were significantly upregulated in vitiligo and HT patients compared with that in normal tissues (Figure 5F and G).

Validation of the Sensitivity and Specificity

ROC analysis of each hub gene was conducted and the gene with AUC >0.8 was considered as the potential diagnostic gene with good specificity and sensitivity. The AUC values of *IFNG, IL1B, STAT1*, and *CXCL10* were 0.8578, 0.7956, 0.8889, and 1 in the vitiligo dataset while 0.8077, 0.8846, 0.7846, 0.8692, and 1 in the HT dataset (Figure 6A and B). Therefore, *IFNG, STAT1*, and *CXCL10* had good sensitivity and specificity in distinguishing vitiligo or HT patients from healthy controls. Among them, *CXCL10* had the highest score, and we chose it for further analysis.

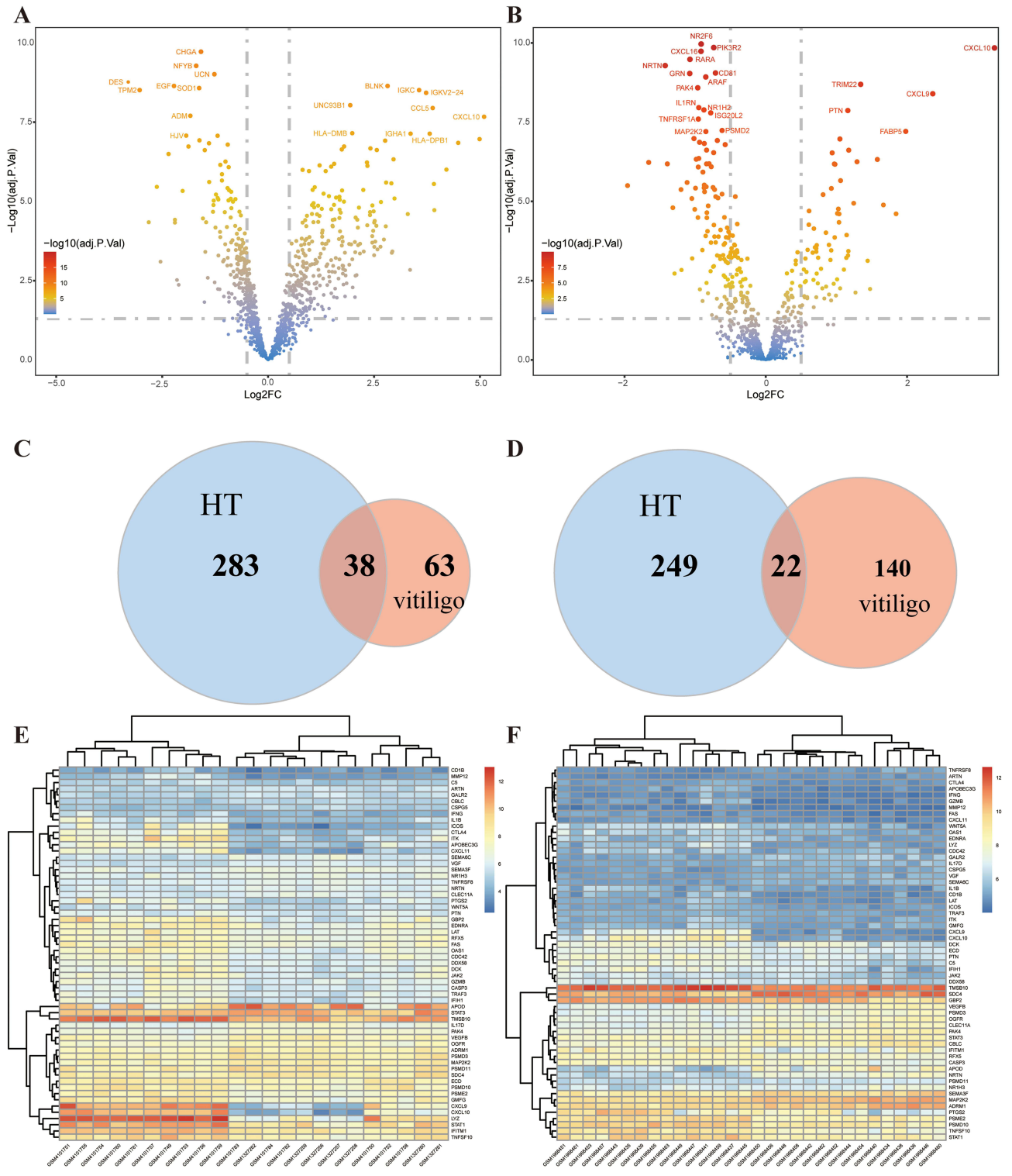

Figure 3 Volcano diagram, Venn diagram, and heatmap of IRDEGs. (**A**) Volcano plot of IRDEGs in HT dataset. (**B**) Volcano plot of IRDEGs in vitiligo dataset. Venn diagram of the up-regulated (**C**) and down-regulated (**D**) IRDEGs. The two Venn diagrams showed an overlap of 60 IRDEGs in total. Heatmaps of 60 IREDGs in HT (**E**) and vitiligo (**F**) datasets. HT, Hashimoto's thyroiditis; IRDEGs, immune-related differentially expressed genes.

Construction of ceRNA Network

miRNAs of *CXCL10* were predicted in the TargetScan, miRDB, starBase, and miRWalk databases. We took the intersection and obtained one miRNA, hsa-miR-219a-2-3p (Figure 6C). Sixty-eight lncRNA of miR-219a-2-3p were

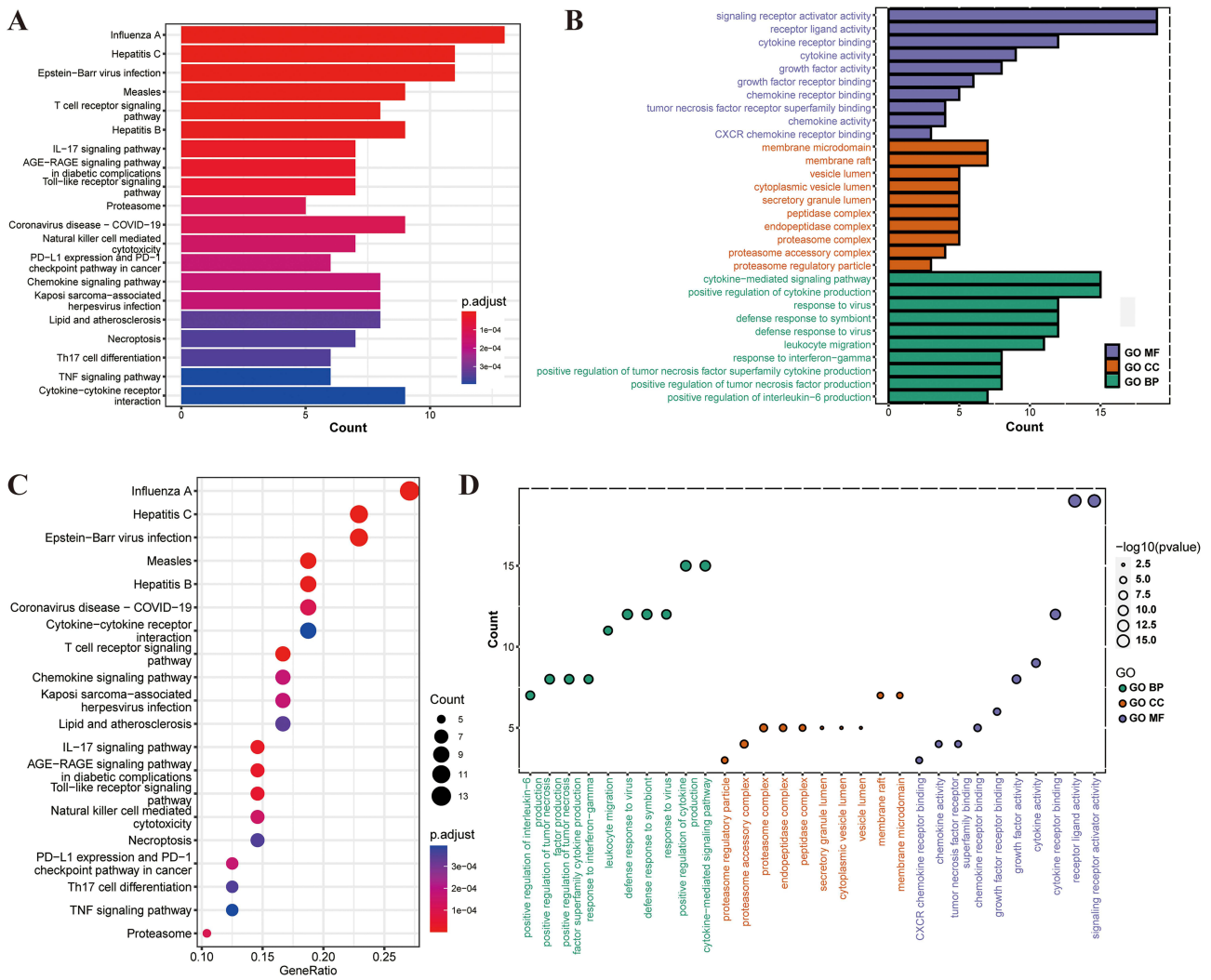

Figure 4 GO and KEGG enrichment analysis of IRDEGs. Bar plots of enriched KEGG pathways (**A**) and GO enrichment (**B**) of IRDEGs. Dot plots of enriched KEGG pathways (**C**) and GO enrichment (**D**) of IRDEGs. IRDEGs, immune-related differentially expressed genes.

acquired using the starBase database. We further constructed the ceRNA regulatory network based on the *CXCL10*-miRNA-lncRNA axis using Cytoscape (Figure 6D).

Evaluation of Immune Cell Infiltration

The compositions of 22 kinds of immune cells in vitiligo and HT datasets were identified by CIBERSORT and visualized on R software (Figure 7A). The infiltration bar plot of thyroid gland tissue showed upregulation of memory B cells, activated $CD4^+$ memory T cells, follicular helper T cells, M1 macrophages, and M2 macrophages in HT patients compared with HC (Figure 7B). In the lesion of vitiligo patients, resting $CD4^+$ memory T cells and M1 macrophages were upregulated, while Tregs, M0 macrophages, and activated dendritic cells were downregulated compared with normal skin (Figure 7C).

Correlation Analysis

The correlations among the hub IREDGs, infiltrated immune cells, and specific antigens were calculated by Spearman correlation rank. In HT patients, *STAT1* was found positively correlated with activated $CD4^+$ memory T cells. Activated NK cells and resting mast cells were negatively correlated with *IFNG, IL1B, STAT1*, and *CXCL10* (Figure 8A). In vitiligo patients,

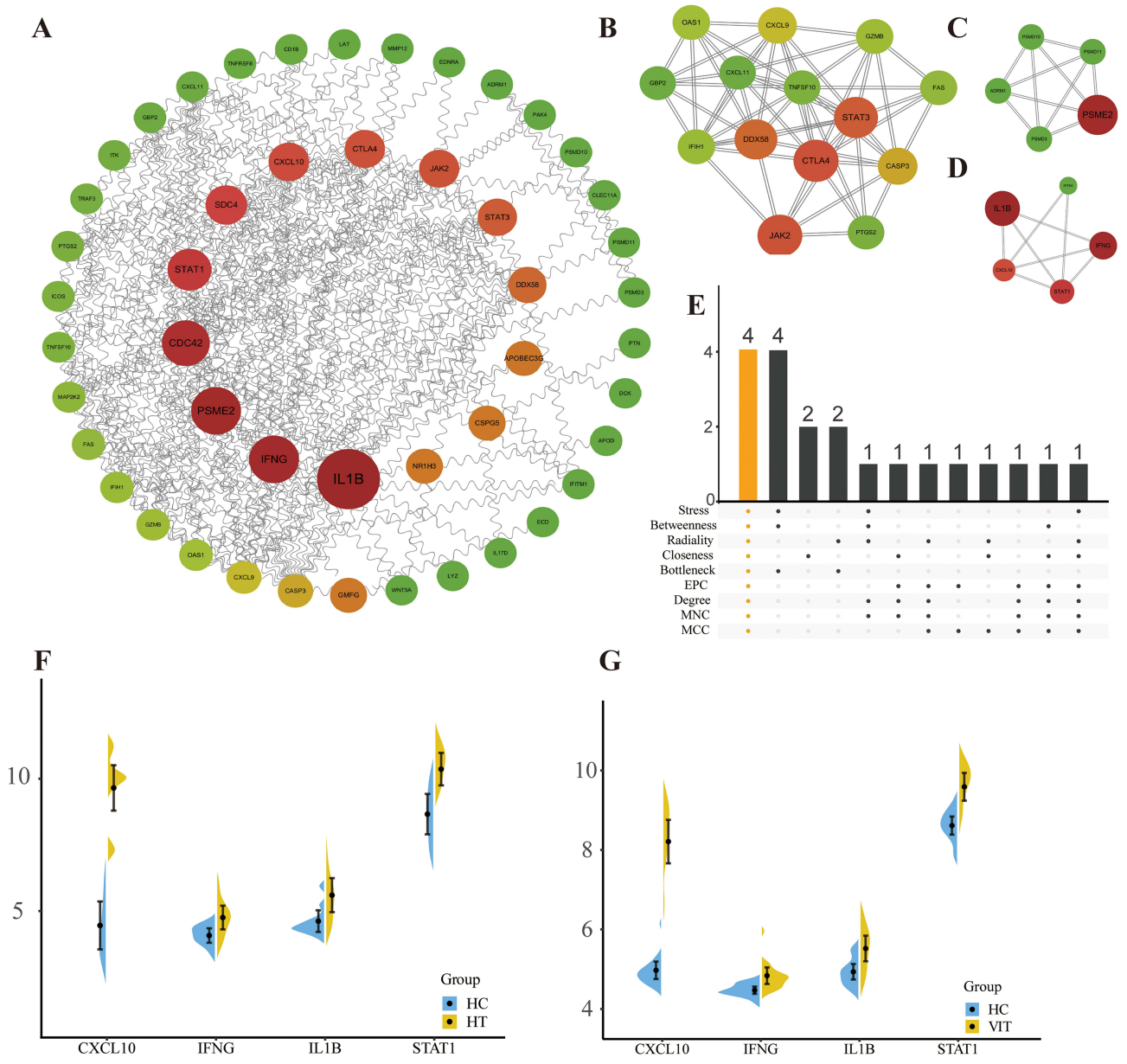

Figure 5 PPI network analysis and identification of hub IRDEGs. (**A**) The PPI network of 60 IRDEGs. (**B**–**D**) Enrichment analysis of the modular genes. (**E**) Diagram of unified hub genes in the nine algorithms. The expression level of the four genes in the merged Hashimoto's thyroiditis dataset (**F**) and vitiligo dataset (**G**). IRDEGs, immune-related differentially expressed genes.

STAT1 was positively associated with regulatory T cells. IL1B was found negatively correlated with $CD8^+$ T cells and activated NK cells (Figure 8B).

Melanocyte-specific antigens include *TYR, TYPR1, TYPR2, LAMP1, S100A1, S100B, MITF*, etc. Thyroid-specific epitopes include *TSHR, TPO*, and *TG*. The analysis showed that $CD4^+$ memory T cells were correlated to the expression of *TSHR* and *TPO* in vitiligo (Figure 9A). In HT, the subtypes of macrophages were correlated with *S100A1, S100B, TYRP1* and *TYR*, and the expression of *TYR* was correlated with plasma cells, $CD4^+$ T cells, Th cells and macrophages (Figure 9B).

Validation of Expression by RT-qPCR

The transcriptional changes of the four hub IRDEGs were verified in the PBMC from the patients with comorbidity of vitiligo and HT by RT-qPCR. The expression levels of *IFNG, IL1B, STAT1*, and *CXCL10* were significantly upregulated in the comorbidity group compared with the healthy group (Figure 10).

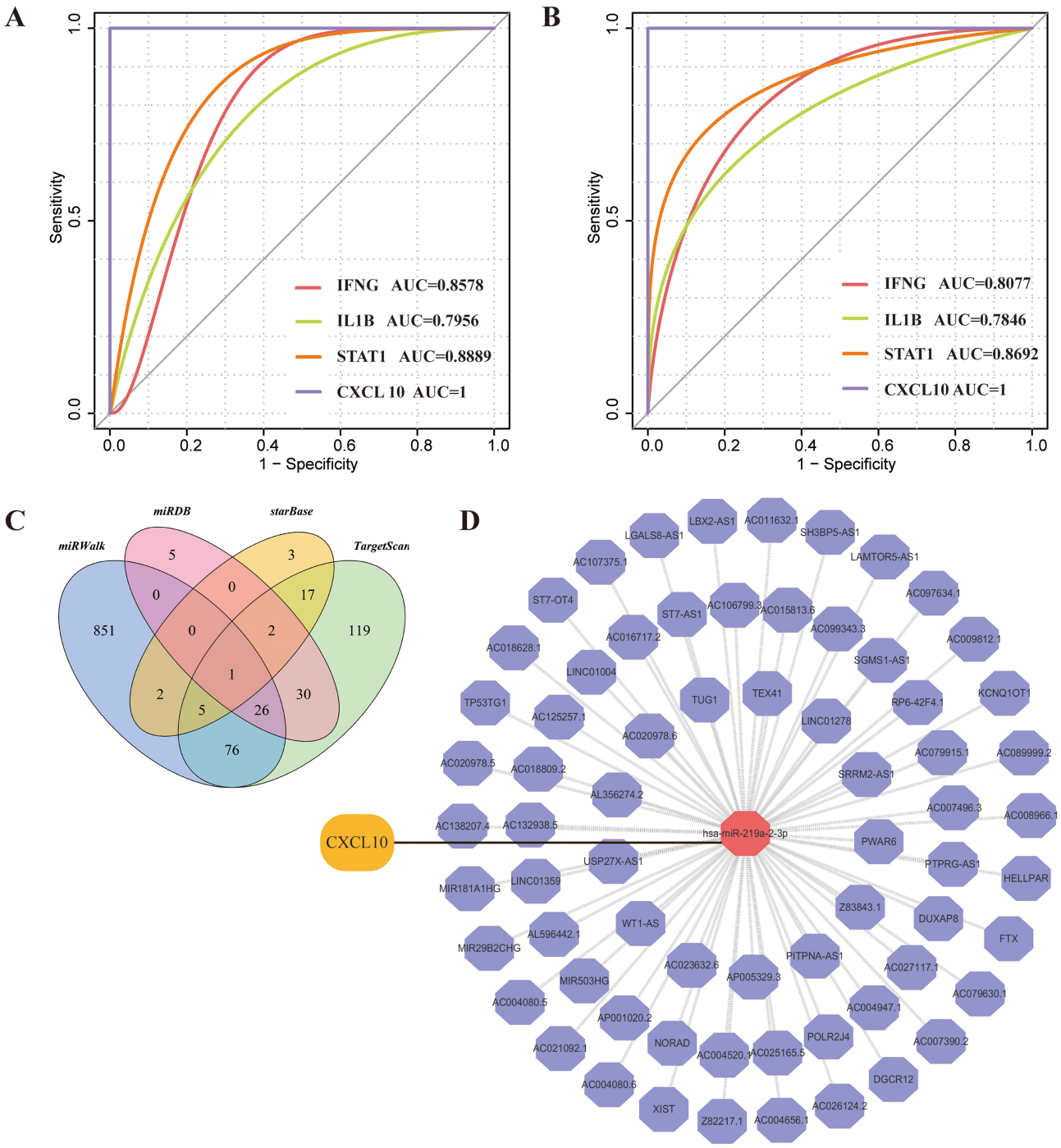

Figure 6 ROC curve and ceRNA network construction. ROC curve of the four hub IRDEGs in vitiligo (**A**) and Hashimoto's thyroiditis (**B**). (**C**) miRNA of CXCL10 predicted by four databases. (**D**) CXCL10-miRNA-lncRNA regulatory network. ROC, receiver operating characteristic; IRDEGs, immune-related differentially expressed genes; AUC, area under the curve.

Discussion

As the commonest cause of acquired disfiguring skin or hair depigmentation, vitiligo has a detrimental influence on a patient's quality of life and mental health.[14] A recent study showed the vulnerability of vitiligo patients to depression, anxiety, skin irritation, and cancer.[15–17] The correlation between vitiligo and autoimmune diseases has been well-established,[6] such as thyroid diseases, alopecia areata, inflammatory bowel disease, type 1 diabetes mellitus, etc. HT,

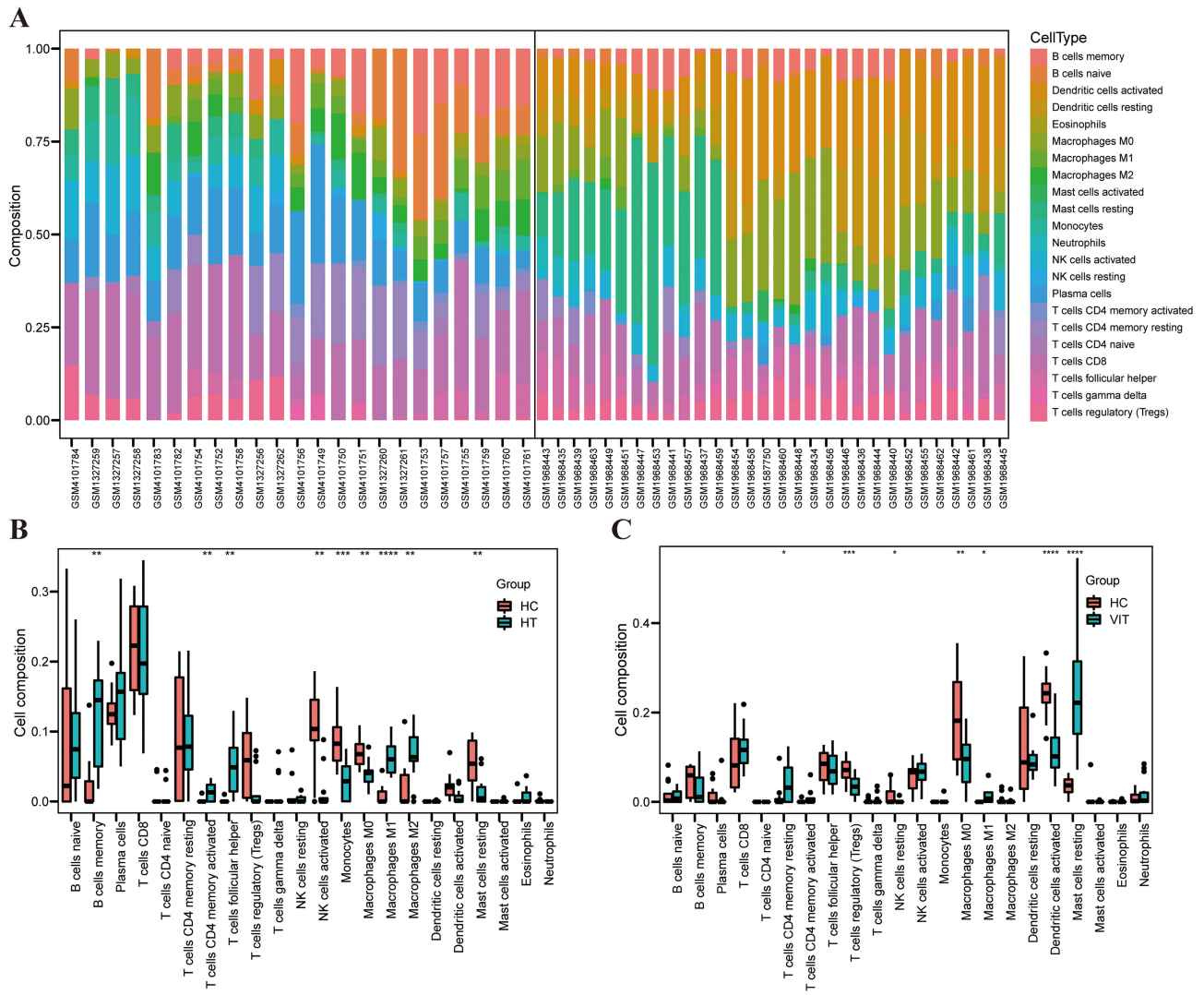

Figure 7 Analysis of the immune landscape. (**A**) Stacked bar chart of immune cell infiltration in HT and vitiligo datasets. The violin plots of the immune cell proportions in the HT dataset (**B**) and vitiligo dataset (**C**). *p < 0.05; **p < 0.01; ***p < 0.001. HT, Hashimoto's thyroiditis.

a familiar cause of hypothyroidism in adults, is also the most common comorbid disease of vitiligo. The comorbidity may bring about a poorer response to the treatment and a higher recurrence rate.[10]

Earlier studies have found multiple vitiligo susceptibility genes encoded proteins involved in immune functions and these genes were shared with autoimmune thyroid diseases.[18] Recent research implied different patterns of melanocyte-specific antigen expression in the thyroid tissue between HT and healthy people,[19] which provided clues to the shared immunological basis for vitiligo and HT. The purpose of our research was to identify the key immune-related genes involved in both vitiligo and HT and investigate the correlation among the hub genes, infiltrating immune cells, and the specific antigens of melanocytes and thyrocytes. Thereby, we might find the overlapped immune process and potential molecular targets to predict and treat this comorbidity.

We extracted the immune-related genes and conducted the differential analysis of vitiligo and HT datasets. Then, we obtained 60 IRDEGs by taking the intersection. GO analysis showed the enrichment of cytokine-associated and receptor ligand-mediated pathways. The enriched KEGG pathways were closely related to T cell-mediated immunity and innate immunity, such as the T cell receptor signaling pathway, IL-17 pathway, Toll-like signaling pathway, and NK cell-mediated cytotoxicity. The results indicated the simultaneous involvement of innate and adaptive immunity.

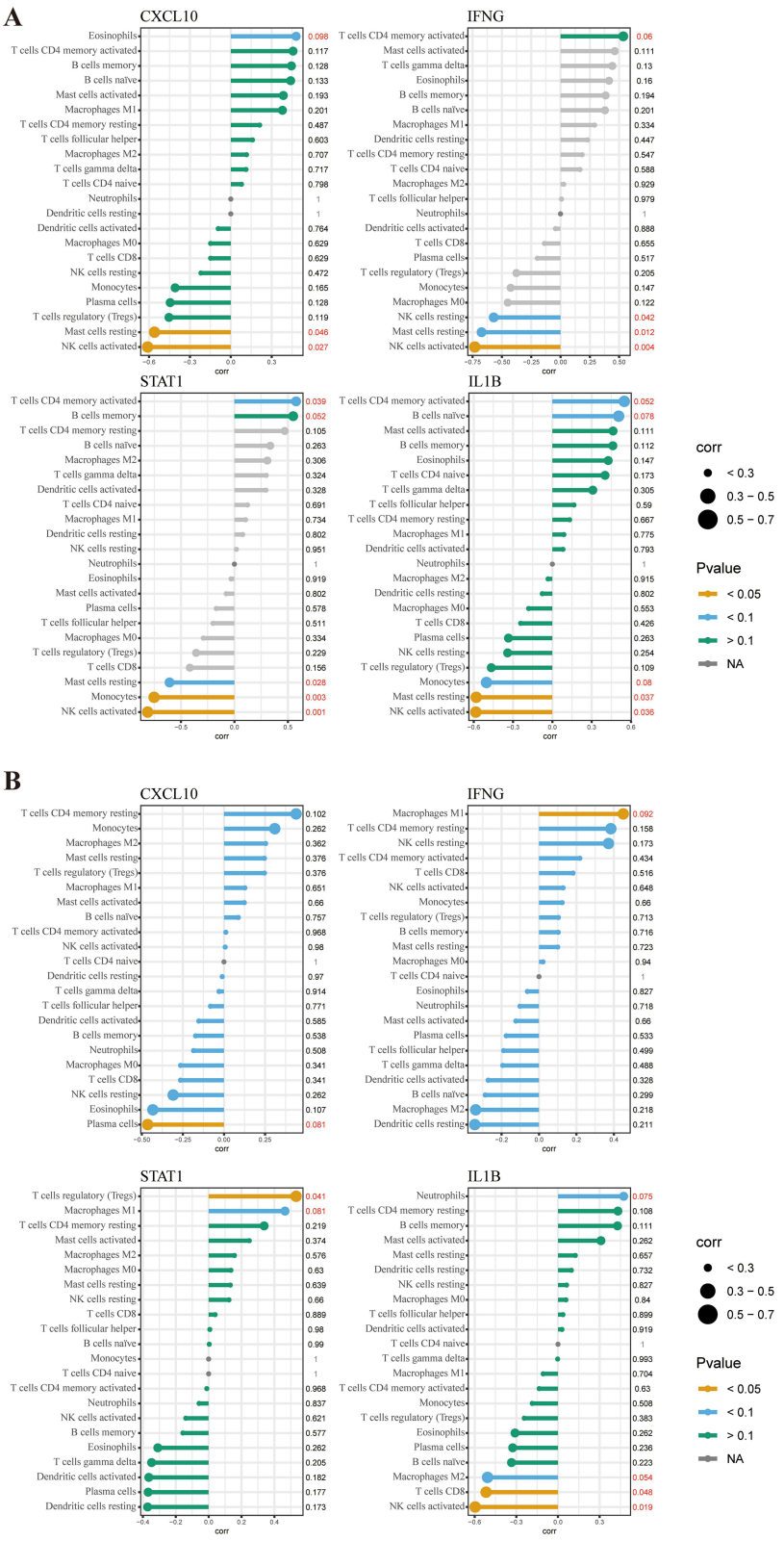

Figure 8 Correlation analysis between the hub immune-related differentially expressed genes and immune cells. (**A**) in Hashimoto's thyroiditis dataset. (**B**) in vitiligo dataset.

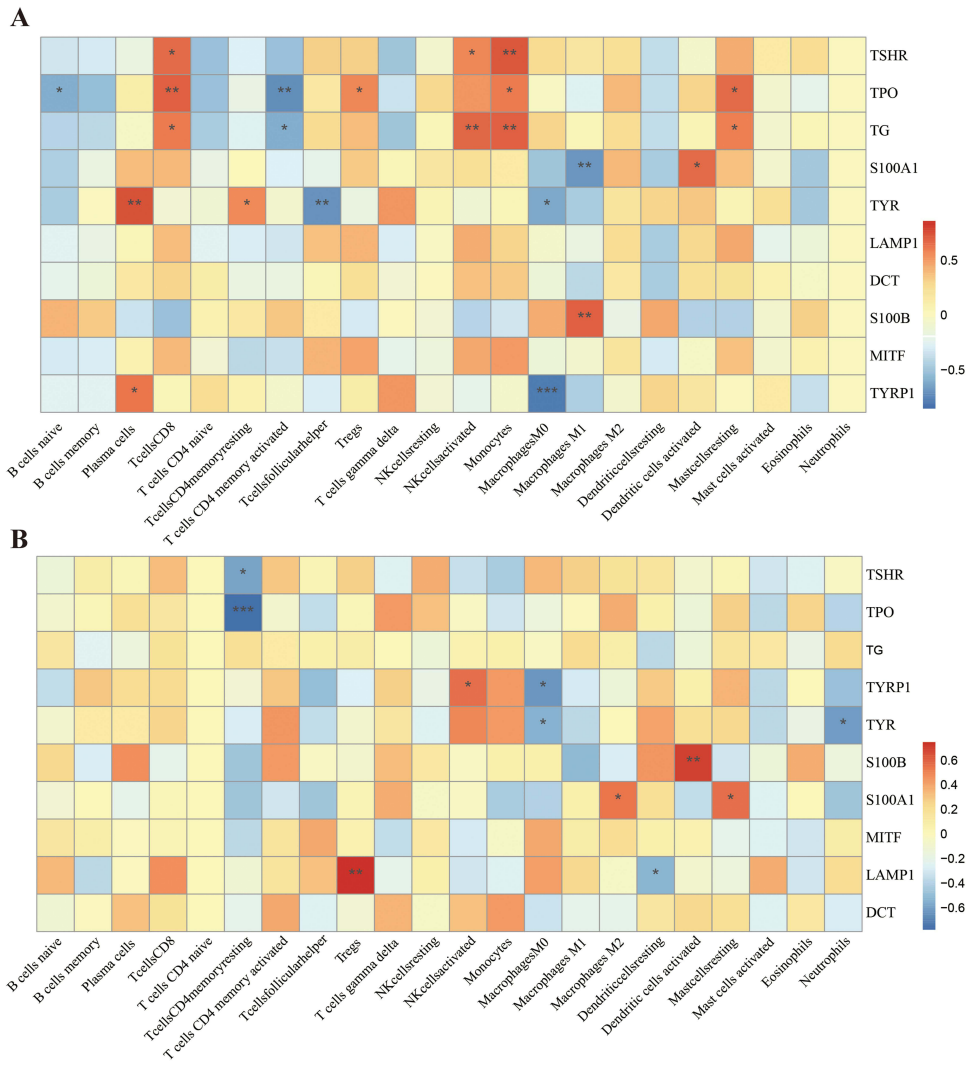

Figure 9 Correlation analysis between antigens and immune cells. (**A**) in Hashimoto's thyroiditis dataset. (**B**) in vitiligo dataset. *p < 0.05; **p < 0.01; ***p < 0.001.

We acquired four hub genes among the IRDEGs (*IFNG, IL1B, STAT1*, and *CXCL10*) as potential biomarkers of the comorbidity, all of which were explored significance to different extents in vitiligo and HT.

Vitiligo patients reflected an IFN-γ-specific signature accompanied by the upregulation of its downstream chemokines CXCL10 and CXCL9 in skin and blood.[20] IFN-γ, a cellular immunity driver and promotor of autoreactive cytotoxic $CD8^+$ T cells, is destructive to melanocytes in vitiligo. It was demonstrated that both natural killer cells and innate lymphoid cells can produce IFN-γ in the presence of reactive oxygen species (ROS), a shared pathogenic factor of HT and vitiligo.[21] In the IFN-γ transgenic mice model, the constitutive expression of IFN-γ in the thyroid follicular cells induced disruption of thyroid architecture and function.[22] It was found in NOD.H-2h4 mice that IFN-γ mediated the development of lymphocytic autoimmune thyroiditis and inhibited the hyperplasia and proliferation of thyrocytes.[23]

CXCR3, the common receptor of CXCL9 and CXCL10, is expressed on pathogenic T cells which are recruited by the CXCL9/10 to the target tissue and subsequently produce IFN-γ, making a positive feedback loop. Expression of CXCR3 was strongly upregulated in the melanocytes of vitiligo patients and the lymphocytes from neoplastic nodules of HT patients.[24] The activation of CXCR3 isoform B was found to be an inducer of cultured melanocyte apoptosis,[21] a vital form of melanocyte loss.

CXCL10 and CXCL9, both combined with CXCR3, have different weights in inflammatory diseases.[25,26] CXCL10 is dominant over CXCL9 in vitiligo and HT.[27,28] CXCL9 recruits the immune cells while CXCL10 mediates the migration

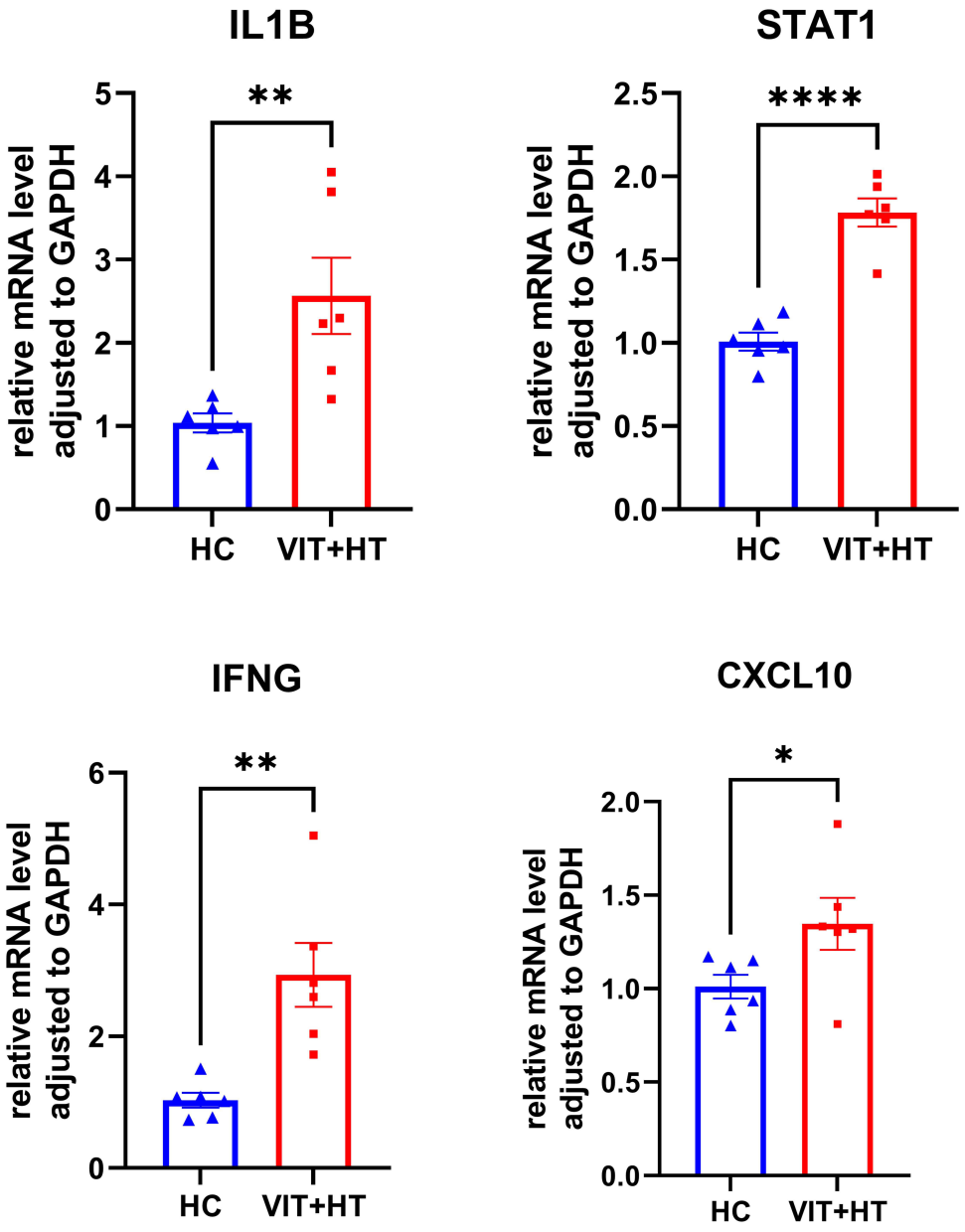

Figure 10 Expression level of the hub genes. The relative expression level of the four hub genes adjusted to GAPDH. *p < 0.05; **p < 0.01; ***p < 0.001.

and localization of melanocyte-specific $CD8^+$ T cells to the epidermis.[20] CXCL10 is correlated with disease severity, activity, treatment response, and outcome in vitiligo.[29] In the mouse model, the neutralization of CXCL10 was able to reverse the depigmentation partially. The upregulation of the level of CXCL10 was also found in the serum and tissue of HT patients[30] with a higher titer in those with a more aggressive thyroid autoimmune process such as hypothyroidism or a hypoechoic ultrasonographic pattern.[31] CXCL10 is absent in normal human thyrocytes but could be triggered by synergistic stimulation of IFN-γ and TNF-α in a dose-dependent pattern.[32]

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway is composed of a rapid membrane-to-nucleus signaling module and a subsequent transduction cascade regulating multiple-immunology processes. IFN-γ is the primary cytokine mediator that activates the JAK/STAT1 pathway. In vitiligo, IFN-γ binds to the keratinocyte-surface receptors and recruits JAKs to transduce the signal. The phosphorylated STAT1 translocates to the nucleus and induces the expression of IFN-γ stimulated chemokines, such as CXCL9 and CXCL10,[33] which recruit cytotoxic T cells to the epidermal,

where melanocytes are destructed. Similarly, activated STAT1 dimers were detected in germinal macrophages and infiltrating lymphocytes in HT.[34]

IL-1β is one of the most prominent messengers of inflammatory signaling and was found upregulated in active vitiligo patients[35,36] and HT patients.[37] It drives the differentiation and activation of Th1 and Th17 cells. IL-17 is the downstream cytokine of Th17 cells. IL-17 signaling pathway was enriched in the KEGG pathway enrichment.[38] IL-1β, with the help of IL-23, promotes the production of innate IL-17 from γδ T cells, which further contributes to the generation of Th17 cells.[39] The pathogenic Th17 cells contribute to the secretion of IFN-γ and then add to the IFN-γ-mediated inflammatory disease.[40] As the inducer of FAS expression on thyroid follicular cells, IL-1β mediates the apoptosis of thyroid follicular cells through FAS/FASL signaling. Grave's disease is another autoimmune thyroid disease and is distinguished from HT histologically with lymphocyte infiltration but no destruction of thyrocytes. The level of IL-1β is lower in the thyroid tissue of Grave's disease than that of HT, which implies a critical role of IL-1β in thyrocyte destruction.[41]

The ROC curve indicated the predominant diagnostic value of *CXCL10* for both vitiligo and HT. We predicted the most essential miRNA, hsa-miR-219a-2-3p, for *CXCL10*, and forecasted the *CXCL10*-miRNA-lncRNA regulatory axis using the starBase database. MiRNA-219a-2-3p has been reported in regulating proliferation and apoptosis in different cells and diseases. The overexpression of miR-219a-2-3p can induce thyroid cancer cell cycle arrest at G0/G1 phase by targeting its downstream gene *HPSE*.[42] miR-219a-2-3p also regulates the apoptosis of pituitary adenoma cells[43] and gastric cancer cells.[44] However, the regulatory role of miR-219a-2-3p in thyrocytes or melanocytes is unknown.

We used CIBERSORT to evaluate the composition of 22 kinds of immune cells of vitiligo and HT. Alteration of T cell subsets and macrophage phenotypes were prominent in both HT and vitiligo patients compared with healthy controls. Studies have found the main role of $CD4^+$ T cells in HT. In addition to Th1 cells, recent research also revealed the enhancement of Th17 cells[45] and unbalanced Th17/Treg ($CD4^+CD25^{+high}FoxP3^+$) cells in HT patients.[46] $CD8^+$ T cells are recognized as the main infiltrated immune cells localized to the basal layer skin of vitiligo. In addition, infiltration of $CD4^+$ T cells and the deficiency of Treg cells were also detected.[47,48] This implies overlapping immune cell infiltration in this comorbidity. The polarization of M0 to M1 macrophages is induced by Th1 cytokines such as IFN-γ and TNF-α. The predominance of STAT1 activation promotes M1 macrophage polarization, too. M1 macrophages are engaged in ROS-induced organelle or tissue damage and result in cytotoxic and proinflammatory functions by secreting cytokines including IL-1β, IL-6, IL-23, CXCL9, CXCL10, etc.[49]

Correlation analysis indicated that hub IRDEGs were significantly associated with the shifts in immune cell populations. Melanocyte-specific antigens are expressed differently in thyroid tissues of HT patients and healthy individuals. Thyroid tissues of HT patients without vitiligo express *TRP-2, LAMP1* and *CD69*, but do not express *NKI-β, Pmel17, TRP1, HMB45* and *S100*.[50] A variety of skin cells, such as melanocytes, keratinocytes, and fibroblasts, can express thyroid-specific epitopes (*TSHR, TG*, and *TPO*).[51,52] With correlation analysis, we found the types of immune infiltrating cells, especially the subsets of T cells and macrophages, were significantly correlated with the expression of the antigens. The activated immune cells in vitiligo patients might cause secondary thyroid damage by attacking the melanocyte-specific antigens expressed in the thyroid tissue.

Patients of vitiligo and HT showed a higher expression level of the four hub genes quantified by RT-qPCR. The consistent results may provide a basis for the novel potential biomarkers or targets for the diagnosis and treatment of this comorbidity.

Limitation

Despite the meaningful results of this study, there were certain limitations. In addition to the relatively small sample size of this study, we have only verified the genes at the transcriptome level. Animal and cell experiments are needed to identify the predicted mRNA-miRNA-lncRNA axis, function of the altered immune cells, and enriched signaling pathways in this study.

Conclusions

In summary, we selected four hub genes, *IFNG, STAT1, IL1B* and *CXCL10*, which might be profoundly involved in the pathogenesis of both vitiligo and HT (Figure 11). The IRDEGs could participate in the comorbidity by reshaping innate and

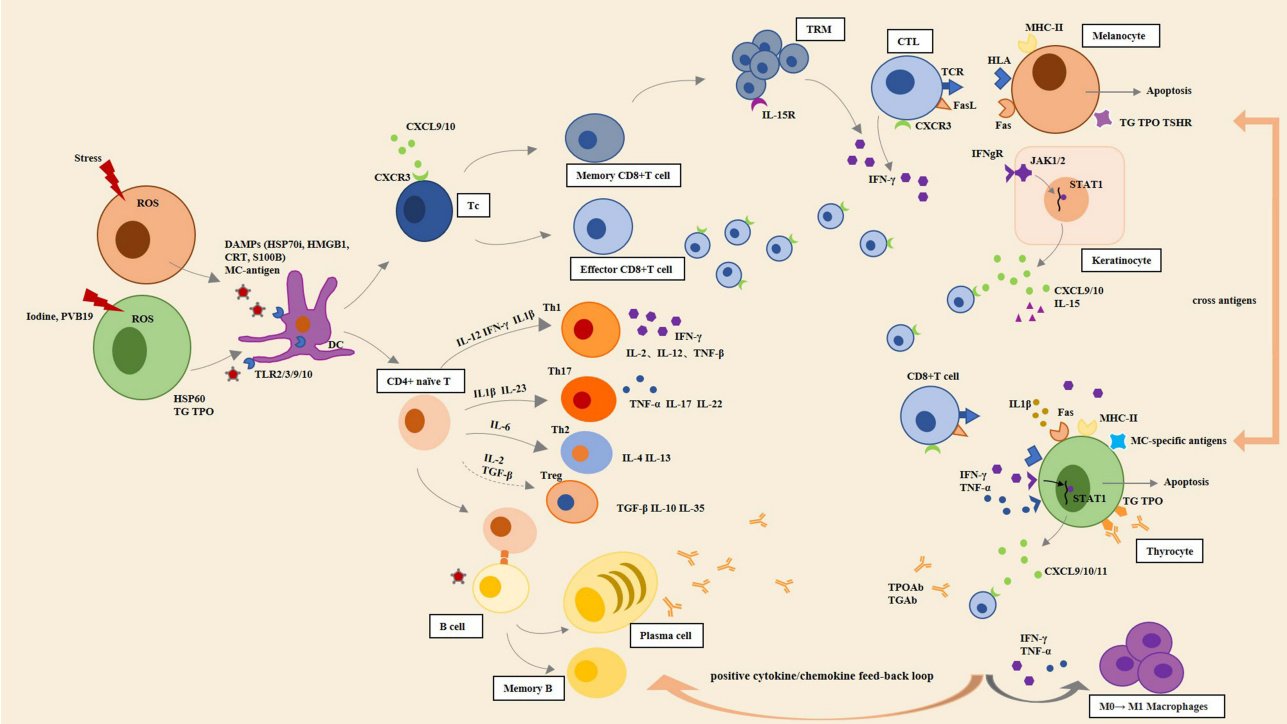

Figure 11 Hypothesis of the pathogenesis of the comorbidities. Intrinsic defective melanocytes and thyroid cells may release DAMPs, such as HSP70i from melanocytes and HSP60 from thyrocytes, exposed to oxidative stress, excessive iodine, and pathogens. Their binding to TLRs on the surface of immune cells can activate innate and adaptive immunity. Effector CTL cells are recruited target tissues by CXCL9 and CXCL10. CTL cells destroy the target cells by releasing cytotoxic particles or through Fas/FasL approach. IFN-γ released by CTL cells activates the JAK/STAT pathway, which stimulates the release of CXCL9/10, forming a positive feedback loop. The activation of memory $CD8^+$ T cells and tissue-resident memory T cells are the main cause of the relapse. With the induction of cytokines such as IL-1β and IFN-γ, T helper cells differentiate into Th1 and Th17 subsets, and release their downstream cytokines, such as IFN-γ, TNF-α, and IL-17. IFN-γ and TNF-α can synergistically activate JAK/STAT pathway in thyroid follicular cells and upregulate the expression of FAS and MHC-II on the cell surface. IFN-γ and TNF-α also induce the polarization of macrophages from M0 to classical M1 macrophages, which produce pro-inflammatory cytokines such as IL-1β, IL-6, IL-12, IL-23, and TNF-α. These cytokines induce the differentiation of Th subsets in turn. Melanocytes and thyrocytes express crossed tissue-specific antigens, which may have contributed to the simultaneous attack of skin and thyroid by circulating immune cells.

Abbreviations: DAMPs, damage-associated molecular patterns; HSP, heat shock protein; TLRs, toll-like receptors; CTL, cytotoxic T lymphocytes; CXCL, chemokine (C-X-C motif) ligand; JAK/STAT pathway, janus kinase/signal transduction and transcription activation pathway; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; Th, T helper; MHC, major histocompatibility complex; ROS, reactive oxygen species; PVB19, Parvovirus B19; DC, dendritic cell; TRM, $CD8^+$ tissue-resident memory T cells; MC, melanocyte; TG, thyroglobulin; TPO, thyroid peroxidase.

adaptive immunology. The simultaneous damage to the skin and thyroid might be attributed to the alteration of the T cell subtypes, macrophage polarization, and the cross-expressed antigens. Large-sample studies and experimental research are needed to further explore the exact role of these key molecules and their possible clinical values.

Ethics Approval and Consent to Participate

The experiment of the study was approved by the Medical Ethics Committee of the first affiliated hospital of Nanjing Medical University (Ethics approval number: 2022-SR-583). Written informed consent was obtained from all participants.

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Disclosure

The authors report no conflicts of interest in this work.

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