Identification and Validation of Immune-Related Genes and Immune Cell Infiltration in Preeclampsia

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Research Article

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Abstract

Background

Preeclampsia (PE) is a complex multisystem disease and its etiology remains unclear. The aim of this study was to identify potential immune-related diagnostic genes for PE, analyze the role of immune cell infiltration in PE, and explore the mechanism underlying PE-induced disruption of immune tolerance at the maternal-fetal interface.

Methods

We used the PE dataset GES25906 from Gene Expression Omnibus and immune-related genes from ImmPort database. The differentially expressed genes (DEGs) were identified using the “limma” package, and the differentially expressed immune-related genes (DEIGs) were extracted from the DEGs and immune-related genes using Venn diagrams. The potential functions of DEIGs were determined by Gene Ontology and Kyoto Encyclopedia of Genes and Genomes analyses. Furthermore, the protein–protein interaction network was obtained from the STRING database, and it was visualized using Cytoscape software. Least absolute shrinkage and selection operator logistic regression was used to verify the diagnostic markers of PE and build a predicting model. The model was validated using datasets GSE66273 and GSE75010. Finally, CIBERSORT was used to evaluate the infiltration of immune cells in PE tissues.

Results

Six genes (*ACTG1*, *ENG*, *IFNGR1*, *ITGB2*, *NOD1*, and *SPP1*) enriched in Th17 cell differentiation, cytokine-cytokine receptor interaction, innate immune response, and positive regulation of MAPK cascade pathways were identified, and a predicting model was built. Datasets GSE66273 and GSE75010 were used to validate the model, and the area under the curve was 0.8333 and 0.8107, respectively. Immune cell infiltration analysis revealed an increase in plasma cells and gamma delta T cells and a decrease in resting natural killer cells in the high score group according to the predictive model risk values.

Conclusions

We developed a risk model to predict PE and proved that immune imbalance at the maternal-fetal interface plays a key role in the pathogenesis of PE.

Background

Preeclampsia (PE) is the second leading cause of death in pregnant women in developing countries[1], affecting approximately 2%-8% pregnancies[2]. It is a complex multisystem disease, and despite extensive research, the exact etiology of PE remains unclear. The pathophysiology of PE includes defective deep placentation, oxidative stress, endothelial dysfunction, presence of an anti-angiogenic state, and intravascular inflammation[3]. In normal pregnancies, proper functioning of the maternal immune system response is essential to maintain immune tolerance to the allogeneic fetus and to protect the fetus from immune rejection[4]. This requires a good balance between effector cells and regulatory immune cells for successful implantation and maintenance of pregnancy until delivery[5]. Many studies have shown that PE is characterized by quantitative and qualitative modifications of both the systemic and local immune cell responses[3]. To explore the mechanism of how PE disrupts immune tolerance at the maternal-fetal interface, we downloaded microarray datasets of PE from the Gene Expression Omnibus (GEO) database and performed differentially expressed gene (DEG) analysis. Moreover, immune-related genes were downloaded from the ImmPort database and intersected with DEGs to obtain differentially expressed immune-related genes (DEIGs). We used protein-protein interaction (PPI) network and least absolute shrinkage and selection operator (LASSO) logistic regression to further screen the diagnostic markers of PE and build a prediction model. Subsequently, we used CIBERSORT to analyze the difference in immune infiltration between high score groups and low score groups according to predictive model risk values in 22 immune cell subsets. The objectives of this study were to better understand the molecular immune mechanisms underlying the development of PE and to determine new markers for the prediction and immunotherapy of PE.

Materials And Methods

Data download and preprocessing

The GSE25906 dataset, which contains transcriptional profiles of preeclamptic human placentas, and clinical data of 23 preeclampsia patients and 37 normotensive controls were downloaded from the GEO database. The downloaded datasets were independently pre-processed for quantile normalization and log2 transformation. The corresponding genes transformed into a probe were converted into a symbol according to the annotation information on the platform.

Screening of DEIGs

DEGs were screened using the R package limma. The volcano plot was plotted using the R package ggplot2. DEGs with $p < 0.05$ and $|log2FC|>1$ were considered statistically significant. We downloaded a list of 2483 immune relevant genes from ImmPort database (https://www.immport.org). DEIGs were extracted from the DEGs and immune-related genes using Venn diagrams.

Enrichment analysis of DEIGs

We used DAVID (https://david.ncifcrf.gov/) to carry out Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes pathway (KEGG) enrichment analysis of the DEIGs.

PPI network construction and hub gene identification

DEIGs were imported into the STRING (https://string-db.org/) database. The PPI network was visualized via Cytoscape 3.7.2. The MCODE plugin was used to analyze the interaction relationships of the DEIGs with their encoded proteins and to screen the hub genes.

Identification and verification of diagnostic markers

We used LASSO logistic regression for feature selection to screen diagnostic markers for PE. The LASSO algorithm was applied with the “glmnet” package. The joint diagnostic efficiency of the obtained diagnostic markers was verified based on the GSE66273 and GSE75010 datasets.

Evaluation of immune cell infiltration

We calculated the risk value of PE for each sample according to the predictive model and divided samples into high and low score groups. We uploaded the gene expression matrix data to CIBERSORT, filtered out the samples with $p < 0.05$, and obtained the immune cell infiltration matrix. Next, “ggplot2” package was used to generate violin diagrams to visualize the differences in immune cell infiltration between the high and low score groups.

Results

Identification of DEIGs

Based on the cut-off criteria of p□0.05 and |log2FC| □1, we identified a total of 929 DEGs between normotensive and PE pregnancies from GSE25906, as shown in the volcano map (Figure 1). We obtained 53 DEIGs by crossing the DEGs with 1793 immune relevant genes downloaded from the ImmPort database, which were used to generate a Venn diagram (Figure 2).

Enrichment analysis of DEIGs

We used DAVID website to analyze the DEIGs in the GO and KEGG pathways. As shown in Figure 3A, the top five KEGG enriched terms were cytokine-cytokine receptor interaction, human T-cell leukemia virus 1 infection, Th17 cell differentiation, osteoclast differentiation, and influenza A. Figure 3B shows the first 15 enrichment results of biological processes, mainly enriched in the regulation of innate immune response, regulation of leukocyte activation, regulation of lymphocyte activation, positive regulation of

mitogen-activated protein kinase (MAPK) cascade, and innate immune response–activating signal transduction. DEIGs linked with cellular components were significantly enriched in adherens junction, receptor complex, side of membrane, focal adhesion, and cell–substrate adherens junction (Figure 3C). As for molecular function, DEIGs were significantly enriched in receptor ligand activity, receptor regulator activity, cytokine activity, cytokine binding, and growth factor activity (Figure 3D).

PPI network construction and hub gene identification

STRING database was used to construct the PPI network of 53 DEIGs. The interaction network consisted of 41 nodes and 71 edges, and it was visualized using Cytoscape version 3.7.2 (Figure 4A). Then, top 15 hub genes were identified based on the degree algorithms (each node had more than three interactions) using Cytohubba plugin with Cytoscape v3.7.2 (Figure 4B).

Screening and verification of diagnostic markers

R package 'glmnet' was utilized for LASSO regression analysis. LASSO logistic regression analysis was performed on the 15 hub genes and regression coefficients were calculated (Figure 5A). The model performed best when six genes were included (Figure 5B). *ACTG1*, *ENG*, *IFNGR1*, *ITGB2*, *NOD1*, and *SPP1* were identified and the value of lambda.min was 0.02382411. We constructed receiver operating characteristic curves of six hub genes for prediction of PE. The model formula was: index=ACTG1*(-0.9263502)+ENG*1.6130049+IFNGR1*0.7304779+ITGB2*(-0.2842581)+NOD1*1.8807506+SPP1*(-0.5979777). Receiver operating characteristic curve analysis indicated that the area under the curve (AUC) of the immune gene-related model was 0.9929. We used GSE66273 and GSE75010 datasets to validate the predictive ability of the model, and the AUC was 0.8333 and 0.8107, respectively, which showed that the model had a strong predictive ability in PE (Figure 5C).

Immune cell infiltration in preeclampsia

The 60 GSE25906 samples were divided into the high score and low score groups (n = 26 and 34, respectively) based on the median scores for predicting the risk of PE. The CIBERSORT algorithm was used to predict the proportion of 22 types of infiltrated immune cells. The Violin plot was used for the CIBERSORT estimation of immune cell infiltration. Compared to that in the low score group, the number of plasma cells and gamma delta T cells increased (p□0.01), whereas the number of resting natural killer (NK) cells decreased (p□0.05) in the high score group (Figure 6).

Discussion

Appropriate placental formation depends on the mutual adaptation of the chorionic trophoblast and maternal immune system to produce immune tolerance. Excessive immune activation and inflammatory

response play a key role in the pathophysiological mechanisms of PE. Imbalance in immune cell subtypes, cytokines released by imbalanced cells, and interactions between pathways cause dysfunctional trophoblast invasion, endothelial dysfunction, inflammatory damage, and ultimately PE. We explored the effect of changes in the immune system at the maternal-fetal interface on the development of PE using bioinformatics methods.

In this study, we identified 53 DEIGs. KEGG enrichment analysis showed that these genes were mainly enriched in cytokine-cytokine receptor interaction, Th17 cell differentiation, and other pathways. In normal pregnancy, Th1 cells are upregulated early in gestation to support early invasion of trophoblast cells into the spiral arteries of the uterus. After placental implantation, immune cells shift from Th1 to Th2 phenotype to support a more anti-inflammatory state until delivery. Regulatory T cell (Treg) and Th2 cell polarization favors a more suppressive immune tolerance state. Conversely, Th1 and Th17 cell polarization favors a pro-inflammatory state. Pro-inflammatory Th cell bias is strongly associated with the development of PE[6]. S.M. Scroggins et al.[7] injected vasopressin into mice to induce PE resulting in an increase in Th1 and Th17 cells and a decrease in Treg and Th2 cell production, suggesting that the Treg/Th17 ratio is imbalanced and that Th17 cell conversion leads to PE. Other studies have also found a significantly higher proportion of circulating Th17 cells in women with PE than that in normal pregnant women[8–9]. In addition, the placenta of pregnant women with PE showed a decrease in Treg cells and an increase in Th17 cells[10]. Wallace et al.[11] induced an increase in CD4[+] T cells in a reduced uterine perfusion pressure (RUPP) rat model, which was characterized by increased Th17 cells and decreased Treg cells, similar to that in patients with PE. The percentage of Th17 cells in peripheral blood was 7% in normal pregnant rats and 22% in RUPP rats. Th17 cells secrete the pro-inflammatory cytokine IL-17[12]. Therefore, plasma IL-17 levels increase significantly in pregnant women with PE. Transfer of CD4[+] T cells from RUPP rats to normotensive rats induced an increase in angiotensin II type I receptor (AT1-AA) and oxidative stress, leading to hypertension, similar to that observed in RUPP rats[11]. IL-17 also activates the MAPK pathway, which promotes the expansion of Th17 cells[13]. Blockade of the IL17 signaling cascade via administration of soluble IL-17 receptor C in the RUPP rat model attenuates Th17 cell levels, oxidative stress, AT1-AA, and hypertension in the model[14].

GO enrichment analysis showed that DEIGs were mainly enriched in regulation of innate immune response, positive regulation of MAPK cascade, adherens junction, and other pathways. The MAPK signaling pathway is involved in the regulation of eukaryotic gene expression and can be activated by growth factors and inflammatory factors. Through intercellular signaling molecular interactions, the "MAPKKK-MAPKK-MAPK" is sequentially activated to transmit extracellular signals from the surface of the cell membrane to the nucleus, regulating different physiological and pathological responses. Conventional MAPKs comprise the extracellular signal-regulated kinases 1/2, c-Jun amino (N)-terminal kinases1/2/3, p38 isoforms, and extracellular signal-regulated protein kinase 5[15]. The innate immune system comprises cells that express a series of receptors known as the toll-like receptors (TLRs). TLRs have the ability to recognize pathogen-associated molecular patterns, such as bacterial lipopolysaccharide (LPS). LPS is recognized by the TLR4 receptor[16]. LPS stimulates excessive activation

of TLR4/p38 MAPK signaling and increases the levels of cytokines IL-6 and IL-8, leading to insufficient trophoblast invasion and uterine spiral artery recasting, which form the basis of PE pathogenesis; blocking this signaling pathway can prevent PE-like symptoms and the development of PE[17]. The above research results are consistent with ours, suggesting that the results of our study are accurate.

In this study, 15 hub genes were screened with the PPI network, and LASSO regression was used to construct a prediction model of immune-related risk of PE. LASSO regression is suitable for fitting models with numerous variables and a small number of instances of each variable. LASSO regression can treat all independent variables simultaneously compared to traditional stepwise regression, and this improvement makes the model much more stable[18]. The final prediction model was: index=ACTG1*(-0.9263502)+ENG*1.6130049+IFNGR1*0.7304779+ITGB2*(-0.2842581)+NOD1*1.8807506+SPP1*(-0.5979777). Our model predicts the incidence of PE with an AUC of 0.9929. External validation of the model with data from GSE66273 and GSE75010 showed an AUC of 0.8333 and 0.8107, respectively, indicating that the model has good predictive power.

ACTG1 encodes gamma actin. Actins are a major component of cytoskeleton and play a central role in nearly all aspects of cellular processes. Gamma actin is one of the two cytoplasmic 'nonmuscle' actins[19]. Actin expression in PE is downregulated compared with that in normal pregnancy[20]. A modest decrease in gamma cyto actin levels induced by siRNA knockdown causes severe cell migration defects[21]. P38 MAPK is activated in response to interferon-gamma (IFN-γ) and leads to actin rearrangement and cell morphological changes. This then mediates hyperpermeability of endothelial cells, which is one of the pathophysiological bases of PE[22].

ENG encodes endoglin (Eng), which is a transmembrane glycoprotein and an accessory receptor for the transforming growth factor-beta (TGF-β)[23]. Soluble endoglin (sEng) is produced by matrix metalloproteinase 14-mediated protein hydrolysis cleavage of Eng[24]. Endoglin and sEng levels significantly increase in the placenta of severe PE patients[25]. Non-pregnant mice continuously exposed to high circulating levels of sEng exhibit PE-like symptoms, such as hypertension and proteinuria. Trophoblast cells subjected to hypoxia, oxidative stress, or inflammation increase the release of sEng[26]. Arterial remodeling occurs when extravillous cytotrophoblasts, which are found around the spiral arteries, invade the vessel wall and reach their inner face, temporarily substituting the endothelial cells and leading to vessels with lower resistance, and thus higher blood flow, in a process called pseudovasculogenesis. High levels of sEng modify the pseudovasculogenesis process, maintaining the epithelial phenotype, while weakening the endothelial-like phenotype of trophoblast cells, thereby reducing their invasive ability and leading to PE[27].

IFN-γ exerts its activities by binding to its specific cell surface receptor. The IFN-γ receptor consists of a and b subunits, which are encoded by *IFNGR1* and *IFNGR2*, respectively. The subunit plays a critical role in ligand-receptor interaction and intracellular signal transduction that follows[28]. IFN-γ is abundantly produced by pregnancy-associated uterine natural killer (uNK) cells in the maternal endometrium. IFN-γR1

is expressed by human uterine epithelium[29]. Sheibak N found a significant increase in the expression of IFN-γ in the placental syncytiotrophoblast cells of PE and placenta previa groups compared with that in normal pregnancies group[30]. High levels of IFN-γ inhibit the invasion of extravillous trophoblast cells, and the spiral artery remains a narrow vessel, resulting in reduced circulating placental blood flow, placental tissue hypoxia, and release of anti-angiogenic factors, ultimately leading to PE[30].

Integrins are transmembrane protein receptors that mediate cell-matrix and cell-cell adhesion. They consist of non-covalently linked heterodimers, which are composed of α and β subunits. The β2 integrin subfamily consists of the β2 subunit (also termed CD18) in combination with one of four different α (CD11) subunits: αL (CD11a), αM (CD11b), αX (CD11c), or αD (CD11d) [31]. The β2 integrin is encoded by *ITGB2*. Integrins act as receptors for osteopontin (OPN) and they are expressed on the surface of endothelial and trophoblast cells [32]. β2 integrins limit TLR signaling by inhibiting NF-κB pathway activation and promoting p38 MAPK activation, thereby inhibiting TLR-induced inflammatory responses. β2 integrin (Itgb2[-/-]) deficiency leads to hyperresponsiveness to TLR stimuli and promotes the development of PE [33].

Nucleotide-binding oligomerization domains 1 and 2 (NOD1 and 2) belong to the nucleotide-binding oligomerization leucine-rich repeat (NLR) family. The ligands of NOD1 and NOD2 regulate the invasion of trophoblasts via the MAPK/p38 pathway. Blockage of this pathway attenuates the depressant effects of NOD1 and NOD2[34]. Co-stimulation of NOD1 with TLR agonists promotes the initiation of immune responses in Th1 and Th17 cells in mice[35]. PE is associated with increased decidual NOD1 expression. Activation of NOD1 is involved in the physiological inflammation that occurs during pregnancy through a cellular communication mechanism at the maternal-fetal interface and persists throughout pregnancy[36].

SPP1, also known as OPN, is located in the placental syncytiotrophoblasts and in the cytoplasm of capillary endothelial cells[37]. SPP1 is the most highly upregulated extracellular matrix adhesion molecule in the human uterus, as it allows for easy embryo implantation[38]. SPP1 has potential to influence tissue remodeling at the maternal interface by affecting cell-cell and cell-extracellular matrix communication, increasing cell proliferation, migration, and survival, and regulating local cytokine networks [32]. Therefore, it can be used as a marker of placental remodeling. Reduced OPN expression is associated with the pathogenesis of PE[37]. J Xia et al.[39] found significantly lower OPN expression in placental tissue in the PE group than that in the normal pregnancy group ($p<0.05$). Ke R et al. [40] co-cultured smooth muscle cells and endothelial cells to mimic the decidua and myometrium interface, and found that OPN promotes the invasive capacity of trophoblasts via targeting integrin avβ3 in the endothelial-smooth muscle cell co-culture system. Therefore, downregulation of SPP1 in the placenta affects the invasive capacity of trophoblast cells, leading to PE.

We used CIBERSORT to conduct a comprehensive evaluation of 22 types of immune cell infiltration in PE. Plasma cells and gamma delta (γδ) T cells were increased in the high score group. γδT cells are a small and unique subset of T cells that bridge the innate and adaptive immune systems. These cells highly

express TLRs and they can be either regulatory or cytotoxic depending on the presence of cytokines and TLR activation[41]. Placentas of mice treated with TLR3/7/8 agonists show a significant increase in IL-2, IL-6, and IL-17A levels, and enhanced Th17 cell immune response, inducing hypertension, endothelial dysfunction, elevated γδ T cell levels, and placental necrosis[42]. The expression of the γδ T cell receptor(TCR) was increased in the placenta of women with PE compared that in the placenta of women without PE. Levels of γδ T cells are elevated in mice with PE-like features and in pregnant women with PE. Treatments that either block or attenuate PE-like features decrease γδ T cell levels, and depletion of γδ T cells improves PE[41]. Liao A et al.[43] found that the percentage of plasma cells produced in the PE group is significantly higher than that in the normal pregnancy group ($p < 0.05$). Pregnant women with PE produce more antibody-producing cells than those in normal pregnancy group after stimulation with pokeweed mitogen ($p < 0.01$) [43]. Various TLRs are expressed in B cells [44]. TLRs induce B1 cells to differentiate into plasma cells. P38 signaling acts as a key regulator that modulates the expression of plasma cell differentiation transcription factors. The decreased phospho-p38 levels contribute to impaired plasma cell differentiation[45]. As mentioned above, TLR and P38 MAPK are involved in the induction of PE.

The result of immune cell infiltration indicated that resting NK cells were decreased in the high score group. Human NK cells are divided into two distinct subsets based on the expression of CD56 and CD16: CD56dimCD16+ cytolytic NK cells and CD56brightCD16- regulatory NK cells[46]. During normal pregnancy, regulatory NK cells, which are poorly cytolytic, increase in the periphery and decidua, while cytolytic NK cells decrease. PE is associated with a shift in the NK population to the pro-inflammatory cytolytic NK phenotype[47]. Decidual natural killer (dNK) cells are the predominant lymphocytes accumulated at the maternal-fetal interface. dNKs are regulatory NK cells with the phenotype CD56birghtCD16-, and they are mainly distributed in the decidua. Higher numbers of dNK cells were found in PE than in normal term pregnancy. In the presence of elevated levels of TGF-β, the functional heterogeneity (cytotoxicity and angiogenesis) of dNKs is reduced, while blockade of TGF-β signaling improves dNK-mediated angiogenesis[48].

The validity of our conclusions mainly rests on the reliability of the original expression dataset. We applied placental tissues obtained in preeclampsia (GSE25906) to explore differentially expressed genes, however, the results were limited since the small sample size.

Conclusion

In conclusion, the findings of this study revealed that immune imbalance at the maternal-fetal interface plays a key role in the pathogenesis of PE from a bioinformatics perspective. An immune-related gene prediction model for predicting PE was developed, providing a new direction for early diagnosis of PE and an immunomodulatory strategy for future PE treatment.

Abbreviations

AUC, area under the curve; DEG, differentially expressed gene; DEIG, differentially expressed immune-related gene; dNK, decidual natural killer; ENG, endoglin; γδ T cells, gamma delta T cells; GEO, Gene Expression Omnibus; GO, Gene Ontology; IFN-γ, interferon gamma; KEGG, Kyoto Encyclopedia of Genes and Genomes; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NK, natural killer; NOD, nucleotide-binding oligomerization domain; OPN, osteopontin; PE, preeclampsia; PPI, protein-protein network; RUPP, reduced uterine perfusion pressure; sENG, soluble endoglin; TGF-β, transforming growth factor beta; TLR, toll-like receptor; uNK, uterine natural killer

Declarations

Ethics approval and consent to participate

Not aquired.

Consent for publication

The article is original, and has neither been published nor under consideration in another journal.

Availability of data and materials

The data of this study are openly available in GEO (https://www.ncbi.nlm.nih.gov/geo/).

Competing interests

The authors declare no conflicts of interest.

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Authors' contributions

Study design: J.Y.and R.C.; data collection and analysis: R.C.□Q.H. and H.Z.; manuscript writing and figure preparation: R.C.; paper revision: J.Y.and R.C.

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Figures

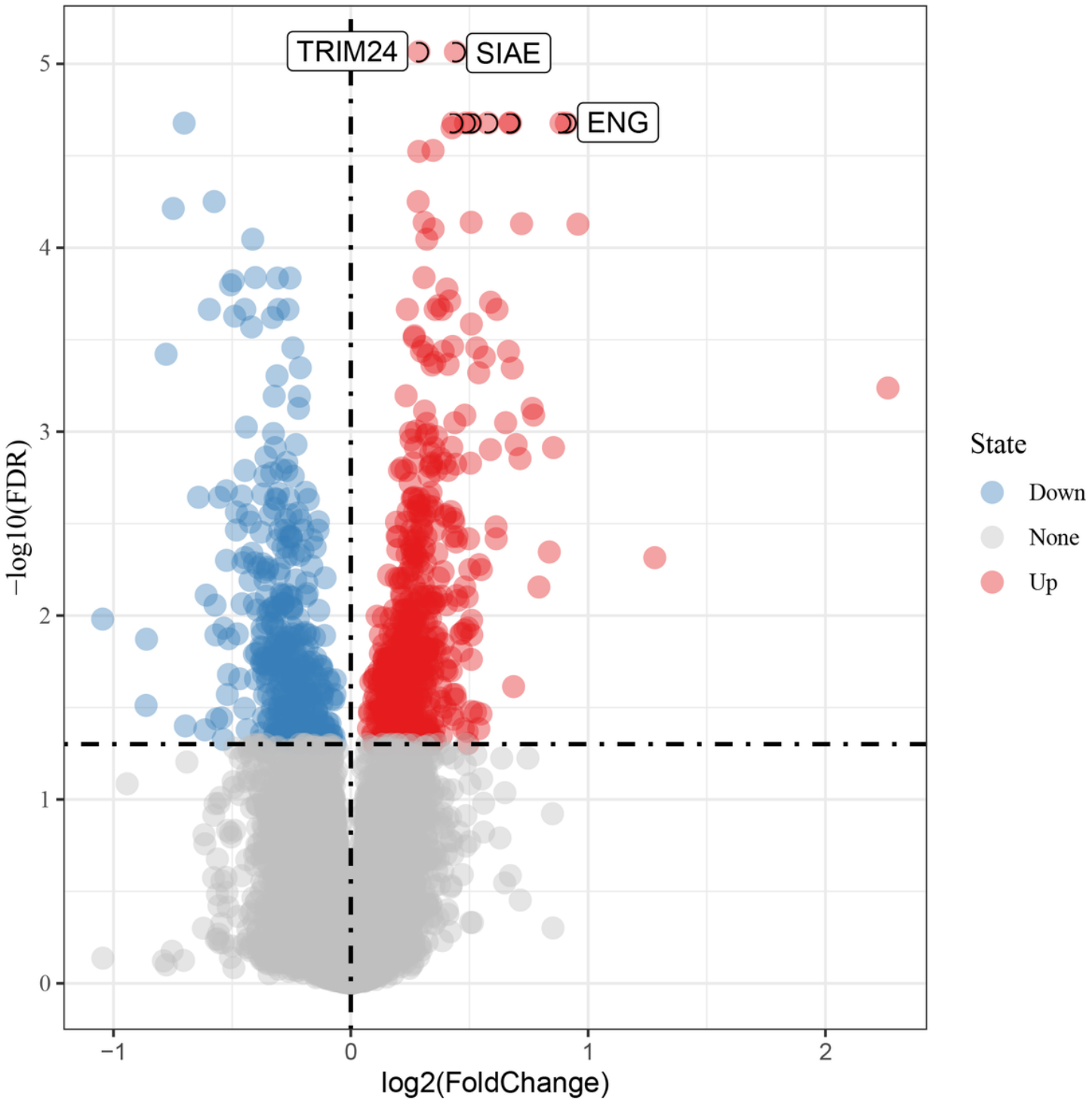

Figure 1

Volcano map of DEGs; red represents up-regulated differential genes, grey represents no significant difference genes, and blue represents down-regulated differential genes.

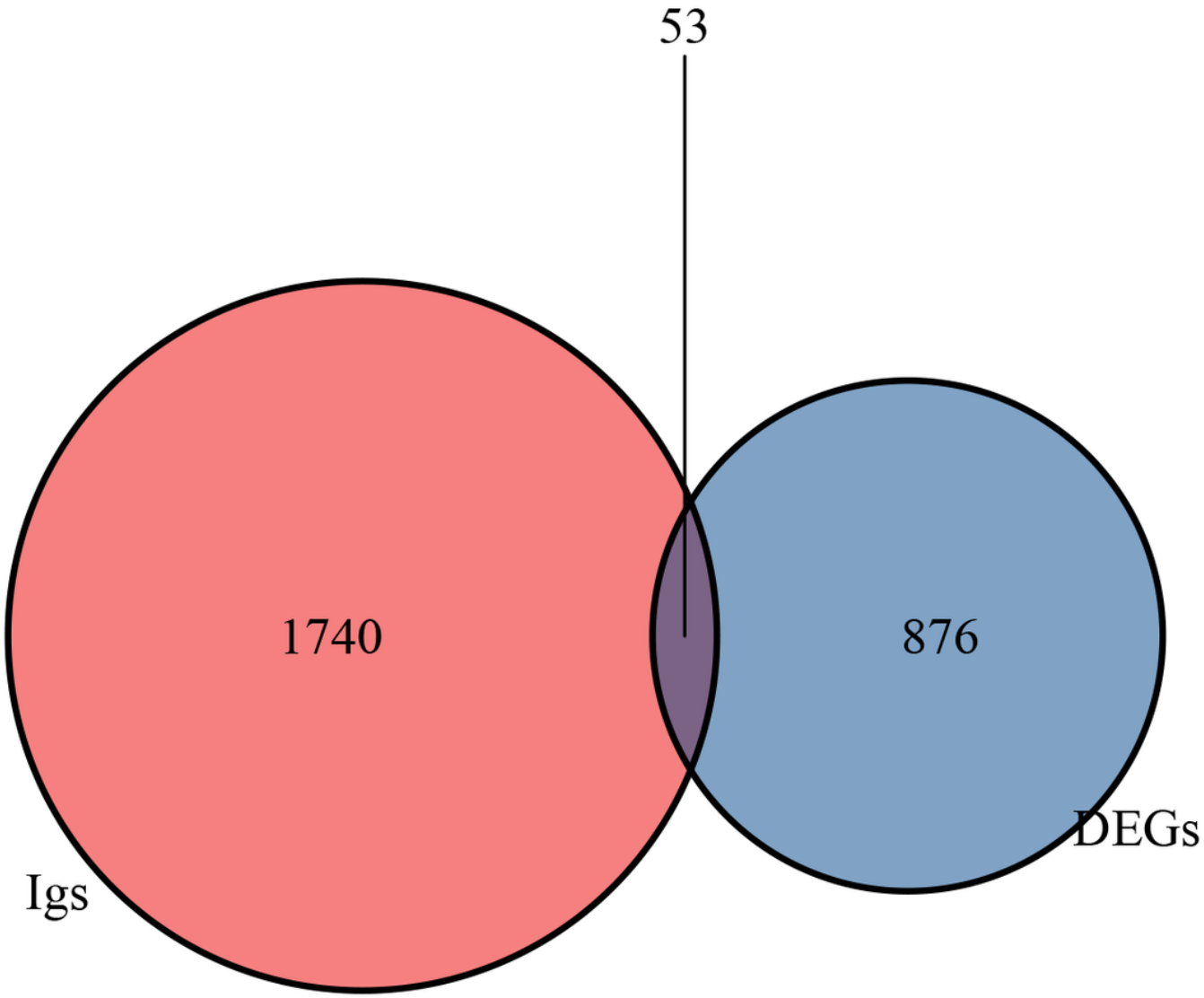

Figure 2

Venn diagram shows the intersection of DEGs and immune genes downloaded from Immport Database.

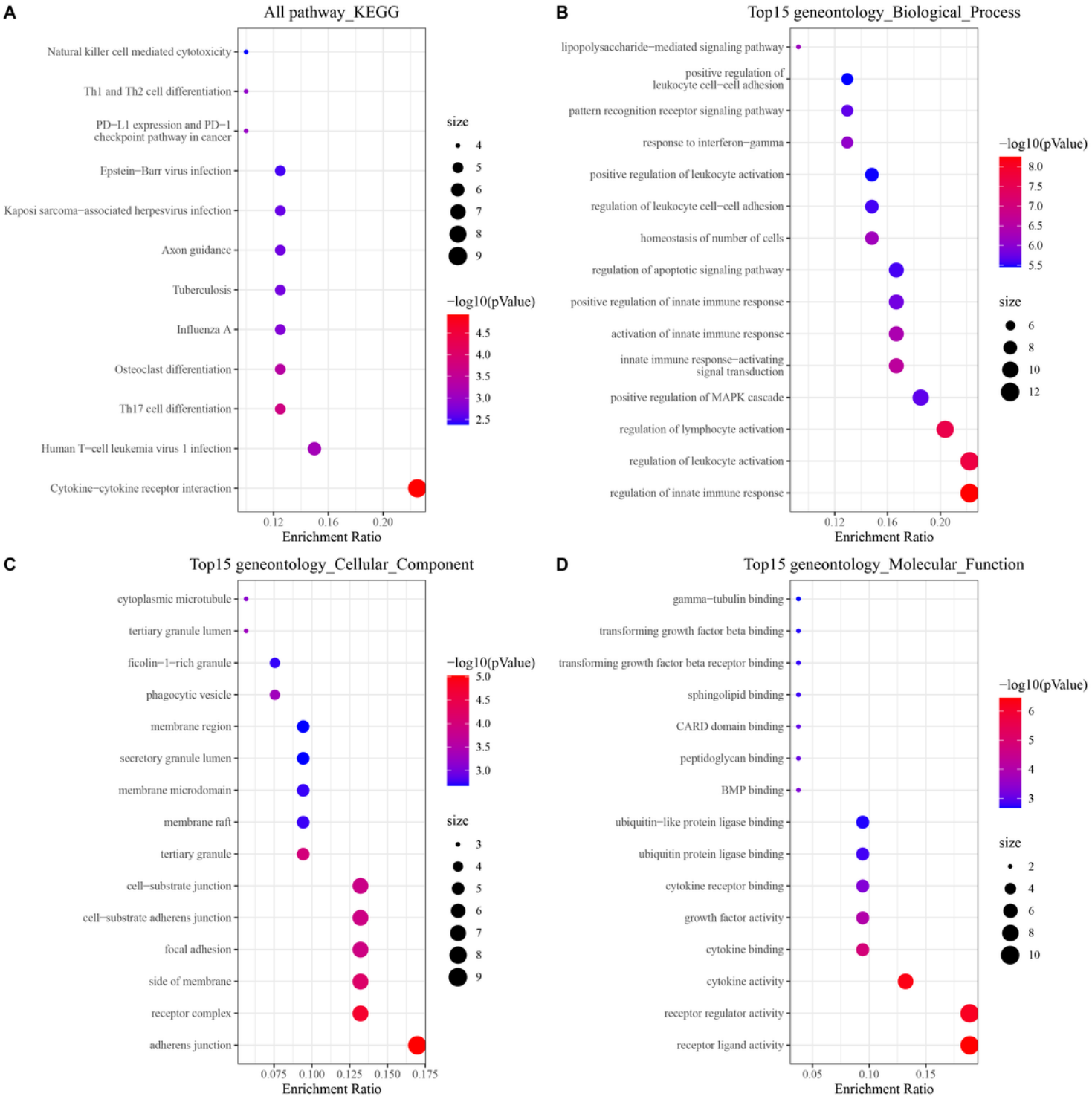

Figure 3

KEGG and GO analysis. All KEGG terms（A）and Top 15 Go terms（B,C,D）of DIEGs.

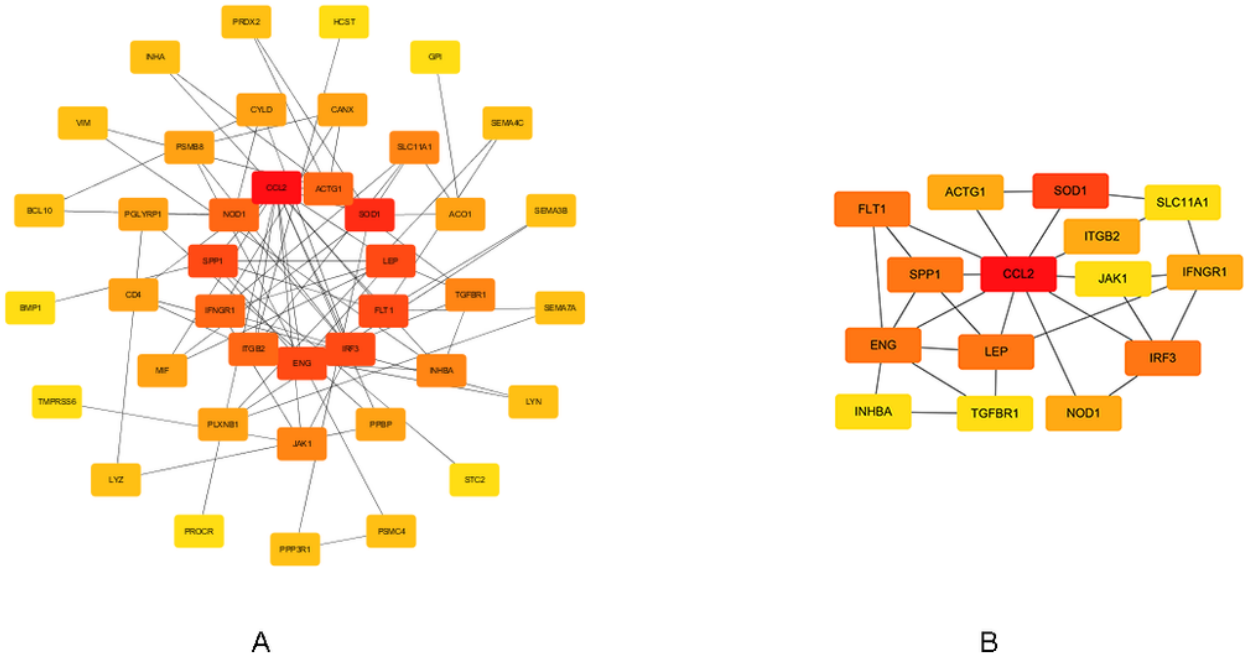

Figure 4

PPI network of DEIGs（A） and Top 15 hub genes（B）.

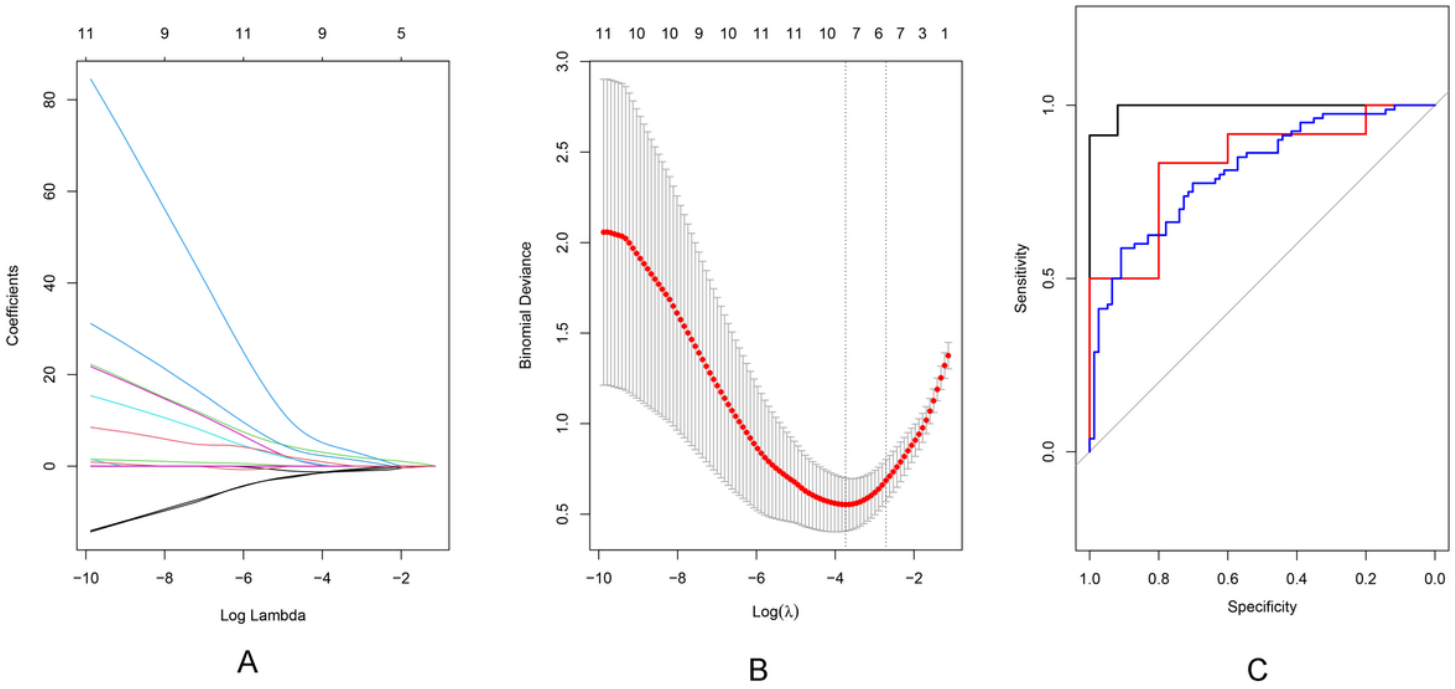

Figure 5

Screening and verification of diagnostic markers. (A) LASSO coefficient profiles of the 15 Hub genes. (B) A coefficient profile plot was produced against the log (lambda) sequence in the LASSO model. The optimal parameter (lambda) was selected as the first black dotted line indicated. C The predicted model ROC curve (black), AUC was 0.9929. Datasets GSE66273 (red) and GSE75010 (blue) validate the model with AUC of 0.8333 and 0.8107, respectively.

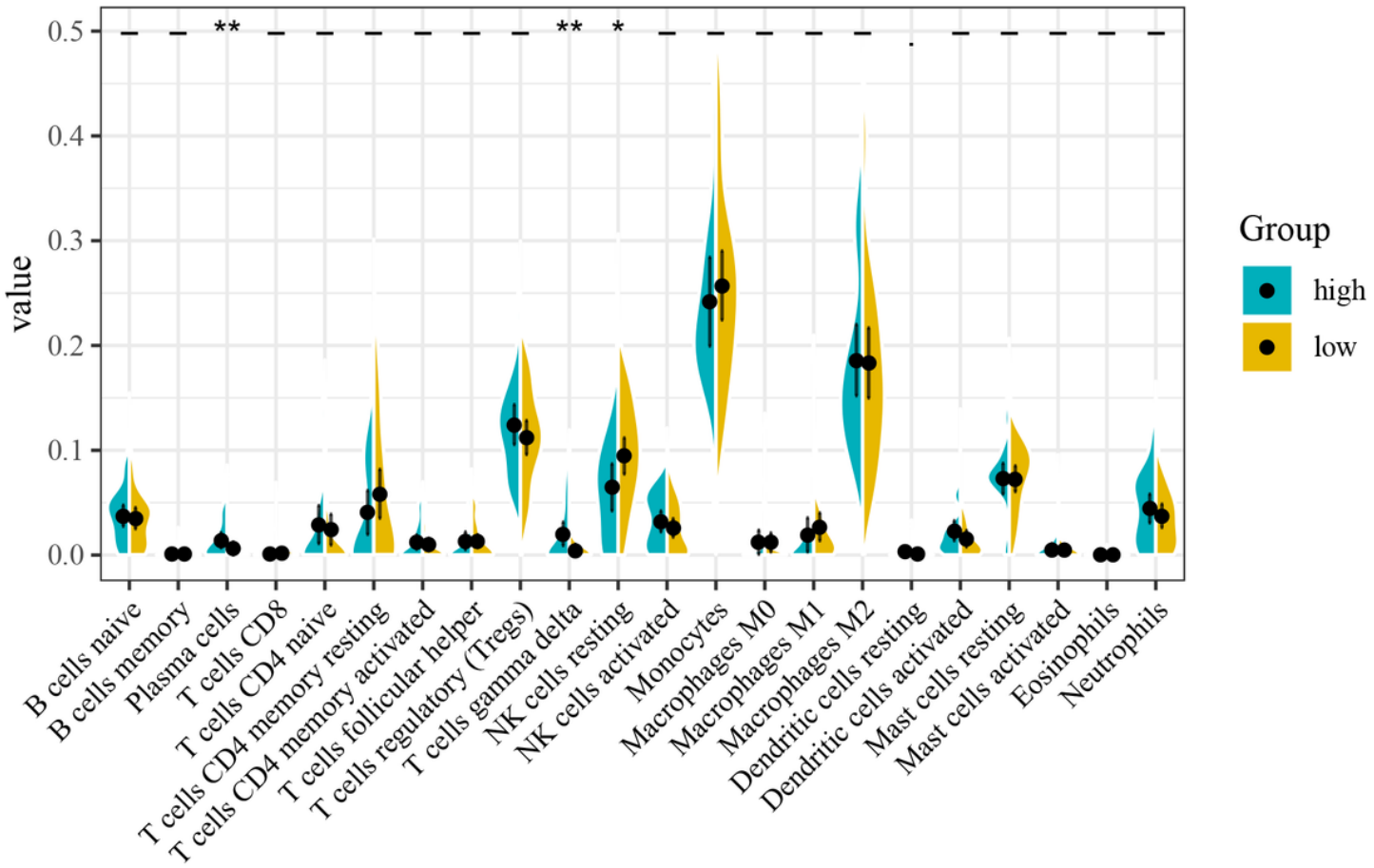

Figure 6

Violin plot of the proportion of 22 immune cells in the high score group versus the low score group. **P 0.01 *P 0.05