International Journal of General Medicine

Dovepress
open access to scientific and medical research

Open Access Full Text Article

ORIGINAL RESEARCH

Elucidating the Gene Signatures and Immune Cell Types in HIV-Infected Immunological Non-Responders by Bioinformatics Analyses

Qing Xiao[1,2], Junyan Han[3–6], Fengting Yu[1–3], Liting Yan[1,2], Qun Li[1,2], Xiaojie Lao[1,2], Hongxin Zhao[1,2], Fujie Zhang[1,2]

[1]Department of Infectious Diseases, Capital Medical University Affiliated Beijing Ditan Hospital, Beijing, People's Republic of China; [2]Clinical Center for HIV/AIDS, Capital Medical University, Beijing, People's Republic of China; [3]Beijing Key Laboratory of Emerging Infectious Diseases, Institute of Infectious Diseases, Beijing Ditan Hospital, Beijing, People's Republic of China; [4]National Center for Infectious Diseases, Beijing Ditan Hospital, Capital Medical University, Beijing, People's Republic of China; [5]Beijing Institute of Infectious Diseases, Beijing Ditan Hospital, Capital Medical University, Beijing, People's Republic of China; [6]Biomedical Innovation Center, Beijing Shijitan Hospital, Capital Medical University, Beijing, People's Republic of China

Correspondence: Fujie Zhang, Beijing, Ditan Hospital, Capital Medical University, Beijing, 100015, People's Republic of China, Tel +86 10 84322581, Email treatment@chinaaids.cn

Objective: Numerous studies have reported on the pathogenesis of poor immune reconstitution (PIR) after antiretroviral treatment in human immunodeficiency virus (HIV) patients. However, fewer studies focused on both immune-related genes (IRGs) and immune cells, and the correlation between IRGs and immune cells was evaluated via bioinformatics analyses.

Methods: Gene expression profiling of GSE143742 from the Gene Expression Omnibus (GEO) database was analyzed to get differentially expressed immune-related genes (DEIRGs). The enrichment analysis and protein-protein interaction (PPI) networks of DEIRGs were established. The relative fractions of 22 immune cell types were detected using the "CIBERSORT". The correlation analysis between DEIRGs and immune cells was constructed to discover the potential IRGs associated with immune cells. A logistic regression diagnostic model was built, and a receiver operating characteristic (ROC) curve was performed to evaluate the model's diagnostic efficacy. The CMap database was used to find molecules with therapeutic potential. RT-qPCR was used to verify the expression of the hub DEIRGs.

Results: We identified eight types of significantly changed immune cells and five hub IRGs in INRs. The DEIRGs were mainly enriched in lymphocyte activation, receptor-ligand activity, and T cell receptor signaling pathway. The correlation analysis showed that the expression of TNF, CXCR4 and TFRC correlate with CD8 cells, resting mast cells, activated NK cells, and naïve CD4 cells in INRs. Meanwhile, TFRC and IL7R relate to activated NK cells and resting memory CD4 cells respectively in IRs. A diagnostic model was constructed using multiple logistic regression and nine small molecules were identified as possible drugs.

Conclusion: In this study, we suggested that the process of PIR might be related to TNF, CXCR4, TFRC, CD48, and IL7R. And these IRGs play roles in regulating immune-competent cells. And our constructed diagnostic model has excellent effectiveness. Moreover, some small-molecule drugs are screened to alleviate PIR.

Keywords: HIV, immunological non-responders, immune-related genes, immune cells, bioinformatics

Introduction

Highly active antiretroviral therapy (HAART) can dramatically repress HIV replication, increase $CD4^+$ T cell (CD4 cell) counts and functions, and is recommended in current clinical guidelines as part of the most effective treatment regimen.[1,2] But not all patients can achieve optimal immune reconstitution with HAART.[3] An estimated 20% of patients, even after long-term regular HAART treatment and complete viral suppression, are not immune reconstituted.[4] These patients are recognized as immunological non-responders (INRs), and are more likely to develop serious complications.[5] Presently, there is no worldwide consensus on the definition of INRs. In different studies, INRs have been defined as

Received: 27 September 2022
Accepted: 29 November 2022
Published: 7 December 2022

failure to achieve diverse CD4 cell count thresholds (like 200, 250, 350, 400, or 500/μL), and the duration of HAART varies considerably among studies, ranging from 6 to 144 months. This inconformity certainly adds to the difficulty of research on poor immune reconstitution.[6]

The mechanisms of poor immune reconstitution are complicated, including reduced bone marrow haematopoiesis, inadequate thymic output, intestinal flora disorder, abnormal immune activation, residual viral replication, disturbance of inflammatory factors, and so on.[6–8] Current researches mainly focus on the production, destruction, and migration of CD4 cells, and there exist few studies focusing on other immune cells. It has been covered that high $CD8^+$ T cell (CD8 cell) counts are more conducive to sustained viral decline and recovery of CD4 cells in immune-restored patients.[9,10] An increased $CD56^{dim}CD16^{dim/-}$ NK cell subpopulation in INRs may play a detrimental role in immune reconstitution.[11] In the meantime, previous studies have shown that genetic factors in the host may affect the regeneration of CD4 cells during suppressive HAART treatment, such as TLR4, IL19, CCR2, etc. But there is still no firm conclusion about the pivotal genes that link to poor CD4 cell recovery in HIV-1 infected individuals.[12–14]

Integrated bioinformatics allows researchers to speedily identify differentially expressed genes (DEGs) and quantify immune cell types from a large number of whole blood or tissue gene expression profiles.[15] It has been widely used in many diseases, such as ischemia-reperfusion injury and an extensive variety of tumors.[16–18] However, so far, no studies have employed bioinformatics to analyze immune cell types in HIV inadequate immunological responders. The clarification of this issue will contribute to the understanding of the immune deficiency state in INRs.

In this study, we performed comprehensive bioinformatics analyses of the GSE143742 dataset and detected potential target immune-related genes (IRGs) and immune cells for inadequate immunological response treatment from the perspective of big data analysis.

Materials and Methods

Data Source

The gene expression dataset was obtained from the Gene Expression Omnibus (GEO) database.[19,20] A total of 1154 series about HIV were retrieved from the database (on June 1st, 2021). After a careful review, one whole blood gene expression dataset GSE143742 was selected, which was based on platform GPL10558 Illumina HumanHT-12 V4.0 expression beadchip. And this dataset included 41 immune non-responders (INRs) and 17 immune responders (IRs). The standard adopted in this study for INRs is HAART treatment for at least three years, CD4 cells<350/μL; and the criteria for IRs is: HAART treatment for at least three years, CD4 cells>500/μL.[21] In addition, the GSE106792 dataset included 12 INR samples and 12 IR samples. INR subjects were defined as having $CD4^+$ T cell counts below 350 cells/μL and IRs as having $CD4^+$ T cell counts above 350/μL after at least two years of HAART with virologic control.[22] All of the data are freely available online. The analysis flow chart was shown in Figure 1.

Patients and Collection of Clinical Samples

This study was approved by the Committee of Ethics at Beijing Ditan Hospital (approval number: 2021–022-01), Capital Medical University, Beijing, China. This study complies with the Declaration of Helsinki. All participants provided written informed consent prior to enrollment. The study enrolled 32 HIV-1-infected patients on HAART with undetectable viremia (HIV-RNA < 40 copies/mL) for more than 4 years, consisting of 17 IRs (with $CD4^+$ T cell counts>500 cells/μL) and 15 INRs (with $CD4^+$ T cell counts< 350 cells/μL) in the Beijing Ditan Hospital, Capital Medical University. The exclusion criteria included coinfection with active opportunistic infections, tuberculosis, pregnancy, as well as malignant tumors.

Identification of Differentially Expressed Immune-Related Genes (DEIRGs)

The genes that showed different expressions between INR and IR samples were identified by the R package "linear models for microarray analysis (LIMMA)". The criteria were as follows: (1) the adjusted $p<0.05$, a moderate *t*-test corrected by Benjamini and Hochberg's method; (2) log fold change (FC) of genes ≥0.3. Then the heat maps and volcano maps of the dataset were generated by the "ggplot" and "pheatmap" package. The immune-related gene set was extracted

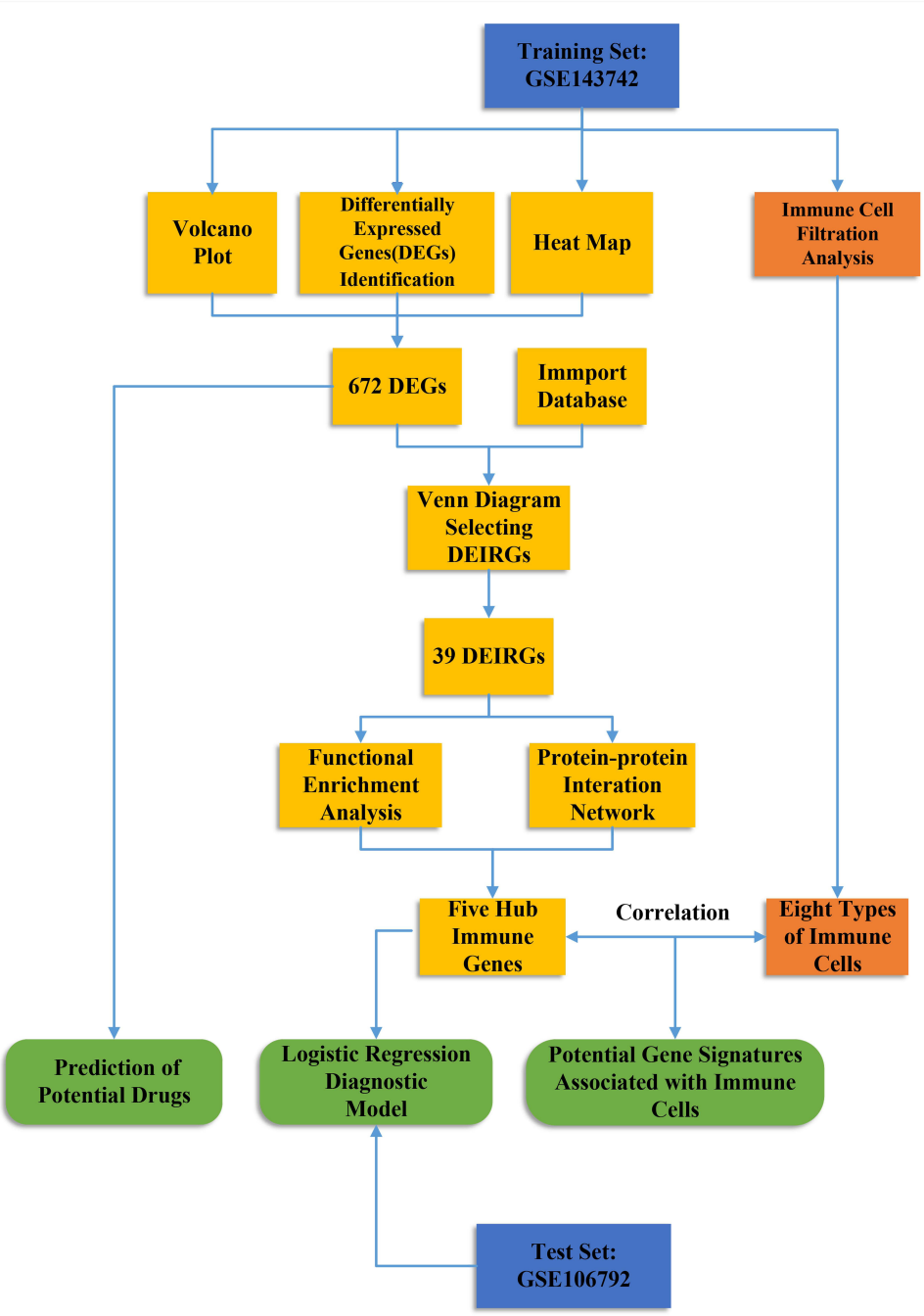

Figure 1 Flowchart of the integrated analysis.

from the Immunology Database and Analysis Portal (ImmPort) database, and the Venn Diagram was used to identify differentially expressed immune-related genes (DEIRGs) between INR and IR groups.

Gene Ontology and pathway Enrichment Analysis

Gene ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were used to evaluate the function and biological processes of DEIRGs. GO is a leading bioinformatics tool for the annotation of genes and the analysis of the biological processes involved in these genes.[23] KEGG is a database resource for gaining insights into high-level functional and biological systems from large-scale molecular datasets.[24] GO and KEGG analyses were performed by using the "Clusterprofiler" package. Gene counts≥3 and p-value<0.05 were set as the screening threshold.

Construction of the PPI Network

The STRING database (http://string-db.org) (version 11.5) is an online search tool used to analyze known proteins and predict protein-protein interaction (PPI) networks, including direct and indirect interactions between proteins and their functional correlations. Molecular interactions and PPI networks can promote the exploration of molecular targets, signaling, metabolic pathways, and network functions involved in the immune response process. Reliable protein-protein interactions (combined score≥0.9) were selected for PPI network construction.

Critical Subnetworks and Hub Genes

Based on the PPI network, hub genes were screened according to network topology. Cytoscape software (version 3.8.2, Cytohubba, and MCODE plug-ins) was used to discover the key targets or subnetworks of complex networks. The critical subnetworks and hub genes during the immune response process were analyzed. The criteria for selecting modules were as follows: MCODE score>5; degree cutoff>2; node score cutoff >0.2; and k-score>2. CytoHubba, a plug-in in Cytoscape, was used to calculate the degree of each protein node. The expression levels of the DEIRGs were plotted into box plots.

Identifying Immune Cell Types

The "CIBERSORT" package is used to estimate the abundance ratio of member cell types in mixed cell populations based on gene expression data. The Wilcoxon test was used to examine the differences in the immune cells between INRs and IRs. Then, we used the "ggplot2" package to draw columnar stack diagrams to show the formation of every sample and violin diagrams to visualize the differences in immune cell types.

Correlation Analysis of Immune Cells with Immune-Related Genes

Spearman correlation test analysis was carried out to illustrate the relationship between the relative percentages of immune cells and immune genes. Based on the paired *t*-test, $P<0.05$ was considered statistically significant. $|r| > 0.45$ and $P < 0.05$ meant significant correlation.

Construction of Logistic Regression Diagnostic Model

As the immune response is a complex process, its etiopathogenesis involves compound gene expression and multiple interactions. To evaluate the diagnostic value of the hub DEIRGs more comprehensively, the diagnostic model was built by fitting different genes into a binary logistic regression model (glm package, R). The optimal threshold was determined by Youden's J index. The training cohort was used for model building and the testing cohort for evaluating the performance of the models, and the "ROCR" package within R was used to calculate the area under the curve (AUC).

RNA Extraction and Quantification Real-Time Polymerase Chain Reaction

The High Purity Total RNA Rapid Extraction Kit (BioTeke, Beijing, China) was used to extract total RNA from the 16 INRs and 16 IRs whole blood samples according to the manufacturer's instructions. The All-in-One cDNA Synthesis SuperMix was then used to reverse transcribe the whole RNA into cDNA (Bimake). Sangon Biotech (Sangon, Shanghai, China) developed gene-specific primers (Table 1). The 2×SYBR Green qPCR Master Mix was then used to perform qPCR (Bimake). Melting curve analysis was done on all final PCR products to determine the amplification specificity. Using the relative quantification (ΔΔCt) approach, relative changes in gene expression were computed and reported as fold changes. Each transcript's relative abundance was compared to that of β-actin.

Statistical Analysis

All statistical analyses were carried out in R version 4.0.5 or Graphpad Prism version 8.0 software. The moderate *t*-test was used to identify DEGs. Fisher's exact test was applied to perform GO and KEGG analysis. The Wilcoxon test was applied to immune cell analysis. Spearman's rank analysis was applied to analyze the correlations between genes and cells.

Table 1 Sequences of Primers Used for Quantitative Real-Time PCR

Gene	Sequence(5'-3')
TFRC (human)	Forward: 5'- GGCTACTTGGGCTATTGTAAAGG-3'
	Reverse: 5'- CAGTTTCTCCGACAACTTTCTCT-3'
CXCR4 (human)	Forward: 5'- ACTACACCGAGGAAATGGGCT-3'
	Reverse: 5'- CCCACAATGCCAGTTAAGAAGA-3'
TNF-α (human)	Forward: 5'-GAGGCCAAGCCCTGGTATG-3'
	Reverse: 5'-CGGGCCGATTGATCTCAGC-3'
IL7R (human)	Forward: 5'- TGTCGTCTATCGGGAAGGAG −3'
	Reverse: 5'- CGGTAAGCTACATCGTGCATTA-3'
CD48 (human)	Forward: 5'- AGGTTGGGATTCGTGTCTGG-3'
	Reverse: 5'- AGTTGTTTGTAGTTCTCAGGCAG-3'
β-actin (human)	Forward: 5'-GCCGACAGGATGCAGAAGG-3'
	Reverse: 5'-TGGAAGGTGGACAGCGAGG-3'

Abbreviations: TFRC, transferrin receptor; CXCR4, C-X-C Motif Chemokine Receptor 4; TNF-α, tumor necrosis factor-α; IL7R, interleukin-7 receptor; CD48, CD48 Molecule; β-actin, beta actin.

Results

Identification of Differentially Expressed Immune-Related Genes (DEIRGs)

The Gene Expression Omnibus (GEO) dataset GSE143742 was included in our study, which had 61 samples, including 44 immunological non-responders (INRs) and 17 immunological responders (IRs). The heatmap demonstrated the expression of the top 50 differentially expressed genes (DEGs), and the volcano plot showed the correlation of all DEGs from the GSE143742 expression profiling microarrays (Figure 2A and B). In this study, we identified 672 DEGs. After screening with the Venn Diagram, 39 differentially expressed immune genes (DEIRGs) were identified (Figure 1C). The list of the DEIRGs were shown in Table 2. Among them, 29 DEIRGs were down-regulated, and the remaining 10 DEIRGs were upregulated (Table 2).

GO and KEGG Pathway Analysis

The most enriched Gene Ontology (GO) terms in the biological process category were "lymphocyte activation", "leukocyte activation involved in immune response", and "cell activation involved in immune response". As for the cellular component category, the DEIRGs were enriched in "side of membrane", "cytoplasmic vesicle membrane", and "ficolin-1-rich granule". Molecular function analysis demonstrated that the DEIRGs were significantly enriched in processes associated with "receptor-ligand activity", "signaling receptor activator activity", and "receptor regulator activity" (Figure 3A). The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway was enriched in "T cell receptor signaling pathway", "Epstein-Barr virus infection", and "Cytokine-cytokine receptor interaction" (Figure 3B). The enrichment map of GO analysis results was shown in Figure 3C, the gene names were on the left and GO terms were under the beneath (Figure 3C).

Construction of PPI Networks and Identification of Hub Immune-Related Genes (IRGs)

Analyzing the functional interactions between proteins may provide insights into the mechanisms of the generation or development of diseases. Protein-protein interaction (PPI) networks were constructed using STRING and Cytoscape. Following the removal of the six isolated genes that are not correlated with other genes, thirty-three DEIRGs were filtered from the thirty-nine DEIRGs to construct the PPI networks, which contained 33 nodes/genes and 77 edges (Figure 4A). Among these 33 genes, five central genes in module 1 were identified by the MCODE plug-in (Figure 4B). And the five genes were also the hub genes, which were obtained by the "Cytohubba" plug-in, namely "TNF", "CXCR4", "IL7R", "CD48", and "TFRC". TNF, a pro-inflammatory cytokine, plays a critical role in HIV infection.[25]

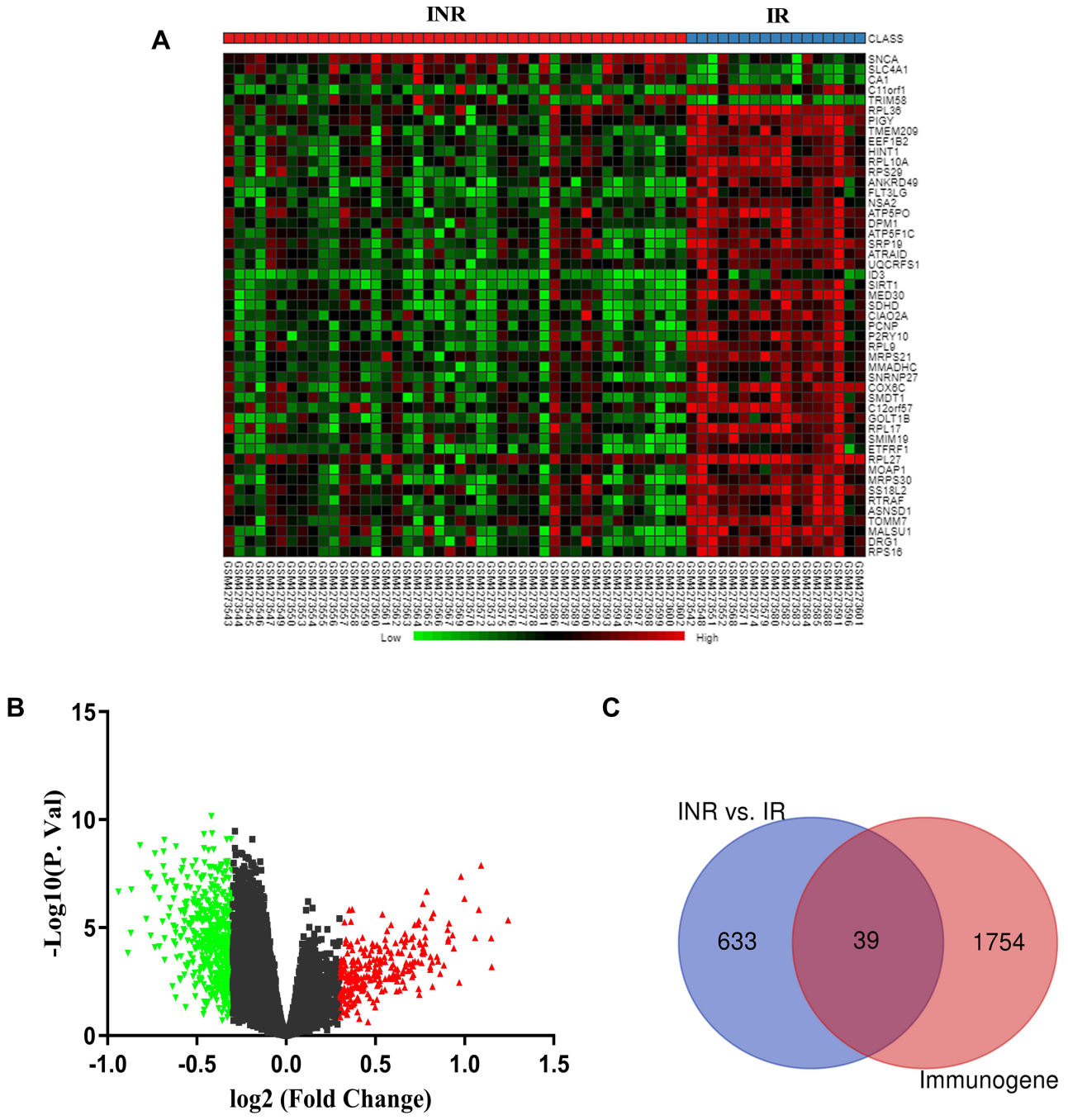

Figure 2 (**A**) Heatmap of the top 50 DEGs from the total 672 DEGs between INRs and IRs. (**B**) Volcano map of all DEGs between INRs and IRs. Red represents up-regulated, black represents no significant difference genes, and the green represents downregulated DEGs in INRs. (**C**) Venn diagram of DEGs and immune-related genes. Blue represents the DEGs between INRs and IRs in GSE143742 datasets, and red represents immune-related genes in the ImmPort Datasets.
Abbreviations: INRs, immunological non-responders; IRs, immunological responders; DEG, differentially expressed gene.

CXCR4 is predominantly distributed on the surface of immune cells and is a co-receptor for T-cellophilic HIV-1.[26] IL7R is expressed exclusively on the membranes of almost all naive lymphocytes and acts on naive lymphocytes to promote lymphocyte proliferation, differentiation and maintenance of the homeostatic function of peripheral mature T cells.[27] CD48 is a protein-coding gene, the X-Linked Lymphoproliferative Syndrome 1 and Acute Myeloid Leukemia are two conditions linked to CD48.[28] TFRC is an indispensable component of the body, not only for the transport and metabolism

Table 2 The 39 Differentially Expressed Immune-Related Genes (DEIRGs) Were Identified in the Whole Blood in INRs

DEIRGs	Gene Names
Upregulated	ADIPOR1, CXCR2, NR1D1, CTSB, ACKR1, PRDX2, IRF3, HDGF, GDF1, NEO1
Downregulated	CD48, CD3D, JUN, PSMC2, IL7R, HLA-DQA1, LTB, TNF, SOD1, TFRC, HSPA8, ITK, MIF, FLT3LG, TNFRSF25, VIM, CXCR4, SBDS, GMFB, CD247, S100A12, BCL3, NFKBIZ, RHOA, GMFG, TMSB10, PPP3CC, RASGRP1, MAP3K8

of iron, but also for the regulation of respiration, cell proliferation and the immune system, as well as for the regulation of iron ion homeostasis and energy balance.[29]

Immune Cell Type Analysis

We first used the CIBERSORT algorithm to investigate the relative proportion of the 22 subpopulations of immune cells among INR and IR samples (Figure 5A). And we found that CD8 cells, activated CD4 memory cells, resting NK cells, and resting Mast cells in INRs were present at higher fractions than in IRs. The results showed that T cell activation was consistent with increased immune activation in INRs.[6] However, naive CD4 cells, resting CD4 memory cells, activated NK cells, and activated Mast cells showed the opposite (Figure 5B).

Correlation Analysis of Immune-Related Genes and Immune Cells

We also explored the correlation between hub genes and the abundance ratios of 22 types of immune cells. We found that multiple hub IRGs were correlated to the abundance ratio of certain types of immune cells in INRs (Figure 6A) and IRs (Figure 6C). As shown, TNF and CD8 cells, TNF and Mast cells resting, CXCR4 and NK cells activated, TFRC and CD8 cells, and TFRC and naive CD4 cells represented good correlation in INR samples (Figure 6B). Moreover, TFRC has a close correlation with NK cells activated, and IL7R is strongly associated with resting memory CD4 cells (Figure 6D).

Diagnostic Model Development and Its Validation

A multivariate logistic regression model was adopted to establish the diagnostic model for INR. The performance of the model can be assessed utilizing the receiver operating characteristic (ROC) curve and the area under the ROC curve (AUC). The "ROCR" package was used to conduct the ROC analysis. The expression of the five hub DEIRGs was included as independent variables. The diagnostic model was established with the training data, and the test data were used to assess model performance with AUC. The diagnostic regression equation was as follows: Logit (P) = −62.6662 + 1.8366*TNF + 0.4966*CXCR4 + 1.1845*TFRC + 0.9928*IL7R + 2.8514*CD48. GSE143742 was used as the training set, and GSE106792 was taken as the test set. GSE143742 contained 41 INRs and 17 IRs. And GSE106792 dataset included 12 INRs and 12 IRs. The area under the ROC curve (AUC) of the training data was 0.922 (95% CI, 0.845–1.000) (Figure 7A). For external validation, the area under the ROC curve was 0.889 (95% CI, 0.694–1.000) (Figure 7B).

Abbreviations: ROC, receiver operating characteristic.

Screening of Potential Drugs for INRs

To identify novel drugs targeting INR patients, we performed CMap analysis on the 300 diagnostic gene candidates. We searched for negatively-correlated gene expression patterns associated with drug-treated cells in the CMap database using the cut-off criteria of percent non-null≥90 and mean<−0.4. The analyses screened out nine drug candidates; including trihexyphenidyl, isoflupredone, isoxicam, viomycin, lisuride, chenodeoxycholic acid, timolol, canadine, and adiphenine; which were not reported to play anti-INR effect before. The chemical structures of nine drugs were obtained through PubChem, which are shown in Figure 8.

Validation of the Expression of the Hub DEIRGs with RT-qPCR

The mRNA expression of the hub DEIRGs in 17 IRs and 15 INRs samples was detected with RT-qPCR. As shown in Figure 9, the expressions of IL7R, TFRC, TNF, CXCR4, and CD48 in INRs were significantly down-regulated in

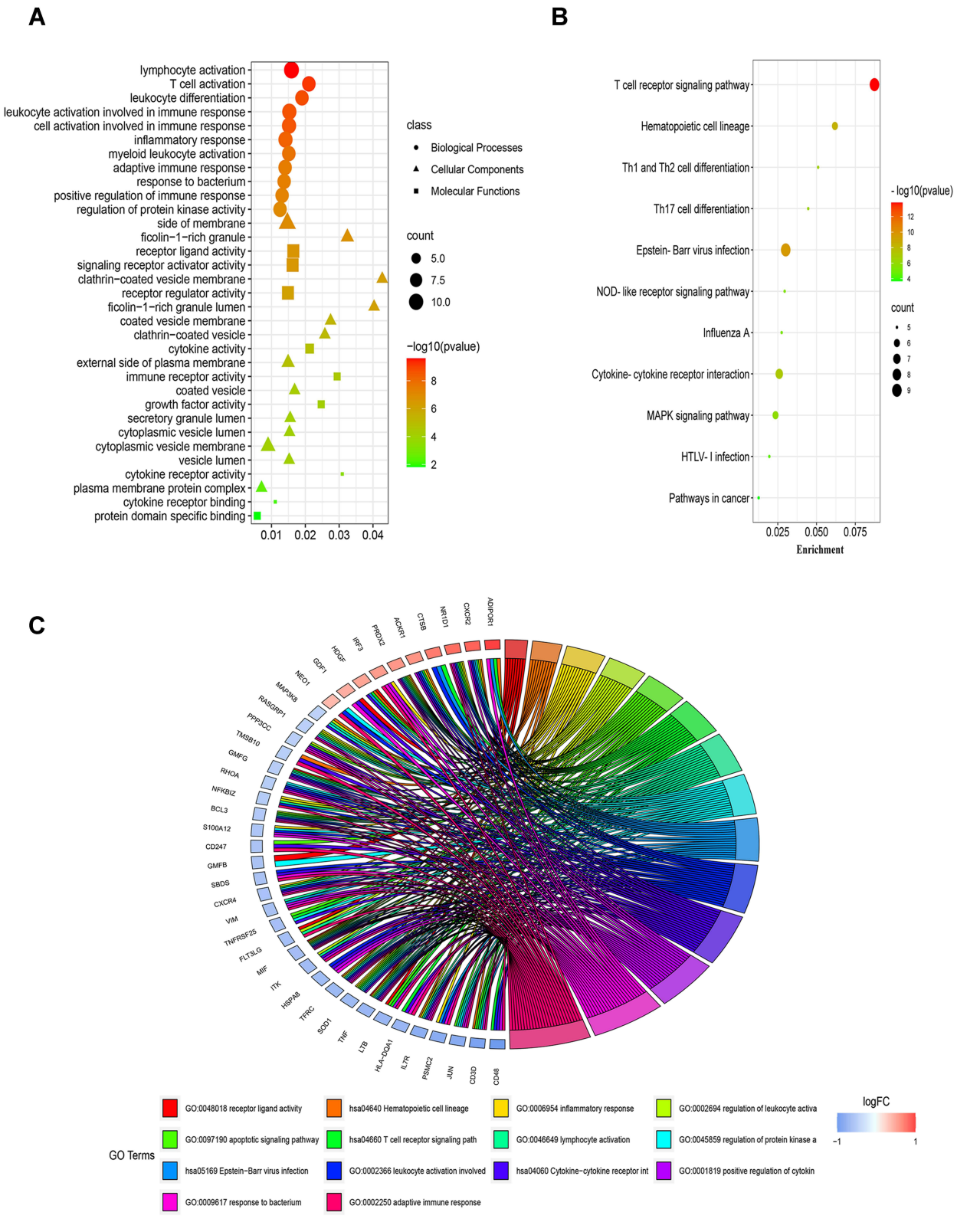

Figure 3 GO and KEGG functional enrichment of the DEIRGs between INRs and IRs. (**A**) Bubble diagram of GO analysis results. (**B**) Bubble diagram of KEGG pathway analysis results. The dots represent biological process (BP), the triangles represent cellular component (CC), the squares represent molecular function (MF), and the sizes of the three shapes represent the numbers of genes in each GO and KEGG category. Red indicates a higher p-value. (**C**) Enrichment map of GO analysis results. Gene names on the left and pathway names under the beneath.

Abbreviations: GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

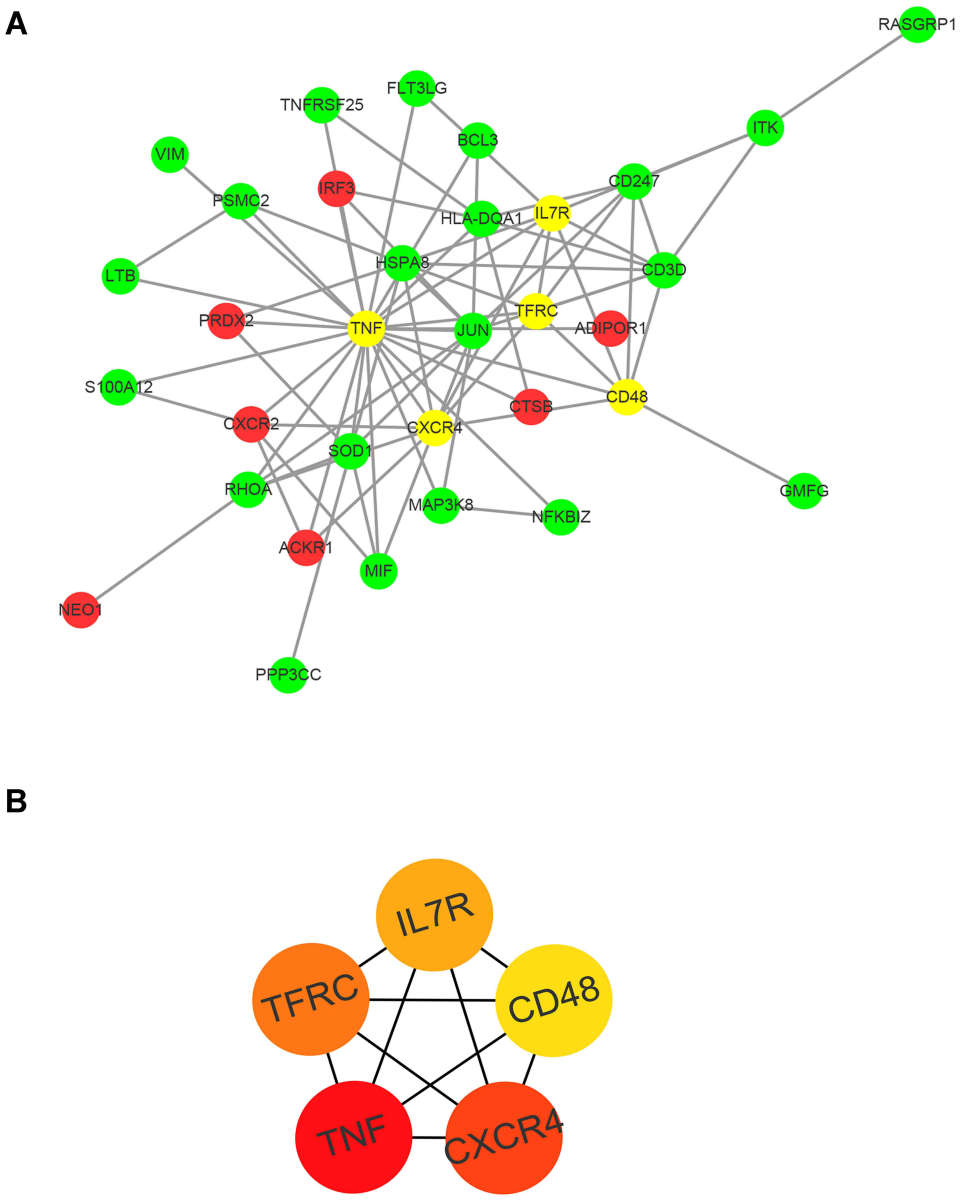

Figure 4 PPI network of identified DEIRGs constructed using Cytoscape software. (**A**) DEIRGs in the INRs vs IRs . Red nodes represent up-regulated genes, green nodes represent down-regulated genes, and yellow nodes represent hub genes in INRs. (**B**) The top module and the top five DEIRGs between INRs and IRs.
Abbreviations: DEIRG, differentially expressed immune-related genes; INRs, immunological non-responders; IRs, immunological responders.

comparison with IRs (Figure 9A). And we then verified the expression of the DEIRGs in the merged datasets from the public database (Figure 9B).

Discussion

The poor recovery of the $CD4^+$ T cell (CD4 cell) population after highly active antiretroviral therapy (HAART) is associated with a significant increase in the mortality and morbidity of acquired immunodeficiency syndrome (AIDS),[30] and receiving HAART timely may have a beneficial effect on immune reconstitution.[31] It has been reported that specific therapies targeting T cell immune activation, such as the extract of Tripterygium wilfordii Hook F (TwHF) and Vitamin,[32,33] are correlated with CD4 cell recovery. And the therapies spotting pro/anti-inflammatory cytokines, like interleukin-7 (IL-7) and interleukin-2 (IL-2), have also been reported to be beneficial for reconstituting T cell counts. IL-7 signals to the cognate receptor IL-7R or CD127, and induces T cell survival and proliferation.[34] IL-2 can increase the

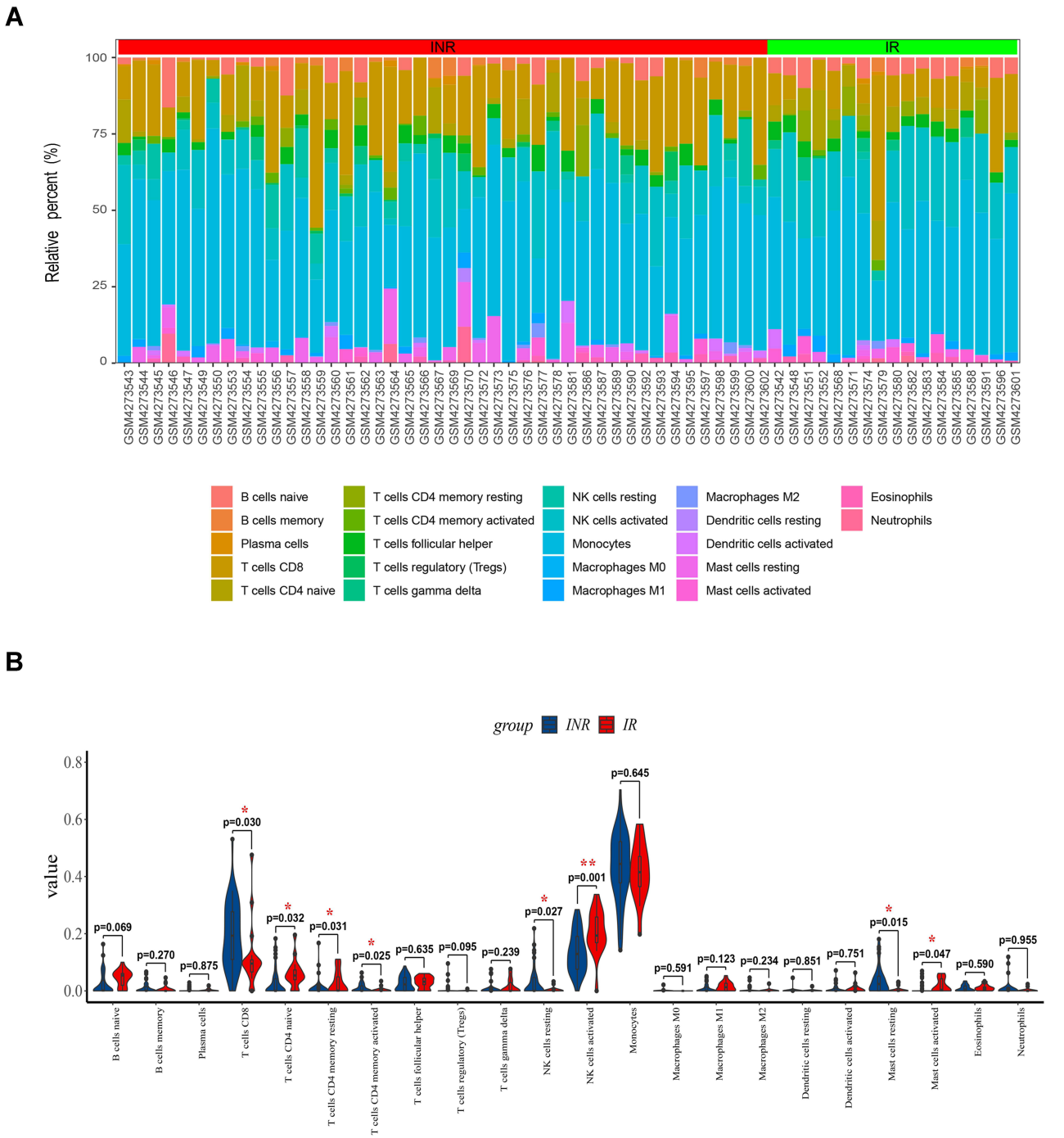

Figure 5 (**A**) Relative proportions of 22 types of immune cells in INRs and IRs. (**B**) Significant changes in immune cells in INRs compared to the IRs. *p < 0.05, **p < 0.01.
Abbreviations: INRs, immunological non-responders; IRs, immunological responders.

proliferation of both CD4 and CD8 cells, and are capable of prolonging the survival of CD4 cells.[35] Synthetically, research on the immunological mechanisms of defective immune response can boost insights into the kinetics of immunological remodeling. In this study, we tried to determine the diagnostic markers of immune non-responders (INRs) and examine the proportion of immune cells in INRs in more detail.

We first identified 39 differentially expressed immune-related genes (DEIRGs) in INRs compared with immune responders (IRs). These genes were enriched in"lymphocyte activation", "leukocyte activation involved in immune response", and "cell activation involved in immune response" biological processes. And they are enriched in the "T cell

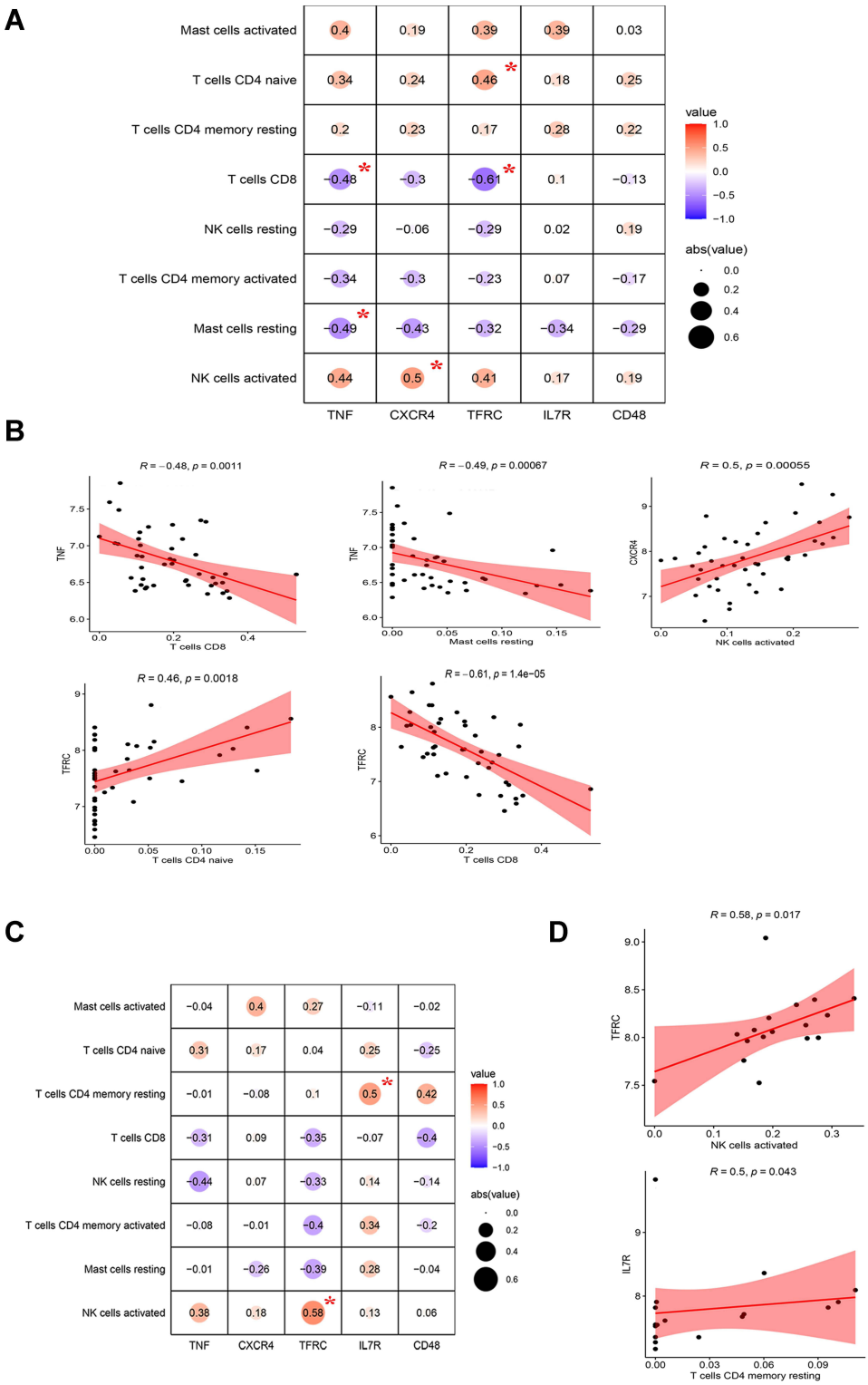

Figure 6 The correlation between pivotal immune-related genes and critical immune cells. (**A**) Correlation between expressions of five IRGs and the relative percentages of eight immune cells in INRs. (**B**) Scatterplots illustrate the relationship between TNF and CD8 cells, TNF and resting Mast cells, CXCR4 and activated NK cells, TFRC and naïve CD4 cells, and TFRC and CD8 cells in INRs. (**C**) Correlation between expressions of five immune genes and the relative percentages of eight immune cells in IRs. (**D**) Scatterplots illustrate the relationship between TFRC and activated NK cells, and IL7R and resting memory CD4 T cells in IRs. Red colors represent positive correlations, while blue represents negative correlations. The darkness of color indicates the strength of the correlation. |r| > 0.45 and P < 0.05 meant significant correlation, and marked with asterisk (*p < 0.05). Red-shaded areas in scatterplots represent the 95% confidence interval of the regression lines.

Abbreviations: IRGs, immune-related genes; r, correlation coefficient; TNF, tumor necrosis factor; CXCR4, C-X-C motif chemokine receptor 4; TFRC, transferrin receptor; IL7R, interleukin 7 receptor; INRs, immunological non-responders; IRs, immunological responders.

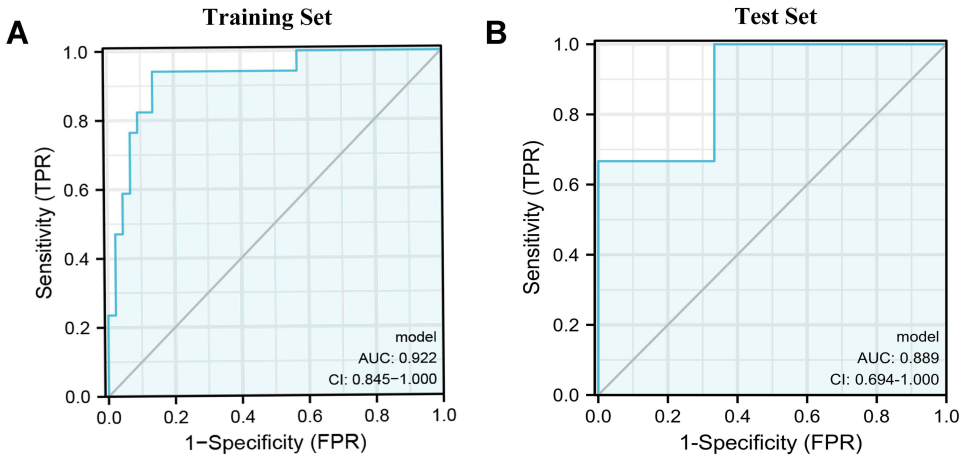

Figure 7 The ROC curve was used to evaluate the diagnostic value of the logistic regression model in the (**A**) training set, and (**B**) test set.

receptor signaling pathway", "Epstein-Barr virus infection", and "Cytokine-cytokine receptor interaction" pathway. The results are supported by a great many published studies.[36,37] It has been generally accepted that one of the vital mechanisms for the occurrence of poor immune response is the presence of abnormal immune activation. The persistence of immune activation can contribute to the endurance of HIV reservoirs, CD4 cell deterioration, and disease progression.[8]

After the construction of the protein-protein interaction (PPI) networks, five hub immune-related genes (IRGs) in INRs were sorted out, including tumor necrosis factor-α (TNF), C-X-C Motif Chemokine Receptor 4 (CXCR4), interleukin-7 receptor (IL7R), CD48 molecule (CD48), transferrin receptor (TFRC). These genes were all altered in INRs, which implies that they may get involved in immune rebuilding in HAART patients. Recent studies have found that TNF is chiefly secreted by macrophages, and involved in the regulation of cell proliferation, differentiation, apoptosis, and lipid metabolism.[38] TNF is immunologically active and causes changes in lymphocyte subsets in HIV patients, and activation of pro-inflammatory and anti-inflammatory cytokines has an invaluable role in immune system dysfunction.[39] CXCR4 encodes a CXC chemokine receptor specific for stromal cell-derived factor-1 and can work with CD4 protein to support HIV entry into cells.[40] IL7R has been shown to act in V(D)J recombination during lymphocyte evolution. Defects in it may be related to severe combined immunodeficiency (SCID).[41] CD48 is found on the surface of many immune cells and participates in activation and differentiation pathways in these cells.[28] And TFRC encodes a cell surface receptor that is indispensable for cellular iron uptake by the receptor-mediated endocytosis process. Diseases associated with TFRC include Immunodeficiency 46 and Combined Immunodeficiency.[42,43]

Concurrently, following the operation of the CIBERSORT algorithm, we found that eight types of immune cells had statistical differences between INR and IR groups. The ratios of resting mast cells, activated memory CD4 cells, resting natural killer (NK) cells, and $CD8^+$ T cells (CD8 cells) were relatively higher in INRs. Nevertheless, the proportions of activated NK cells, resting memory CD4 cells, naïve CD4 cells, and activated mast cells were lower in INRs. In short, INRs had less activated intrinsic immune cells but more over-activated adaptive immune cells. This result was consistent with the literature that has been reported. It has been issued that there was an increased proportion of $CD56^{bright}$ subpopulations of NK cells, monocytes, and dendritic cells (DCs), and a significant increase in cell activation ($CD38^+HLA\text{-}DR^+$, $CD69^+$) in INRs.[44–46] Persistent T cell activation has been reported to be associated with reduced CD4 cell increases in HIV patients during HAART.[47–49] For every 5% increase in the percentage of activated CD4 cells, the CD4 cell count decreased by 45 cells/μL in the first three months of HAART and by 35 cells/μL after three months.[47]

Besides, we also analyzed the interaction of the 8 types of key immune cells with 5 hub immune-related genes in INRs and IRs. We found that naïve CD4 cells, CD8 cells, resting mast cells, and activated NK cells had complicated associations with TNF, CXCR4, and TFRC in INRs. Activated NK cells positively related to TFRC, and resting memory CD4 cells correlated consistently to IL7R in IRs. Recent studies have found that TNF was predominantly secreted by CD8 cells, and some dysmature CD8 cells in HIV-infected patients produce significantly lower levels of perforin,

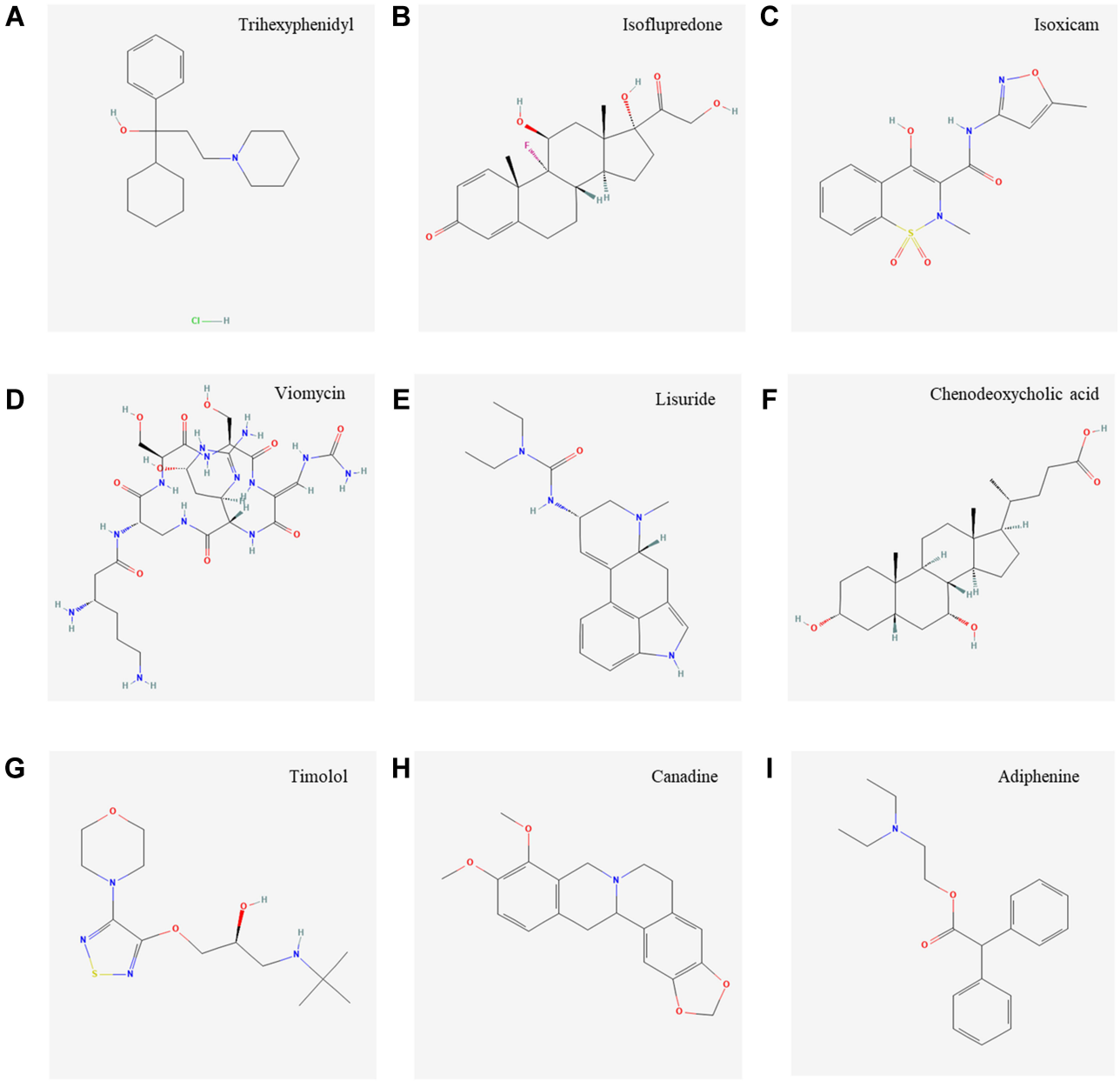

Figure 8 The chemical structure of the targeted therapeutic drugs of the diagnostic signature in INRs. (**A**) Trihexyphenidyl; (**B**) Isoflupredone; (**C**) Isoxicam; (**D**) Viomycin; (**E**) Lisuride; (**F**) Chenodeoxycholic acid; (**G**) Timolol; (**H**) Canadine;(**I**) Adiphenine.

granulysin, and TNF.[50] This can partially explain the correlation between TNF and CD8 cells. CXCR4 has been shown to mediate NK-cell trafficking to the bone marrow microenvironment and may involve in NK cell recruitment.[51,52] TFRC is involved in the transport and storage of iron and the synthesis of substances essential for cell production, which has been reported to positively regulate the proliferation of T and B cells.[42] At the same time, it has been addressed that IL7R gene polymorphisms were associated with the rate of CD4 cell recovery in a large cohort of HAART recipients, a finding consistent with the known role of IL-7 in establishing CD4 lymphocyte homeostasis.[53] Although many studies have identified the relevance of these genes and cells, the association of these genes with the immune system is still unclear. It will need more research to study the underlying mechanism of these genetic codes in immune reconstitution for HIV patients after HAART therapy.

And we also constructed a diagnostic model based on the five hub immune genes. The area under the curve (AUC) values in the training set and testing set were both greater than 0.85, which showed that our risk model had a strong

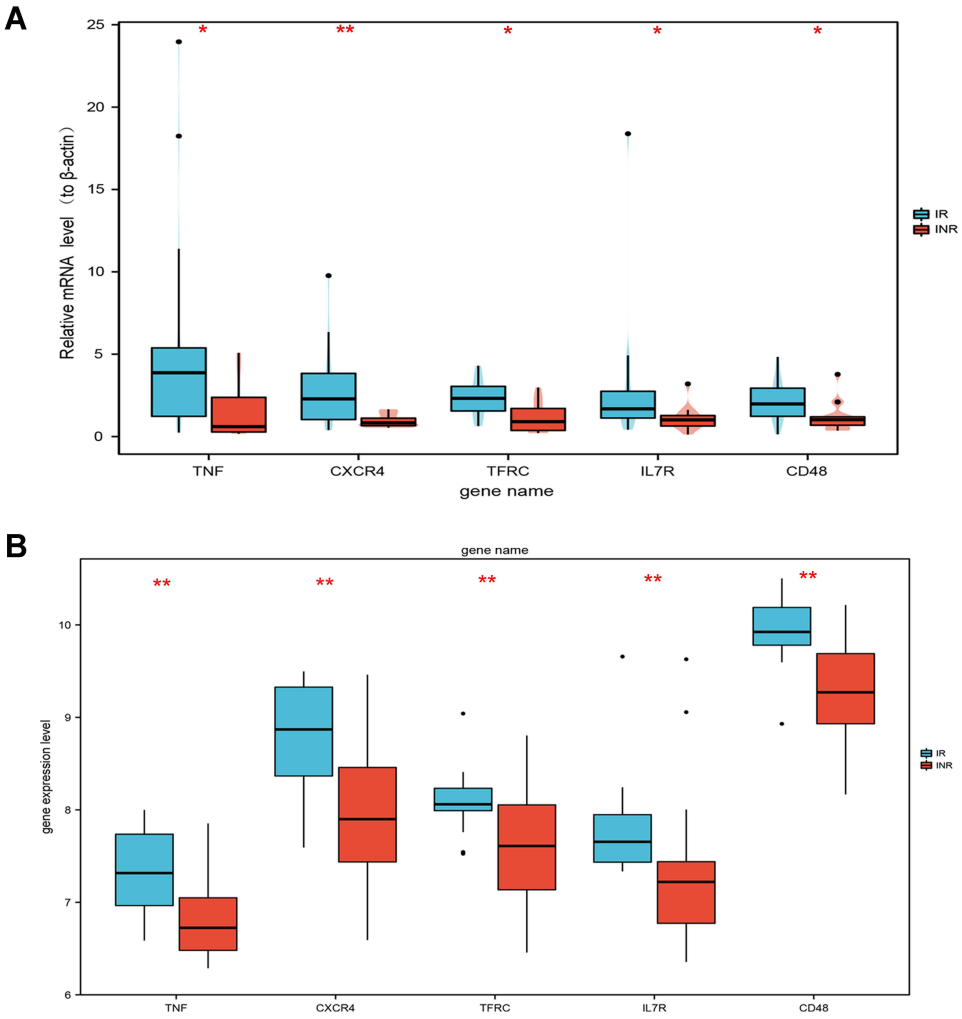

Figure 9 The mRNA expressions of DEIRGs in clinical 17 IR and 15 INR samples were detected by RT-qPCR. The expression of TNF, CXCR4, TFRC, IL7R, and CD48 (**A**) between the INR and IR samples. (**B**) The expression of the DEIRGs in merged GEO series. *p < 0.05, **p < 0.01.

clinical application value. As far as we know, there is no study building the diagnostic model based on gene expression, so it is an innovative and exportable diagnostic model in laboratory practice. In the meantime, we screened trihexyphenidyl, isoflupredone, isoxicam, viomycin, lisuride, chenodeoxycholic acid, timolol, canadine, and adiphenine from many small-molecule drugs. These nine drugs showed a significant adverse association with the highly-expressed or lowly-expressed genes in INRs, which may alleviate or reverse the status of the infiltration of immune cells, thus improving the prognosis of INR patients. Among the drugs mentioned above, chenodeoxycholic acid has been reported to selectively inhibit the replication of human immunodeficiency virus type 1 (HIV-1) in vitro,[54] and topical timolol was shown to be effective in treating penile Kaposi sarcoma in HIV-negative patients.[55]

The innovation of this study is to screen out varied immune cells and gene signatures associated with these immune cells in INRs, which has not been reported. Our findings offer the possibility to regulate the interactions of these genes with membrane receptors or ligands to identify new treatments for INRs. Nevertheless, there also exist some inevitable deficiencies. For instance, the HIV-related datasets are fewer and not as easily acquired as cancer datasets, which causes a lack of verification of other chipsets. Given that the sample size and experimental applications are limited, additional molecular and cellular experiments should be performed to further explore more in-depth mechanisms. Conclusively, our study has provided a novel perspective on the immunomodulation mechanisms underlying poor immune reconstitution in HIV patients. And the ideas and findings in this study are helpful for further and future investigation.

Conclusions

In summary, eight types of significantly changed immune cells and five hub IRGs were identified in INRs. The process of poor immune reconstitution in INRs might be related to TNF, CXCR4, TFRC, CD48, and IL7R. And these IRGs play roles in regulating immune-competent cells. And our constructed diagnostic model, based on immune-related genes, has excellent effectiveness. Moreover, some small-molecule drugs are screened to alleviate poor immune response. These results will enable studies of the potential genes associated with immune cells, as well as provide insight for discovering new treatments and drugs for INRs.

Abbreviations

HIV, human immunodeficiency virus; AIDS, acquired immunodeficiency syndrome; IRGs, immune-related genes; IRs, immunological responders; INRs, immunological non-responders; GEO, Gene Expression Omnibus; DEIRGs, differentially expressed immune-related genes; PPI, protein-protein interaction; HAART, Highly active antiretroviral therapy; ImmPort, Immunology Database and Analysis Portal; GO, Gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; ROC, receiver operating characteristic; HIV-1, human immunodeficiency virus type 1.

Data Sharing Statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE143742 and https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE106792.

Acknowledgments

The authors would like to acknowledge Beijing Ditan Hospital, Capital Medical University, and the Beijing Key Laboratory of Emerging Infectious Disease to support the study. Simultaneously, the authors sincerely thank Professor Jiang Xiao for his help in the linguistic review.

Funding

This work was supported by the 13th Five-year Plan, Ministry of Science and Technology of China (2018ZX10302-102), Beijing Municipal Administration of Hospitals' Ascent Plan (DFL20191802), and Beijing Municipal Administration of Hospitals Clinical Medicine Development of Special Funding Support (ZYLX202126).

Disclosure

The authors declare no conflicts of interest in this work.

References

1. Yu F, Hao Y, Zhao H, et al. Distinct mitochondrial disturbance in CD4+T and CD8+T cells from HIV-infected patients. *J Acquir Immune Defic Syndr*. 2017;74(2):206–212. doi:10.1097/QAI.0000000000001175
2. Zhang F, Dou Z, Ma Y, et al. Effect of earlier initiation of antiretroviral treatment and increased treatment coverage on HIV-related mortality in China: a national observational cohort study. *Lancet Infect Dis*. 2011;11(7):516–524. doi:10.1016/S1473-3099(11)70097-4
3. Sun L, Zhang L, Yang K, et al. Analysis of the causes of cervical lymphadenopathy using fine-needle aspiration cytology combining cell block in Chinese patients with and without HIV infection. *BMC Infect Dis*. 2020;20(1):224. doi:10.1186/s12879-020-4951-x
4. Ruiz-Briseño MDR, De Arcos-Jiménez JC, Ratkovich-González S, et al. Association of intestinal and systemic inflammatory biomarkers with immune reconstitution in HIV+ patients on ART. *J Inflamm*. 2020;17:32. doi:10.1186/s12950-020-00262-4
5. Lederman MM, Calabrese L, Funderburg NT, et al. Immunologic failure despite suppressive antiretroviral therapy is related to activation and turnover of memory CD4 cells. *J Infect Dis*. 2011;204(8):1217–1226. doi:10.1093/infdis/jir507
6. Yang X, Su B, Zhang X, Liu Y, Wu H, Zhang T. Incomplete immune reconstitution in HIV/AIDS patients on antiretroviral therapy: challenges of immunological non-responders. *J Leukoc Biol*. 2020;107(4):597–612. doi:10.1002/JLB.4MR1019-189R
7. Xiao Q, Yu F, Yan L, Zhao H, Zhang F. Alterations in circulating markers in HIV/AIDS patients with poor immune reconstitution: novel insights from microbial translocation and innate immunity. *Front Immunol*. 2022;13:1026070. doi:10.3389/fimmu.2022.1026070
8. Gaardbo JC, Hartling HJ, Gerstoft J, Nielsen SD. Incomplete immune recovery in HIV infection: mechanisms, relevance for clinical care, and possible solutions. *Clin Dev Immunol*. 2012;2012:670957. doi:10.1155/2012/670957
9. Zhang LX, Jiao YM, Zhang C, et al. HIV reservoir decay and CD4 recovery associated with high CD8 counts in immune restored patients on long-term ART. *Front Immunol*. 2020;11:1541. doi:10.3389/fimmu.2020.01541

10. Puronen CE, Ford ES, Uldrick TS. Immunotherapy in people with HIV and cancer. *Front Immunol*. 2019;10:2060. doi:10.3389/fimmu.2019.02060
11. Zhang QY, Zhang X, Su B, et al. Increased early activation of CD56dimCD16dim/- natural killer cells in immunological non-responders correlates with CD4+ T-cell recovery. *Chin Med J*. 2020;133(24):2928–2939. doi:10.1097/CM9.0000000000001262
12. Veloso Carvalho-Silva WH, Andrade-Santos JL, Dos Santos Guedes MC, Guimarães RL. Genetics and immunological recovery with antiretroviral treatment for HIV. *Pharmacogenomics*. 2020;21(14):979–983. doi:10.2217/pgs-2020-0083
13. Corbeau P, Reynes J. Immune reconstitution under antiretroviral therapy: the new challenge in HIV-1 infection. *Blood*. 2011;117(21):5582–5590. doi:10.1182/blood-2010-12-322453
14. Rb-Silva R, Goios A, Kelly C, et al. Definition of immunological nonresponse to antiretroviral therapy: a systematic review. *J Acquir Immune Defic Syndr*. 2019;82(5):452–461. doi:10.1097/QAI.0000000000002157
15. Newman AM, Liu CL, Green MR, et al. Robust enumeration of cell subsets from tissue expression profiles. *Nat Methods*. 2015;12(5):453–457. doi:10.1038/nmeth.3337
16. Deng R, Li J, Zhao H, et al. Identification of potential biomarkers associated with immune infiltration in papillary renal cell carcinoma. *J Clin Lab Anal*. 2021;35:e24022. doi:10.1002/jcla.24022
17. Chen B, Khodadoust MS, Liu CL, Newman AM, Alizadeh AA. Profiling Tumor Infiltrating Immune Cells with CIBERSORT. *Methods Mol Biol*. 2018;1711:243–259.
18. Lv J, Zhu Y, Ji A, Zhang Q, Mining LG. TCGA database for tumor mutation burden and their clinical significance in bladder cancer. *Biosci Rep*. 2020;40(4). doi:10.1042/BSR20194337
19. Edgar R, Domrachev M, Lash AE. Gene expression omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res*. 2002;30(1):207–210. doi:10.1093/nar/30.1.207
20. Barrett T, Wilhite SE, Ledoux P, et al. NCBI GEO: archive for functional genomics data sets--update. *Nucleic Acids Res*. 2013;41:D991–D995. doi:10.1093/nar/gks1193
21. Ghneim K, Sharma AA, Ribeiro SP, et al. Passive Transfer of Vaccine-Elicited Antibodies Protects against SIV in Rhesus Macaques. *Cell*. 2020;183(1):185–196.e14. doi:10.1016/j.cell.2020.08.033
22. Younes SA, Talla A, Pereira Ribeiro S, et al. Cycling CD4+ T cells in HIV-infected immune nonresponders have mitochondrial dysfunction. *J Clin Invest*. 2018;128(11):5083–5094. doi:10.1172/JCI120245
23. Ashburner M, Ball CA, Blake JA, et al. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat Genet*. 2000;25 (1):25–29. doi:10.1038/75556
24. Kanehisa M, Furumichi M, Tanabe M, Sato Y, Morishima K. KEGG: new perspectives on genomes, pathways, diseases and drugs. *Nucleic Acids Res*. 2017;45(D1):D353–D361. doi:10.1093/nar/gkw1092
25. Planès R, Serrero M, Leghmari K, BenMohamed L, Bahraoui E. HIV-1 envelope glycoproteins induce the production of TNF-α and IL-10 in human monocytes by activating calcium pathway. *Sci Rep*. 2018;8(1):17215. doi:10.1038/s41598-018-35478-1
26. Costa JM, Torres TS, Coelho LE, Luz PM. Adherence to antiretroviral therapy for HIV/AIDS in Latin America and the Caribbean: systematic review and meta-analysis. *J Int AIDS Soc*. 2018;21:1. doi:10.1002/jia2.25066
27. Lundström W, Highfill S, Walsh ST, et al. Soluble IL7Rα potentiates IL-7 bioactivity and promotes autoimmunity. *Proc Natl Acad Sci U S A*. 2013;110(19):E1761–E1770. doi:10.1073/pnas.1222303110
28. McArdel SL, Terhorst C, Sharpe AH. Roles of CD48 in regulating immunity and tolerance. *Clin Immunol*. 2016;164:10–20. doi:10.1016/j.clim.2016.01.008
29. Daniels TR, Delgado T, Helguera G, Penichet ML. The transferrin receptor part II: targeted delivery of therapeutic agents into cancer cells. *Clin Immunol*. 2006;121(2):159–176. doi:10.1016/j.clim.2006.06.006
30. Meissner EG, Chung D, Tsao B, Haas DW, Utay NS. IFNL4 genotype does not associate with CD4 T-cell recovery in people living with human immunodeficiency virus. *AIDS Res Hum Retroviruses*. 2021;37(3):184–188. doi:10.1089/aid.2020.0104
31. Noiman A, Esber A, Wang X, et al. Clinical factors and outcomes associated with immune non-response among virally suppressed adults with HIV from Africa and the United States. *Sci Rep*. 2022;12(1):1196. doi:10.1038/s41598-022-04866-z
32. Li T, Xie J, Li Y, et al. Tripterygium wilfordii Hook F extract in cART-treated HIV patients with poor immune response: a pilot study to assess its immunomodulatory effects and safety. *HIV Clin Trials*. 2015;16(2):49–56. doi:10.1179/1528433614Z.0000000005
33. Eckard AR, O'Riordan MA, Rosebush JC, et al. Vitamin D supplementation decreases immune activation and exhaustion in HIV-1-infected youth. *Antivir Ther*. 2018;23(4):315–324. doi:10.3851/IMP3199
34. Camargo JF, Kulkarni H, Agan BK, et al. Responsiveness of T cells to interleukin-7 is associated with higher CD4+ T cell counts in HIV-1-positive individuals with highly active antiretroviral therapy-induced viral load suppression. *J Infect Dis*. 2009;199(12):1872–1882. doi:10.1086/598858
35. Kovacs JA, Lempicki RA, Sidorov IA, et al. Induction of prolonged survival of CD4+ T lymphocytes by intermittent IL-2 therapy in HIV-infected patients. *J Clin Invest*. 2005;115(8):2139–2148. doi:10.1172/JCI23196
36. Utay NS, Overton ET. Immune activation and inflammation in people with human immunodeficiency virus: challenging targets. *J Infect Dis*. 2020;221(10):1567–1570. doi:10.1093/infdis/jiz351
37. Paiardini M, Müller-Trutwin M. HIV-associated chronic immune activation. *Immunol Rev*. 2013;254(1):78–101. doi:10.1111/imr.12079
38. Kumar A, Coquard L, Herbein G. Targeting TNF-alpha in HIV-1 infection. *Curr Drug Targets*. 2016;17(1):15–22. doi:10.2174/1573399811666150615145824
39. Fiske CT, de Almeida AS, Shintani AK, Kalams SA, Sterling TR. Abnormal immune responses in persons with previous extrapulmonary tuberculosis in an in vitro model that simulates in vivo infection with Mycobacterium tuberculosis. *Clin Vaccine Immunol*. 2012;19 (8):1142–1149. doi:10.1128/CVI.00221-12
40. Zhang C, Zhu R, Cao Q, Yang X, Huang Z, An J. Discoveries and developments of CXCR4-targeted HIV-1 entry inhibitors. *Exp Biol Med*. 2020;245(5):477–485. doi:10.1177/1535370220901498
41. Crawley AM, Angel JB. Expression of γ-chain cytokine receptors on CD8+ T cells in HIV infection with a focus on IL-7Rα (CD127). *Immunol Cell Biol*. 2012;90(4):379–387. doi:10.1038/icb.2011.66
42. Jabara HH, Boyden SE, Chou J, et al. A missense mutation in TFRC, encoding transferrin receptor 1, causes combined immunodeficiency. *Nat Genet*. 2016;48(1):74–78. doi:10.1038/ng.3465

43. Aljohani AH, Al-Mousa H, Arnaout R, et al. Clinical and immunological characterization of combined immunodeficiency due to TFRC mutation in eight patients. *J Clin Immunol*. 2020;40(8):1103–1110. doi:10.1007/s10875-020-00851-1
44. Miedema F. Immunological abnormalities in the natural history of HIV infection: mechanisms and clinical relevance. *Immunodefic Rev*. 1992;3 (3):173–193.
45. Massanella M, Negredo E, Pérez-Alvarez N, et al. CD4 T-cell hyperactivation and susceptibility to cell death determine poor CD4 T-cell recovery during suppressive HAART. *Aids*. 2010;24(7):959–968. doi:10.1097/QAD.0b013e328337b957
46. Luo Z, Li Z, Martin L, et al. Increased natural killer cell activation in HIV-infected immunologic non-responders correlates with CD4+ T cell recovery after antiretroviral therapy and viral suppression. *PLoS One*. 2017;12(1):e0167640. doi:10.1371/journal.pone.0167640
47. Hunt PW, Martin JN, Sinclair E, et al. T cell activation is associated with lower CD4+ T cell gains in human immunodeficiency virus-infected patients with sustained viral suppression during antiretroviral therapy. *J Infect Dis*. 2003;187(10):1534–1543. doi:10.1086/374786
48. Vajpayee M, Kaushik S, Sreenivas V, Mojumdar K, Mendiratta S, Chauhan NK. Role of immune activation in CD4+ T-cell depletion in HIV-1 infected Indian patients. *Eur J Clin Microbiol Infect Dis*. 2009;28(1):69–73. doi:10.1007/s10096-008-0582-7
49. Zhang X, Hunt PW, Hammer SM, Cespedes MS, Patterson KB, Bosch RJ. Immune activation while on potent antiretroviral therapy can predict subsequent CD4+ T-cell increases through 15 years of treatment. *HIV Clin Trials*. 2013;14(2):61–67. doi:10.1310/hct1402-61
50. Gougeon ML, Ledru E, Naora H, Bocchino M, Lecoeur H. HIV, cytokines and programmed cell death. A subtle interplay. *Ann N Y Acad Sci*. 2000;926:30–45. doi:10.1111/j.1749-6632.2000.tb05596.x
51. Benson DM Jr, Bakan CE, Mishra A, et al. The PD-1/PD-L1 axis modulates the natural killer cell versus multiple myeloma effect: a therapeutic target for CT-011, a novel monoclonal anti-PD-1 antibody. *Blood*. 2010;116(13):2286–2294. doi:10.1182/blood-2010-02-271874
52. Yamamoto Y, Miyazato K, Takahashi K, Yoshimura N, Tahara H, Hayakawa Y. Lung-resident natural killer cells control pulmonary tumor growth in mice. *Cancer Sci*. 2018;109(9):2670–2676. doi:10.1111/cas.13703
53. Ceausu A, Rodríguez-Gallego E, Peraire J, et al. IL-7/IL-7R gene variants impact circulating IL-7/IL-7R homeostasis and ART-associated immune recovery status. *Sci Rep*. 2019;9(1):15722. doi:10.1038/s41598-019-52025-8
54. Baba M, Schols D, Nakashima H, et al. Selective activity of several cholic acid derivatives against human immunodeficiency virus replication in vitro. *J Acquir Immune Defic Syndr*. 1989;2(3):264–271.
55. Sainz-Gaspar L, Suárez-Peñaranda JM, Pousa-Martínez M, Vázquez-Veiga H, Fernández-Redondo V. Topical timolol for treatment of penile Kaposi sarcoma in HIV-negative patient. *Dermatol Ther*. 2017;30:5. doi:10.1111/dth.12519