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Integrated immune and programmed cell death analysis identifies NR4A2 as a candidate blood biomarker in asthma

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Article

Keywords: Asthma, Programmed Cell Death, Immunity, NR4A2, Bioinformatics, Biomarker

Posted Date: March 25th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-9114966/v1

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Additional Declarations: No competing interests reported.

Abstract

Background

Asthma is a heterogeneous chronic airway disease in which both immune dysregulation and programmed cell death (PCD) contribute to disease pathogenesis. However, the molecular crosstalk linking these processes remains incompletely understood. This study aimed to identify key genes associated with immune-PCD interactions in asthma and to evaluate their potential clinical relevance.

Methods

Public transcriptomic datasets were analyzed using single-sample gene set enrichment analysis (ssGSEA) to quantify immune-cell infiltration and PCD-related signatures in asthma and control samples. Candidate genes were identified by intersecting module genes, differentially expressed genes (DEGs), immune-related genes, and PCD-related genes. Key genes were further screened using least absolute shrinkage and selection operator (LASSO), support vector machine-recursive feature elimination (SVM-RFE), and Boruta algorithms. GSEA and single-cell RNA sequencing analyses were performed to explore potential biological pathways, cell-type distribution, and intercellular communication. Peripheral blood samples from 57 asthmatic patients and 30 non-asthmatic controls were collected for qPCR validation, and associations with fractional exhaled nitric oxide (FeNO) and eosinophil counts were assessed.

Results

11 immune cell types and 7 PCD categories differed significantly between asthma and control groups. Nine candidate immune-PCD genes were identified, from which three key genes (NRAS, JUN, and NR4A2) were subsequently identified by overlapping the outputs of LASSO, SVM-RFE, and Boruta, and showed high diagnostic performance for asthma (AUC: 1.00, 0.97, and 0.99, respectively). GSEA suggested that these key genes may be involved in asthma-related biological processes through natural killer (NK) cell-mediated cytotoxicity and Toll-like receptor signaling pathways. Single-cell analysis indicated that the key genes were predominantly expressed in T cells and NK cells, with potential intercellular communication via multiple HLA–CD8 ligand–receptor pairs. In peripheral blood validation, NR4A2 expression was significantly reduced in asthma and was negatively correlated with FeNO and eosinophil levels, whereas no significant expression differences were observed for NRAS or JUN.

Conclusion

NR4A2 was identified as a key immune-PCD-associated gene in asthma and may serve as a candidate diagnostic biomarker. These findings provide a framework for investigating immune-PCD crosstalk in asthma and support further mechanistic and translational studies of NR4A2.

Background

Asthma is a heterogeneous chronic airway disease characterized by variable respiratory symptoms, reversible airflow limitation, and persistent airway inflammation[1]. As a prevalent global health issue, asthma affects an estimated 300 million people worldwide causing over 450,000 deaths annually[2]. Despite major advances in disease phenotyping and the development of targeted therapies, the molecular basis underlying asthma heterogeneity remains incompletely understood [3, 4]. Increasing evidence indicates that asthma pathogenesis is shaped not only by dysregulated immune responses, but also by multiple forms of PCD, including apoptosis, autophagy, pyroptosis, ferroptosis, and necroptosis, which are critical for maintaining tissue homeostasis, eliminating damaged or infected cells, and supporting developmental processes[5, 6] and may influence airway epithelial injury, inflammatory amplification, and airway remodeling[7]. However, these processes are often investigated separately, and a systematic understanding of their interaction in asthma is still lacking.

Immune dysregulation is a central feature of asthma and involves both innate and adaptive immune compartments [1]. In addition to the established roles of eosinophils, mast cells, and T-helper-cell subsets, recent studies suggest that broader immune-cell networks and intercellular communication patterns also contribute to disease initiation, persistence, and heterogeneity[8, 9]. At the same time, aberrant regulation of PCD-related pathways may alter immune-cell survival, epithelial integrity, and inflammatory signaling, thereby promoting chronic airway inflammation[7]. The interconnection between PCD and immunity is profound; for instance, apoptotic cells can be cleared by phagocytes, thereby modulating immune tolerance and preventing autoimmunity, while immune effectors like cytotoxic T lymphocytes and natural killer cells can induce PCD in target cells to combat infections and malignancies[10, 11]. In asthma, a chronic inflammatory airway disease, dysregulated PCD and immune activation contribute to pathogenesis, with excessive apoptosis of bronchial epithelial cells exacerbating inflammation and immune cells such as eosinophils, neutrophils and lymphocytes, promoting airway inflammation and remodeling through PCD-related pathways [7, 12–14]. Consequently, exploring biomarkers associated with PCD and immunity could elucidate asthma mechanisms and unveil novel therapeutic targets. Because immune regulation and PCD are biologically interconnected, identifying genes located at the intersection of these two processes may improve our understanding of asthma pathobiology and provide clinically relevant biomarkers or therapeutic targets.

With the increasing availability of public transcriptomic resources and single-cell datasets, integrated bioinformatics approaches offer an opportunity to dissect complex disease-associated molecular networks at both bulk and cellular resolution[9]. Machine-learning algorithms can further prioritize robust candidate genes from high-dimensional data, whereas single-cell RNA sequencing can localize these genes to specific cell populations and infer potential cell-cell communication patterns [4]. Nevertheless, many computational studies in asthma remain limited by insufficient experimental or clinical validation, which restricts their translational relevance. Given the clinical value of inflammatory biomarkers such as FeNO[15], there remains a need to identify molecular markers that are mechanistically informative and clinically accessible.

In the present study, we aimed to identify key genes linking immune dysregulation and PCD in asthma through an integrated analytical framework combining bulk transcriptomic analysis, machine-learning-based feature selection, pathway enrichment, and single-cell RNA sequencing. We further validated candidate genes in peripheral blood samples from patients with asthma and non-asthmatic controls by quantitative PCR and examined their associations with clinical inflammatory indicators. By integrating computational discovery with clinical validation, we sought to identify biologically meaningful and clinically relevant immune-PCD-associated biomarkers in asthma, with particular attention to the potential role of NR4A2.

Methods

Data source

The datasets used in this study were downloaded from the publicly available GEO database (https://www.ncbi.nlm.gov/geo/). The GSE165934 dataset (10 asthma and 9 control) was used as the training set for gene screening. The GSE104468 dataset (12 asthma and 12 control) was used as the testing set to validate the results obtained from the training set. The single-cell level analysis was performed on 5 asthma and 3 control samples in the GSE172495 dataset. Immune genes were obtained from the previous literature[16]. Meanwhile, gene sets corresponding to each pattern of programmed cell death were referenced to the published literature[17]. The small-molecule drug structures were downloaded from the PubChem database (https://pubchem.ncbi.nlm.nih.gov).

Identification of immune and PCDs involved in asthma

The ssGSEA algorithm was employed to calculate the enrichment of 28 immune cells, including activated CD8 T cells, central memory CD8 T cells, effector memory CD8 T cells, activated CD4 T cells, central memory CD4 T cells, effector memory CD4 T cells, T follicular memory CD4 T cells, gamma delta T cells, type 1 T helper cells, type 17 T helper cells, type 2 T helper cells, regulatory T cells, activated B cells, immature B cells, memory B cells, natural killer cells, CD56 bright natural killer cells, CD56 dim natural killer cells, myeloid derived suppressor cells, natural killer T cells, activated dendritic cells, plasmacytoid dendritic cells, immature dendritic cells, macrophage, eosinophil, mast cells, monocyte, neutrophil and 18 PCDs, including apoptosis, anoikis, autophagy, alkaliptosis, cuproptosis, entosis, entotic cell death, immunogenic cell death, ferroptosis, lysosome-dependent cell death, methuosis, necroptosis, netotic cell death, NETosis, oxeiptosis, pyroptosis, parthanatos, paraptosis for each sample in the GSE165934 dataset. Differences in PCDs and immune cells between asthma and control samples were then compared using the Wilcoxon test.

Identification and functional analysis of candidate immune-PCD genes

After detecting outliers and screening the optimal soft threshold, a weighted gene co-expression network analysis (WGCNA) network was constructed based on the GSE165934 dataset. Using Pearson correlation analysis, we calculated the correlation coefficients and p-values between modules and differentially distributed immune cells to select immune cell-correlated modules, in which genes were further screened by |GS| > 0.4 and |MM| > 0.7. At the same time, the DEGs between asthma and control groups were obtained by using adjusted p-value < 0.05 and $|\log_2 FC| > 1$ as criteria. Next, candidate immune-PCD genes were obtained by overlapping genes involved in the differentially enriched PCD types, immune genes, WGCNA module genes and DEGs, followed by GO and KEGG enrichment analyses by ClusterProfiler package.

Machine learning analysis

Three machine learning algorithms, LASSO, SVM-RFE and Boruta, were utilized to screen immune-PCD feature genes in asthma. Then key immune-PCD genes were identified by overlapping feature genes obtained from LASSO, SVM-RFE and Boruta. Subsequently, a diagnostic nomogram based on key immune-PCD genes was developed by the rms package. The accuracy and clinical unitality of this nomogram were evaluated using calibration curve, ROC curve and decision curve analysis (DCA). In addition, the individual diagnostic value of key immune-PCD genes were evaluated by ROC curves in both training and testing cohorts.

Characterization of key PCD-immune genes

Based on the expression profiles of key genes, we divided samples into high- and low-score groups, and performed GSEA to investigate the function of key genes. Subsequently, the functional similarity among the key genes was analyzed by GOSemSim package. In addition, we compared the enrichment of immune functions (ccr, inflammation-promoting, parainflammation, T_cell_co-stimulation, type_I_IFN_response) and expressions of HLAs between asthma and control samples. Thereafter, Spearman's correlation analysis was conducted to assess the relationship between key genes and immunity.

Construction of transcription factor (TF)-key gene and drug-key gene networks

To investigate the molecular regulatory mechanisms of key genes, the miRNet database (https://www.mirnet.ca/) was first used to search for TFs that target key genes. From these results, transcription factors that were differentially expressed between the asthma and control groups were screened, followed by the construction of TFs-key genes regulatory network.

Drugs targeting key genes were first screened using the Drug-Gene Interaction database (DGIdb) (https://dgidb.org). Next, the protein structures of the key genes were downloaded from the Uniprot

database (https://www.uniprot.org). Molecular docking analysis was then performed using CBdock (https://cadd.labshare.cn/cb-dock/php/manual.php) and visualized by PyMol.

Single-cell data processing and analysis

For the single-cell dataset GSE172495, we downloaded the raw data for quality control and data filtering using the R package Seurat. Cells with 100 < n.features < 2500, n.Count < 10000 and percent.mt < 5% were retained. The data were normalized and scaled to identify highly variable genes and principal components. Dimensionality reduction of the cells was achieved through principal component analysis (PCA), and clustering of the cells was performed using uniform manifold approximation and projection (UMAP) with a resolution of 0.4. Then, the FindALLMarkers function was used to identify marker genes for each cluster and to annotate cell types. Cell types exhibiting differential expressions of key genes were defined as key cell types. Furthermore, GO and KEGG pathway enrichment analyses of key cells were performed. Additionally, cell-cell interactions were explored using the CellChat. Finally, key cells were subclustered, followed by a pseudotime analysis across subclusters using the Monocle 2 package.

Sample collection

57 patients with asthma in non-acute exacerbation stage and 30 healthy individuals were enrolled in this study. All the asthmatic patients have a doctor's diagnosis of asthma according to the 2024 update of the Global Strategy for Asthma Management and Prevention [18]. We collected peripheral blood samples from all participants and blood eosinophils count, IgE level and FeNO data were collected in asthmatic group. The study protocol was approved by the medical ethics committee of Qilu Hospital of Shandong University. All subjects gave their written informed consent. Patients' characteristics were shown in Table 1.

Table 1
Subjects' characteristics.

Gender(F/M)	**Control**	**Asthma**
	13/17	**26/31**
Age	46.38 ± 3.356	47.02 ± 2.193
Eosinophils count(*10^9/L)	0.118 ± 0.023	0.481 ± 0.080
IgE(IU/ml)	NA	608.5 ± 226.0
NA: Not available		

Key PCD-immune genes expression in asthmatic peripheral blood.

Total RNA was isolated according to the manufacturer's instructions (E.A.N.A.@ Blood RNA kit, Omega Bio-Tek). RNA concentration was quantified with a Nanodrop 2000 Spectrophotometer (NanoDrop

Technologies) before reverse transcription was performed (RevertAid First strand cDNA Synthesis Kit, ThermoFisher Scientific). Gene expression profiles were detected using SYBR Green qPCR Master Mix (GLPBIO) in a BIO-RAD CFX connect system with specific primers (Table 2). GAPDH was used as the reference to normalize the relative gene expressions. The expressions of key genes were calculated using the $2^{-\Delta\Delta Ct}$ method.

Table.2 Primer sequence for key PCD-immune genes expression

Primer name		Primer sequence
NR4A2	Forward	AGTGGTTCGCACAGACAGTT
	Reverse	GGAGCCAGTCAGGAGATCATAG
JUN	Forward	GGACCTTATGGCTACAGTAACC
	Reverse	CCGTTGCTGGACTGGATTATC
NRAS	Forward	CCTCCTCACTTGGCTGTCTG
	Reverse	TCACGTTTGCGGTTTGGTTC
GAPDH	Forward	AGAAGGCTGGGGCTCATTTG
	Reverse	AGGGGCCATCCACAGTCTTC

Statistical analysis

All statistical results were analyzed through R (version 4.3.1). The regulatory network was constructed by Cytoscape (version 3.9.1). Q-PCR data were statistically analyzed using GraphPad Prism(version6). Differences between the two groups were tested using the Wilcoxon test or t-test and one-way ANOVA were used to compare differences among three groups with p-value < 0.05 indicating statistical significance. Pearson correlation analysis was used to analyze whether there was a correlation.

Result

The immune cell infiltrations and PCDs were altered in asthma patients

The ssGSEA results indicated that 11 immune cells, including activated CD4 T cells, eosinophils, gamma delta T cells, macrophages, mast cells, natural killer cells, natural killer T cells, neutrophils, plasmacytoid dendritic cells, regulatory T cells and type 2 T helper cells were significantly decreased in asthma samples (Fig. 1). Meanwhile, the enrichment scores of seven PCDs, including anoikis, apoptosis, cuproptosis, immunogenic cell death, necroptosis, NETosis and pyroptosis, were also significantly lower in asthma samples (Fig. 2). Those results indicated that immune cell infiltration and PCDs might

participate in the development of asthma. Thus, it is of great significance to identify key immune-PCD related genes involved in asthma.

Nine candidate immune-PCD genes associated with immune and metabolism

were identified in asthma

Firstly, sample cluster analysis by Hclust function was conducted, and no outlier sample was detected, and all samples were included in the network construction (Fig. 3A). We set the soft threshold to 8 to ensure a scale-free topology of the network (R^2 > 0.85, Fig. 3B). By assessing gene correlation, we constructed a gene hierarchy clustering dendrogram (Fig. 3C), which allowed us to identify 14 distinct gene modules. Among these modules, turquoise and pink modules showed the highest correlations with differentially distributed immune cells (Fig. 3D). Then, 980 genes related to immune cells were further selected from turquoise and pink modules by using |GS|≥0.4 and |MM|≥0.7 as criteria. Meanwhile, a total of 794 DEGs were identified between asthma and controls. Among those DEGs, 156 genes exhibited an upregulation trend, while 638 genes showed a downregulation trend in asthma samples (Fig. 4A). Next, 9 candidate immune-PCD genes (JUN, NRAS, IL1B, NR4A2, TNFAIP3, IFNG, PTGS2, IL1RAP and TLR2) were obtained by overlapping 980 module genes, 794 DEGs, 956 PCDs genes and 1793 immune genes (Fig. 4B). The enrichment results of GO revealed that those genes were associated with neuroinflammatory response, glial cell activation, interleukin-6 production, regulation of interlrukin-6 production, nitric oxide metabolic process, NAD+ nucleosidase activity, NAD(P)+ nucleosidase activity, NAD+ nucleotidase, cyclic ADP-ribose generating, interleukin-1 receptor binding, hydrolase activity, hydrolyzing N-glycosyl compounds (Fig. 4C). The enrichment results of KEGG also showed that those genes had close relationship with immune and inflammatory response and metabolism, such as IL-17 signaling pathway, PD-L1 expression and PD-1 checkpoint pathway in cancer, rheumatoid arthritis, Th17 cell differentiation, TNF signaling pathway, lipid and atherosclerosis (Fig. 4D).

Machine learning identified NRAS, JUN and NR4A2 as key immune-PCD genes

To identify key genes from 9 candidate genes, LASSO, SVM-RFE and Boruta algorithms were employed. By LASSO, 5 genes (JUN, NRAS, IL1B, NR4A2 and IL1RAP) were screened as feature genes (Fig. 5A). By SVM-RFE, 4 genes (JUN, NRAS, NR4A2 and IFNG) were identified as important gene signatures (Fig. 5B). All 9 candidate genes were retained by Boruta algorithm (Fig. 5C). Finally, by overlapping feature genes selected by three algorithms, NRAS, JUN and NR4A2 were obtained and defined as key genes (Fig. 5D). Three key genes were involved in two PCDs (anoikis and apoptosis) and five immune-related functions (TCR signaling pathway, antimicrobials, cytokine_receptors, natural killer_cell_cytotoxicity and BCR

signaling pathway) (Fig. 5E). Furthermore, a nomogram was constructed based on key genes (Fig. 6A). Results of the calibration curve and ROC curve (AUC = 1.000) indicated that the model exhibited excellent predictive performance (Fig. 6B-6C). Meanwhile, DCA curve showed the good clinical utility (Fig. 6D).

Key genes might regulate the immune microenvironment of asthma

To further elucidate the characteristics and functions of key genes, we first observed their expressions in asthma. It was found that the expressions of key genes were significantly downregulated in the asthma group in both training and testing sets (Fig. 7A-7B). Furthermore, the AUCs of NRAS, JUN and NR4A2 were above 0.7 in both training and testing cohorts (Fig. 7C-7D), indicating their individual diagnostic potential in asthma. The functional similarity among the three genes using the GOSemSim package showed that JUN and NR4A2 have a high functional similarity (Fig. 7E). To unveil the relevant mechanisms of key genes in asthma, we then performed GSEA. Interestingly, natural killer cell-mediated cytotoxicity and toll-like receptor signaling pathway were significantly enriched in NRAS/ JUN/ NR4A2-high expression groups. In addition, key genes might regulate asthma through other signaling, such as B cell receptor, mitogen-activated protein kinase (MAPK) and p53 pathways (Fig. 8A-8C). Besides, we found that CCR, inflammation-promoting, parainflammation, T cell co-stimulation and type I IFN response existed significant differences between asthma and control groups (Fig. 8D), which were positively or negatively correlated with three key genes (Fig. 8E). Also, significant differences in two HLA genes, namely HLA-DOA and HLA-E, were observed between asthma and control groups (Fig. 8F), and remarkably positive correlations were detected between key genes and HLAs (Fig. 8G).

Single-cell data reveals NK cells and T cells as key cells

After quality control and preprocessing, 4,283 cells were used for the downstream analyses (Figure S1A). Cells were reduced dimensionally by PCA, in which 10 PCs were selected (Figure S1B and S1C), and were divided into 7 clusters (Figure S1D). Those clusters were then annotated T cells, NK cells, B cells and Monocytes (Fig. 9A). The proportions of cells in asthma and control groups revealed that T cells comprised the largest proportion, followed by NK cells, and the monocytes had the lowest proportion. In addition, asthma group had higher abundance of T cells and monocytes, but less B cells and NK cells (Fig. 9B). We found that three key genes had expressions in all cell types (Fig. 9C), but JUN and NR4A2 showed significantly differentially expressions in T cells and NK cells, respectively, between asthma and control groups (Fig. 9D). Thus, NK cells and T cells were selected as key cells for subsequent analyses.

The GO analysis revealed that NK cells were mainly associated with cell killing, leukocyte mediated cytotoxicity, leukocyte mediated immunity, while T cells were mainly relevant to T cell differentiation,

regulation of T cell activation and lymphocyte differentiation (Fig. 10A). The KEGG analysis revealed that NK cells functioned mainly through natural killer cell-mediated cytotoxicity, graft-versus-host disease, and apoptosis, while genes expressed in T cells were mainly enriched in viral protein interaction with cytokine and cytokine receptor, cytokine-cytokine receptor interaction, and adherens junction (Fig. 10B). Furthermore, we found the close cross-talk between NK cells and T cells in asthma (Fig. 10C). NK cells and T cells communicate with each other via multiple ligand-receptor pairs, such as HLA-E - CD8A, HLA-B - CD8A, HLA-A - CD8A (Fig. 10D). Further clustering of T cells and NK cells was conducted, dividing both NK cells and T cells into three subpopulations (Fig. 11A). Pseudotime analysis showed that the differentiation trajectory of NK cells encompassed seven states, with variations in the expression levels of JUN and NR4A2 across these temporal states (Fig. 11B and 11C). The analysis of T cells indicated that the differentiation proceeds through five states, during which the expression level of JUN fluctuated across these temporal stages (Fig. 11D and11E).

Key genes are regulated by multiple transcription factors and targeted by small molecule drugs

To investigate the regulatory mechanism of these three genes in asthma, 20 TFs were identified. Among those TFs, the expressions of 9 TFs showed significant differences between asthma and control groups (Fig. 12A). Finally, a TF-key genes network, which included the three key genes and 9 TFs, was constructed (Fig. 12B). To explore the therapeutic value of key genes, chemical drugs, including holacanthone, compound_108 and chelidonine, were predicted to target those genes in asthma. Molecular docking results showed that JUN had highest binding energy value of -6.9 Kcal/mol with holacanthone (Fig. 12C), NR4A2 had better binding activity with compound_108 (-5.7 Kcal/mol) (Fig. 12D) and NRAS had the highest binding energy value of -8.8 Kcal/mol with chelidonine (Fig. 12E), indicating that holacanthone, compound_108 and chelidonine may be potential drugs for the treatment of asthma.

The expression of NR4A2 was altered in peripheral blood of asthmatic patients and was negatively correlated with FeNO and peripheral blood eosinophil counts.

We have identified key genes that might regulate the immune microenvironment of asthma through bioinformatics analysis and performed qPCR to clarify the expressions of the above three genes in peripheral blood of asthma patients. The expression of NR4A2 was significantly decreased in asthma which was consistent with the results of bioinformatics analysis (Fig. 13A), while no significant difference was found in the expression of NRAS and JUN between the two groups (Fig. 13B-C). Since NR4A2 was the only index with differential expression between the two groups, we performed a subgroup analysis on its expression based on eosinophil counts and total serum IgE levels. NR4A2 was reduced in the eosinophilic asthma (EA) group (Fig. 13D) while the expression of JUN (Fig. 13E) and NRAS (Fig. 13F) exhibited no statistically significant differences in the EA and non-eosinophilic asthma (NEA) subgroups compared with the control group. In the subgroup of asthmatic patients with elevated

IgE levels, the expression of NR4A2 (Fig. 13G) and JUN (Fig. 13H) was decreased, whereas no significant difference in NRAS (Fig. 13I) expression was observed.

To investigate the diagnostic value of NR4A2 in asthma, we studied its correlation with key indicators of asthma. Correlation analysis revealed a significant negative correlation between NR4A2 expression levels and FeNO(r=-0.3765, p = 0.0216,n = 37, Fig. 13L), but no significant association with serum total IgE levels(Fig. 13K). In EA group, NR4A2 expression was similarly negatively correlated with peripheral blood eosinophil counts (r=-0.4264, p = 0.0236, n = 30, Fig. 13J). While no statistically significant correlations were observed between the expression levels of JUN and NRAS and FeNO, total serum IgE, or blood eosinophil count (Figure S2A-F).

Discussion

In the present study, we integrated immune-related and programmed cell death-related signatures to identify key molecular determinants of asthma. By combining bulk transcriptomic analysis, machine-learning-based feature selection, pathway enrichment, single-cell RNA sequencing, and peripheral blood validation, we identified NRAS, JUN, and NR4A2 as candidate immune-PCD-associated genes. Among these, NR4A2 emerged as the most robust candidate, as it was significantly downregulated in patients with asthma and showed negative correlations with FeNO and eosinophil levels. These findings suggest that NR4A2 may represent a clinically relevant molecular indicator linking immune dysregulation with cell death-associated processes in asthma, especially in eosinophilic asthma.

Asthma is increasingly recognized as a heterogeneous inflammatory airway disorder shaped by complex interactions among structural cells, immune cells, and stress-response pathways[1]. Although immune dysregulation is central to asthma pathogenesis[19, 20], accumulating evidence indicates that aberrant activation of PCD-related programs also contributes to epithelial injury, inflammatory amplification, and airway remodeling[7]. However, these two biological dimensions are often investigated in parallel rather than within an integrated framework. Existing evidence indicates the intricate interplay between PCD and immune dysregulation constitutes a fundamental mechanism in asthma pathogenesis[21]. Various PCD modalities, such as apoptosis, autophagy, pyroptosis, ferroptosis, necroptosis, NETosis and pyroptosis, contribute significantly to airway inflammation and remodeling through the release of damage-associated molecular patterns and pro-inflammatory cytokines, which subsequently activate and recruit immune cells such as T lymphocytes and eosinophils [22–24]. This creates a self-perpetuating cycle of chronic inflammation and epithelial damage that characterizes asthma pathophysiology. Our analysis supports the concept that immune perturbation and PCD are coordinately involved in asthma and that genes at this intersection may better capture disease-associated molecular changes than either dimension alone.

Among the identified candidates, NR4A2 was of particular interest. Unlike NRAS and JUN, which were prioritized by the computational pipeline but were not confirmed in the peripheral blood validation cohort, NR4A2 demonstrated both differential expression and clinically relevant associations. This finding may

be explained by several factors pertaining to the inherent biological characteristics of these genes. Both JUN and NRAS are involved in active signal transduction and transcriptional regulation, which makes their expression highly dynamic and sensitive to external stimuli, transient inflammatory states, and changes in the proportions of various cell types in a heterogeneous peripheral blood sample. In contrast, NR4A2, a nuclear receptor transcription factor[25], exhibits more stable basal expression in peripheral blood mononuclear cells (PBMCs). It is relatively insensitive to short-term environmental fluctuations or shifts in cellular composition. Furthermore, NR4A2 expression is distributed more uniformly across PBMC populations. Consequently, observed differential expression trends for NR4A2 are likely more robust and reliable in analyses of mixed-cell samples, such as those derived from whole blood or PBMC isolates.

NR4A2 is a member of the nuclear family 4 subgroup A receptor (NR4A) super family, which has been implicated in transcriptional regulation, immune-cell differentiation, inflammatory control, and cellular stress responses [3, 4]. Previous studies have shown that NR4A family members promote regulatory T-cell differentiation, restrain effector T-cell responses, and suppress allergic airway inflammation, supporting the biological plausibility of NR4A2 as an immune-regulatory node in autoimmune disorders[8, 26, 27]. Takashi Yamamura found that NR4A2 orchestrates Th17 cell-mediated autoimmune inflammation via IL-21 signaling[28]. NR4A2 has emerged as a critical transcriptional regulator at the nexus of inflammation, immunity, and cellular homeostasis[29], with significant implications for the pathophysiology of asthma. In the context of airway inflammation, NR4A2 exerts a predominantly anti-inflammatory influence, though its role can be context- and cell-type-dependent. In macrophages and dendritic cells, NR4A2 activation can suppress the production of key pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β, often by antagonizing the NF-κB pathway[30]. This regulatory function is crucial in asthma, where unchecked NF-κB signaling drives chronic airway inflammation. Furthermore, in T lymphocytes, particularly T helper 2 (Th2) cells—central players in allergic asthma—NR4A2 expression can be induced by T cell receptor engagement[31]. Therefore, it may modulate the expression of cytokines like IL-5 and IL-13, thereby influencing the Th2-polarized immune response that characterizes asthma. NR4A2 also interacts with pathways involved in glucocorticoid receptor(GR) signaling and GR is a positive co-regulator of NR4A2 activity in PC12 cells on various NR4A2 response elements, potentially contributing to steroid sensitivity or resistance, a major clinical challenge in severe asthma management[32]. Sadia Ashraf reported that overexpression of NR4A2 in cardiomyocytes leads to changes in cell phenotype and function[33], suggesting that NR4A2 regulates proliferation and functional synthesis in structural cells. Its role extends to structural cells of the airway. In airway smooth muscle cells, for instance, it may regulate proliferative and synthetic functions, impacting airway remodeling and hyperresponsiveness.

Beyond inflammation and adaptive immunity, NR4A2 is a pivotal player in regulating cell survival and PCD, processes intimately linked to immune resolution and tissue damage in asthma[31, 34]. We found NR4A2 showed significantly differentially expressions in NK cells between asthma and control groups through Single-cell data. The GO analysis revealed that NK cells were mainly associated with cell killing, leukocyte mediated cytotoxicity, leukocyte mediated immunity, The KEGG analysis revealed that NK cells

functioned mainly through natural killer cell-mediated cytotoxicity, graft-versus-host disease, and apoptosis. Tim Vierbuchena et al. found the lncRNA LUCAT1 restrains inflammation by regulating the splicing and stability of NR4A2 in inflammatory disease, such as inflammatory bowel disease as well as asthma and chronic obstructive pulmonary disease[31]. The observed correlations between NR4A2 expression and immune cell infiltration scores are particularly striking in osteoarthritis and normal cartilage tissues[35]. Furthermore, they discovered NR4A2 attenuated IL-1β-induced chondrocyte apoptosis. Silencing NR4A2 enhanced the proliferative capacity of chondrocytes with IL-1β-induced apoptosis. NR4A2 can function as a pro-survival factor in certain cell types, including neurons and potentially inflammatory leukocytes, by inducing anti-apoptotic genes like Bcl-2[36]. However, under specific stress signals, it can translocate to mitochondria, induce cytochrome c release, and promote apoptosis, demonstrating a dual, context-dependent role in cell fate decisions[37, 38]. In asthma, the balance between airway epithelial cells, eosinophil, neutrophil, and lymphocyte apoptosis is critical for resolving inflammation. Dysregulation of these processes can lead to persistent inflammatory cell infiltration[7]. NR4A2's involvement in these pathways suggests it may help determine the lifespan of inflammatory cells within the airway mucosa[39]. Moreover, its interaction with autophagy pathways and other forms of PCD [40], represents a nascent area of research with potential relevance to asthma pathogenesis. The net effect of NR4A2 signaling in the asthmatic lung likely represents a complex summation of its actions across epithelial, immune, and mesenchymal cells, aiming to restrain excessive inflammation while also contributing to the clearance of effector cells[31]. Therefore, targeting NR4A2 activity—either by enhancing its anti-inflammatory functions or modulating its role in cell death—presents a novel, though complex, therapeutic strategy for achieving better disease control, especially in steroid-refractory asthma phenotypes.

The pathway analyses provided additional biological context for these observations. GSEA implicated natural killer cell-mediated cytotoxicity and Toll-like receptor signaling, both of which are biologically relevant in asthma[41–43]. NK cells have been reported to participate in allergic sensitization, effector responses, virus-related exacerbations, and the resolution of airway inflammation, underscoring their context-dependent role in asthma pathobiology[41, 42]. Likewise, Toll-like receptor signaling is increasingly recognized as a key interface between innate and adaptive immunity in asthma and may modulate inflammatory amplification and disease exacerbation[43]. At single-cell resolution, the enrichment of key genes in T cells and NK cells in our study is therefore biologically plausible and consistent with the emerging view that lymphocyte-associated immune circuits contribute to asthma heterogeneity.

In addition, our cell-cell communication analysis suggested intercellular interactions involving multiple HLA-CD8-related ligand-receptor pairs. Although this finding requires further validation, it is consistent with recent single-cell studies showing that asthma is characterized by altered cellular composition and intensified intercellular communication within inflamed airway tissues[9, 44]. These observations support the notion that immune-cell crosstalk, rather than isolated changes in single cell populations, may underlie disease-associated transcriptional reprogramming. Our data therefore extend current

single-cell observations by nominating NR4A2 as a candidate molecular component of the broader immune-PCD interaction network in asthma.

The comprehensive analysis conducted on the DGIdb, coupled with the intricate process of molecular docking, revealed that NR4A2 demonstrated a notably superior binding activity with compound_108. This particular compound is characterized as a fatty acid mimetic agonist, which has been documented to possess a degree of selectivity for the NR4A2 nerve growth factor IB-like receptor, indicating its potential therapeutic relevance[45]. The establishment of direct binding to the receptor was confirmed through the precise methodology of isothermal titration calorimetry (ITC), which provided robust evidence of the interaction. Furthermore, compound_108 has been predicted to specifically target NR4A2 in the context of asthma, suggesting its possible role in managing this condition. It is also noteworthy that multiple endogenous fatty acid metabolites serve as essential signaling molecules, playing significant roles in various inflammatory processes, thereby interacting with a range of receptors and other proteins within the body. In addition to its primary actions, compound_108 exhibited a slight activation of Peroxisome Proliferator-Activated Receptor Gamma (PPARγ) and demonstrated substantial agonistic activity towards RXRα, further highlighting its multifaceted biological effects and potential implications in therapeutic applications.

From a translational perspective, our findings support NR4A2 as a candidate blood-based biomarker in asthma. Compared with airway sampling, peripheral blood assessment is more feasible in routine clinical settings and may offer value for biomarker development and patient stratification. In particular, the association of NR4A2 with FeNO and blood eosinophils, two commonly used markers of type 2 airway inflammation, enhances its potential clinical relevance[15, 46]. Nonetheless, several limitations should be acknowledged. First, the high diagnostic performance observed in the public datasets may partly reflect dataset-specific characteristics and possible overfitting, and therefore requires confirmation in larger independent cohorts. Second, although single-cell and enrichment analyses suggested a link between NR4A2, immune dysregulation, and programmed cell death-related pathways, direct functional validation was not performed. Third, the validation cohort was based on peripheral blood rather than airway tissue and thus may not fully reflect compartment-specific mechanisms within the airway microenvironment. Fourth, the predicted NR4A2-targeting compound was identified by computational analysis only and should be regarded as hypothesis-generating until pharmacologic and functional studies are available. Finally, the sample size of the GEO datasets used is relatively limited, which may introduce statistical bias or reduce the power of our conclusions. Although we have validated the expression patterns of the identified genes using independent datasets, further in vitro and in vivo experimental studies are essential to confirm their functional roles and the underlying molecular mechanisms in asthma pathogenesis. We will continue to focus on these mechanisms to provide more robust evidence and deeper insights in future research.

Conclusion

This study identifies NR4A2 as a key immune-PCD-associated gene in asthma and supports its potential as a clinically relevant biomarker. By integrating transcriptomic, single-cell, and clinical validation data, our work provides a framework for understanding immune-PCD crosstalk in asthma and a rationale for further investigation of NR4A2 in disease stratification and mechanistic studies.

Abbreviations

PCD: Programmed cell death

ssGSEA: Single-sample gene set enrichment analysis

DEGs: Differentially expressed genes

LASSO: Least absolute shrinkage and selection operator

SVM-RFE: Support vector machine-recursive feature elimination

FeNO: Fractional exhaled nitric oxide

WGCNA: Weighted gene co-expression network analysis

DCA: Decision curve analysis

TF: Transcription factor

DGIdb: Drug-Gene Interaction database

PCA: Principal component analysis

UMAP: Uniform manifold approximation and projection

EA：Eosinophilic asthma

PBMCs: Peripheral blood mononuclear cells

NR4A: Nuclear family 4 subgroup A receptor

GR: Glucocorticoid receptor

ITC: Isothermal titration calorimetry

PPARγ: Peroxisome Proliferator-Activated Receptor Gamma

Declarations

Ethics approval and consent to participate

This human study was performed in line with the principles of the Declaration of Helsinki and approved by the medical ethics committee of Qilu Hospital of Shandong University (Approval number: KYLL-2020(KS)-430). All subjects gave their written informed consent.

Consent for publication

Not applicable

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

Funding

The authors declare that financial support was received for the research and/or publication of this article. This work was supported by National Natural Science Foundation of China (82000031,82000025) and Natural Science Foundation of Shandong Province (ZR2020QH002, ZR2022QH342, ZR2024MH349).

Author contributions

Tian Liu and Junfei Wang designed the study. Tian Liu drafted the manuscript. Xiaofei Liu, Yun Pan, Jiawei Xu and Rong Zeng performed the analysis. Tao Li Jiping Zhao, Shuang Luo, Xiaoyan Lu, Yufan Duan, Xiaoxuan Fan and Baoxiang Yu collected clinical specimens. Tian Liu, Peihang Jing and Junfei Wang contributed to interpretation of the results. Yuwen Xue and Junfei Wang reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

Acknowledgements

We thank Research Center for Basic Medical Science of Qilu hospital affiliated to Shandong University for consultation and instrument availability that supported this work.

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Figures

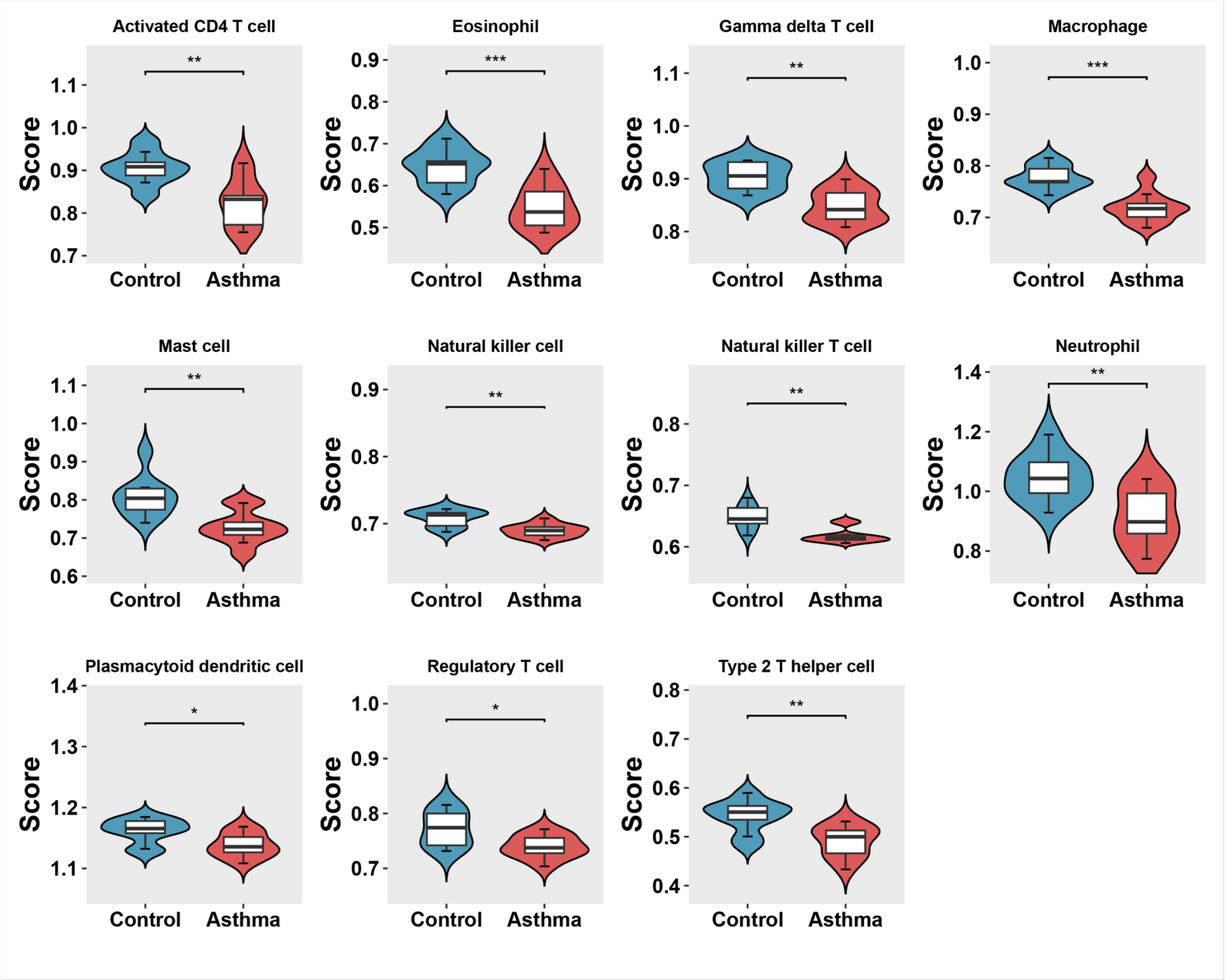


Figure 1

The differentially infiltrated immune cells between asthma and control groups. *P ≤ 0.05, **≤ 0.01, ***≤0.001.

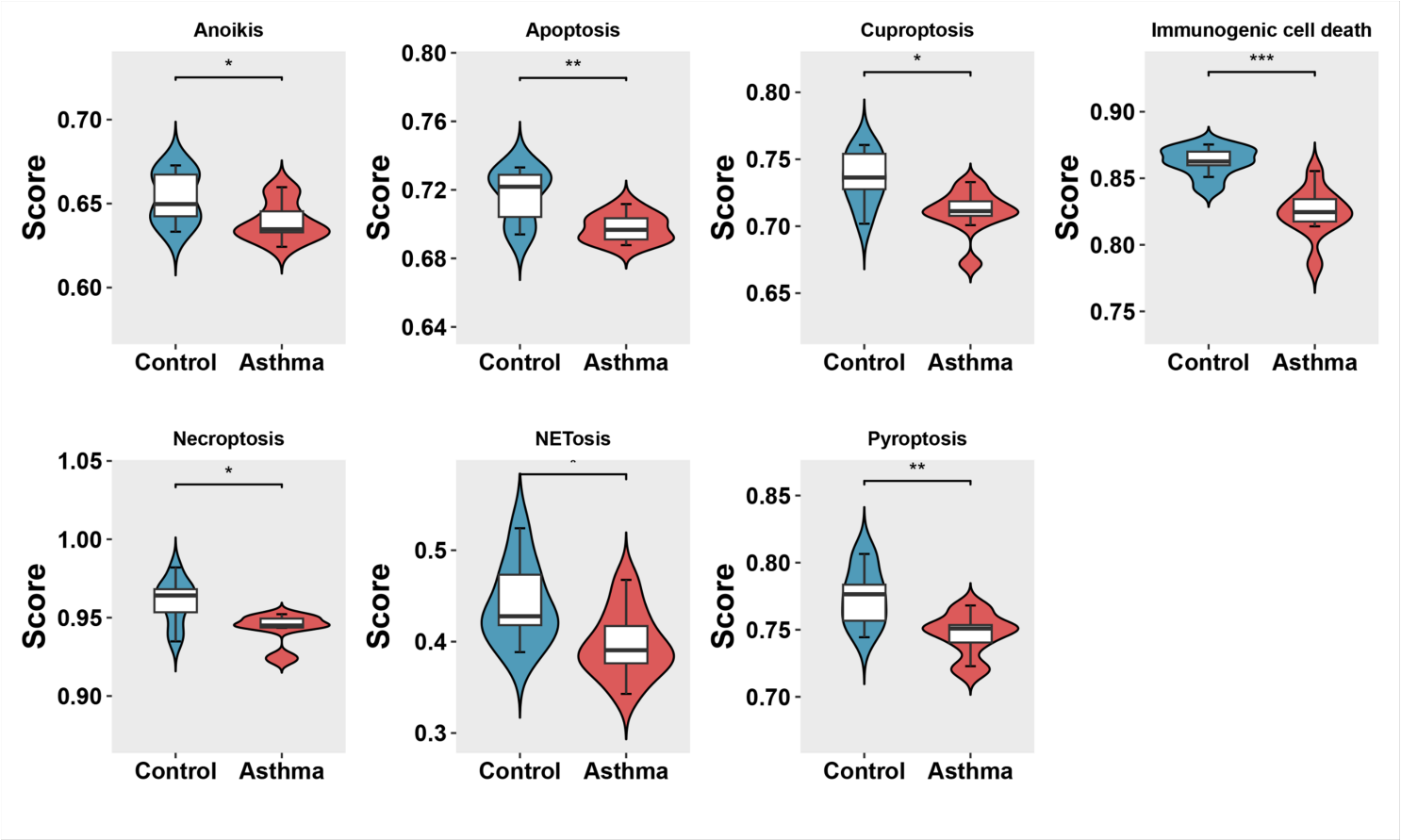


Figure 2

The different PCD types between asthma and control groups. *P ≤ 0.05, **≤ 0.01, ***≤0.001.

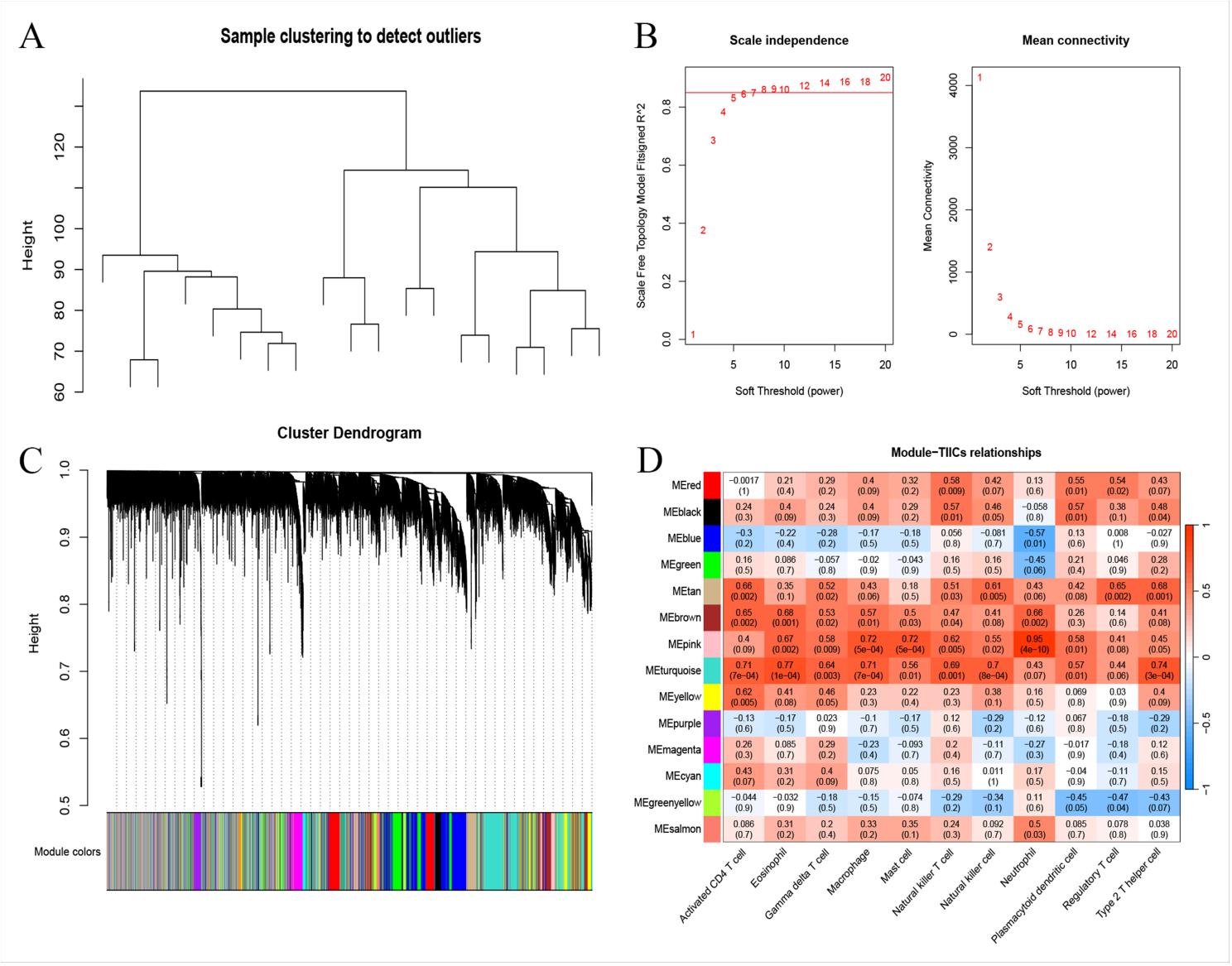


Figure 3

Weighted gene co-expression network analysis (WGCNA). (A) Clustering dendrogram of samples. (B) Analysis of network topology for various soft-thresholding powers. (C) Clustering dendrogram of genes. (D) Heatmap of the association between modules and differentially infiltrated immune cells.

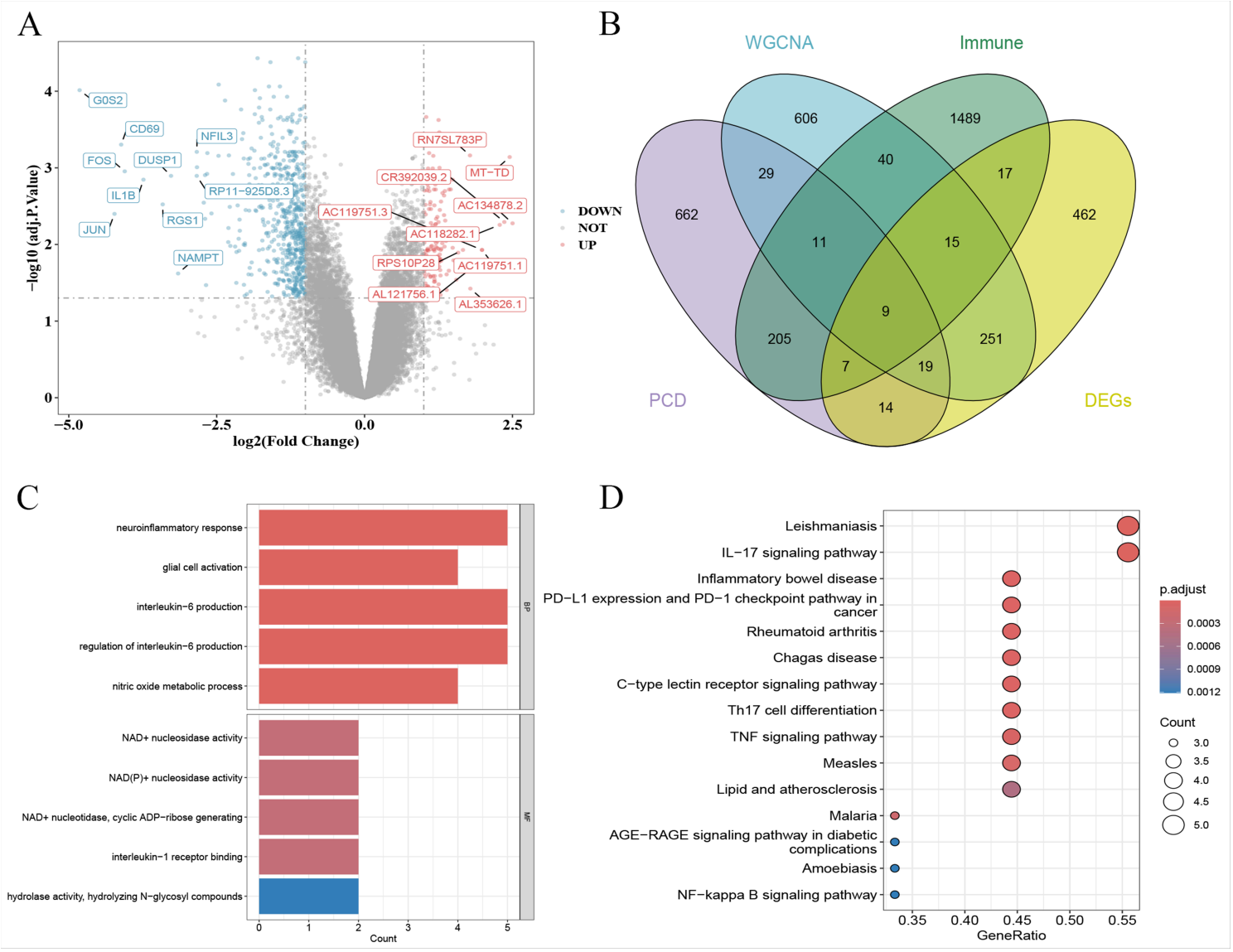


Figure 4

Candidate immune-PCD genes associated with immune and metabolism. (A) Volcano diagram showing differentially expressed genes (DEGs) between asthma and control groups. (B) Venn diagram showing common genes between the DEGs, WGCNA module genes, PCDs genes and immune genes. (C) Gene Ontology (GO) enrichment analysis of candidate genes. (D) Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of candidate genes.

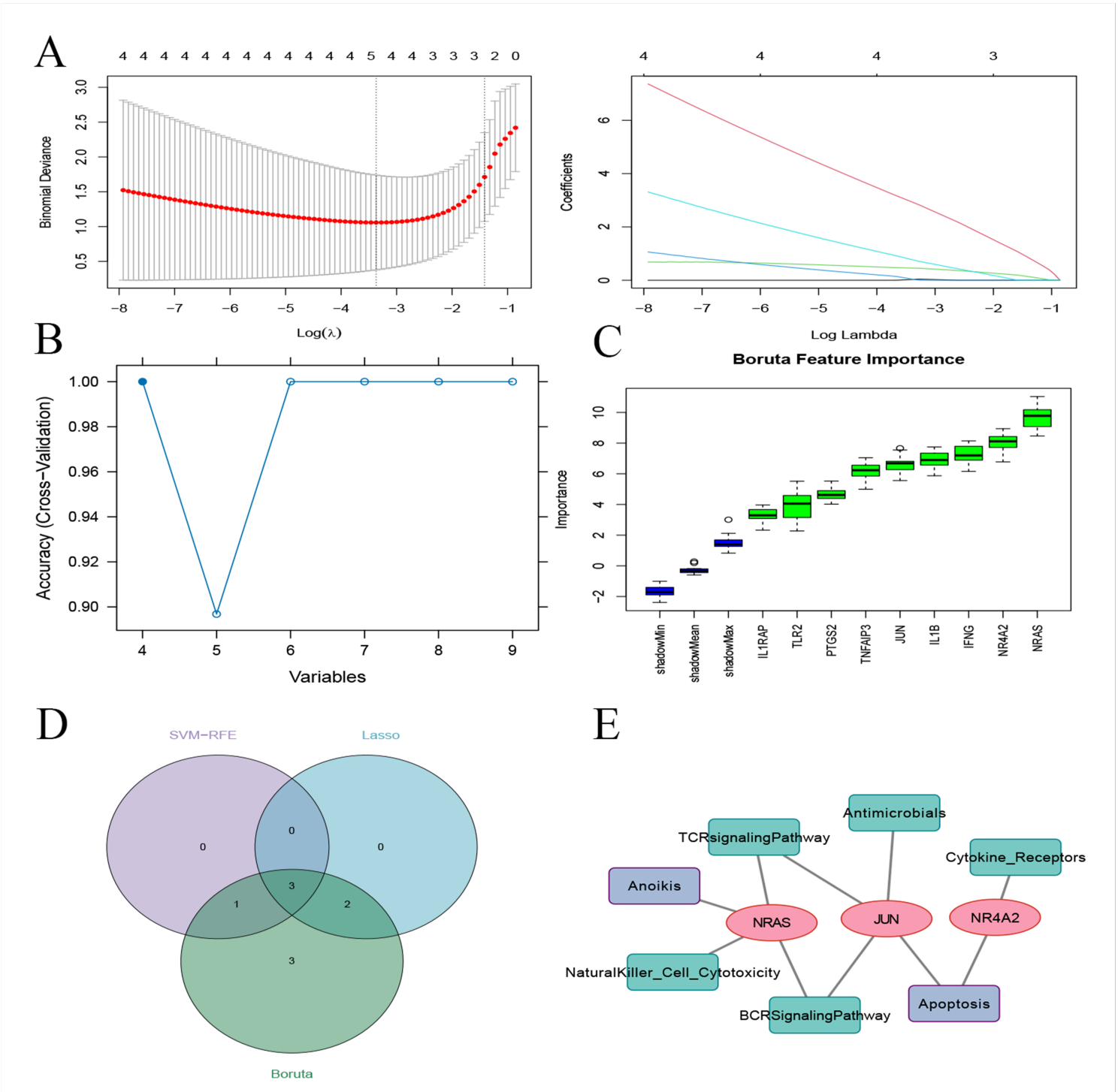


Figure 5

Identification of key immune-PCD genes by machine learning. (A) LASSO regression selected key genes (λ_{min} = 0.03445091). (B) SVM–RFE selected key genes. (C) Boruta selected key genes. (D) Venn diagram of key genes selected by three machine learning methods. (E) The PCD types and immune-related functions of key genes.

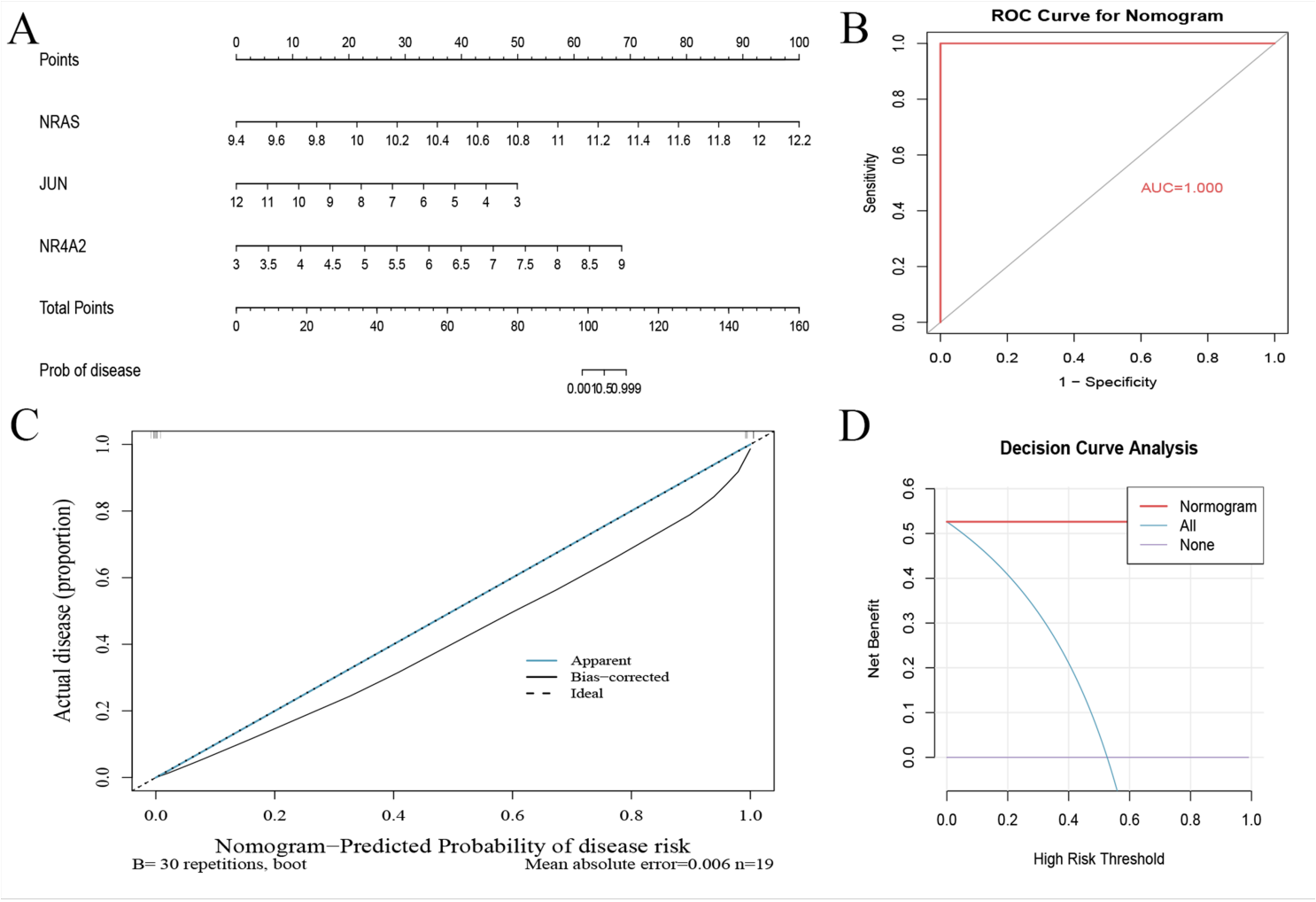


Figure 6

Construction and evaluation of diagnostic model. (A) Nomogram for asthma diagnosis based on key genes. (B) Receiver operating characteristics (ROC) curve for the training set showing the diagnostic performance of the nomogram. (C) Calibration curve assessing nomogram accuracy. (D) Decision curve analysis (DCA) evaluating the net benefit of diagnostic decisions based on the nomogram.

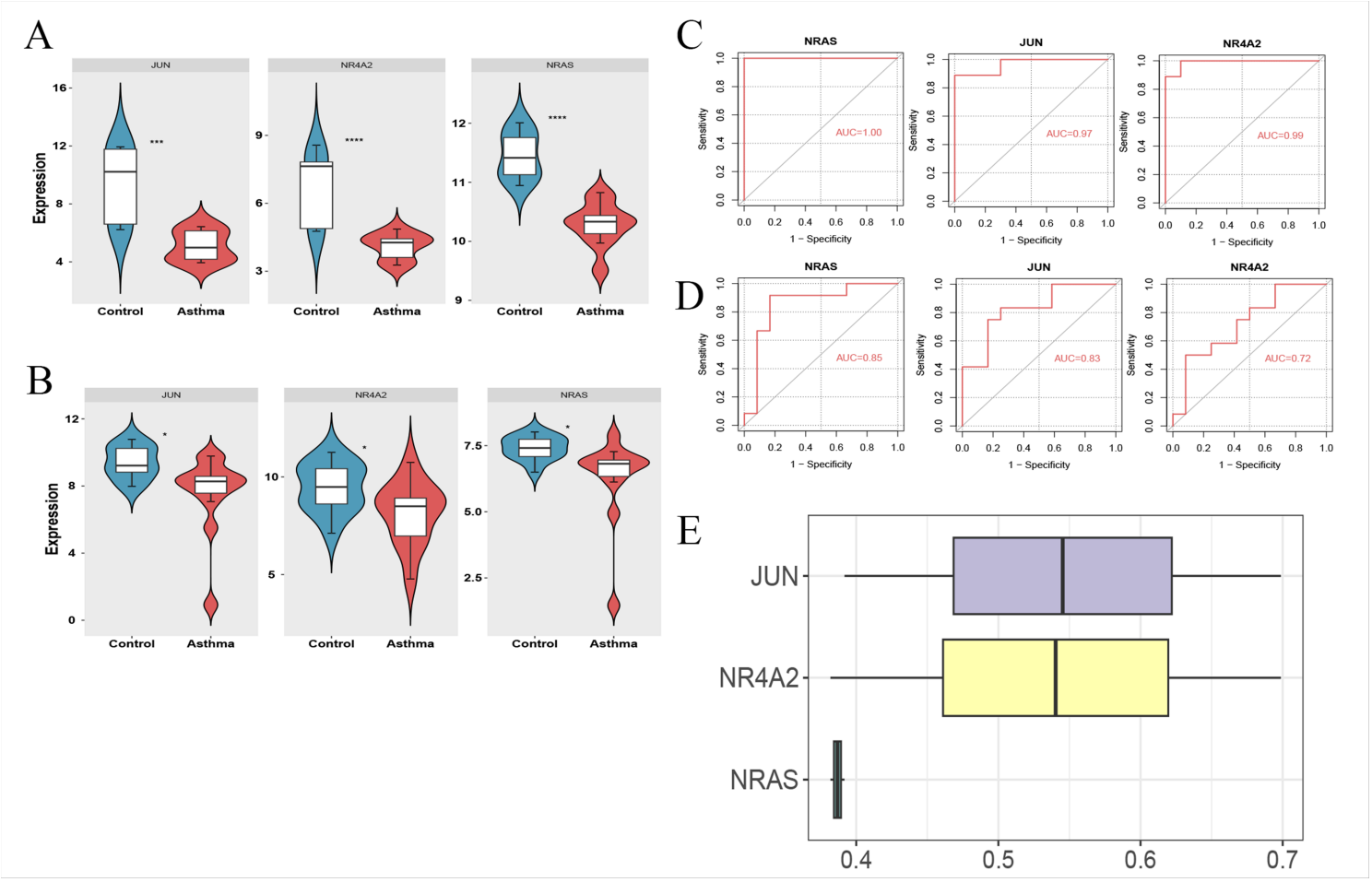


Figure 7

Characteristics of key genes. (A) The expression levels of key genes in control and asthma groups in training set. (B) The expression levels of key genes in control and asthma groups in testing set. (C) ROC curves of key genes in training set. (D) ROC curves of key genes in testing set. (E) The functional similarity analysis of key genes. *P ≤ 0.05, **≤ 0.01, ***≤0.001.

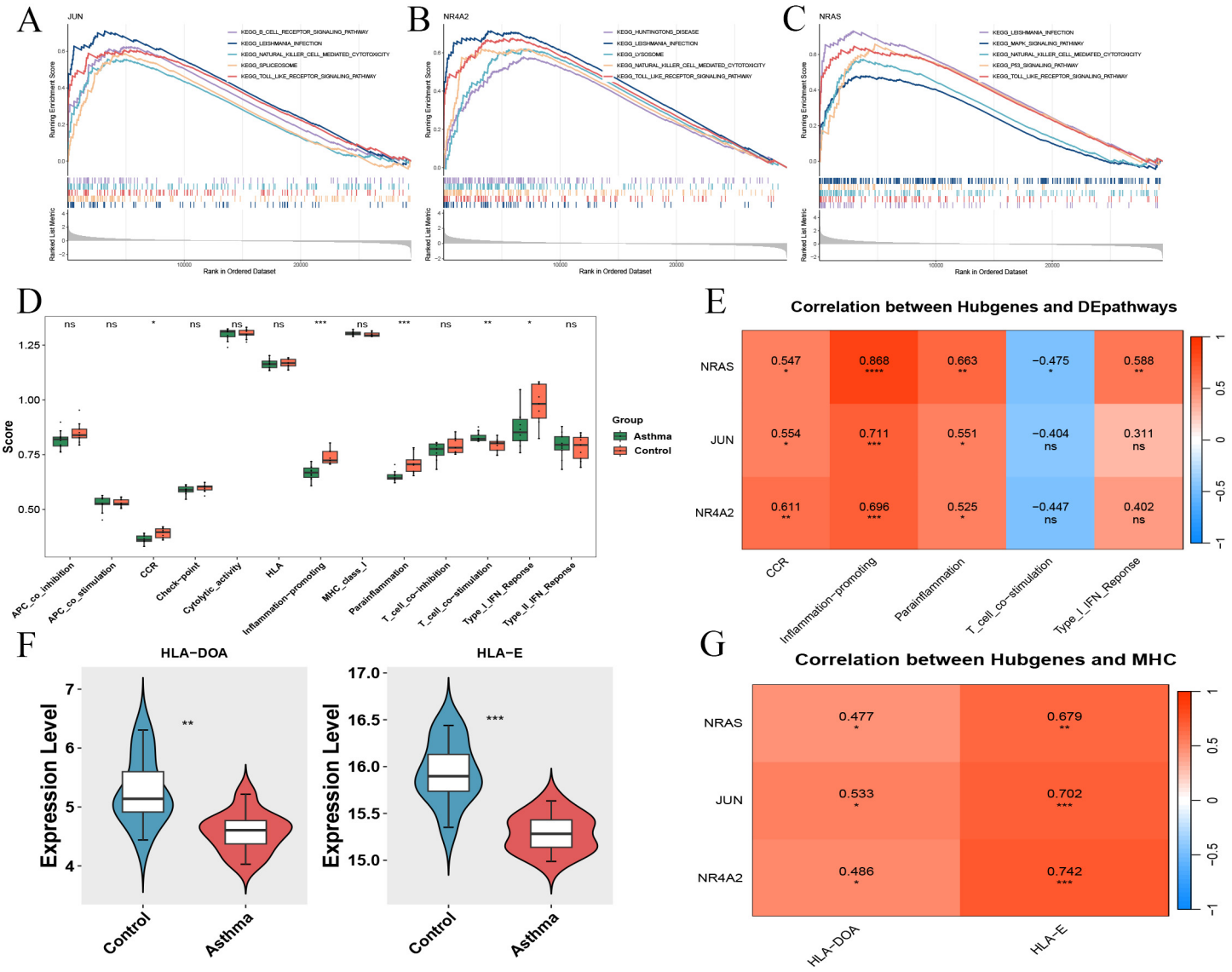


Figure 8

Molecular mechanisms of key genes. (A) Gene set enrichment analysis (GSEA) of JUN. (B) GSEA of NR4A2. (C) GSEA of NRAS. (D) Different immune pathways between asthma and control groups. (E) Correlation between key genes and different immune pathways. (F) Different HLA genes between control and asthma groups. (G) Correlation between key genes and different HLA genes. ns P >0.05, * ≤ 0.05, **≤ 0.01, ***≤0.001.

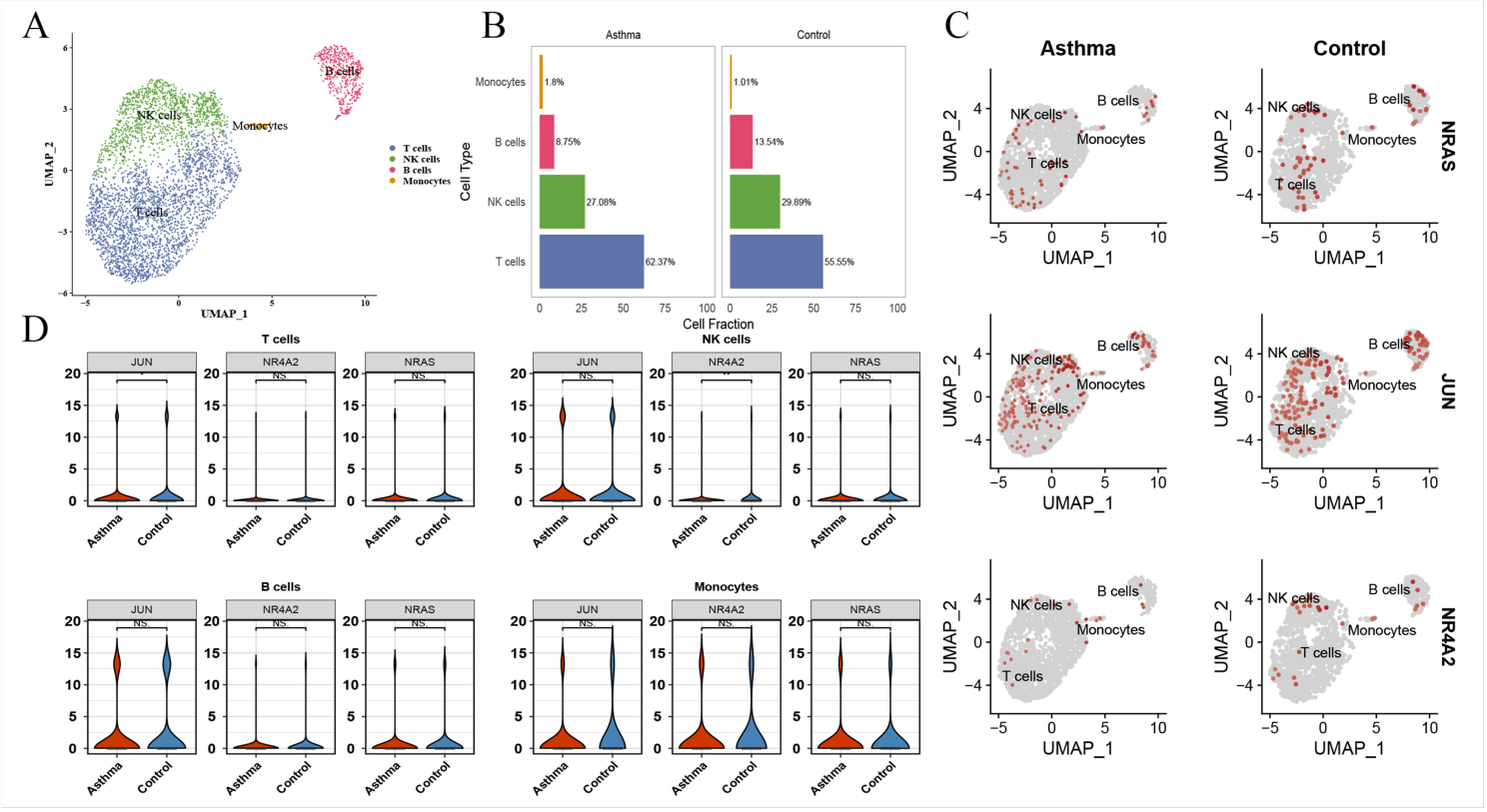


Figure 9

Single-cell analysis. (A) Single-cell data were annotated into 4 cell clusters. (B) The proportions of cell populations of each cell type within each sample group. (C-D) The expression of key genes across the different cell types. ns P >0.05, * ≤ 0.05, **≤ 0.01, ***≤0.001.

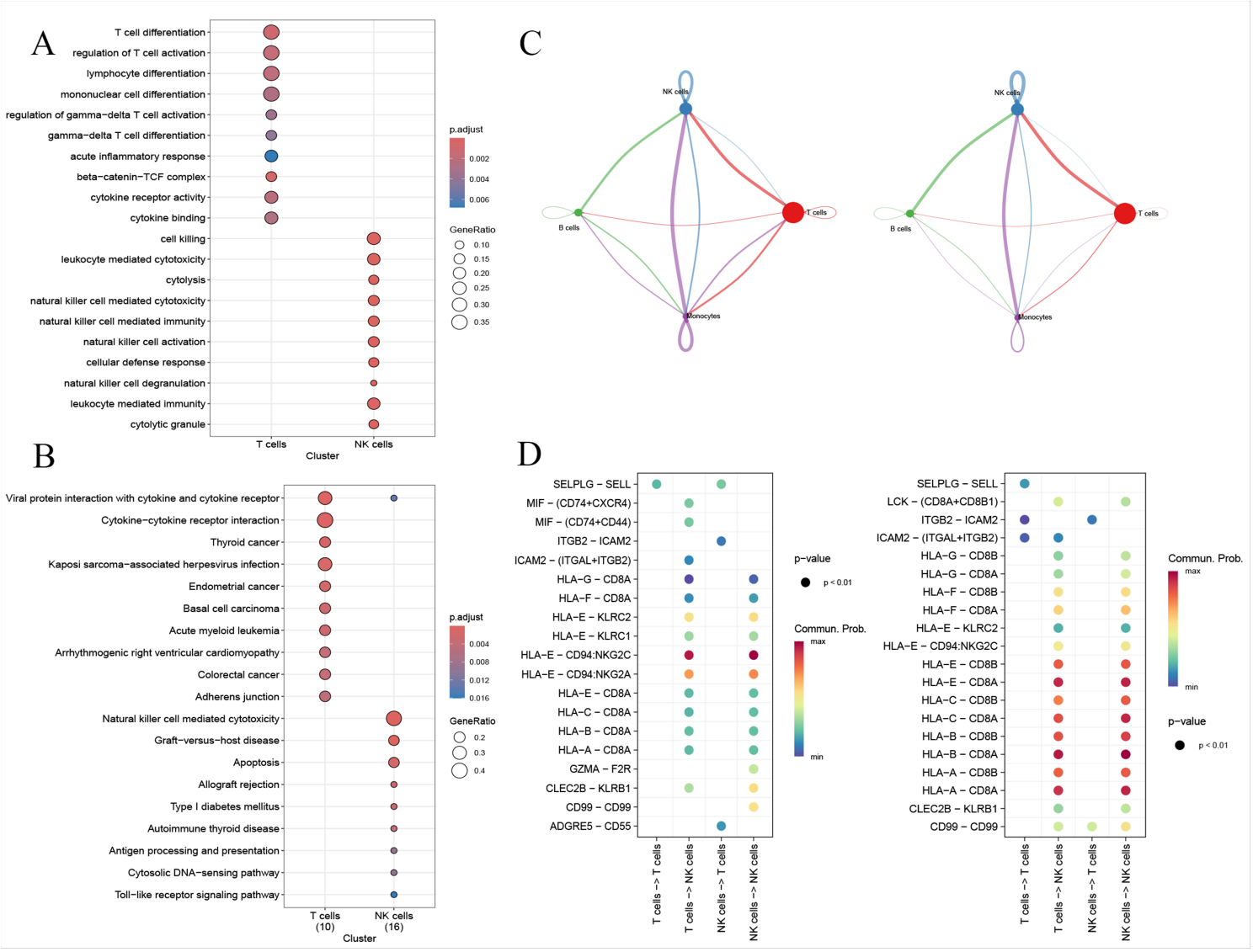


Figure 10

Functions and communications of T cells and NK cells. (A) GO analysis of T cells and NK cells. (B) KEGG analysis of T cells and NK cells. (C) The number and strength of cell communication between T cells and NK cells. (D) Ligand-receptor pairs involved in cell communication between T cells and NK cells.

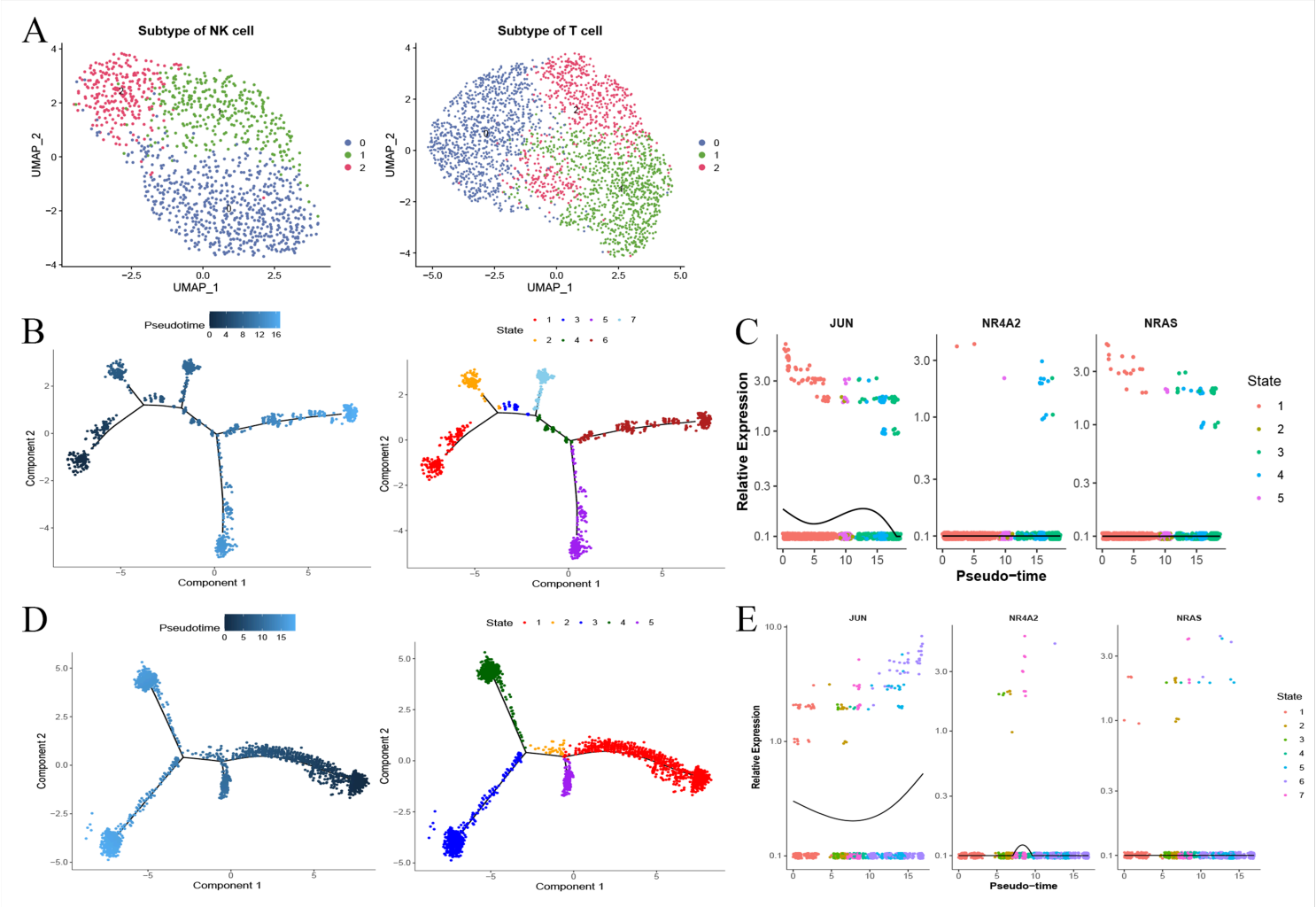


Figure 11

Pseudotime analysis of T cells and NK cells. (A) Both NK cells and T cells into three subpopulations. (B) Pseudotime analysis of NK cells. (C) The expression levels of key genes in NK cells temporal states. (D) Pseudotime analysis of T cells. (E) The expression levels of key genes in T cells temporal states.

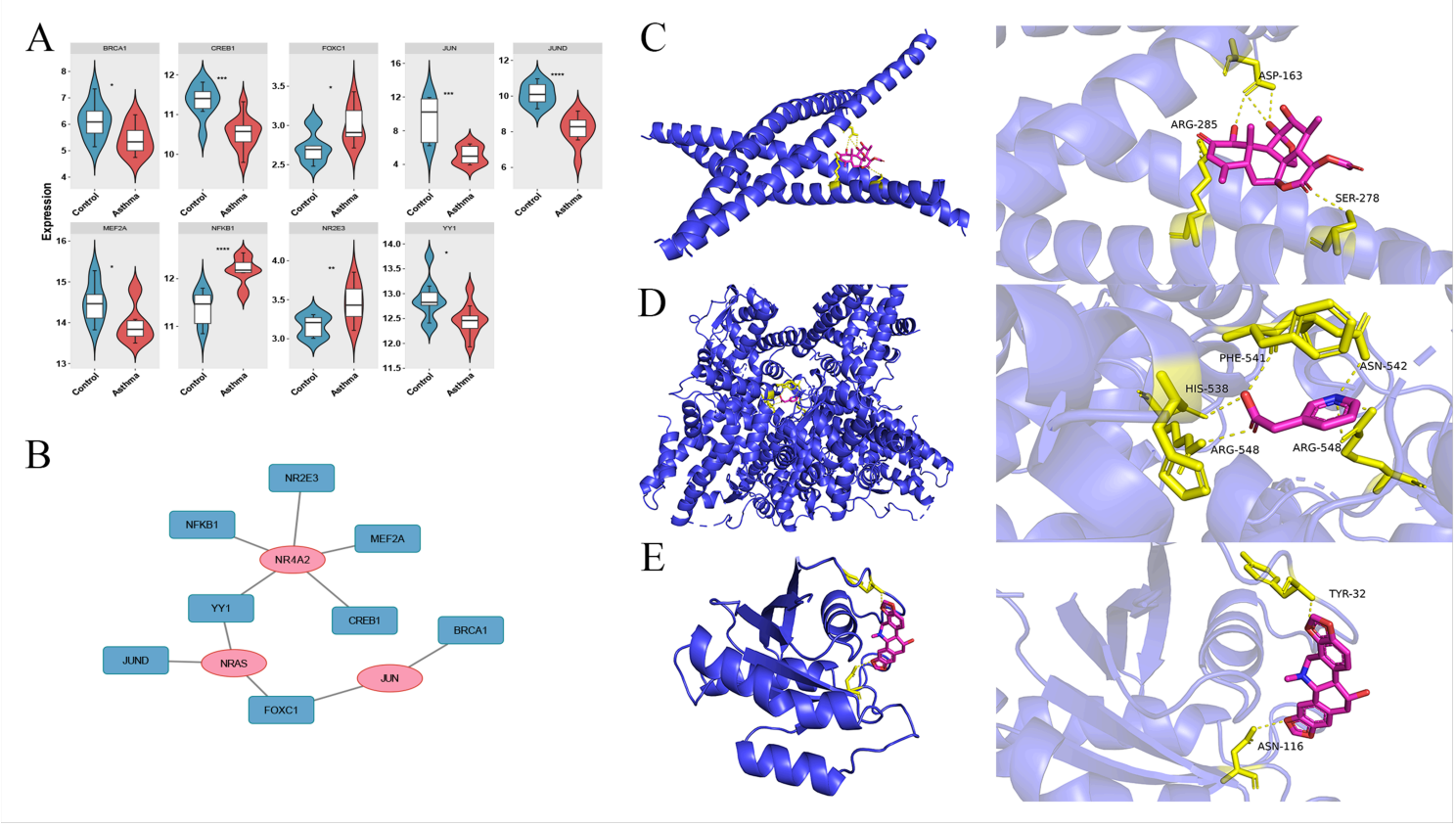


Figure 12

Transcription factors (TFs) and molecular docking. (A) Differentially expressed TFs between control and asthma groups. (B) TF-key gene network. (C) Molecular docking between JUN and holacanthone. (D) Molecular docking between NR4A2 and compound_108. (E) Molecular docking between NRAS and chelidonine.

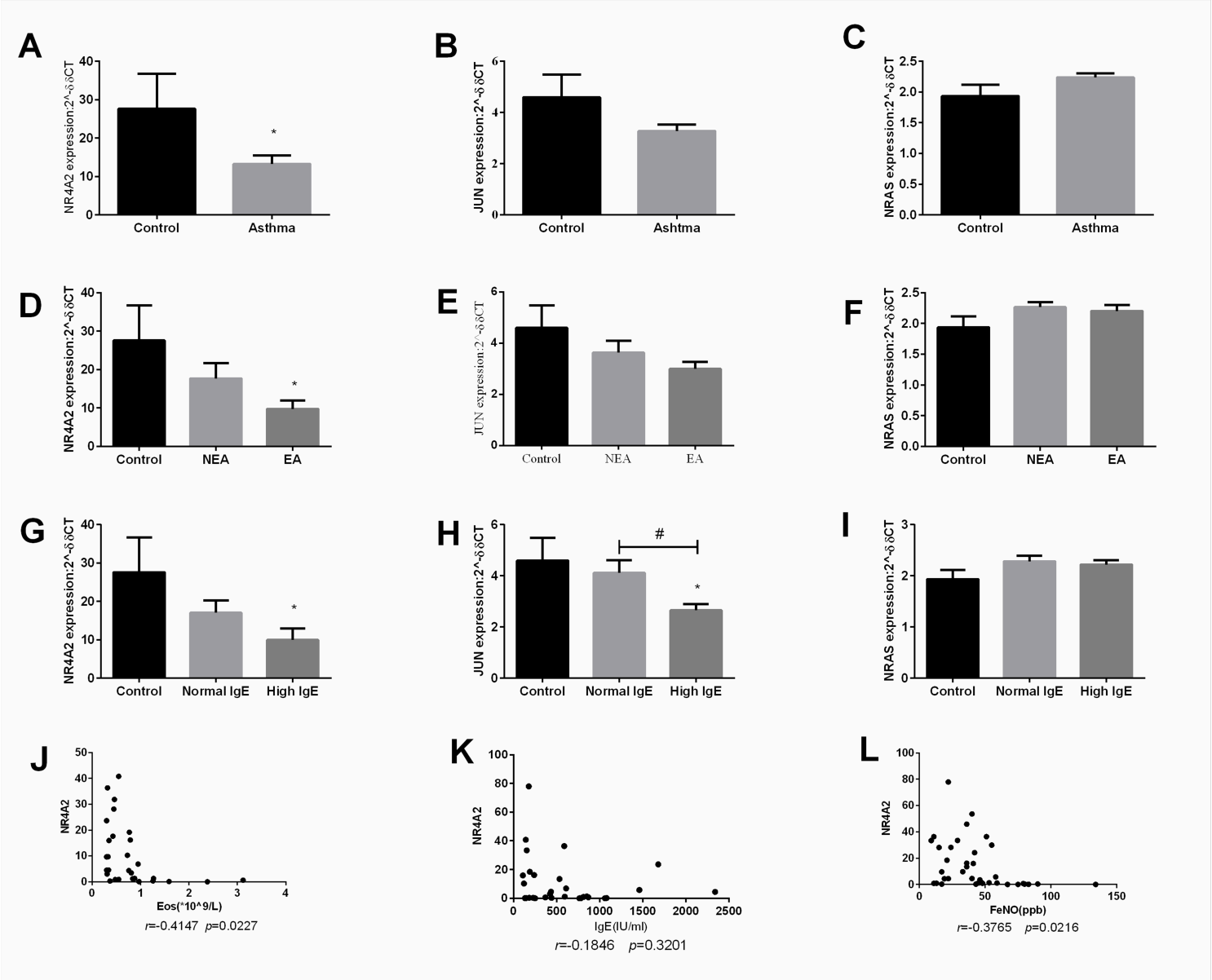


Figure 13

Key PCD-immune gene expression in asthmatic peripheral blood. (A) NR4A2, (B) Jun and (C) NRAS mRNA expression was assessed in asthmatic patients and healthy individuals. Subgroup analysis on NR4A2(D, G), Jun (E, H) and NRAS (F, I) mRNA expression was conducted based on blood eosinophils (Eos) and IgE levels. Correlation between NR4A2 and Eos (J), IgE (K) and FeNO (L) were Analyzed. (EA, eosinophilic asthma. NEA, non-eosinophilic asthma. *p<0.05, denotes significance between control and asthma. #p<0.05 indicates significant difference between normal IgE and High IgE group)

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