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ORIGINAL RESEARCH

Identification of Novel Biomarkers Associated with Corticosteroid Resistance in Asthma by Bioinformatics Analysis and Experimental Validation

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Background: Corticosteroid resistance in asthma, marked by reduced glucocorticoid response, is significantly influenced by cigarette smoke (CS). We sought to explore potential novel biomarkers and therapeutic targets associated with CS-induced corticosteroid resistance in asthma.

Methods: GSE230048 (related to corticosteroid resistance) and GSE13896 (from CS-exposed macrophages) were obtained from GEO. Initially, we performed differential gene expression analysis and weighted gene co-expression network analysis (WGCNA) to discover key genes involved in corticosteroid resistance in asthma. Gene Ontology (GO) term and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were conducted. Receiver operating characteristic (ROC) analysis was used to assess the diagnostic accuracy of these markers. CIBERSORT was applied to clarify immune cell infiltration. Expression of the key biomarker was validated in asthma patients and asthma murine models.

Results: Five overlapping genes upregulated in corticosteroid-resistant asthma patients and smokers' alveolar macrophages were identified. Subsequently, using WGCNA, the most relevant modules were identified and intersected with differentially expressed genes. Tensin 1 (TNS1), ATP-binding cassette subfamily C member 4 (ABCC4), and TNF receptor superfamily member 21 (TNFRSF21) were identified as critical biomarkers for corticosteroid resistance in asthma. ROC analysis showed an AUC of 0.718 for the three-marker combination. Single-cell RNA sequencing confirmed their expression in macrophages from asthmatic patients. Elevated levels of TNS1, ABCC4, TNFRSF21, and M2 macrophage markers (CD301 and CD206) were observed in CS-exposed murine lung tissues and CS condensate-treated Raw264.7 cells. TNS1 knockdown significantly reduced CD301 and CD206 expression, suggesting its role in promoting macrophage M2 polarization.

Conclusion: In conclusion, we identified three hub genes (TNS1, ABCC4, and TNFRSF21) associated with CS-induced corticosteroid resistance in asthma. Additionally, TNS1 may be involved in CS-induced corticosteroid resistance in asthma by promoting macrophage M2 polarization.

Keywords: corticosteroid resistance, asthma, TNS1, ABCC4, TNFRSF21, macrophage

Introduction

Asthma is a chronic inflammatory condition of the airways marked by inflammation and structural changes within the airways, which contribute to the development of airway hyperresponsiveness (AHR). Corticosteroid remains the mainstay in the management of all types of asthma due to its potent anti-inflammatory properties.[1–3] However, asthma is a heterogeneous disease. Not all people respond well to corticosteroid therapies.[4] Approximately 5–10% of asthmatics do not respond satisfactorily to corticosteroid therapy,[5] requiring higher doses of inhaled corticosteroid,[6] oral

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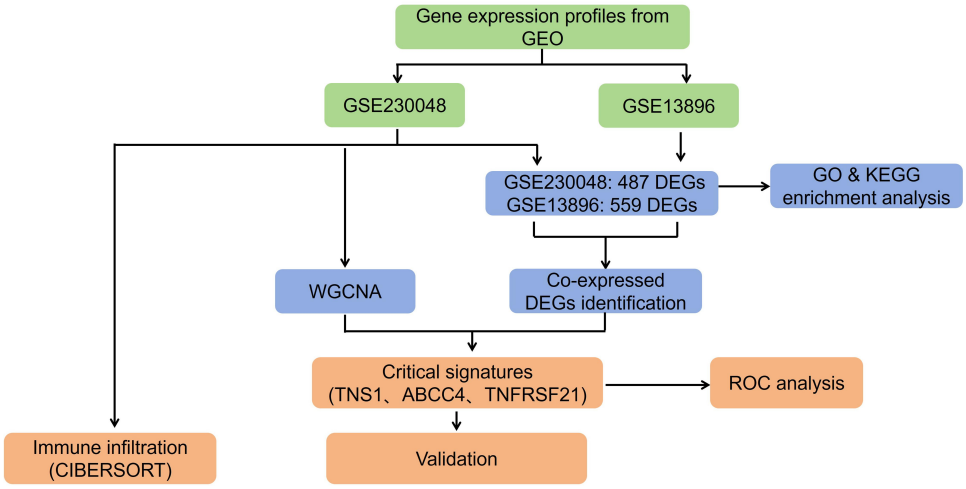
Graphical Abstract



corticosteroid,[6] additional immunotherapy,[7] or biologic therapies[8] for asthma control. These patients are typically with more severe disease and are referred to as having corticosteroid-resistant asthma.[9] Severe asthma, particularly corticosteroid-resistant asthma, is associated with more frequent exacerbations, greater lung function impairment, and increased healthcare costs.[4] In asthma management, patients with corticosteroid resistance may require higher doses of corticosteroid to control their symptoms.[6] This can lead to a variety of side effects, such as osteoporosis,[10] weight gain,[11] and hypoglycemia.[12] The early identification of corticosteroid resistance is essential for preventing the various side effects of high-dose corticosteroid use and for initiating alternative therapies that can better control asthma symptoms. Moreover, understanding mechanisms underlying corticosteroid resistance of asthma may facilitate the development of novel therapeutic strategies.

Several molecular mechanisms underlying the reduced anti-inflammatory efficacy of corticosteroids in asthmatic patients have been identified, including modifications to the glucocorticoid receptor (GR), elevated expression of GRβ, increased levels of pro-inflammatory transcription factors, upregulated pro-inflammatory genes, and impaired histone acetylation.[13] Corticosteroid resistance is influenced by a complex interplay between genetic predispositions and environmental exposures.[14] Cigarette smoke (CS), a major indoor and outdoor air pollutant, is a well-established contributor to corticosteroid resistance in asthma, significantly complicating disease management.[15] CS contains numerous free radicals, oxidants, and toxic chemicals, as well as microbial components, all of which can induce airway inflammation and oxidative stress, leading to lung tissue damage.[16] CS primarily mediates corticosteroid resistance by promoting oxidative stress and nitrative stress responses,[17] and by inhibiting histone deacetylase 2 expression and activity.[18] Studies showed that CS exposure in an asthma murine model increased the infiltration of macrophages in the lung tissue.[19,20] Moreover, numerous studies have provided compelling evidence that CS significantly alters the function and phenotype of alveolar macrophages in asthma patients.[21] Exposure to CS increases the generation of reactive oxygen species, matrix metalloproteinases and chemokines by alveolar macrophages.[11] All these studies emphasize the essential role macrophages play in CS-induced corticosteroid resistance in asthma. Currently, in clinical practice, there are no widely accepted biomarkers or models to guide the use of corticosteroids in asthma. Identification of novel biomarkers related to smoking-induced corticosteroid resistance is crucial for asthma management, particularly in asthmatic patients who are smokers. Moreover, elucidating the functional roles of these genetic markers may enable the development of personalized therapeutic strategies.

This study sought to identify potential biomarkers and therapeutic targets linked to CS-induced corticosteroid resistance in asthma by integrating bioinformatics analysis with in vivo experimental validation.

Methods

Bioinformatics Analysis

Datasets Collection and Processing

The high-throughput RNA sequencing dataset GSE230048 and the microarray dataset GSE13896 were from the GEO database. GSE230048 comprised the gene expression profiling of whole blood samples from 22 corticosteroid non-responsive asthma patients and 24 corticosteroid responsive asthma patients, based on the GPL16791 platform. The GSE13896 dataset contained the gene expression profiling of alveolar macrophages from 24 normal non-smokers and 34 normal smokers, based on GPL570 platform. In addition, the GSE27002 dataset, which contains gene expression profiles of alveolar macrophages from 10 normal non-smokers and 13 normal smokers, and the GSE74986 dataset, which includes bronchoalveolar lavage cell transcriptome data from 46 severe asthma patients and 12 healthy controls, were used as validation datasets to confirm the expression levels of the signature. GSE74986 specifically included bronchial alveolar lavage cell transcriptome data from severe asthma patients and healthy controls. Severe asthma is often characterized by corticosteroid resistance, making this dataset particularly relevant for validating our findings in a population with similar clinical characteristics. GSE27002 includes alveolar macrophages from both normal non-smokers and normal smokers. It provides a baseline comparison for gene expression profiles in healthy individuals and those with potential inflammation due to smoking. Since no single publicly available dataset can simultaneously compare corticosteroid response in asthma patients with and without CS exposure, the combination of GSE27002 and GSE74986 provides a diverse range of samples, including normal individuals, smokers, and severe asthma patients.

Initially, we performed differential gene expression analysis and weighted gene co-expression network analysis (WGCNA) to discover key genes involved in corticosteroid resistance in asthma. We also carried out Gene Ontology (GO) term and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses. Next, diagnostic performance of these markers was evaluated using receiver operating characteristic (ROC) analysis. The hub gene was also validated using external datasets. Subsequently, we applied CIBERSORT to clarify the variations in immune cell infiltration between the corticosteroid non-responsive and responsive groups. Lastly, we validated the expression of the key biomarkers in asthma patients and asthma murine models.

Identification of Differentially Expressed Genes (DEGs)

We used the online analysis tool GEO2R to screen the differential genes in dataset GSE230048 and dataset GSE13896. The screening criteria were adj. P-value < 0.05 and $|\log_2(FC)| > 0.58$. "ggplot2" was utilized to generate volcano plots for the visualization of DEGs. The "pheatmap" package was utilized to generate heatmaps for top 50 DEGs from the GSE230048 and GSE13896 datasets. DEGs that were common to dataset GSE230048 and dataset GSE13896 were identified using the VennDiagram package (version 1.6.20) in R.

Functional Enrichment Analysis of DEGs

To explore the biological roles and pathways linked to DEGs in GSE230048 and GSE13896, we employed the functional enrichment analysis provided by the clusterProfiler package and the GOplot package.

Screening and Validation of Critical Gene Signatures

The R package WGCNA was used to build a gene co-expression network. We calculated the average expression level of each gene. To ensure computational efficiency and biological relevance, the top 20000 genes were chosen for WGCNA based on their average expression levels. High-expression genes are more likely to play significant roles in cellular functions and disease processes. Therefore, prioritizing these genes increases the likelihood of identifying meaningful gene co-expression modules and pathways. Low-expressed genes are often filtered out to improve the reliability of the network.[22] The R software was used to compute a soft threshold power β in a preliminary step. We calculated an

appropriate soft threshold using the "pickSoftThreshold" function in the WGCNA package. We selected a range of candidate powers for β from 1 to 30. We assessed the scale-free topology fit index by plotting the log-log relationship between node connectivity and rank and computed the R^2 value. Then, we determined the optimal soft threshold power β based on the scale independence of R^2 greater than 0.8 and mean connectivity tending to zero. The adjacency was transformed into a topological overlap matrix (TOM). Using the TOM-based dissimilarity metric, a hierarchical clustering algorithm was applied to cluster genes exhibiting similar expression profiles into modules. Each module contains at least 30 genes. Samples were divided into corticosteroid non-responders and corticosteroid responders. Perform an in-depth analysis correlating gene expression patterns (modules) with the phenotype. Identify the gene module that has the strongest relationship with corticosteroid non-responders. The intersection of the genes in the modules that were most relevant to corticosteroid non-responders and the identified DEGs was obtained. To assess the diagnostic efficacy of the signatures for corticosteroid resistance in the GSE230048 dataset, a binary logistic regression model was constructed. ROC curves and the area under the curve (AUC) were computed.

Analysis of Immune Cells Infiltration

CIBERSORT was applied to assess the infiltration of 22 distinct types of immune cells in GSE230048. We compared the percentage of distinct immune cell types between corticosteroid non-responders and corticosteroid responders group. A heatmap was used to display the association between different types of immune cells.

Validation of the Key Genes in Asthmatic Patients

Subjects

A total of 3 patients, diagnosed with asthma and treated at the outpatient clinic of Beijing Chao-Yang Hospital, were enrolled in this study. Asthma diagnoses were made by respiratory physicians following the GINA criteria. Participants were excluded if they were: pregnant or lactating; had malignancies; had systemic autoimmune diseases; or were receiving systemic oral corticosteroids.

Table 1 summarizes the clinical features of the 3 participants for scRNA-seq. The Research Ethics Committee of Beijing Chao-Yang Hospital approved this study (Approval No. 2022-ke-642, approved January 2023, study period: January 2023-January 2024; Approval No. 2025-ke-597, approved July 2025, study period: July 2025-July 2026). Regarding scRNA-seq, prior to enrollment, each participant received detailed information about the study objectives, procedures (eg, collection of 3–4 mL peripheral blood in EDTA tubes, storage at 4 °C, and immediate processing for

Table 1 Clinical Characteristics of Recruited Donors for scRNA-Seq

	P1	P2	P3
Demographics			
Age (y)	42	29	52
BMI (kg/m^2)	26.73	23.3	33.8
Sex (male/female)	Female	Male	Female
Clinical outcomes			
ACQ-5 score	1.6	1.4	0.4
Inflammatory outcomes			
Total cell count (millions)	7.02	6.37	10.15
Peripheral eosinophil (%)	1.3	6.8	2.3
Peripheral neutrophil (%)	65.2	59.9	75.1
Peripheral lymphocytes (%)	25.8	28.1	18.5
Peripheral monocytes (%)	7.4	4.4	3.9
FeNO point (ppb)	23	214	37

(*Continued*)

Table 1 (Continued).

	P1	P2	P3
Functional outcomes			
Baseline FEV1 (% predicted)	85.2	90.3	97.2
Baseline FVC (% predicted)	124.3	112.8	97.4
Medication use	ICS	ICS+LABA SABA	ICS

Abbreviations: BMI, body mass index; FENO, fraction of exhaled nitric oxide; FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; ICS, inhaled corticosteroid; LABA, long-acting bronchodilator; SABA, short-acting bronchodilator.

scRNA-seq), risks, benefits, voluntary participation, and the right to withdraw at any time without penalty to clinical care or impact on their rights as a participant. Participants signed a written informed consent form after confirming their understanding of the study details. Signed consent forms were kept securely and were accessible only to authorized personnel. For each participant undergoing scRNA-seq, 3–4 mL of peripheral blood was drawn into EDTA-coated tubes and transported to the laboratory on ice. Peripheral blood mononuclear cells (PBMCs) were isolated using Histopaque-1077 (Sigma-Aldrich, St Louis, MO, USA. Cat. NO. 10771).

Single-Cell Preparation and RNA Sequencing

Using single cell 3 'Library and Gel Bead Kit V3.1 (10× Genomics, Pleasanton, CA, USA. Cat. NO. 1000075) and Chromium Single Cell B Chip Kit (10× Genomics, Pleasanton, CA, USA. Cat. NO. 1000074), the cell suspension (300–600 living cells per microliter determined by Count Star) was loaded onto the Chromium single cell controller (10× Genomics) to generate single-cell gel beads in the emulsion according to the manufacturer's protocol. Single cells were suspended in PBS containing 0.04% BSA. Approximately 6000 cells were loaded per channel, with an estimated recovery of approximately 3000 cells. Captured cells were lysed and the released RNA was barcoded through reverse transcription in individual GEMs. Reverse transcription was performed on a S1000TM Touch Thermal Cycler (Bio Rad) at 53 °C for 45 min, followed by 85 °C for 5 min, and held at 4 °C. The cDNA was generated and then amplified, and quality assessed using an Agilent 4200 (performed by CapitalBio Technology, Beijing, China). The libraries were finally sequenced using an Illumina Novaseq 6000 sequencer with a sequencing depth of at least 100,000 reads per cell with paired-end 150 bp (PE150) reading strategy (performed by CapitalBio Technology, Beijing, China).

10 × genomics raw data were processed by the Cellranger pipeline. The cellranger count module was used to perform alignment, filtering, barcode counting, and unique molecular identifier (UMI) counting, generating a feature-barcode matrix and determining clusters. Analysis of the gene-barcode matrix was conducted with Seurat 3.0. Cells with fewer than 200 genes detected,[23,24] those with a gene number in the top 1% percentile, or those with a mitochondrial gene ratio exceeding 25%[25] were considered abnormal and were filtered out. Cells with fewer than 200 genes detected are often of low quality. This threshold helps to exclude cells that may have been damaged during the sample preparation process or that contain insufficient RNA for reliable analysis. A high mitochondrial gene ratio (exceeding 25%) is often a sign of cellular stress or damage, particularly in the context of apoptosis. The criteria for filtering out cells with fewer than 200 genes detected and a mitochondrial gene ratio exceeding 25% are commonly used in single-cell transcriptomics to ensure that the data is of high quality and that the subsequent analysis is reliable and biologically meaningful.[23,25] Dimensionality reduction was performed using principal component analysis (PCA), and visualization was realized by uniform manifold approximation and projection (UMAP). We identified PBMCs clusters based on the dominant expression patterns of cell markers sourced from an online database. Clusters sharing common canonical markers were merged into a single cluster.

Validation of the Key Genes in Asthma Murine Model and Raw264.7 Cell Line

Ovalbumin (OVA)-Induced Asthma Murine Model and CS Exposure

Specific pathogen free male BALB/c mice (18–20 g) from Beijing HFK Bioscience Co., Ltd. (Beijing, China) were kept under a 12 h light-dark cycle at 20–26 °C with free access to food and water. Approval for animal experiments was granted by the Committee on Ethics in the Care and Use of Laboratory Animals at Beijing Chao-Yang Hospital (No. 2023-dong-336). The mice were grouped as control, OVA (Sigma-Aldrich, St Louis, MO, USA. Cat. NO. A5503), OVA/CS, OVA/dexamethasone (DEX), and OVA/CS/DEX groups (n = 7 per group).

On days 1 and 7, mice in the OVA, OVA/CS, OVA/DEX, and OVA/CS/DEX groups received an intraperitoneal injection of a mixture containing OVA (100 μg) and aluminum hydroxide gel (Thermo Fisher Scientific, Waltham, MA, USA. Cat. NO. 77161). The mice were then challenged on days 12–14 with 100 μg OVA through intratracheal administration. For CS exposure, mice in the OVA/CS and OVA/CS/DEX groups were placed in a smoke chamber of the exposure system (Sibata, Kyoto, Japan). The mice were exposed to CS for 1 h on days 1–14. To assess responsiveness to corticosteroid treatment, the mice in the OVA/DEX and OVA/CS/DEX groups were treated with DEX (1 mg/kg, i.p.) from days 12 to 14. Lung function measurement and hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) staining were performed as described.[26] Malondialdehyde (MDA) and superoxide dismutase (SOD) levels in the lung tissues were determined using a MDA assay kit (Beyotime, China. Cat. NO. S0131) and total SOD assay kit with WST-8 (Beyotime, China. Cat. NO. S0101).

siRNA Transfection

Raw264.7 cells were acquired from the Cell Culture Centre of the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Beijing, China). The cells were transfected with siRNA specific for TNS1 (sense: GCAACUACCUGCUAUUCAATT, antisense: UUGAAUAGCAGGUAGUUGCTT) and negative control siRNA (sense: UUCUCCGAACGUGUCACGUTT, antisense: ACGUGACACGUUCGGAGAATT) using Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific, Waltham, MA, USA. Cat. NO. 13778). Cigarette smoke condensate (CSC) was prepared as described previously.[27] 48 h after transfection, the cells were treated with 100 mg/L CSC for 24 h. TNS1, CD206, and CD301 levels were determined by reverse transcription-quantitative PCR (RT-qPCR).

Reverse Transcription-Quantitative PCR (RT-qPCR)

RNA from murine lung tissues and Raw264.7 cells was converted into cDNA using FastKing RT kit (Tiangen, China. Cat. NO. KR116). PCR amplification was carried out using SYBR Premix Ex Taq kit (Tiangen, China. Cat. NO. FP205). Primers: murine GAPDH: AGGTCGGTGTGAACGGATTTG (F), TGTAGACCATGTAGTTGAGGTCA (R); murine TNS1: CTGGTGTATGTCACCGAACG (F), GTTCAGAGAGGTTGAATAGCAGG (R); murine ABCC4: CATCGCGGTAACCGTCCTC (F), CCGCAGTTTTACTCCGCAG- (R); murine TNFRSF21: GCCATGTTGACCGTACCACT (F), CTATGCCGTTCTCGTGCCTG (R); murine CD206: CTCTGTTCAGCTATTGGACGC (F), CGGAATTTCTGGGATTCAGCTTC (R); murine CD301: CAGCTTGCTCCCCTCTACCT (F), TCCAACGACCATCGTAAGAAAAG (R). The $2^{-\Delta\Delta Ct}$ method was applied.

Statistical Analysis

RStudio V4.4.1 was used to analyze data from the GEO database. DEGs were identified using the online analysis tool GEO2R. GO and KEGG pathway enrichment analysis was performed with clusterProfiler package and GOplot package. WGCNA was utilized to construct a gene co-expression network. CIBERSORT algorithm was used to conduct an immune infiltration analysis. We applied the Shapiro–Wilk test to every continuous variable that underwent a parametric test. All variables with a Shapiro–Wilk P-value ≥ 0.05 were considered sufficiently normal. We categorized and presented our data as follows: normally distributed variables were expressed as means ± SEM, while non-normally distributed variables were reported as medians (interquartile ranges). To compare the means between two groups, we employed a t-test. For datasets that did not meet the assumptions of normality, we applied nonparametric tests for

statistical analysis. Comparison of means among three or more groups was conducted using a one-way ANOVA followed by Bonferroni correction. $P < 0.05$ was considered statistically significant.

Results

Identification of DEGs

To explore the key genes involved in CS-induced corticosteroid resistance in asthma, we conducted an in-depth analysis of two datasets, GSE230048 and GSE13896. Box plots of the normalized data showed a good degree of consistency across samples in both GSE13896 and GSE230048 (Figure 1A and B). In the GSE230048 dataset, compared with corticosteroid responsive asthma patients, we obtained 468 upregulated and 19 downregulated genes in corticosteroid non-responsive asthma patients (Figure 1C and D). When comparing gene expression profiles in alveolar macrophages of normal non-smokers and normal smokers, we identified 559 DEGs, comprised of 266 upregulated and 293 down-regulated genes were identified in GSE13896 (Figure 1E and F). The heatmap shows the top 30 DEGs from the two datasets (Figure 1G and H). These heatmaps highlight the expression patterns of these genes across different samples, providing a clear visual representation of the differential expression. Five DEGs were shared between the two datasets (Figure 1I). Figure 1I presents a Venn diagram showing the overlap of DEGs between the GSE230048 and GSE13896 datasets, emphasizing the common genes that may play an essential role in CS-induced corticosteroid resistance in asthma.

KEGG and GO Pathway Enrichment Analysis of DEGs

To further elucidate biological processes and pathways related to the DEGs identified in the GSE230048 and GSE13896 datasets, we performed KEGG pathway and GO enrichment analyses. The KEGG pathway analysis revealed that the DEGs in GSE230048 were mostly associated with the IL-17 and the chemokine signaling pathway (Figure 2A), indicating their potential role in corticosteroid resistance in asthma. The DEGs in the GSE13896 dataset were mainly enriched in NOD-like receptor and Toll-like receptor signaling pathway (Figure 2B), suggesting their importance in the response to CS exposure in alveolar macrophages. The main enriched BP terms in GSE230048 included cell-substrate adhesion and chronic inflammatory response (Figure 2C). The main enriched CC terms in GSE230048 included cell-substrate junction and stress fiber (Figure 2D). The main enriched MF terms in GSE230048 included phosphoric ester hydrolase activity and phosphatase activity (Figure 2E). The main enriched BP terms in GSE13896 included immune response-activating and immune response-regulating cell surface receptor signaling pathway (Figure 2F). The main enriched CC terms in GSE13896 included external side of plasma membrane and vacuolar membrane (Figure 2G). The main enriched MF terms in GSE13896 included cytokine activity and cytokine receptor binding (Figure 2H).

Identification of Co-Expression Gene Modules

To identify co-expression modules associated with corticosteroid resistance in asthma, we performed WGCNA on the top 20,000 genes with the highest expression levels in GSE230048. As shown in Figure 3A, the soft threshold power β was 7 based on the scale independence of R2 greater than 0.8. Genes from GSE230048 were clustered into 14 modules, each visualized with a unique color (Figure 3B). Each module represents a group of genes with similar expression patterns. To determine the association between these modules and corticosteroid non-responsiveness in asthma, we calculated the module-trait relationships. Tan, turquoise, black and salmon co-expressed gene modules were found to have a strong association with corticosteroid non-responders in asthma (Figure 3C).

Identification and Verification of Key Genes

To identify the hub genes involved in CS-induced corticosteroid resistance in asthma, a Venn diagram was constructed to identify the overlap between modular hub genes and key DEGs. TNS1, ABCC4, and TNFRSF21 were overlapping genes (Table 2). To evaluate the predictive potential of key gene markers in corticosteroid resistance, ROC analysis was performed. The AUC for the combination of the three markers (TNS1, ABCC4 and TNFRSF21) was 0.718 (Figure 4A). The three hub genes may act as biomarkers to estimate the development of corticosteroid resistance in asthma. Given that

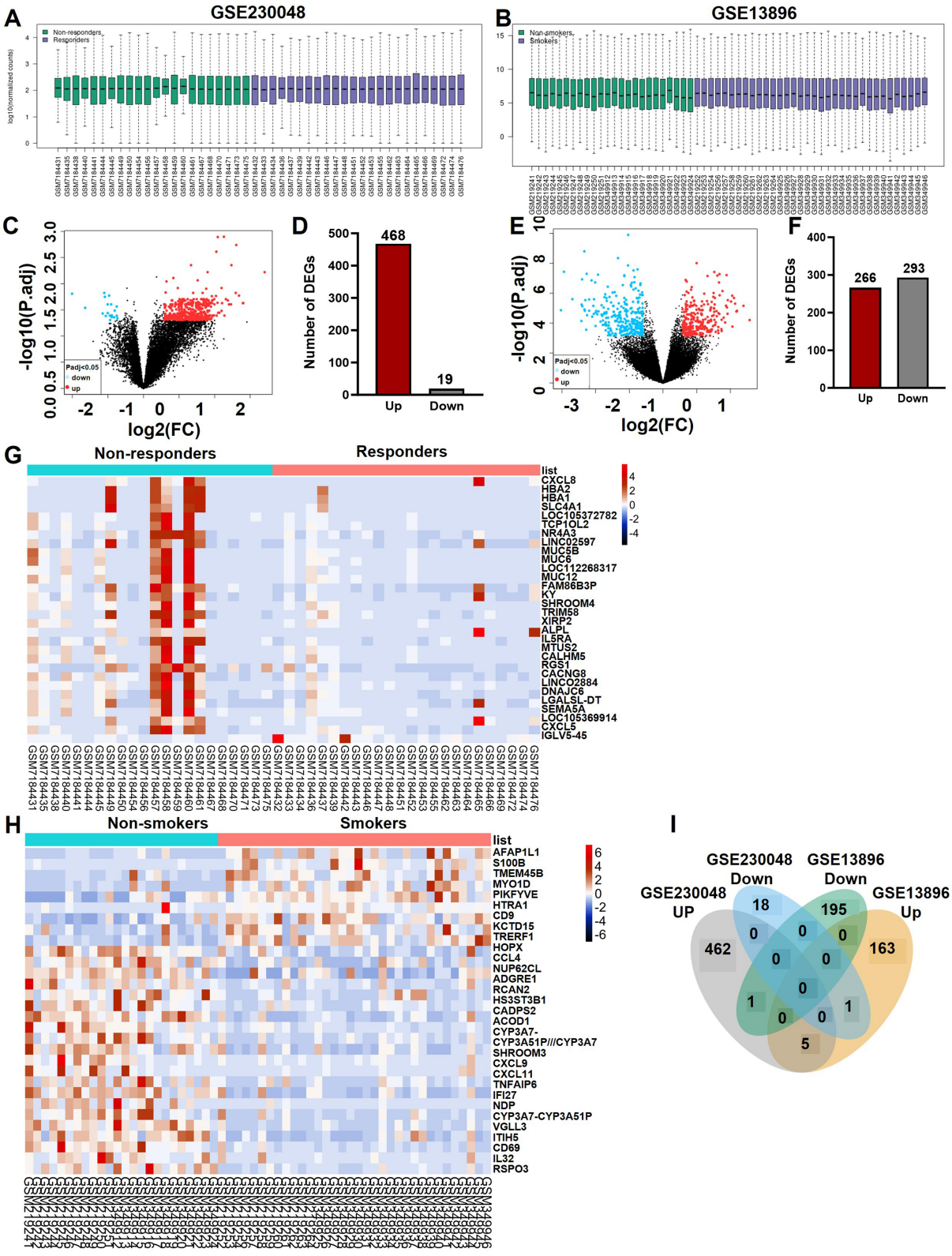


Figure 1 Identification of DEGs. (**A**) Box plots of the normalized data in GSE230048. (**B**) Box plots of the normalized data in GSE13896. (**C**) Volcano plots of DEGs in GSE230048. (**D**) Number of DEGs in GSE230048. (**E**) Volcano plots of DEGs in GSE13896. (**F**) Number of DEGs in GSE13896. (**G**) Heatmap of top 30 DEGs in GSE230048. (**H**) Heatmap of top 30 DEGs in GSE13896. (**I**) Venn diagram of DEGs from GSE230048 and GSE13896.

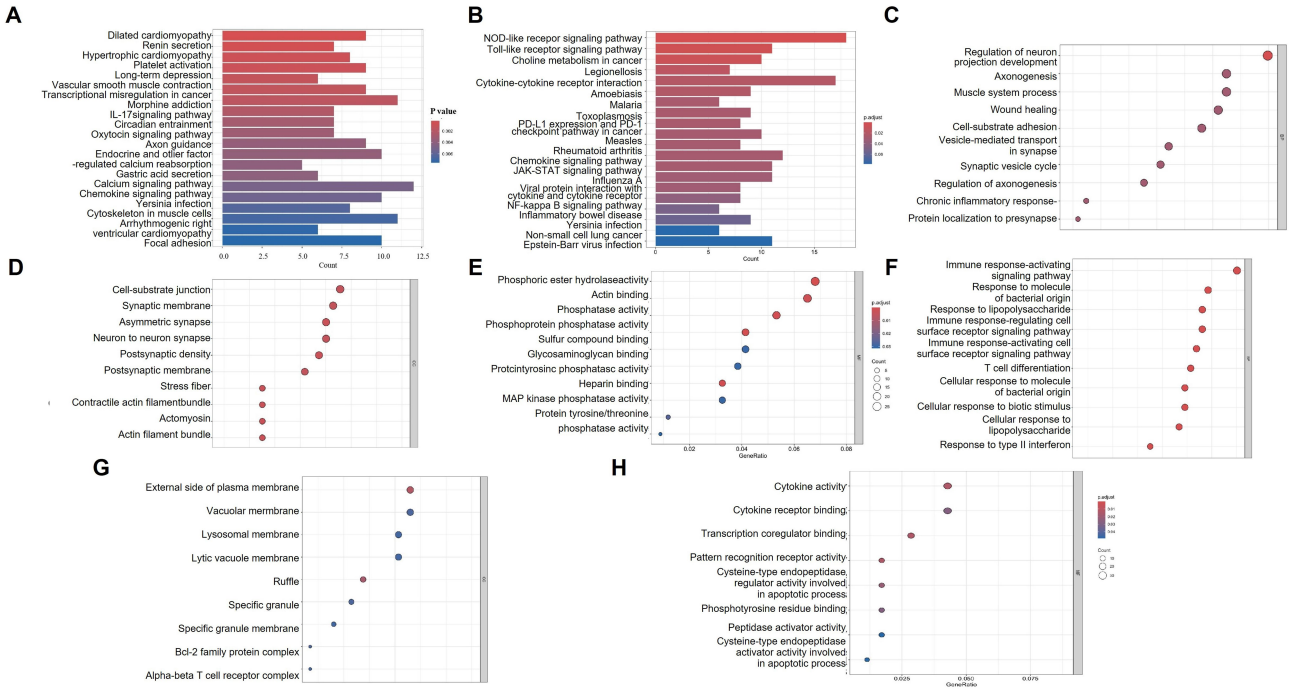


Figure 2 Functional enrichment analysis of the DEGs. (**A**) KEGG analysis of the DEGs from GSE230048. (**B**) KEGG analysis of the DEGs from GSE13896. (**C**–**E**) GO analysis of the DEGs from GSE230048: biological processes (BP, C), cell composition (CC, D), and molecular function (MF, E). (**F**–**H**) GO analysis of the DEGs from GSE13896: BP (**F**); CC (**G**); MF (**H**).

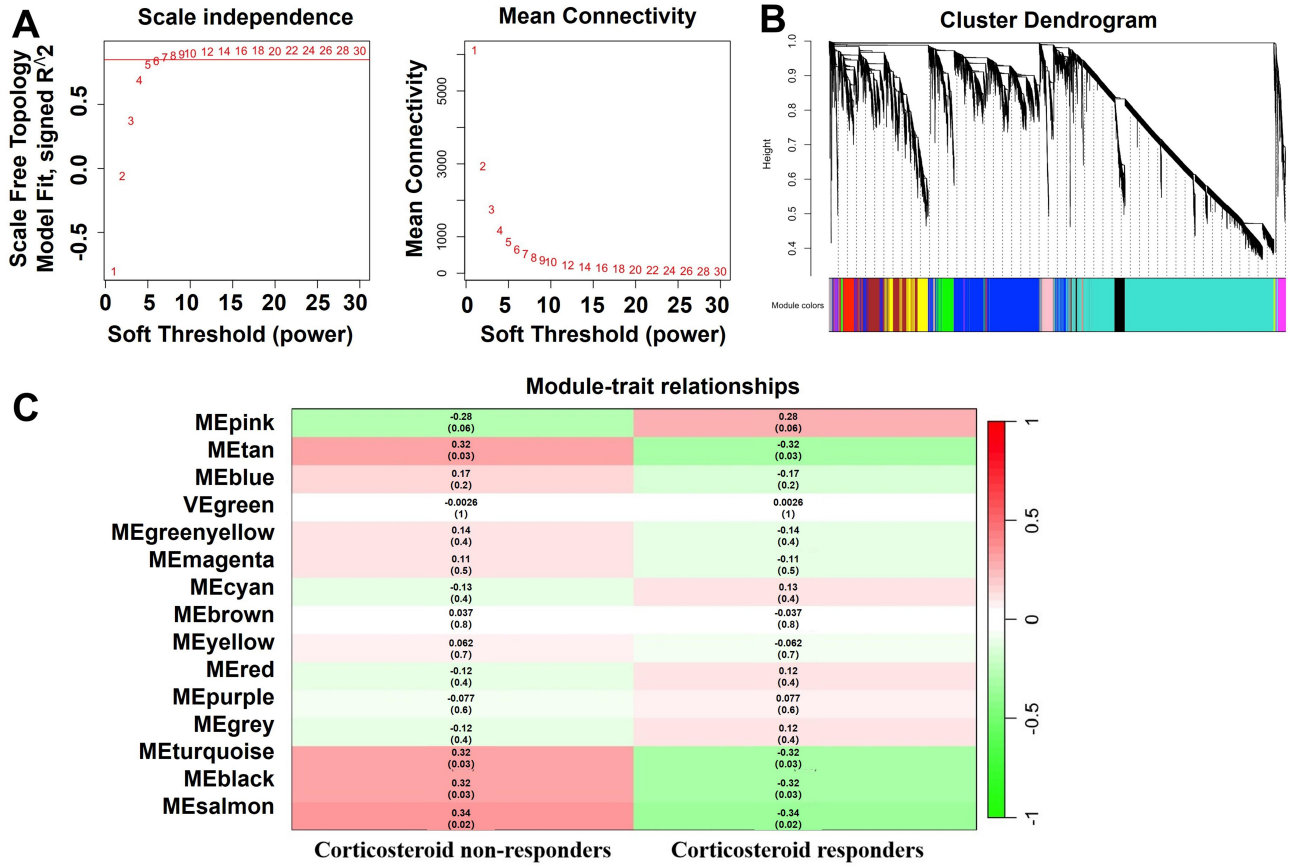


Figure 3 WGCNA analysis of GSE230048. (**A**) The selection of the optimal soft threshold. (**B**) The dendrogram of clustering and its associated module colors. (**C**) The correlation between module eigengenes and clinical features (corticosteroid non-responders or responders).

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Table 2 Expression Pattern of Potential Hub Genes

Gene symbol	Log_2(FC) (GSE230048)	Adj.P.Val (GSE230048)	Log_2(FC) (GSE13896)	Adj.P.Val (GSE13896)
TNS1	1.78	0.025	1.66	0.0004
ABCC4	1.00	0.032	0.86	0.006
TNFRSF21	1.02	0.049	0.85	0.014

TNS1 was the most significantly upregulated gene in the GSE230048 dataset and the GSE13896 dataset (Table 2), TNS1 was selected for further analysis.

Next, verification of TNS1 expression levels was conducted. Upregulated TNS1 expression was confirmed in the whole blood cells from corticosteroid non-responsive asthma patients and in the macrophages from smokers, when compared to corticosteroid responsive asthma patients and macrophages from non-smokers, respectively (Figure 4B and C). In the validation dataset, TNS1 expression was significantly upregulated in bronchial alveolar lavage cell of severe asthma patients when compared with healthy controls in the GSE74986 dataset (Figure 4D). Furthermore, TNS1 expression was significantly elevated in alveolar macrophages from heavy smokers compared to non-smoking controls (Figure 4E).

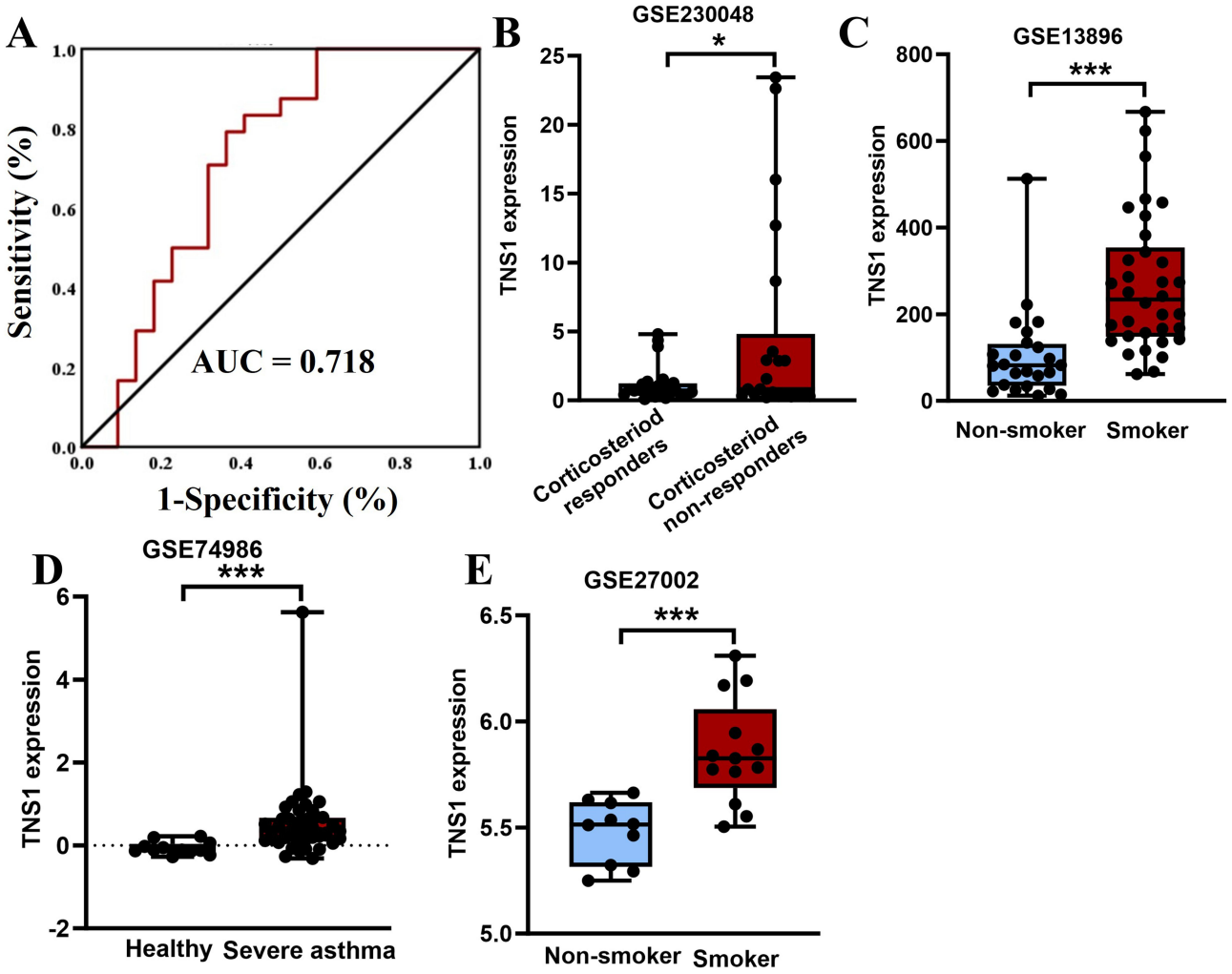

Figure 4 Expression validation and ROC analysis of the identified hub genes in GSE230048. (**A**) ROC curves of TNS1, ABCC4 and TNFRSF21 combined in GSE230048. (**B**–**E**) The expressions of TNS1 in GSE230048 (**B**), GSE13896 (**C**), GSE74986 (**D**), and GSE27002 (**E**). *$P < 0.05$; ***$P < 0.001$.

Immune Cell Infiltration Analysis

Immune dysregulation is involved in corticosteroid resistance in asthma. To investigate the immune cell infiltration profiles in corticosteroid-resistant asthma, CIBERSORT algorithm was used to conduct an immune infiltration analysis in the GSE230048 dataset. Compared to corticosteroid responsive asthma patients, corticosteroid non-responsive asthma patients showed a greater abundance of eosinophils and memory B cells (Figure 5A). The findings also indicated a significant correlation between the various types of immune cells (Figure 5B and C). As revealed by the correlation heatmap of 17 immune cell types (Figure 5B and C), significant positive correlations were observed between the following pairs: T cells follicular helper and B cells memory, Tregs and B cells memory, T cells CD8 and B cells naive, T cells CD4 naive and B cells naive, T cells CD4 memory activated and B cells memory, T cells CD4 memory activated and Plasma cells, T cells follicular helper and Plasma cells, Tregs and Plasma cells, T cells CD4 naive and T cells CD8, T cells CD4 memory resting and T cells CD8, T cells CD8 and T cells CD4 naive, NK cells resting and T cells CD4 memory resting, T cells follicular helper and T cells CD4 memory activated, Tregs and T cells CD4 memory

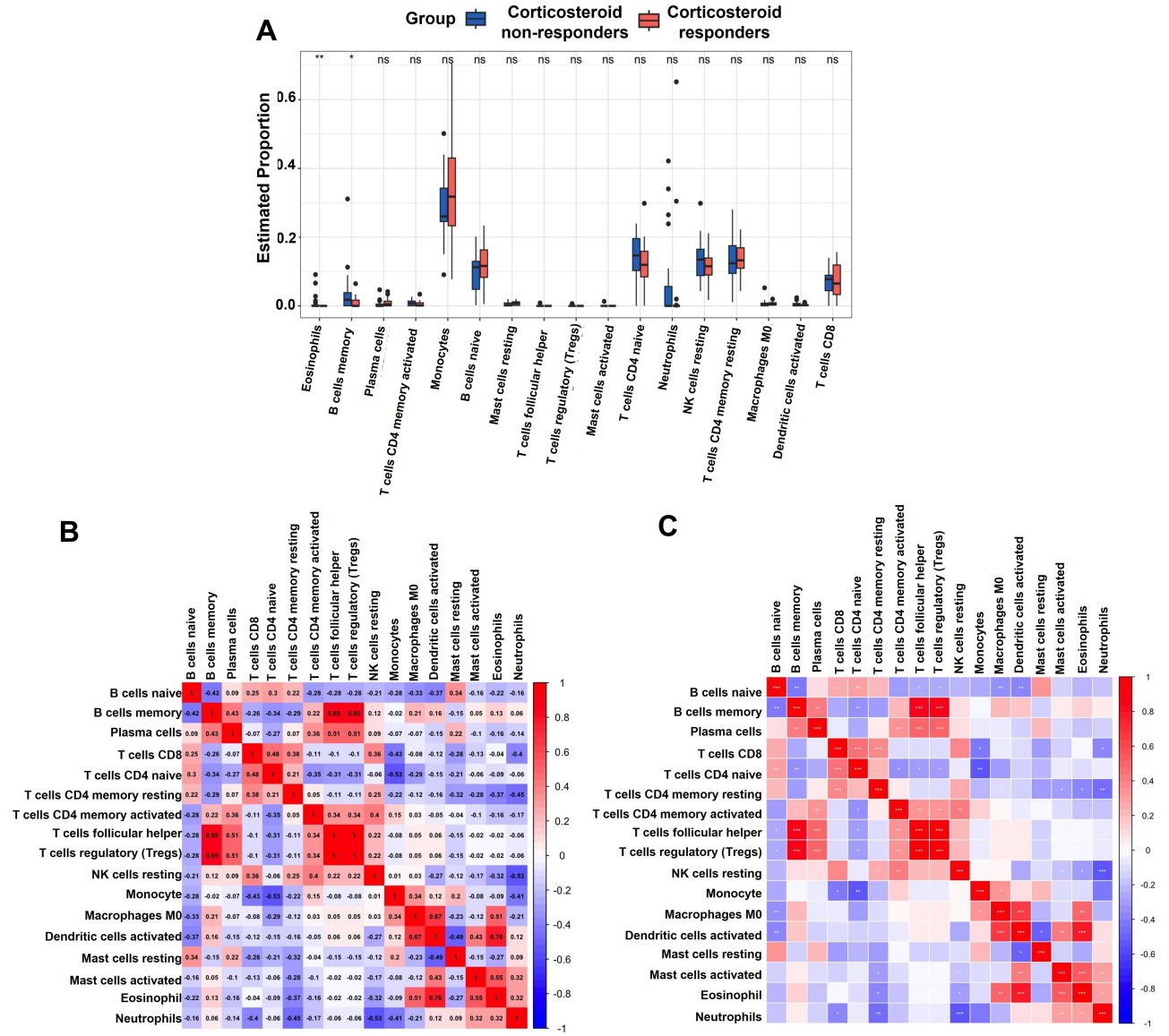

Figure 5 Immune cells infiltration analysis in GSE230048. (**A**) The different expression levels of 22 immune cell types between corticosteroid non-responder and responder samples. (**B**–**C**) The heatmap depicting the relationship between 22 immune cells in corticosteroid non-responder and responder samples. *$P < 0.05$; **$P < 0.01$.

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activated, NK cells resting and T cells CD4 memory activated, Tregs and T cells follicular helper, Macrophages M0 and Monocyte, Macrophages M0 and Dendritic cells activated, Macrophages M0 and Eosinophils, Dendritic cells activated and Mast cells activated, Dendritic cells activated and Eosinophils, Mast cells activated and Eosinophils, Mast cells activated and Neutrophils, and Eosinophil and Neutrophils. In contrast, significant negative correlations were found between: B cells naive and B cells memory, B cells naive and T cells follicular helper, B cells naive and Tregs, B cells naive and Macrophages M0, B cells naive and Dendritic cells activated, B cells memory and T cells CD4 naive, T cells CD8 and Monocytes, T cells CD8 and Neutrophils, T cells CD4 naive and T cells CD4 memory activated, T cells CD4 naive and T cells follicular helper, T cells CD4 naive and Tregs, T cells CD4 naive and Monocytes, T cells CD4 memory resting and Mast cells activated, T cells CD4 memory resting and Eosinophils, T cells CD4 memory resting and Neutrophils, NK cells resting and Mast cells activated, NK cells resting and Eosinophils, NK cells resting and Neutrophils, and Dendritic cells activated and Mast cells resting. The heatmap highlights the complex interrelationships among immune cells, suggesting that the immune microenvironment in corticosteroid-resistant asthma is highly interconnected and dysregulated.

Validation of TNS1, ABCC4, and TNFRSF21 in Asthmatic Patients and in Corticosteroid-Resistant Asthma Murine Models

Data from the Human Protein Atlas shows that TNS1, ABCC4 and TNFRSF21 are highly expressed on macrophages in lung tissues (Figures S1A, S2A, and S3A). Moreover, in human immune cells, TNS1 was mostly expressed in monocytes (Figure S1B and C). In human immune cells, ABCC4 and TNFRSF21 were moderately expressed in monocytes (Figures S2B, S2C and S3B, S3C). To more accurately determine the expression of TNS1, ABCC4, and TNFRSF21 on immune cells in asthma, we performed scRNA sequencing of PBMCs from asthmatic patients. Subpopulations were depicted in the UMAP plot.

Major cell types captured by single-cell RNA sequencing included monocytes, natural killer (NK) cells, $CD8^+$ T cells, B cells and other T cells. As expected, TNS1 and TNFRSF21 were mainly expressed in monocytes of PBMCs from asthmatic patients (Figure 6A–C). However, ABCC4 was expressed in various cell types of PBMCs, including monocytes, T cells, B cells and NK cells.

To further confirm the role of TNS1, ABCC4, and TNFRSF21 on CS-induced corticosteroid resistance in asthma, OVA-induced asthma mice were exposed to CS as detailed in methods. sRaw was significantly elevated in mice sensitized/challenged with OVA. CS exposure led to a slight decrease in sRaw, although it was not statistically significant. DEX treatment caused a decrease in sRaw in the OVA-sensitized/challenged mice but had no effect on CS exposed mice (Figure 7A). CS exposure induced a significant increase in inflammatory cell infiltration and mucus secretion (Figure 7B). CS exposure in mice significantly elevated MDA (Figure 7C) and concurrently lowered SOD activity (Figure 7D), underscoring enhanced oxidative stress. Additionally, CS exposure increased the macrophage numbers in bronchoalveolar lavage fluid compared to OVA-sensitized and challenged mice (Figure 7E). DEX treatment significantly attenuated these changes in mice sensitized/challenged with OVA alone. However, DEX treatment showed no significant effect on these changes in the OVA-sensitized/challenged mice exposed to CS (Figure 7A–E). These results indicated that CS exposure could induce corticosteroid resistance in murine models of asthma.

We confirmed the expression of TNS1, ABCC4, and TNFRSF21 in lung tissues of OVA plus CS-induced corticosteroid-resistant murine models by RT-qPCR. CS exposure increased TNS1, ABCC4 and TNFRSF21 mRNA levels from 1.37 ± 0.04, 2.71 ± 0.15, and 5.02 ± 1.66 to 2.05 ± 0.13 ($P < 0.001$), 4.72 ± 0.76 ($P = 0.016$), and 7.35 ± 1.38, respectively (Figure 7F–H). While CS exposure significantly increased the expression of TNS1 and ABCC4, the increase in TNFRSF21 mRNA levels did not reach statistical significance ($P > 0.05$). This lack of significance may reflect individual variability or insufficient statistical power due to limited sample size.

TNS1 Is Involved in CS-Induced M2 Polarization

Studies have highlighted the critical role of macrophage activation and polarization states in the progression of asthmatic airway inflammation and corticosteroid resistance. We examined the impact of CS exposure on levels of M2 macrophage

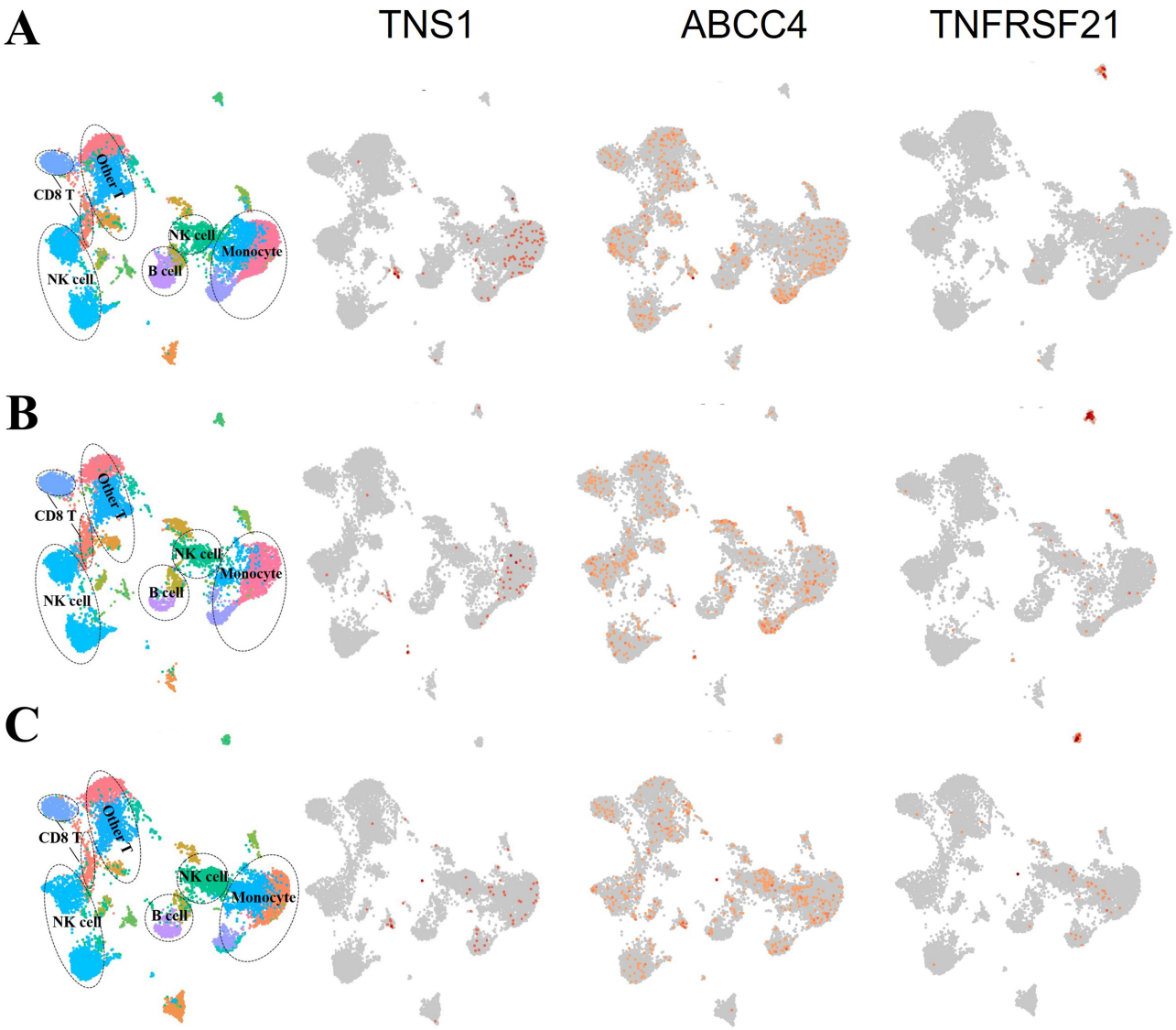

Figure 6 Expression of TNS1, ABCC4, and TNFRSF21 in asthmatic patients. (**A**–**C**) The expression of TNS1, ABCC4, and TNFRSF21 across the different cell types of asthmatic patients determined by scRNA sequencing.

markers, CD301 and CD206, in both Raw264.7 macrophage cell lines and the lung tissues of OVA plus CS-induced murine models of corticosteroid-resistant asthma. In Raw264.7 cells, CS exposure increased the mRNA levels of CD206 and CD301 from 1.00 ± 0.04 and 1.00 ± 0.01 to 2.07 ± 0.13 and 5.07 ± 0.14, respectively (Figure 8A). OVA challenge increased the mRNA levels of CD206 and CD301 from 1.05 ± 0.13 and 1.02 ± 0.08 to 2.44 ± 0.14 and 2.59 ± 0.21, respectively (Figure 8B). Moreover, CS exposure further increased the mRNA levels of CD206 and CD301 to 3.78 ± 0.34 and 4.40 ± 0.35, respectively (Figure 8B).

In Raw264.7 cells, CS exposure increased the mRNA levels of TNS1 from 1.01 ± 0.07 to 1.70 ± 0.11 ($P < 0.01$; Figure 8C). To identify whether TNS1 is essential for macrophage M2 polarization, we developed siRNA targeting TNS1 in Raw264.7 cells (Figure 8D). Knockdown of TNS1 reversed the mRNA levels of CD206 (from 10.31 ± 1.06 to 6.33 ± 0.95, $P < 0.05$) and CD301 (from 1.89 ± 0.16 to 1.24 ± 0.10, $P < 0.05$) in Raw264.7 cells (Figure 8E).

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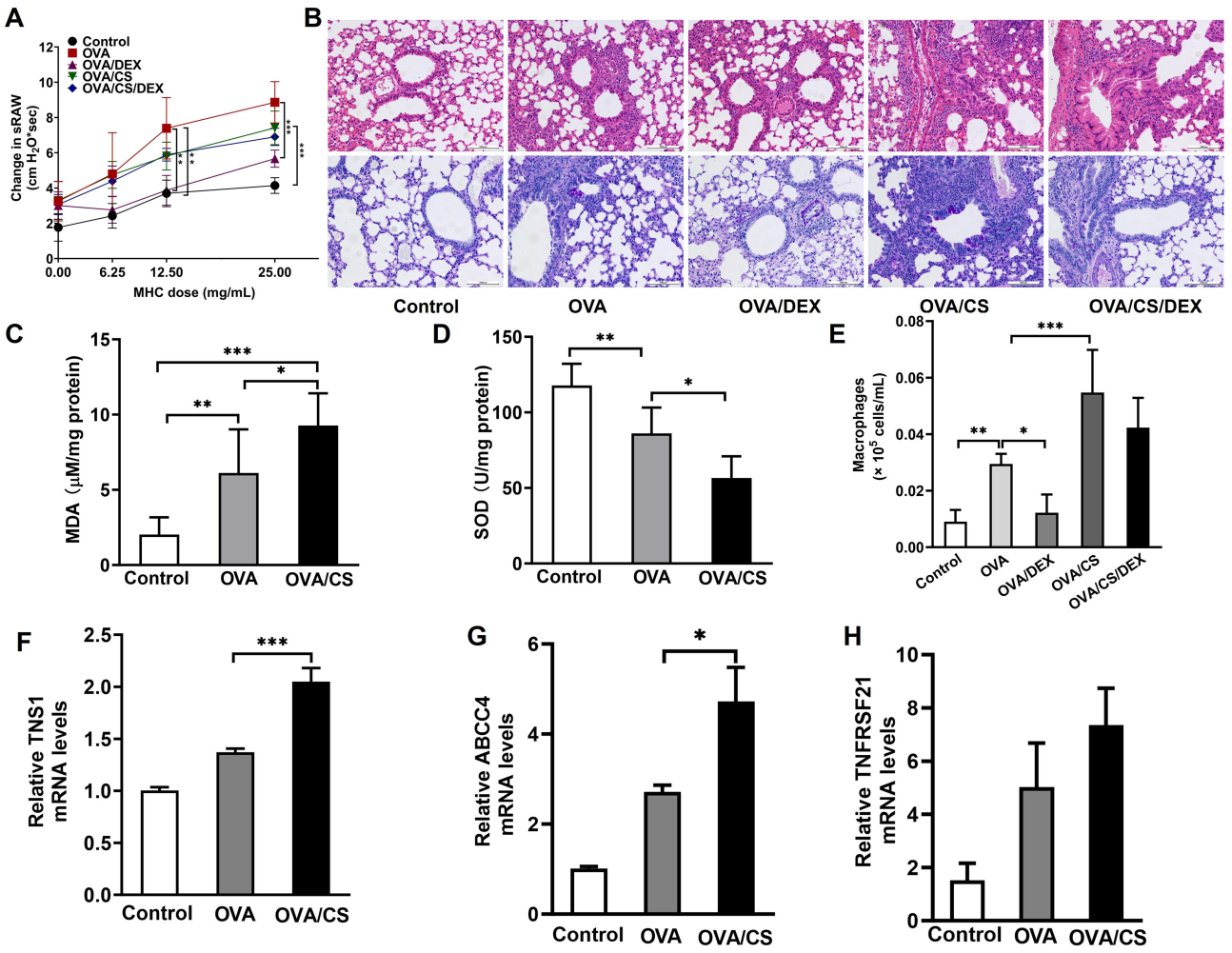

Figure 7 Expression of TNS1, ABCC4, and TNFRSF21 in asthma murine models. Mice were sensitized and challenged with OVA and exposed to CS as described in methods. (**A**) The sRaw values. n = 4. (**B**) Representative images of H&E and PAS (200 ×). n = 7. (**C**) MDA levels in the lung tissues. n = 7. (**D**) SOD levels in the lung tissues. n = 7. (**E**) Bronchoalveolar lavage fluid macrophage cell counts. n = 7. (**F–H**) TNS1, ABCC4, and TNFRSF21 mRNA levels in lung tissues. n = 7. Data were expressed as mean ± SEM. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Discussion

Corticosteroid resistance is a significant clinical challenge in asthma management, characterized by reduced responsiveness to corticosteroid. Patients with corticosteroid resistance experience more frequent exacerbations, increased hospitalizations, and a higher likelihood of needing emergency care.[4] Early recognition of corticosteroid resistance in severe asthma is essential to prevent the side effects of high-dose corticosteroid and initiate more effective treatments. Moreover, understanding mechanisms of corticosteroid resistance in asthma could facilitate the development of new therapeutic approaches. Research has shown that both genetic and environmental factors contribute to corticosteroid resistance in asthma.[28] Tobacco use remains one of the major public health threats, and efforts to control tobacco continue to be a global health priority. Despite experiencing chronic respiratory symptoms, some individuals with asthma start and continue to smoke, with prevalence rates in asthmatic individuals being similar to those observed in the general population. Exposure to second-hand smoke, third-hand smoke, or second-hand aerosol from e-cigarettes, also poses a significant risk for people with asthma, even if they do not smoke themselves.[29] Previous studies showed that asthmatic smokers tend to have poorer control and may exhibit reduced responsiveness to corticosteroid.[30]

Dysregulation of multiple immune cell types, such as Th17 cells, basophils, neutrophils, and macrophages, contributes to the development of refractory corticosteroid-resistant asthma.[28] Severe corticosteroid-resistant asthma is most often linked to non-eosinophilic endotypes. In murine models, adoptively transferred Th17 cells elicit pronounced airway

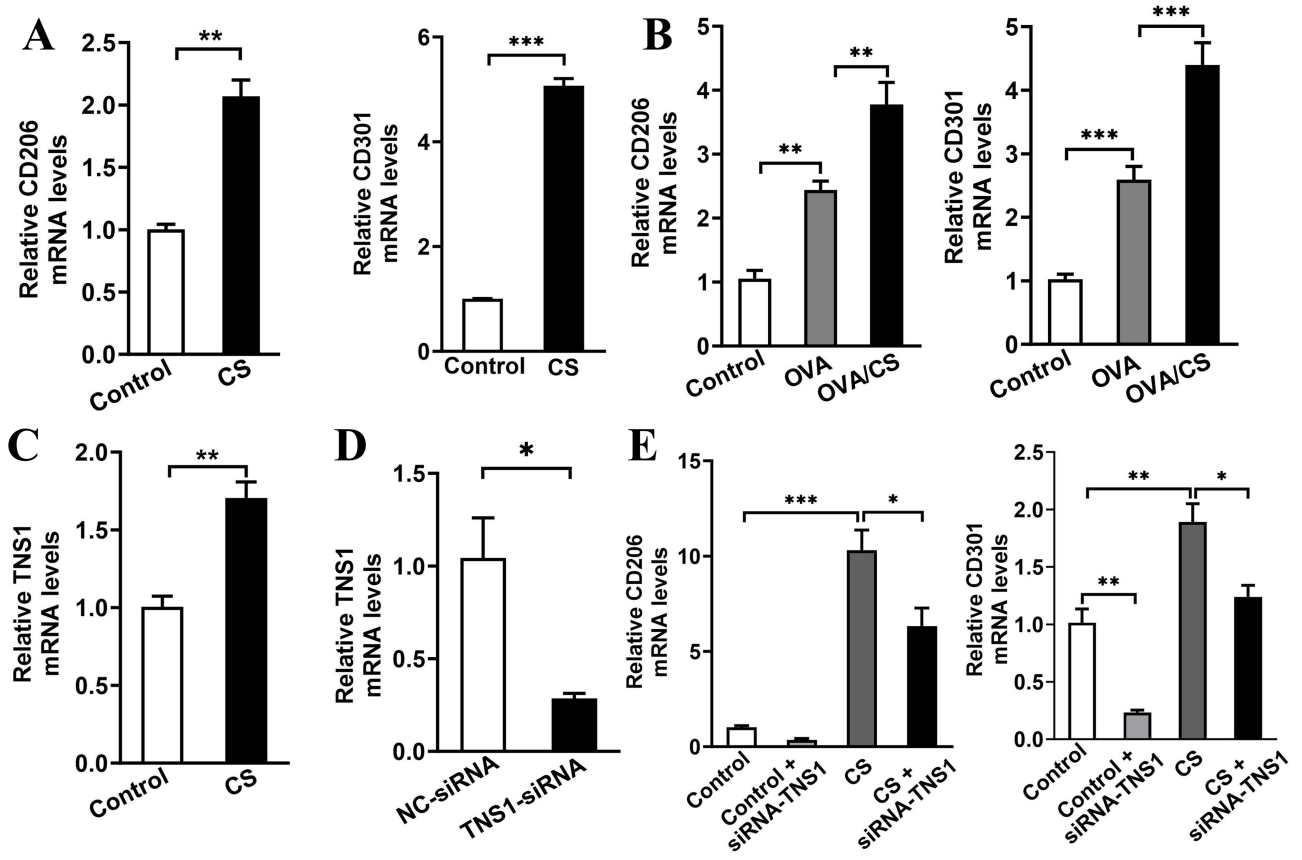

Figure 8 TNS1 is involved in CS-induced M2 polarization. (**A**) The mRNA levels of CD206 and CD301 in CSC-exposed Raw264.7 cells. (**B**) Mice were sensitized and challenged with OVA and exposed to CS as described in methods. The mRNA levels of CD206 and CD301 in the lung tissues. n = 7. (**C**) The mRNA levels of TNS1 in CSC-exposed Raw264.7 cells. (**D**) The mRNA levels of TNS1 in Raw264.7 cells transfected with negative control siRNA (NC-siRNA) or TNS1-siRNA. (**E**) Raw264.7 cells transfected with NC-siRNA or TNS1-siRNA was exposed to 100 mg/L CSC for 24 h. CD206 and CD301 levels were determined by RT-qPCR. Data were expressed as mean ± SEM. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

neutrophilia and exaggerated bronchial hyper-responsiveness while exhibiting markedly diminished sensitivity to corticosteroid treatment.[31] Corticosteroid-resistant patients display pronounced over-expression of GRβ-the dominant-negative splice variant of the GR-in circulating mononuclear cells, lymphocytes, and neutrophils.[32] Airway basophils are increased and activated in eosinophilic asthma.[33] Asthmatic airway basophil levels correlate positively with eosinophils and negatively with neutrophils.[34] However, a study by Wang et al showed that certain basophil subpopulations serve as major producers of IL-4 and IL-13, and notably, corticosteroid treatment with DEX fails to suppress their expression.[35]

Macrophages represent the largest population of immune cells in lung tissue and play a critical role in airway inflammation, particularly in asthma that is resistant to corticosteroid therapy.[36] In corticosteroid-insensitive asthma, bronchoalveolar lavage macrophages display markedly elevated cytoplasmic and nuclear GRβ; silencing GRβ mRNA in these cells restores DEX-driven GRα transactivation.[37] In the OVA/LPS model of corticosteroid-resistant asthma, depletion of airway macrophages-but not neutrophils-attenuated AHR, suggesting macrophages as the key drivers of corticosteroid-resistant AHR.[38] The effect of CS on corticosteroid resistance in asthma, particularly through its impact on macrophages, is an important area of investigation. Macrophages exposed to CS showed increased levels of proinflammatory cytokines and reduced histone deacetylase 2 expression, which can contribute to a heightened inflammatory response that is less responsive to corticosteroids.[39] CS can also alter the polarization and function of macrophages,[40] which is a critical mechanism contributing to corticosteroid resistance in asthma. To identify potential biomarkers and pathways related to CS-induced corticosteroid resistance in asthma, we conducted bioinformatics analysis on GEO datasets and validated the results through in vivo experiments.

Using the datasets GSE230048 and GSE13896, we identified five overlapping genes that were upregulated in corticosteroid non-responsive asthmatic patients and alveolar macrophages of smokers. Subsequently, the application of WGCNA helped us identify the most relevant modules, which when intersected with DEGs, resulted in the discovery of three potential biomarkers for diagnosing corticosteroid resistance in asthma: TNS1, ABCC4, and TNFRSF21. ROC analysis demonstrated that the combination of these three markers could significantly distinguish corticosteroid non-responsive asthmatic patients from corticosteroid-responsive asthmatic patients, underscoring the potential of TNS1, ABCC4, and TNFRSF21 as biomarkers for the diagnosis of corticosteroid resistance in asthma. Currently, in clinical practice, there are no widely accepted biomarkers or models to identify corticosteroid resistance. Some researchers have also conducted studies on biomarkers related to corticosteroid resistance in asthma. Nagasaki et al demonstrated the reliability of using FENO and serum periostin to identify inhaled corticosteroids-insensitive patients.[41] Leukocyte redistribution has also been reported as potential bioindicator of corticosteroid resistance in severe asthma.[42] In this study, the diagnostic efficacy of the identified markers is moderate, with an AUC of 0.718. The relatively low AUC highlights the limitations of these markers when considered independently and suggests that it might benefit from being combined with other biomarkers or clinical data, such as FENO and leukocyte redistribution, to enhance its predictive capability. Future research will focus on integrating these markers with additional clinical parameters to develop a more robust diagnostic tool. Additionally, the current marker set can serve as a complementary tool to existing diagnostic methods. Our results showed that TNS1 was the most significantly upregulated gene in the GSE230048 dataset and the GSE13896 dataset. Data from the HPA and single-cell RNA sequencing analysis of PBMCs from asthmatics confirmed the expression of TNS1, ABCC4, and TNFRSF21 in macrophages. Furthermore, TNS1, ABCC4, and TNFRSF21 expression was significantly upregulated in the lung tissues of a CS-induced corticosteroid-resistant asthma murine model.

Previous research has demonstrated that short-term CS exposure can induce significant inflammatory and immunological changes relevant to asthma pathogenesis. Studies showed that exposing mice to CS for 14 days reduced sensitivity to corticosteroids in an OVA-induced asthma murine model.[19] Additionally, several studies indicate that mice exposed to CS for 1 hour per day exhibit good tolerance without mortality.[43,44] These findings support the relevance of our chosen exposure duration (1 hour per day for 14 days) for examining the effects of CS on corticosteroid resistance in asthma. Our present study showed that mice sensitized and challenged with OVA exhibited a significant increase in sRaw, inflammatory cell infiltration, and mucus hypersecretion compared to the saline control group. This increase indicates heightened airway resistance and airway inflammation, which are hallmarks of allergic asthma. DEX treatment caused a decrease in sRaw, inflammatory cell infiltration, and mucus hypersecretion in the OVA-sensitized/challenged mice, which are typically steroid-sensitive. Our study also showed that CS exposure led to a slight decrease in sRaw, although this decrease was not statistically significant. Published data about the effect of CS co-exposure are varied, with some reporting decreases and others showing increases in airway resistance in asthma murine models.[45–47] These inconsistencies may be due to variations in CS co-exposure protocols and the different time points for measuring airway resistance. However, our results showed that DEX treatment did not affect sRaw, inflammatory cell infiltration, or mucus hypersecretion in the OVA-sensitized/challenged mice exposed to CS (1 hour per day for 14 days). This confirms the impact of CS exposure on corticosteroid resistance in asthma. Chronic exposure is more representative of human smoking behavior and this exposure duration (1 hour per day for 14 days) may not fully recapitulate the chronic exposure seen in human smokers. However, it allows for the examination of early-stage effects of CS exposure on corticosteroid resistance and establishes a foundation for understanding the mechanisms involved. Future studies will aim to address the effects of prolonged CS exposure to provide a more comprehensive understanding. Moreover, additional studies utilizing knockout or overexpression mice will be conducted to validate the effects and mechanisms of these biomarkers on corticosteroid resistance in asthma.

TNS1 is a member of the tensin family. Tensin proteins are adaptor proteins that play a role in cell adhesion and signaling. They are known to interact with components of the focal adhesion complex, such as integrins, and participate in the regulation of cell adhesion and migration.[48] Studies showed that TNS1 was highly expressed in heart, lungs, and other organs.[49] Mutations or dysregulation of TNS1 have been implicated in various diseases, including cancer and asthma.[50] A variant in the TNS1 gene has been associated with the lung function parameter FEV1/FVC.[51] A TNS1 single

nucleotide polymorphism was also related to the risk of asthma with hay fever[52] and COPD. Additionally, studies have shown that TNS1 expression was increased in lung tissues of COPD patients.[53] In the present study, TNS1 was identified as genes significantly associated with CS induced corticosteroid resistance in asthma. Analysis of GEO datasets revealed significantly higher expression of TNS1 in corticosteroid non-responsive asthma patients and alveolar macrophages of smokers. Single-cell RNA-seq also confirmed TNS1 expression in macrophages from asthmatic patients. Data from the Human Protein Atlas show that TNS1, ABCC4, and TNFRSF21 are highly expressed on macrophages in lung tissues. Moreover, in human immune cells, TNS1 was mostly expressed in monocytes. Macrophages are categorized into two main phenotypes: the classically activated (M1) and the alternatively activated (M2) macrophages.[54] In asthma, M1 macrophage activation is typically linked to heightened inflammatory responses, while M2 macrophages are implicated in promoting airway remodeling and sustaining chronic inflammation. The polarization balance of macrophages is critical in asthma. Both M1 and M2 macrophages have been associated with the pathogenesis of asthma.[55,56] M1 macrophages in asthma secrete pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α.[57] A higher abundance of $CD206^{+}$ M2 macrophages is correlated with increased asthma severity, and these cells exhibit resistance to inhaled corticosteroid treatment.[58] ROS have been shown to regulate macrophage polarization and are capable of promoting both M1 and M2 phenotypic states depending on the cellular context and redox balance.[57] The imbalance in macrophage polarization driven by oxidative stress contributes to chronic inflammation and tissue remodeling, key features of asthma progression.[57,59] Moreover, studies showed that an imbalance between oxidants and antioxidant responses may contribute to the pathogenesis of corticosteroid resistant asthma.[60,61] Targeting ROS and their signaling pathways is proposed as a promising therapeutic strategy for corticosteroid resistant asthma.[61] TNS1 has been reported to be associated with alternatively activated macrophages. However, current studies have primarily observed the positive correlations of TNS1 with M2 macrophages.[62,63] No study has directly demonstrated the role of TNS1 in macrophage polarization. Our study reveals that CS exposure significantly increases TNS1 expression in both macrophages and lung tissues of asthmatic mice. Notably, when TNS1 levels were knocked down using siRNA, the expression of M2 macrophage markers (CD206 and CD301) was markedly reduced. These findings indicate that TNS1 plays a crucial role in regulating macrophage polarization. However, whether TNS1's regulation of M2 macrophage polarization is a key mechanism underlying its promotion of corticosteroid resistance in asthma remains to be further investigated.

ABCC4 is a transmembrane protein that functions as an efflux transporter. Despite the limited research on ABCC4 in the context of asthma, it is known to be involved in export of various pro-inflammatory molecules, including leukotrienes, cyclic nucleotides (cAMP and cGMP), and sphingosine-1-phosphate.[64] These proinflammatory molecules all play essential roles in asthma. Notably, Palikhe et al demonstrated the association of an ABCC4 polymorphism with airway inflammation in asthmatics by increasing extracellular 15-hydroxyeicosatetraenoic acid and sphingosine-1-phosphate levels.[65] Additionally, the blockade of cAMP efflux by ABCC4 inhibitors can enhance the effects of LABA and glucocorticoid combination therapy in asthma.[66] Collectively, these studies suggest that ABCC4 polymorphisms may be linked to asthma and that inhibiting ABCC4 could augment the anti-inflammatory efficacy of LABA/glucocorticoid therapy. Several studies suggest that activity of cAMP-dependent signaling pathway can enhance glucocorticoid receptor function.[67] By facilitating the efflux of cAMP, ABCC4 may reduce the intracellular concentrations of cAMP, thereby dampening the anti-inflammatory effects of glucocorticoids. To further elucidate the role of ABCC4 in corticosteroid resistance, our future studies will focus on experimental validation of the proposed mechanisms. Future investigations using knockout or overexpression mouse models of ABCC4 are needed to confirm the role and mechanisms of ABCC4 in corticosteroid resistance in asthma.

TNFRSF21 belongs to the TNF receptor family. A recent study by Clay et al demonstrated a significant association between TNFRSF21 and IgE.[68] Rare variants in TNFRSF21 were associated with allergy-related phenotypes in children with mild and severe asthma. Moreover, TNFRSF21 has been reported to regulate Th2 cell differentiation through Jun amino terminal kinase (JNK) pathway activation.[69] JNK activation leads to GR phosphorylation, affecting its nuclear translocation and transcriptional activity. This can result in reduced sensitivity to glucocorticoids, contributing to corticosteroid resistance.[70] Whether TNFRSF21 induces corticosteroid resistance through its effect on JNK activation requires further investigation. Venkataraman et al used TNFRSF21 knockout mice to investigate the role of TNFRSF21 in airway inflammation in OVA-induced asthmatic mice. The results demonstrated that TNFRSF21 knockout

significantly reduced Th2 inflammatory cytokine secretion in mouse lung tissue and markedly suppressed airway inflammation development in asthmatic mice. This effect may be associated with the regulation of T cell function by TNFRSF21.[71] Recently, Clay et al highlighted the association of TNFRSF21 with asthma phenotypes.[68] Nevertheless, whether ABCC4 and TNFRSF21 are involved in corticosteroid resistance is unclear, and would be interesting to be investigated in more detail. In this study, ABCC4 and TNFRSF21, in combination with TNS1, were identified as potential diagnostic biomarkers for corticosteroid resistance in asthma.

However, this study still has some limitations and issues that need to be addressed in future studies. Firstly, single-cell sequencing was only performed on three samples, which limits the generalizability of our findings. Further validation using larger independent cohorts including smoking asthmatic patients, non-smoking asthmatic patients, and smoking non-asthmatic patients would provide more robust evidence for the observed gene expression differences. We observed that TNS1, ABCC4, and TNFRSF21 expression was significantly upregulated in mouse lungs after 14 days of CS exposure compared to controls. Further studies are needed to characterize the time-dependent changes in the expression of these genes and their response to corticosteroid therapy, which could provide insights into their potential roles in the development of corticosteroid insensitivity.

Secondly, while our work focused on macrophage-driven corticosteroid insensitivity, the Human Protein Atlas indicates that TNS1, ABCC4, and TNFRSF21 are also moderately expressed in pulmonary granulocytes and alveolar epithelial cells-populations previously implicated in corticosteroid resistant airway inflammation. ABCC4 expression is, in fact, higher in these cells than in macrophages. The potential contribution of TNS1, ABCC4, and TNFRSF21 to corticosteroid resistance via basophils, alveolar epithelial cells, or other granulocytes remains an open question that we will address in future work.

Thirdly, our analysis primarily focused on gene expression levels. Future investigations using knockout or over-expression mouse models are needed to confirm the role of these genetic biomarkers in corticosteroid resistance in asthma. TNS1 is associated with the mTOR signaling pathway. A recent study by Heung-Woo Park et al identified mTOR activation as a key driver of corticosteroid-resistant asthma.[72] The mTOR inhibitor rapamycin was shown to restore sensitivity to DEX in PBMCs from COPD patients.[73] Given that mTOR also governs macrophage polarisation,[74] we hypothesise that TNS1 may skew macrophages toward a phenotype that compromises corticosteroid efficacy through mTOR-dependent mechanisms. Mechanistic studies are warranted to elucidate the precise molecular pathways involved. Understanding how these genes influence cellular functions and contribute to disease mechanisms will provide deeper insights into asthma biology. Several compounds[75] and miRNAs[76] have been shown to directly or indirectly suppress TNS1 expression-for example, prazosin down-regulates TNS1 and thereby attenuates downstream Akt/mTOR signalling.[75] Pharmacological or genetic TNS1 inhibition might restore corticosteroid sensitivity in CS-exposed asthmatic mice, but this remains to be confirmed in future studies.

Fourthly, in the GSE230048 dataset, the three-gene signature (TNS1, ABCC4, TNFRSF21) derived from peripheral-blood RNA yielded an AUC of 0.718 for distinguishing corticosteroid-resistant from corticosteroid-responsive asthma patients. This level of performance indicates moderate diagnostic efficacy. Future efforts will concentrate on combining these genetic markers with additional clinical and biochemical data to develop a more robust and reliable diagnostic tool and provide personalized treatment recommendations. Prospective studies with larger and more diverse patient cohorts will be essential to validate the utility of this integrated approach in clinical practice. Assessing the expression of these genes in airway samples (eg, sputum) and peripheral blood cells from asthma patients, especially smokers, warrants further investigation as a strategy to stratify patients by risk of corticosteroid resistance.

Conclusion

In conclusion, we employed integrated bioinformatics analyses to identify three hub genes-TNS1, ABCC4, and TNFRSF21-associated with CS-induced corticosteroid resistance in asthma. These genes exhibit potential as diagnostic biomarkers for predicting corticosteroid non-response in asthmatic patients. Notably, TNS1 was found to be predominantly expressed in macrophages. Functional studies demonstrated that TNS1 was upregulated by CSC in macrophages and played a critical role in CS-induced M2 polarization, as its silencing significantly reduced the expression of M2

markers CD206 and CD301. These findings suggest that TNS1 contributes to an immunomodulatory milieu that promotes corticosteroid resistance.

Collectively, our results establish TNS1 as a novel and key regulator of macrophage polarization in smoke-exposed asthma, highlighting its potential as both a biomarker and a therapeutic target for overcoming corticosteroid resistance. Targeting TNS1 in macrophages may represent a promising strategy for the development of personalized treatments in refractory asthma.

Abbreviations

ABCC4, ATP binding cassette subfamily C member 4; AHR, airway hyperresponsiveness; AUC, area under the curve; BMI, body mass index; COPD, chronic obstructive pulmonary disease; CS, cigarette smoke; CSC, cigarette smoke condensate; DEGs, differentially expressed genes; DEX, dexamethasone; FENO, fraction of exhaled nitric oxide; FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity;; GO, Gene Ontology; ICS, inhaled corticosteroid; KEGG, Kyoto Encyclopedia of Genes and Genomes; LABA, long-acting bronchodilator; MDA, malondialdehyde; OVA, ovalbumin; PAS, periodic acid-Schiff; PBMCs, peripheral blood mononuclear cells; PCA, principal component analysis; ROC, receiver operating characteristic; RT-qPCR, reverse transcription-quantitative PCR; SABA, short-acting bronchodilator; SOD, superoxide dismutase; sRaw, specific airway resistance; TNFRSF21, TNF receptor superfamily member 21; TNS1, tensin 1; TOM, topological overlap matrix; UMAP, uniform manifold approximation and projection; UMI, unique molecular identifier; WGCNA, weighted gene co-expression network analysis.

Data Sharing Statement

Data are available from the corresponding author on request.

Ethics Approval and Informed Consent

The Research Ethics Committee of Beijing Chao-Yang Hospital approved this study (No. 2022-ke-642 and 2025-ke-597). And this study was conducted in accordance with the Declaration of Helsinki. All participants provided informed consent.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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