

PLD4, CD247, and TREM2 as Phagocytosis-Related Biomarkers in Traumatic Brain Injury Identified by Integrated Transcriptomics and Single-Cell Sequencing

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1 **Title page**

2 **PLD4, CD247, and TREM2 as Phagocytosis-Related Biomarkers in Traumatic**
3 **Brain Injury Identified by Integrated Transcriptomics and Single-Cell**
4 **Sequencing**

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22 **Abstract**

23 **Background:** Traumatic brain injury (TBI) triggers secondary neuroinflammation,
24 wherein dysregulated phagocytosis plays a critical but poorly understood role. This
25 study sought to identify key phagocytosis-related biomarkers and their cellular origins

in TBI via integrative multi-omics analysis and experimental validation.

Methods: We integrated GEO bulk transcriptomic data (GSE223245, GSE89866) and single-cell RNA-seq data (GSE182398) to examine differential expression, immune infiltration, cellular localization, pseudo-time, and metabolism. Candidate genes consistently identified across datasets were validated in a mouse model of TBI using qPCR, H&E staining, and TUNEL staining.

Results: In TBI, TREM2 was upregulated, while CD247 and PLD4 were downregulated ($P < 0.05$). CD247/PLD4 were positively correlated with regulatory T cells and natural killer cells but negatively correlated with M2 macrophages; TREM2 exhibited the opposite trend. Single-cell analysis revealed that CD247/PLD4 localize to NKT cells, while TREM2 localizes to myeloid cells and microglia. Pseudo-time analysis: CD247 persists throughout NKT cell development; PLD4 is enriched during the early to mid-stages, and PLD4⁺ NKT cells are enriched in phagocytosis and lysosomes. TBI-NKT cells exhibit enhanced oxidative phosphorylation and fatty acid degradation capabilities. Animal experiments confirmed the changes in biomarkers, cortical cell apoptosis, and histopathological alterations.

Conclusion: These findings establish TREM2, PLD4, and CD247 as robust TBI associated biomarkers. To our knowledge, this is the first study to implicate PLD4 in TBI pathophysiology. Furthermore, our findings suggest that PLD4⁺ NKT cell subpopulations may contribute to post-TBI immunometabolic reprogramming by modulating nucleic acid clearance and inflammatory homeostasis.

Keywords: traumatic brain injury, phagocytosis, TREM2, PLD4, CD247, bioinformatics

1. Introduction

Traumatic brain injury (TBI) is one of the most common neurological disorders worldwide. Recent Global Burden of Disease (GBD) estimates indicate that TBI continues to impose a substantial global burden, with moderate-to-severe injuries

accounting for a major share of cases[1]. The associated direct and indirect economic costs are enormous, positioning TBI as a serious global public health crisis[2, 3]. China bears one of the largest populations of TBI patients globally. With an aging population and increasing traffic density, the incidence of TBI continues to rise, posing significant challenges to healthcare systems and social stability[4]. The pathology of TBI encompasses primary mechanical injury and a cascade of secondary events, including neuroinflammatory dysregulation, oxidative stress, blood–brain barrier disruption, calcium homeostasis disturbances, and apoptosis. These interrelated factors form a complex pathological network. Disease progression and prognosis are significantly influenced by injury severity, age, and genetic background[5-7]. Current treatment remains largely limited to surgical decompression and symptomatic supportive care, with a marked lack of targeted neuroprotective agents. Consequently, patients frequently experience complications, a protracted disease course, and poor long-term outcomes[8, 9]. Given that post-TBI neuroinflammation can persistently impair cognitive recovery and elevate the long-term risk of neurodegenerative diseases, identifying molecules that mediate immune clearance may help bridge the gap between acute injury mechanisms and long-term prognostic assessment[1, 10]. Therefore, discovering molecular biomarkers that play key regulatory roles in TBI is critical for elucidating its pathogenesis and developing targeted interventions.

Phagocytosis regulators (PRs) are key molecules that govern the recognition, ingestion, and clearance of target cells by phagocytes. They play a vital role in maintaining tissue homeostasis, limiting inflammatory amplification, and promoting tissue repair[11, 12]. Recent studies have implicated PRs in diverse pathological processes, such as tumor immune evasion[13], autoimmune diseases[14], and neurodegeneration[15, 16]. In TBI, the immune clearance and inflammatory regulatory networks orchestrated by PRs have garnered increasing research attention. For instance, anti-CD3 monoclonal antibodies modulate microglial phagocytosis through the regulatory T cell IL-10 axis, thereby promoting neural functional

recovery[17]. Anti-CD47 antibodies have been shown to accelerate hematoma clearance[18]. Moreover, SIRT1 suppresses aberrant synaptic phagocytosis via the autophagy pathway, thereby alleviating cognitive impairment[19]. Collectively, these findings suggest that PRs may play a crucial role in secondary injury after TBI. However, systematic studies on their expression profiles, key effector cells, and regulatory mechanisms remain limited. For example, Recent evidence suggests that enhancing phagocytic clearance mediated by microglia or astrocytes may improve neuroinflammation and cognitive outcomes following TBI, indicating that PRGs may be involved in the clearance of injury debris and the restoration of immune homeostasis[17, 19].

Most current TBI studies have focused on the TREM2 signaling pathway in microglia[15, 20, 21], whereas the potential role of NKT cells—particularly via novel phagocytic regulators such as PLD4—remains largely unexplored. PLD4 has recently been implicated in nucleic acid recognition and autoimmune diseases[22-24], yet its association with TBI or neuroinflammation has not been reported. Therefore, we integrated peripheral blood transcriptomics with single-cell brain atlases to identify reliable diagnostic biomarkers and uncover previously unrecognized effector cell populations and their regulatory axes. This approach may bridge systemic immune signals with local neuroinflammatory events.

In this study, we integrated transcriptomic and single-cell RNA sequencing data from the GEO database with independent experimental validation in a C57BL/6 mouse TBI model. Our aim was to systematically elucidate the molecular mechanisms by which PRs contribute to TBI, identify robust biomarkers, and characterize key cellular subpopulations. Furthermore, we sought to delineate the dynamic expression patterns of these biomarkers in NKT cells, their metabolic reprogramming features, and the functional differentiation of subpopulations, thereby providing potential new targets for immune-metabolic intervention in TBI.

2. Materials and Methods

2.1 Data Collection

Three TBI-related datasets were obtained from the Gene Expression Omnibus (GEO) database: two bulk transcriptomic datasets (GSE223245 and GSE89866) and one single-cell RNA sequencing (scRNA-seq) dataset (GSE182398). GSE223245 (GPL33038 platform, microarray) contains 12 TBI and 4 control peripheral blood samples and was used as the training set for initial differential expression screening. GSE89866 (high-throughput sequencing) comprises 29 TBI and 29 control peripheral blood samples and served as an independent validation set. GSE182398 (GPL24676 platform) includes six brain tissue samples (three TBI, three control) and was used for single-cell atlas construction and cell-type localization. Phagocytosis-related genes (PRGs) were retrieved from the Molecular Signatures Database (MSigDB) using the keyword "phagocytosis." After removing duplicates, 267 PRGs were obtained (Supplementary Table 1). To minimize platform-related bias, an independent cohort cross-validation strategy was employed rather than merging the two bulk datasets directly. Specifically, bulk transcriptomic data from peripheral blood were used to screen for candidate biomarkers, whereas brain scRNA-seq data were used to identify the potential cell types expressing these biomarkers and their local microenvironmental features.

We deliberately adopted a cross-tissue strategy in this study. Peripheral blood transcriptomic data were used primarily for the identification of clinically accessible biomarker candidates, whereas brain single-cell RNA sequencing data were used to localize these candidates within the lesional microenvironment and to provide cell-type-specific mechanistic context. Rather than directly equating peripheral and intracerebral signals, this design integrates systemic immune alterations with local neuroinflammatory features in a complementary manner, thereby improving biological interpretability while avoiding overstatement of cross-tissue differences.

2.2 Screening and Identification of Differentially Expressed Genes

Differentially expressed genes (DEGs) between the TBI and control groups in

GSE223245 were identified using the “limma” package (v1.40.2)[25] with the thresholds $|\log_2 \text{fold change (FC)}| > 0.5$ and $P < 0.05$. Volcano plots and heatmaps were generated with the “ggplot2” (v3.5.2) and “ComplexHeatmap” (v2.16.0) packages[26]. The five most upregulated and five most downregulated genes (ranked by $|\log_2 \text{FC}|$) were selected for display. Subsequently, GO and KEGG pathway enrichment analyses were performed on the DEGs using the “clusterProfiler” package (v4.10.1)[27] ($P < 0.05$). For GO, the top five significantly enriched terms in each category (BP, CC, MF) were selected based on P value. Similarly, the top five KEGG pathways were selected and visualized.

2.3 Biomarker Screening

Candidate genes were defined as the intersection of DEGs and PRGs, identified using the “ggvenn” package (v0.1.10). Expression levels of the candidate genes were then compared between TBI and control groups in both GSE223245 and GSE89866 using the Wilcoxon rank-sum test ($P < 0.05$). Genes showing significant differential expression in both datasets with consistent direction of regulation (up- or downregulation) were ultimately considered as biomarkers.

2.4 Functional Analysis of Biomarkers

Chromosomal locations of the biomarkers were mapped using the “RCircos” package (v1.2.2)[28, 29]. Subcellular localization information was retrieved from the GeneCards database. The GeneMANIA database was then used to explore the functional associations and co-expression networks of these biomarkers.

2.5 Immune infiltration analysis

Based on the GSE223245 dataset, enrichment scores for 64 immune cell types were calculated using the “xCell” package (v1.1.0; <http://xCell.ucsf.edu>). Next, differential immune cells were identified using the Wilcoxon rank-sum test ($P < 0.05$) and visualized using the “ggplot2” package. Finally, the Spearman correlation coefficient (cor) was calculated using the “psych” package (v2.2.9;

<https://CRAN.R-project.org/package=psych>) to assess the correlations among the differential immune cells and between biomarkers and the differential immune cells ($|\text{cor}| > 0.3$, $P < 0.05$).

2.6 Processing of scRNA-seq Data

Quality control and data integration of the single-cell dataset GSE182398 were performed using the “Seurat” package (v5.3.0)[30]. For each cell, the total UMI count (nCount_RNA), the number of detected genes (nFeature_RNA), and the percentage of mitochondrial reads (percent.mt) were calculated using the “PercentageFeatureSet” function. Cells meeting the following criteria were retained as high-quality: $\text{nCount_RNA} < 50,000$, $200 < \text{nFeature_RNA} < 7,000$, and $\text{percent.mt} < 10\%$. Following sample integration, the data were normalized with the “NormalizeData” function, and the top 2,000 highly variable genes (HVGs) were identified using “FindVariableFeatures”. Representative HVGs were also flagged for visualization ($P < 0.05$). The data were then centered and scaled with the “ScaleData” function, and principal component analysis (PCA) was performed using “RunPCA”. Significant principal components (PCs) were determined by combining the JackStraw permutation test (top 50 PCs) and the elbow plot’s variance trend. Using these PCs, unsupervised clustering was performed with “FindNeighbors” and “FindClusters” (resolution = 0.5), and the cells were visualized through UMAP dimensional reduction using “RunUMAP”. Cell clusters were manually annotated using canonical marker genes[31], and the resulting cell-type distribution was visualized on the UMAP plot. Because the dataset comprised multiple samples, potential batch effects were corrected during sample integration to minimize the influence of individual variation on clustering.

2.7 Identification of Key Cell Types

Key cell types were defined as cell clusters that simultaneously fulfilled two criteria: (1) expression enrichment of the candidate biomarkers and (2) significant disease association, assessed by comparing average expression levels, the proportion of

positive cells, and expression differences between TBI and control groups.

2.8 Analysis of Cell-Cell Communication

Cell-cell communication networks were analyzed with the “CellChat” package (v1.6.1)[32]. The aggregated cell-cell communication (CCC) network was calculated using the “aggregateNet” function, which outputs the number and weights of interactions between cell types.

2.9 Secondary Clustering and Pseudotime Analysis of Key Cell Subpopulations

To further resolve heterogeneity and differentiation trajectories within the key cell types, we performed secondary clustering following the workflow in Section 2.5, with these adaptations: the key cell subset was scaled, and PCA was run on highly variable genes. Significant PCs (PCs 1–50 with JackStraw $P < 0.05$ and those near stabilization in the elbow plot) were used for downstream analysis. Unsupervised clustering was then performed with “FindNeighbors” and “FindClusters” (resolution = 0.1), and the results were visualized via UMAP. Subclusters were annotated according to the expression of the identified biomarkers, defining key subpopulations in distinct functional states. Finally, pseudotime trajectory analysis was conducted with Monocle2 (v2.30.1)[33], and heatmaps were used to display the dynamic expression of the biomarkers across developmental stages.

2.10 Analysis of Functional and Regulatory Features of Key Cells

To compare biological functions between TBI and control key cells, gene set variation analysis (GSVA) was performed using the “GSVA” package (v1.46.0)[34]. The Hallmark gene set (h.all.v2024.1.Hs.symbols.gmt) was used to compute pathway activity scores for each cell. Differential pathway activity among cell subtypes was further evaluated with the “ReactomeGSA” package (v1.16.1). Transcription factors (TFs) regulating the key cells were inferred using the “DoRothEA” algorithm. Metabolic pathway analysis was conducted with the “scMetabolism” package (v0.2.1) [35], and the results were visualized with “ggplot2”.

2.11 Experimental Animals

SPF-grade male C57BL/6 mice (8–10 weeks, 20–25 g) were purchased from Beijing Weitong Lihua Laboratory Animal Co., Ltd. (license no. SCXK(Jing)2021-0006). Mice were housed in standard cages within an SPF facility under a 12-h light/dark cycle, with controlled room temperature, quiet and well-ventilated conditions, and ad libitum access to food and water. After a 5–7-day acclimatization period, surgery was performed to minimize environmental stress. The animal protocol was approved by the Ethics Committee of Guizhou Medical University. All procedures complied with the ARRIVE guidelines and the NIH Guide for the Care and Use of Laboratory Animals.

2.12 Establishment of a Feeney Free-Fall Mouse TBI Model

The TBI model was established as described previously. After weighing and numbering, mice were placed in an induction chamber and anesthetized with isoflurane (7%–8% for induction). Once respiration slowed, muscles relaxed, and there was no response to noxious stimuli, the mice were transferred to a temperature-controlled surgical table. Anesthesia was maintained with 3%–4% isoflurane via a face mask, and body temperature was kept at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ using a heating blanket. The scalp was disinfected sequentially with povidone-iodine and 75% ethanol. A 2-cm midline longitudinal incision was made, and the scalp and subcutaneous tissue were reflected to expose the skull. Using the mouse stereotaxic atlas, a 4×4 mm bone window was drilled 1.5 mm posterior to bregma and 1.5 mm lateral to the midline on the right side. Drilling was performed carefully to preserve the underlying dura. After creating the bone window, bone debris was gently removed with a cotton ball to keep the field clear. A 20-g impactor was dropped from 20 cm in free fall to strike the impactor footplate vertically, inducing the injury. Immediately after impact, the impactor was lifted, and the dura was inspected for integrity and bleeding. Mice with dural rupture or excessive hemorrhage were excluded. After confirming hemostasis, the scalp was closed in layers, and the wound was disinfected again with povidone-iodine. Postoperatively, mice were kept on a 37°C heating pad

until full recovery of consciousness and then returned to their home cages. Daily monitoring included assessment of general appearance, food intake, and wound condition. Sham-operated mice underwent craniotomy without impact. Tissue was collected at 3 days post-injury, a time point corresponding to the peak of acute inflammation and cellular injury after TBI.

2.13 Modified Neurological Severity Score (mNSS)

The mNSS test was performed as previously described. The scale evaluates motor (muscle tone, abnormal movements), sensory (visual, tactile, proprioceptive), reflex, and balance functions. Higher scores reflect greater neurological impairment (severe, 13–18; moderate, 7–12; mild, 1–6). All assessments were performed 24 h after TBI by two investigators blinded to group allocation.

2.14 Hematoxylin and Eosin (H&E)

Brain tissue was fixed in 4% paraformaldehyde. The tissue was dehydrated through a graded ethanol series and xylene, and embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E) and examined for histological changes in the cortex and hippocampus adjacent to the injury site.

2.15 TUNEL

Paraffin sections were deparaffinized, rehydrated, and permeabilized. The sections were incubated with the TUNEL reaction mixture. After the reaction was stopped, the cell nuclei were counterstained with DAPI. Finally, the sections were mounted and observed under a fluorescence microscope; apoptotic cell nuclei exhibited specific green fluorescence, while normal cell nuclei appeared only blue.

2.16 qPCR Assay

Following the kit instructions, total cellular RNA was extracted using the provided reagents, and its concentration and purity were determined. Specific primers for genes such as TREM2, CD247, and PLD4 were used in conjunction with M-MuLV reverse transcriptase to establish the reaction system, converting RNA into cDNA.

Subsequently, real-time quantitative PCR amplification was performed using the cDNA as a template, and the relative expression levels of each gene were calculated using the $2^{-\Delta\Delta C_t}$ method.

2.17 Statistical Analysis

All analyses were performed using R software (v4.2.2). The Wilcoxon rank-sum test was used to compare differences between two independent groups. All independent experiments in this study were repeated at least three times. Statistical analysis was performed using GraphPad Prism 10.0 software. Quantitative data following a normal distribution are expressed as mean $\pm$ standard deviation ($\bar{x} \pm s$). An independent-samples t-test was used for comparisons between two groups, and one-way analysis of variance (ANOVA) was used for comparisons among multiple groups. A P value < 0.05 was considered statistically significant. * indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$, **** indicates $P < 0.0001$, and ns indicates no significant difference.

3. Results

3.1 Functional Enrichment Analysis of DEGs and construction of PPI networks

In the training set GSE223245, 803 DEGs were identified (272 upregulated and 531 downregulated; Fig. 1a, b; Supplementary Table 2). Functional enrichment analysis revealed significant enrichment across 534 terms (Fig. 1c; Supplementary Table 3), comprising 468 biological process (BP), 20 cellular component (CC), and 46 molecular function (MF) terms. Representative enriched terms included immune response activation signaling, lymphocyte-mediated immunity, collagen-containing extracellular matrix, lysosomal granules, nucleic acid exonuclease activity, and phosphatidylserine binding. DEGs were also enriched in 20 KEGG pathways, such as apoptosis, TGF- β signaling, cytokine-cytokine receptor interaction, and natural killer (NK) cell-mediated cytotoxicity ($P < 0.05$; Supplementary Table 4). These findings indicate that post-TBI transcriptomic changes in peripheral blood primarily involve immune and inflammatory activation, regulation of cell death, and remodeling of

immune cell function. PPI analysis showed that genes such as the interferon-induced protein with tetratricopeptide repeats (IFIT) family (IFIT1, IFIT2, IFIT3) and the HECT and RCC1 domain-containing protein (HERC) family (HERC5, HERC6) were highly clustered, indicating high functional interconnection within this network (Fig 1d).

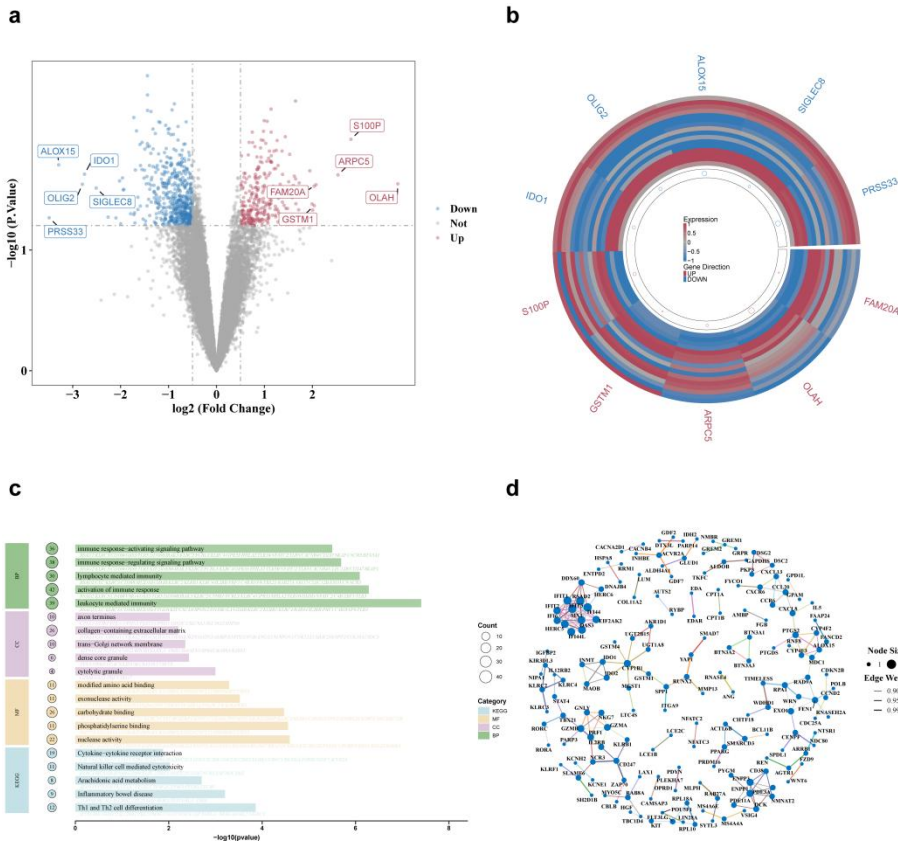


Fig 1. Functional enrichment analysis of DEGs. (a) Volcano plot of DEG distribution. (b) Heatmap of the top five upregulated and downregulated DEGs. (c) Top significantly enriched GO terms and KEGG pathways. (d) Protein-protein interaction (PPI) network of DEGs.

3.2 Screening and Identification of Three Biomarkers

Intersection of the DEGs with the phagocytosis-related genes (PRGs) yielded eight candidate genes (Fig. 2a): ARPC5, SPON2, PLD4, C4B, CD247, PIK3R6, MARCKSL1, and TREM2. In both the training and validation datasets, PLD4 and CD247 were

significantly downregulated in the TBI group, whereas TREM2 was significantly upregulated ($P < 0.05$; Fig. 2b, c). Thus, PLD4, CD247, and TREM2 were identified as biomarkers.

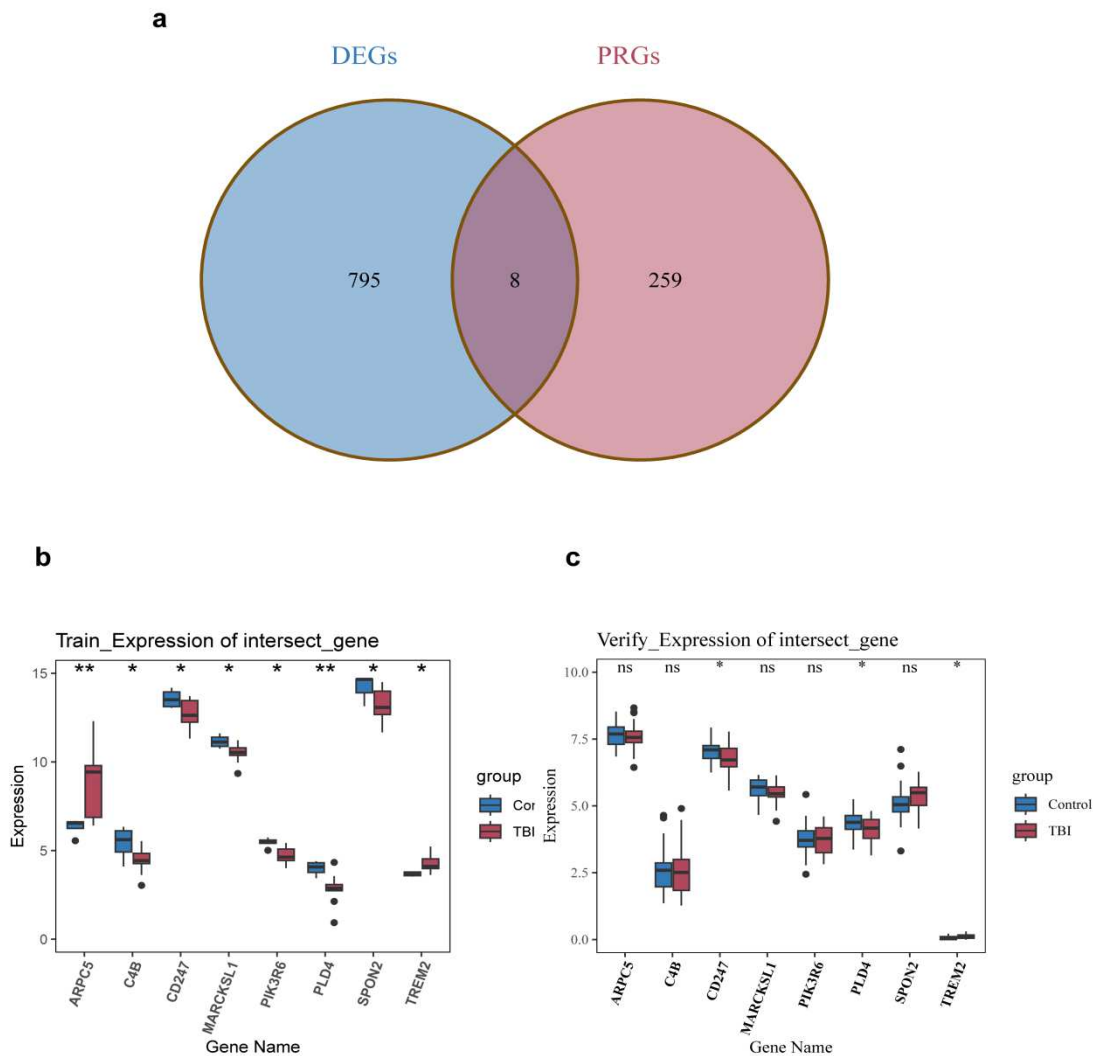


Fig 2. Identification of three biomarkers. (a) Venn diagram of the overlap between DEGs and phagocytosis-regulating genes (PRGs). (b, c) Expression of candidate genes in the (b) GSE223245 and (c) GSE89866 datasets. * $P < 0.05$, ** $P < 0.01$; ns, not significant.

3.3 Functional analysis of biomarkers

Chromosomal localization analysis indicated that CD247, TREM2, and PLD4 were mapped to chromosomes 1, 6, and 14, respectively (Fig 3a). Subcellular localization results showed that CD247 and TREM2 were primarily localized to the plasma

membrane, while PLD4 was primarily localized to the Golgi apparatus and lysosomes (Fig 3b-d). Gene-gene interaction (GGI) network analysis identified 20 interacting genes, including CD28, CD8A, LCK, ZAP70, and NFATC1 (Fig 3e). Further analysis indicated close functional interactions among the three biomarkers, including pathways such as the antigen receptor-mediated signaling pathway, receptor complex, lymphocyte differentiation, plasma membrane signaling receptor complex, and T cell selection.

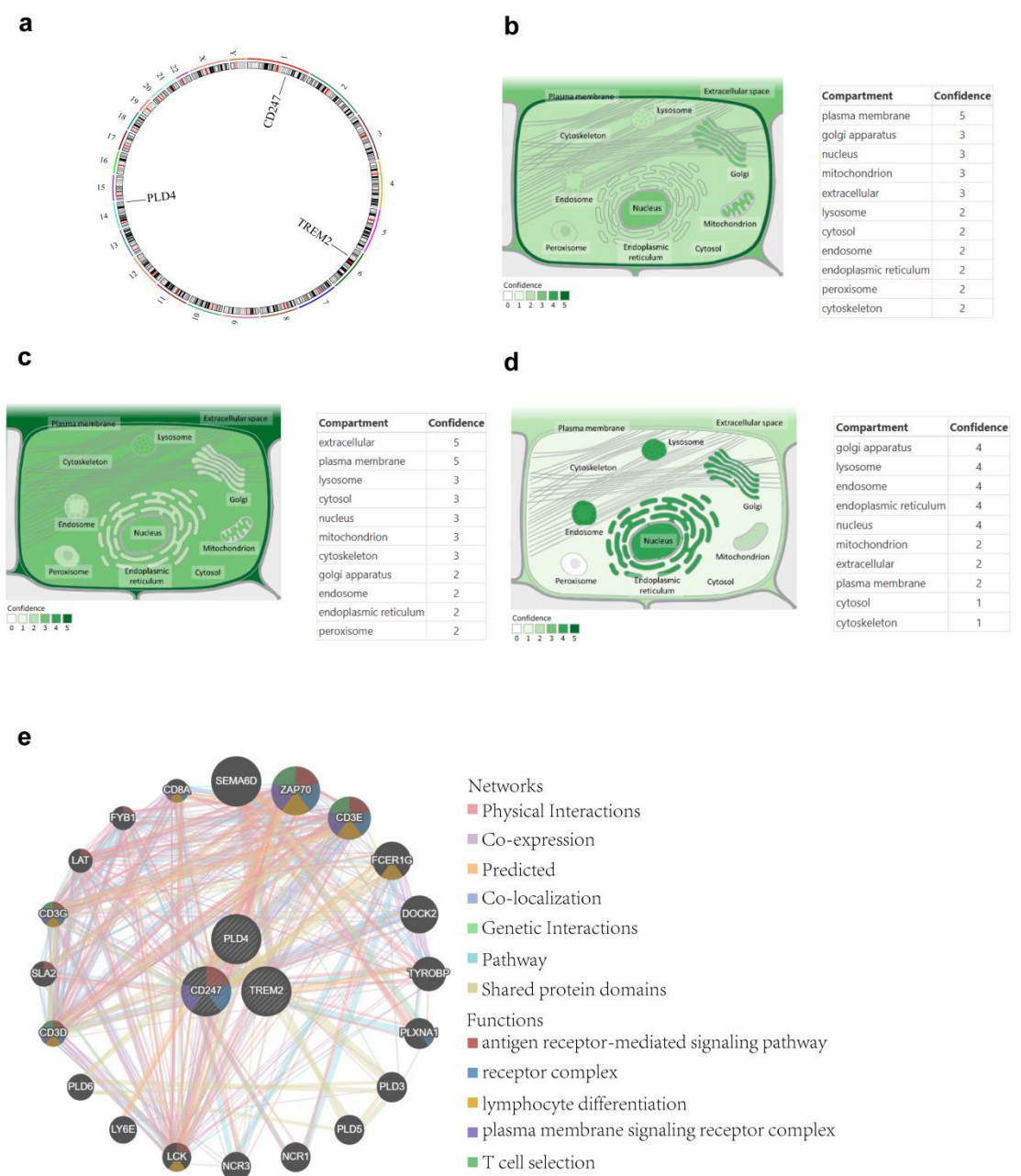


Fig 3. Functional analysis of biomarkers. (a) Chromosomal localization of biomarkers.

(b-d) Subcellular localization of biomarkers. (e) Gene-gene interaction (GGI) network of biomarkers.

3.4 Immune infiltration analysis and correlation with biomarkers

Immune infiltration analysis revealed altered infiltration patterns in TBI samples, with elevated M2 macrophages and microvascular endothelial cells (Fig 4a). Eight differential immune cells were identified (Fig 4b), including CD4⁺ effector memory T cells (CD4⁺ Tem), macrophages, macrophages M2, myocytes, NK cells, T gamma delta (TGD) cells, regulatory T cells (Tregs), and microvascular (mv) endothelial cells. Correlation analysis among these differential immune cells showed significant positive correlations between Tregs and CD4⁺ Tem ($\text{cor} = 0.90, P < 0.001$), as well as between TGD cells and NK cells ($\text{cor} = 0.88, P < 0.001$) (Fig 4c). Macrophages M2 showed significant negative correlations with CD4⁺ Tem ($\text{cor} = -0.77, P < 0.001$), TGD cells ($\text{cor} = -0.67, P < 0.001$), and Tregs ($\text{cor} = -0.63, P < 0.001$). Correlation analysis between biomarkers and differential immune cells further revealed that both CD247 and PLD4 showed positive correlations with Tregs, TGD cells, NK cells, and CD4⁺ Tem (Fig 4d), and showed negative correlations with macrophages M2 and macrophages, whereas TREM2 showed the opposite pattern (all $P < 0.05$).

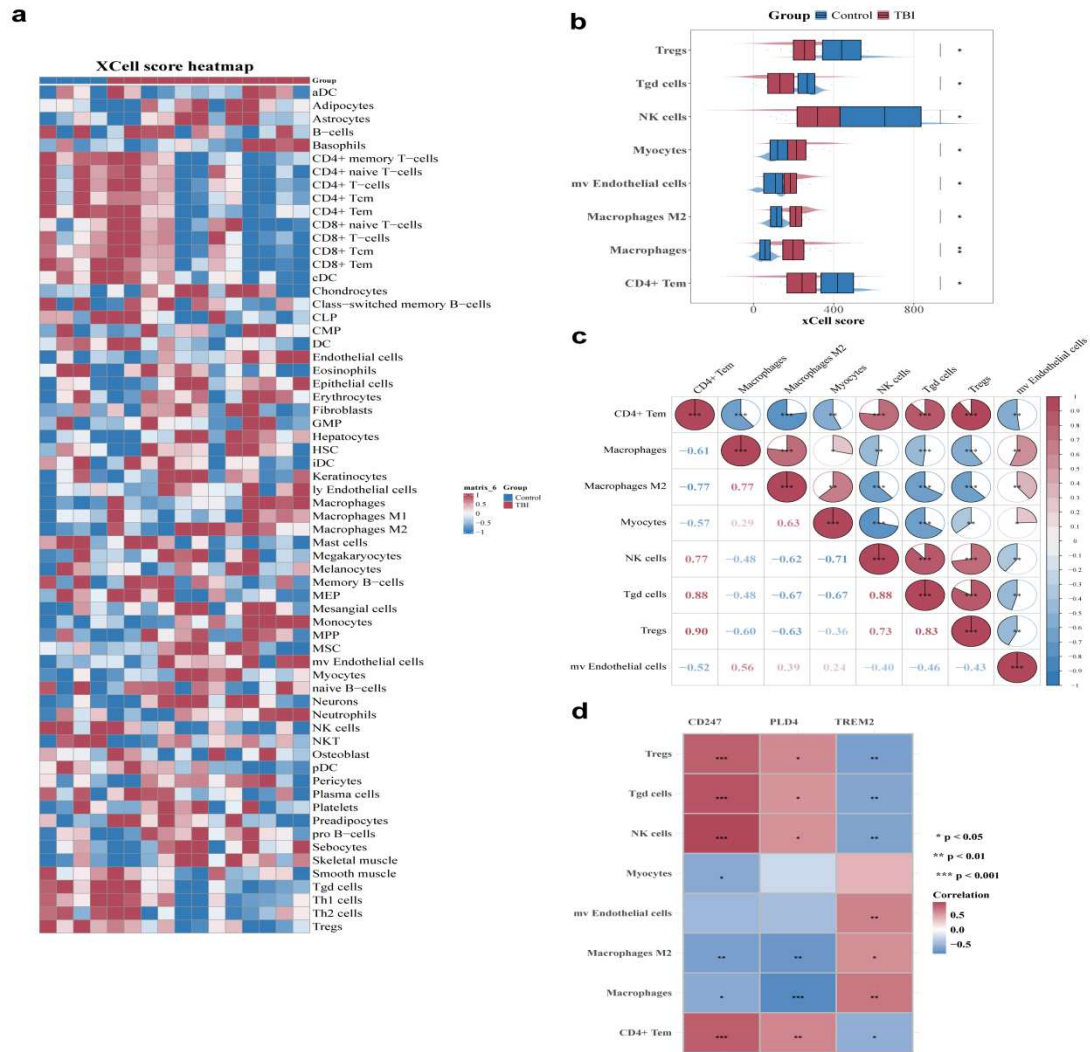


Fig 4. Immune infiltration analysis and correlation with biomarkers. (a) Heatmap of immune cell infiltration levels in the traumatic brain injury (TBI) and control groups. (b) Differential immune cells between the TBI and control groups. (c) Heatmap of correlations among different immune cells. (d) Heatmap showing the association between differential immune cells and biomarkers. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

3.5 Cell Type Identification in the GSE182398 Dataset

After quality control, 1,390 low-quality cells were removed from GSE182398, leaving 11,413 cells and 21,142 genes for downstream analysis (Fig. 5a, b). After normalization, 2,000 highly variable genes (HVGs) were identified (Fig. 5c). Principal component analysis (PCA) showed that the explained variance plateaued

after the first 30 principal components (PCs) (Fig. 5d, Fig. 6a); therefore, these 30 PCs were used for clustering. Unsupervised clustering identified 13 clusters, which were annotated as six cell types: oligodendrocytes, fibroblasts, microglia, epithelial cells, natural killer T (NKT) cells, and B cells (Fig. 6b, c; Table 1). The marker genes showed cell-type-specific expression, confirming the accuracy of the annotation (Fig. 6d).

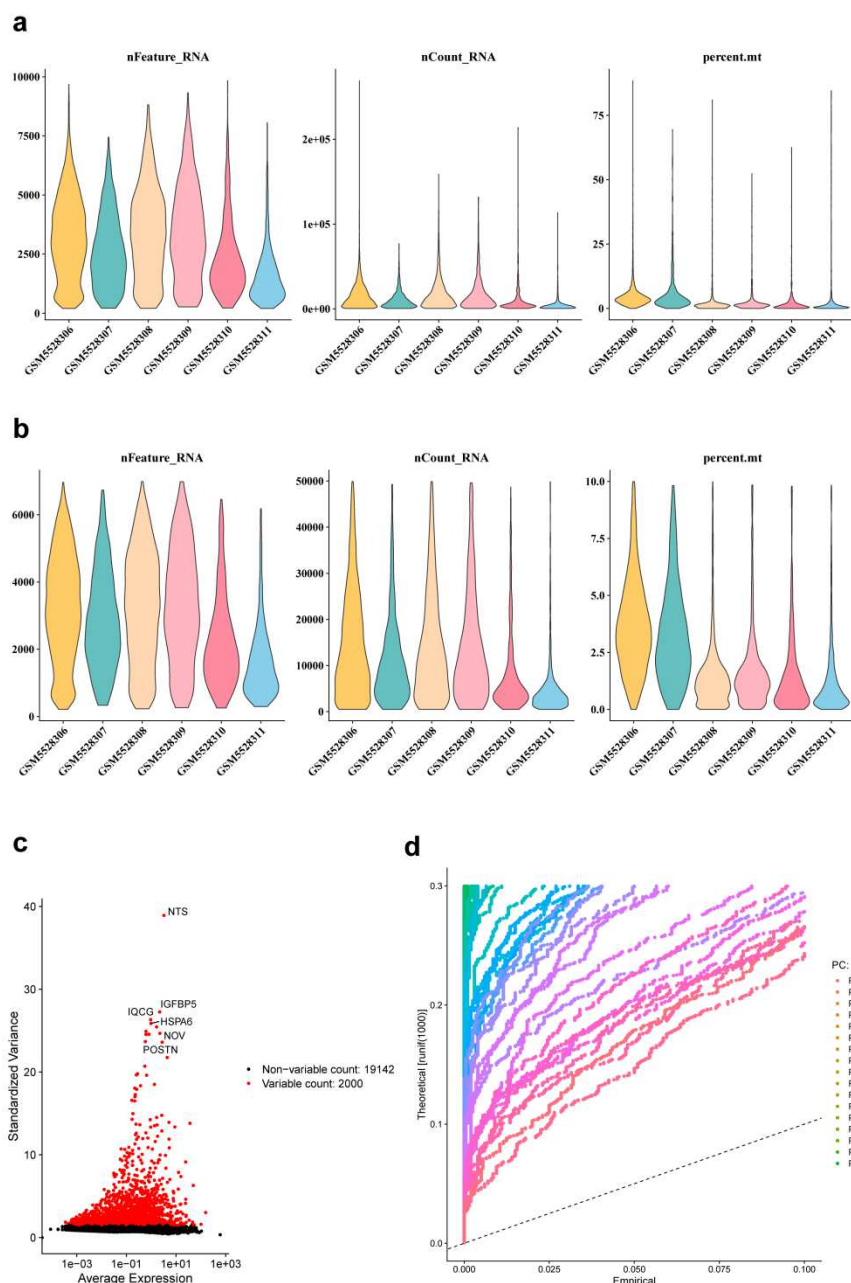


Fig 5. Cell type identification in GSE182398. (a, b) Quality control metrics before (a) and after (b) filtering: `nFeature_RNA`, `nCount_RNA`, and `percent.mt`. (c) Identification of highly variable genes (HVGs). (d) Heatmap of PC loadings (top 50

377 PCs).

378 **3.6 Identification of Key Cells**

379 CD247 and PLD4 were predominantly expressed in NKT cells (Fig.6e). Previous
380 studies have shown that, after TBI, injury-associated molecular patterns and
381 endogenous lipid antigens activate NKT cells via CD1d, triggering rapid secretion of
382 cytokines such as IFN- γ and IL-4, which in turn recruit and activate microglia and
383 infiltrating macrophages, initiating a neuroinflammatory cascade [30]. Together with
384 our finding that these biomarkers are enriched in NKT cells, this suggests that NKT
385 cells may represent a key population involved in phagocytosis regulation after TBI.
386 Therefore, NKT cells were defined as key cells.

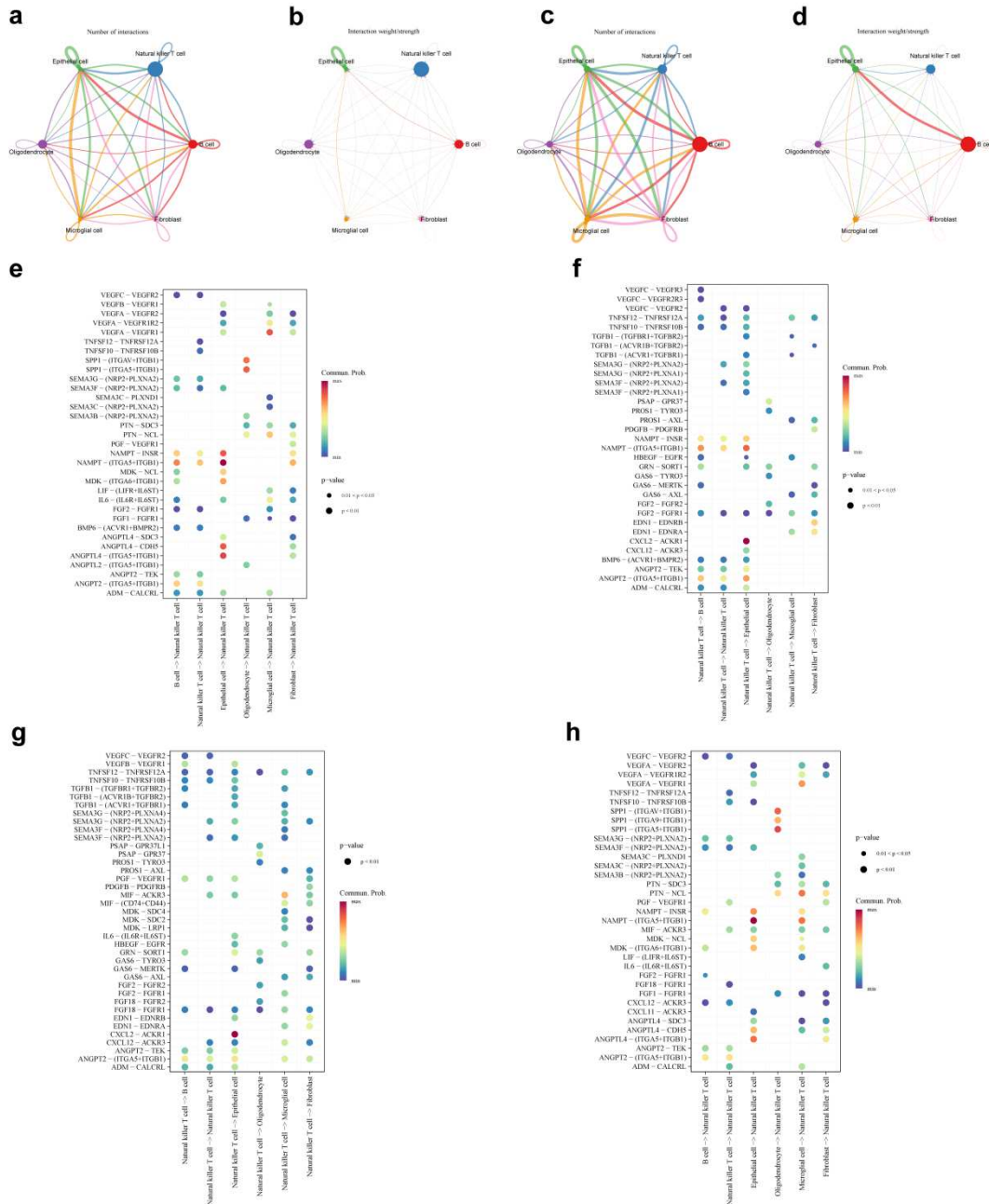


Fig 7. Cell–cell communication (CCC) analysis. (a-d) Number and interaction weights between cell types in the control (a, b) and TBI (c, d) groups. (e-h) Outgoing and incoming ligand–receptor interactions of NKT cells in the control (e, f) and TBI (g, h) groups.

3.8 Pseudotime Trajectory Analysis of NKT Cell Subpopulations

Secondary clustering of NKT cells was performed using the top 20 principal components (PCs) (Supplementary Fig. 1). UMAP clustering revealed two

subpopulations, annotated as CD247+ NKT cells and PLD4+ NKT cells according to biomarker expression (Fig. 8a, b). Pseudotime analysis showed that NKT cells progressed along a trajectory from an initial state (dark blue) to a mature state (light blue; Fig.8c). NKT cells were classified into two subtypes spanning seven developmental stages. Notably, the developmental trajectories differed between the TBI and control groups. CD247 was highly expressed in both early and late developmental stages, whereas PLD4 was predominantly expressed in early and middle stages (Fig. 8d). TREM2 was not detected in NKT cells, further supporting cell-type-specific regulation.

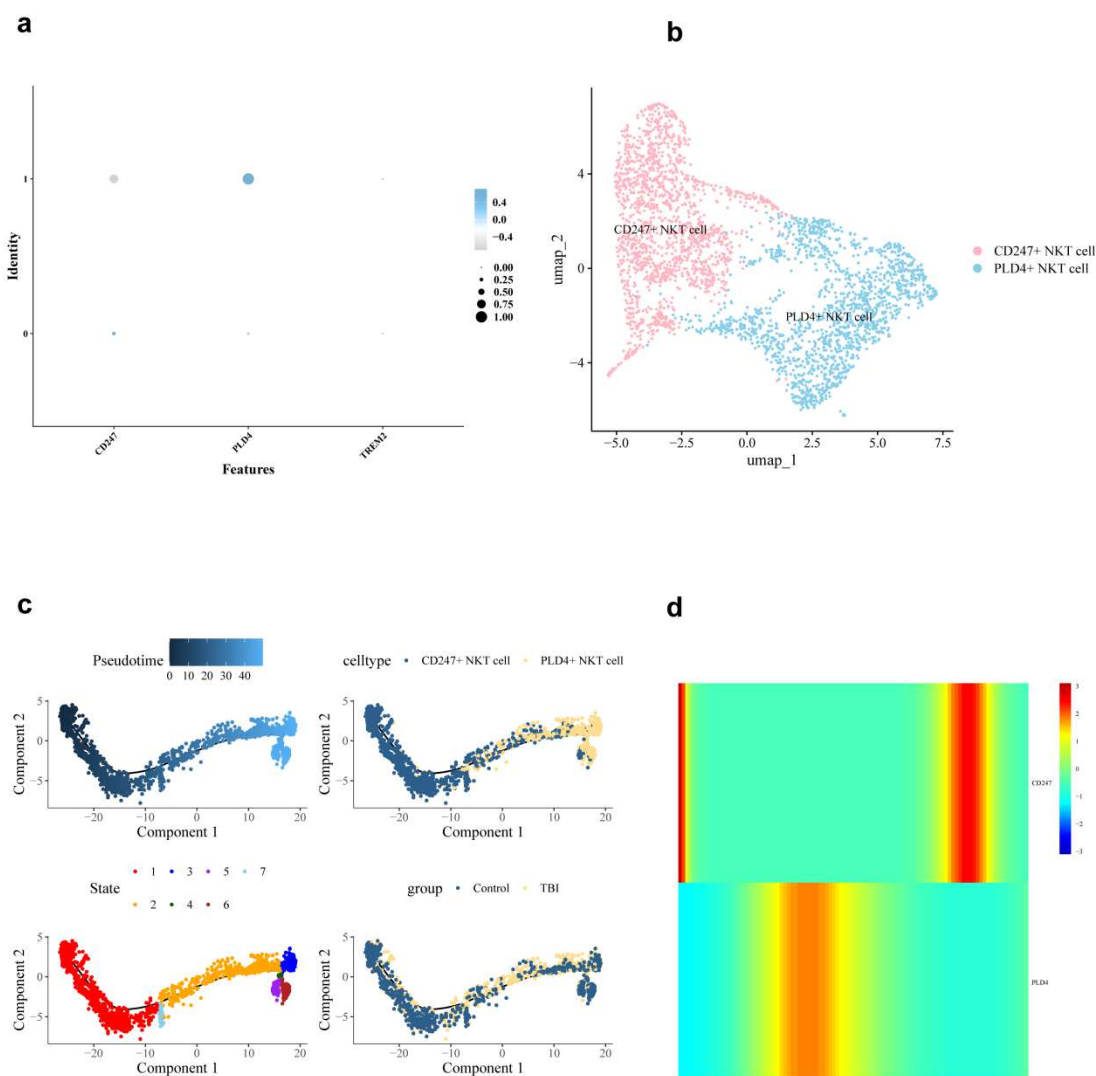


Fig 8. Pseudotime trajectory analysis. (a) Biomarker expression in NKT cells. (b) Subpopulation annotation. (c) Pseudotime trajectory. (d) Dynamic expression of CD247, PLD4, and TREM2 along the pseudotime axis.

3.9 Functional, Transcriptional, and Metabolic Characteristics of NKT Cells

Functional enrichment analysis showed that, in the TBI group, NKT cells were positively enriched for oxidative phosphorylation, epithelial–mesenchymal transition (EMT), and fatty acid metabolism pathways (Fig.9a). Conversely, they were negatively enriched for WNT– β -catenin signaling, allograft rejection, and the interferon- α response. In addition, the acetylation pathway was significantly upregulated in CD247+ NKT cells and downregulated in PLD4+ NKT cells (Fig.9b). Transcription factor (TF) activity analysis revealed group differences: MYC, HHEX, and NR2C2 showed higher activity in the TBI group, whereas TBX21, POU5F1, and PRDM14 showed lower activity (Fig.9c). Metabolic pathway analysis identified oxidative phosphorylation, fatty acid degradation, and the tricarboxylic acid (TCA) cycle as major pathways in NKT cells (Fig.9d).

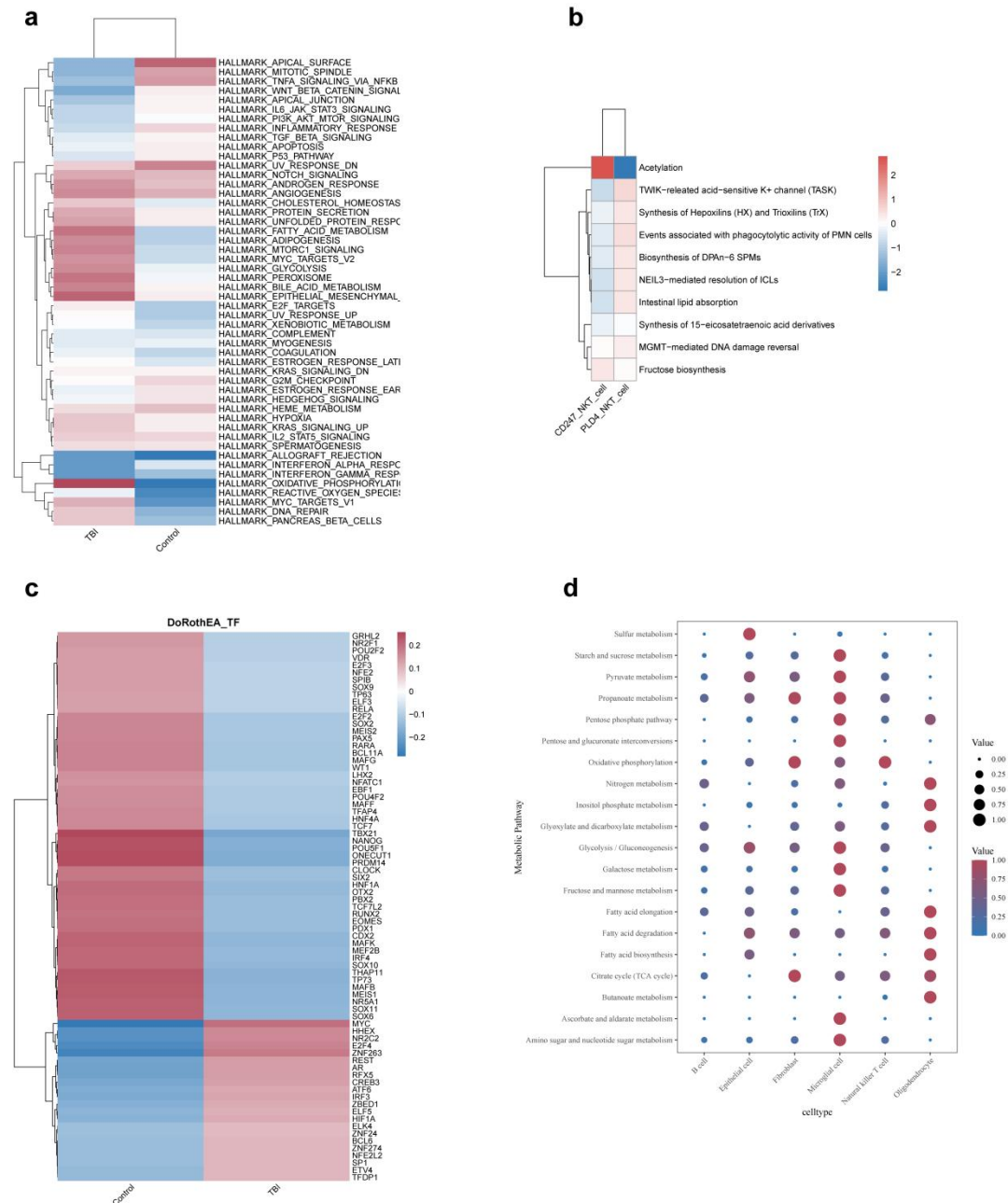


Fig 9. Functional, transcriptional, and metabolic characteristics of NKT cells. (a) GSVA enrichment scores in NKT cells. (b) Differentially enriched pathways between CD247⁺ and PLD4⁺ NKT cells. (c) Predicted transcription factor activity. (d) Metabolic pathway activity (bubble plot).

3.10 Functional Differences Between CD247⁺ and PLD4⁺ NKT Subpopulations

To further characterize functional differences between the CD247⁺ and PLD4⁺ NKT subpopulations, pathway enrichment analysis was performed (Fig.9b). CD247⁺ NKT

cells were specifically enriched in pathways related to acetylation and fructose biosynthesis, whereas PLD4⁺ NKT cells were significantly enriched in pathways associated with TASK potassium channels, HX/TrX synthesis, DPAn-6-specific pro-resolving mediator (SPM) synthesis, polymorphonuclear (PMN) phagocytic and lysosomal activity, NEIL3-dependent DNA damage repair, intestinal lipid absorption, and MGMT-mediated DNA damage repair. Notably, PLD4⁺ NKT cells were significantly enriched in phagocytosis- and lysosome-related pathways. This is consistent with the reported role of PLD4 in nucleic acid processing and suggests that this subpopulation may participate in recognizing and clearing damage-associated nucleic acid fragments, thereby contributing to inflammatory homeostasis.

3.11 Histopathological Changes and mRNA Expression of Key Genes in the TBI Mouse Model

Compared with sham-operated mice, brain tissue from the TBI group exhibited marked morphological changes (Fig.10a). In the TBI group, modified neurological severity scores (mNSS) were significantly higher in the TBI group than in sham-operated mice, confirming successful induction of a moderate-to-severe TBI model (Fig.10b). Concurrently, neurons in the cerebral cortex and hippocampus were disorganized; cell nuclei appeared condensed and hyperchromatic, and the tissue was loose and markedly edematous (Fig.10c). qPCR analysis confirmed that, relative to sham-operated mice, TREM2 mRNA was significantly upregulated in the TBI group, whereas PLD4 and CD247 were significantly downregulated (Fig. 10d–f). These results are consistent with the bioinformatics screening.

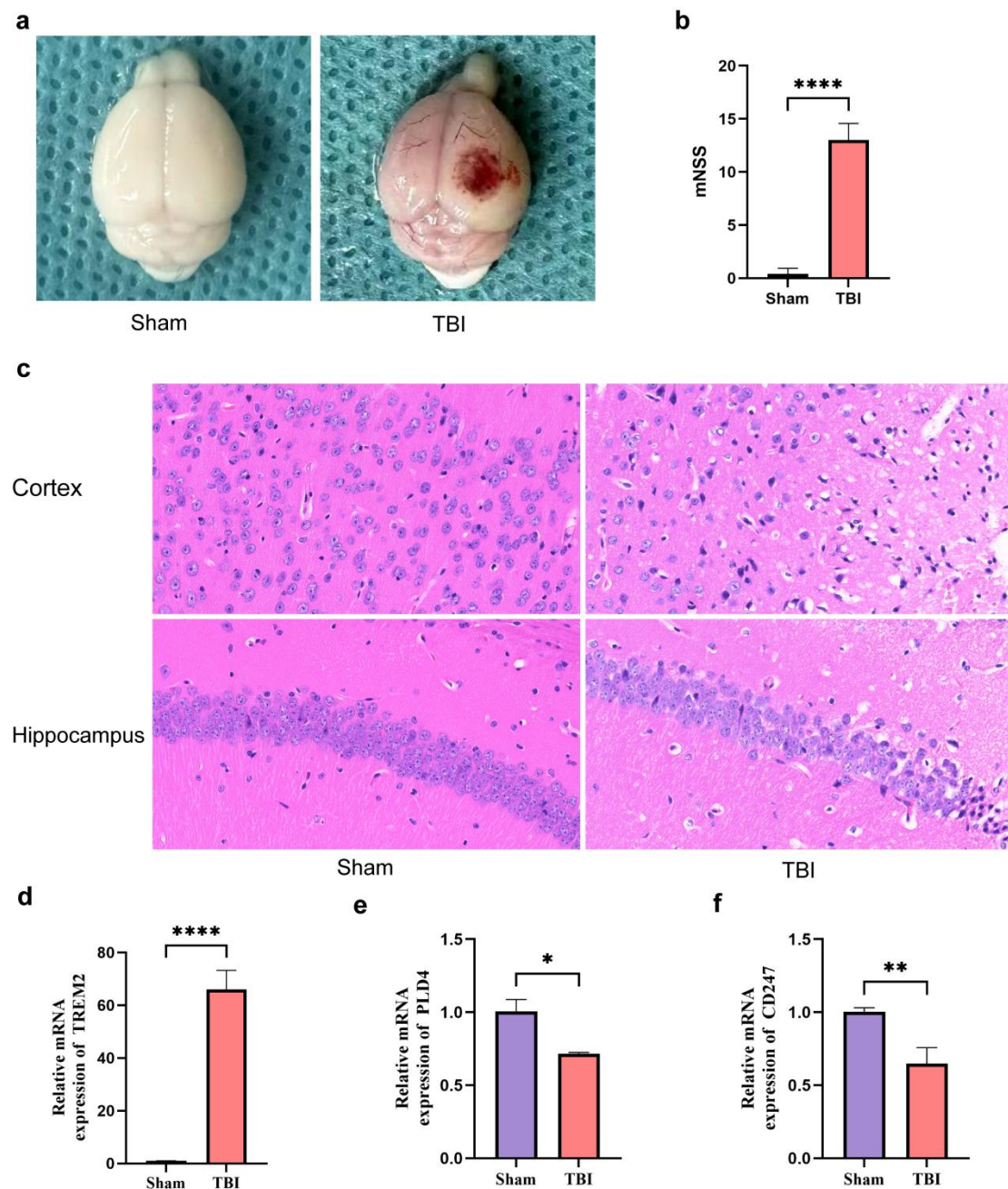


Fig 10. Histopathology and mRNA expression of key genes in the TBI mouse model. (a) Gross brain morphology of sham and TBI mice. (b) mNSS scores. (c) H&E staining of the cerebral cortex and hippocampus. (d-f) Relative mRNA expression of TREM2, CD247, and PLD4 determined by qPCR. Scale bar = 20 μ m. Data are shown as mean $\pm$ SD.(n = 3 mice per group). One-way ANOVA; *P < 0.05, **P < 0.01, ****P < 0.0001.

3.12 Apoptosis in Brain Tissue of the TBI Mouse Model

TUNEL staining revealed significantly more TUNEL-positive cells in the cerebral cortex and hippocampus of the TBI group than in sham-operated mice, indicating that TBI markedly elevates apoptosis (Fig.11a-d). Collectively, these findings demonstrate that the identified key genes are closely associated with TBI-related injury at the histopathological, molecular, and apoptotic levels, providing a foundation for evaluating their potential as diagnostic biomarkers and mechanistic targets.

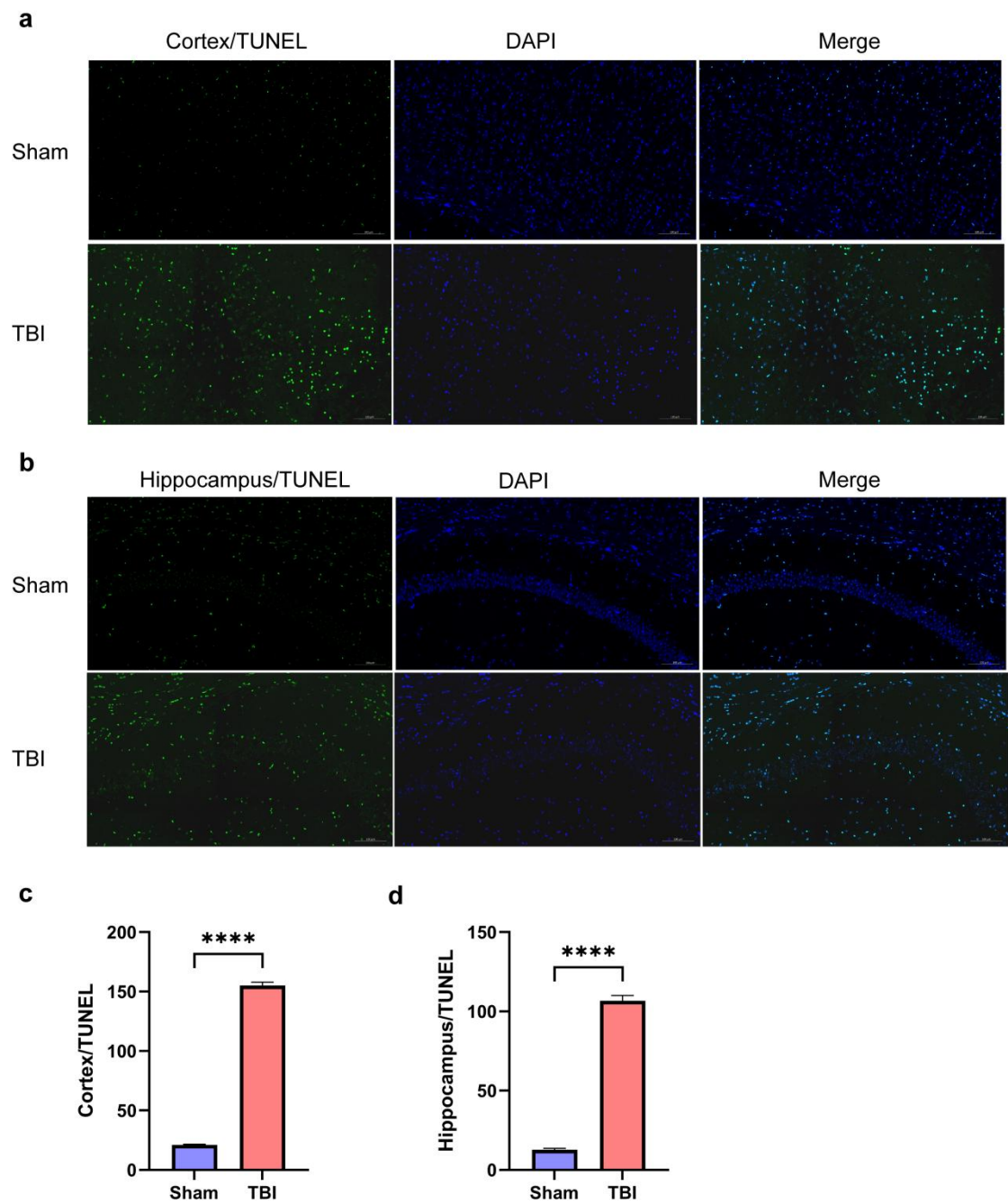


Fig 11. Apoptosis in brain tissue of the TBI mouse model.(a, b) Representative

TUNEL staining images reveal apoptotic cells within the cerebral cortex (a) and hippocampus (b) of sham-operated and TBI mice. Nuclei were counterstained with DAPI; merged images are provided. Scale bar = 100 μ m. Quantitative analysis of TUNEL-positive cells per microscopic field in the cortex (c) and hippocampus (d). Data are expressed as mean $\pm$ SD (n = 3 mice per group).

4. Discussion

TBI is a leading cause of morbidity, disability, and mortality worldwide, affecting individuals of all ages. Accumulating epidemiological evidence indicates that TBI not only causes acute neurological impairment but also represents an independent risk factor for several neurodegenerative diseases, substantially elevating the long-term risk of cognitive decline and dementia[36-38]. Secondary injury after TBI involves a cascade of complex pathological processes, including inflammatory dysregulation, immune microenvironment disruption, cell death, and aberrant tissue repair[39, 40]. Dysregulation of PRGs may serve as a key driver of this pathological cascade[41-43]. The complex pathophysiological cascades following TBI are largely driven by metabolic reprogramming of infiltrating immune cells; however, the immunometabolic status of lymphocyte subsets in neuroinflammation remains poorly understood. By integrating peripheral blood transcriptomics with single-cell data from brain parenchyma, this study employed a cross-tissue triangulation strategy to map phagocytosis-related immune dysregulation across systemic and local compartments. This approach not only confirmed the myeloid-centric action of TREM2 but also identified CD247 and the lysosomal exonuclease PLD4 as key factors. Crucially, single-cell and pseudotime analyses identified NKT cells as a previously unrecognized immunometabolic hub. Under injury conditions, these cells exhibit significant alterations in oxidative phosphorylation and fatty acid degradation and show a specific association with PLD4. Collectively, these findings broaden the understanding of TBI pathophysiology from a microglia-centric view to an adaptive immune-metabolic axis.

Among the three biomarkers, TREM2 is currently the most extensively studied. TREM2 is a membrane receptor expressed primarily on myeloid cells, including microglia, and plays a crucial role in maintaining central nervous system (CNS) homeostasis, clearing injury-related debris, and regulating inflammatory responses[44, 45]. In Alzheimer's disease, TREM2 influences microglial function and also participates in regulating inflammation and metabolism, acting either independently or synergistically with molecules such as APOE[46]. Furthermore, after mild TBI (mTBI), most activated resident microglia are TREM2-positive, and p75NTR expression is significantly elevated in these M1-polarized microglia, which is associated with increased pro-inflammatory cytokine release, demyelination, and cognitive deficits[47]. Our findings confirm that TREM2 is significantly upregulated in TBI, consistent with previous reports, and suggest that TREM2 participates in damage clearance, neural repair, and inflammatory homeostasis regulation after TBI[48], supporting its potential as an immunotherapeutic target.

Unlike TREM2, which primarily reflects myeloid/microglial responses, CD247 may serve as a molecular link between peripheral immunity and central inflammation. CD247 encodes CD3 ζ , a key signaling subunit of T-cell receptors (TCRs) and related immune receptor complexes that mediates antigen receptor signaling in T- and NK-lineage cells[49]. In autoimmune diseases such as ankylosing spondylitis and systemic lupus erythematosus, downregulation of CD247 has been associated with impaired T-cell and NK-cell function[50-52]. CD247 is essential for the normal development and function of T cells, including NKT cells. CD247 deficiency results in severe immunodeficiency, and even when TCR expression is partially restored by somatic reversion, downstream signaling is not fully recovered[53]. We found that CD247 was highly expressed in NKT cells yet significantly downregulated in the TBI group. Pseudotime analysis further revealed that CD247 maintains high expression during both early and late stages of NKT cell development, suggesting it participates in the entire transition from the initial to the effector state and contributes to functional regulation. Given the enriched expression of CD247 in NKT cells and the

emerging link between NKT cells and post-TBI immune remodeling, CD247 represents a promising diagnostic biomarker and may guide immune interventions aimed at restoring NKT cell function.

PLD4, a member of the phospholipase D family, is a glycosylated type II transmembrane protein that localizes primarily to lysosomes[54, 55]. PLD4 is highly expressed in dendritic cells, monocytes, and B cells and is thought to be closely involved in endolysosomal nucleic acid processing[56]. PLD4 plays a key role in immune regulation. It is upregulated in activated microglia, where it contributes to phagocytic function, and has also been implicated in neonatal cerebellar development and the suppression of renal fibrosis[57, 58]. PLD4 single-nucleotide polymorphisms have been associated with several immune-related diseases, including rheumatoid arthritis, systemic lupus erythematosus, and systemic sclerosis[59-61]. PLD4 has also been proposed as a potential biomarker for plasmacytoid dendritic cell tumors[20]. We found that PLD4 was significantly downregulated in both peripheral blood from TBI patients and brain tissue from TBI mice. Single-cell analysis further revealed that PLD4 is predominantly expressed in NKT cells, primarily during the early and middle stages of development. Functional analysis of subpopulations showed that PLD4+ NKT cells were significantly enriched in pathways related to DNA damage repair, synthesis of pro-resolving lipid mediators, and lipid uptake. Taken together, these findings indicate that PLD4 may participate in phagocytosis regulation and maintenance of inflammatory homeostasis after TBI. To our knowledge, this is the first study to report PLD4 as a candidate biomarker in TBI, thereby expanding its potential biological relevance in central nervous system injury.

In summary, by integrating phagocytosis-related gene (PRG) analyses, single-cell transcriptomics, and experimental validation, we identified three TBI-associated biomarkers—TREM2, CD247, and PLD4. Our results suggest that NKT cells are the primary cell population expressing CD247 and PLD4, whereas TREM2 predominantly reflects myeloid/microglial responses. Further analysis revealed marked metabolic reprogramming and functional differentiation of NKT cells after

TBI, with PLD4 emerging as a novel candidate warranting further investigation. Animal experiments corroborated the aberrant expression of these genes in TBI. Collectively, this study provides new theoretical insights and potential avenues for the diagnosis, prognosis, and targeted intervention of TBI. However, several limitations should be acknowledged. First, the single-cell dataset had a limited sample size, which may have restricted a comprehensive assessment of NKT subpopulation stability, heterogeneity, and intercellular communication networks. Therefore, these findings require validation in a larger human TBI single-cell cohort. Second, the bulk transcriptomic data originated from peripheral blood, while the single-cell data were derived from brain tissue; caution is warranted when integrating results from the two tissue types. Third, current validation has focused primarily on mRNA expression changes in a mouse model, and functional intervention studies targeting the identified genes remain to be performed. Future investigations should include paired brain and peripheral blood samples from TBI patients and employ techniques such as flow cytometry, immunofluorescence, gene knockdown, and overexpression to comprehensively characterize the spatiotemporal expression and functional mechanisms of CD247, PLD4, and TREM2 across disease stages, thereby accelerating clinical translation.

5. Conclusion

These findings provide new insights into the immunometabolic mechanisms of TBI and establish a theoretical foundation for developing intervention strategies that target NKT cells or PLD4. Given PLD4's enzymatic activity, our findings may open a new avenue for developing small-molecule modulators to fine-tune the phagocytic-inflammatory balance after TBI.

Supplementary data

Supplementary Materials:

Supplementary Fig 1: Secondary clustering analysis of NKT cells. (a) Contribution of the first 50 principal components (PCs) to the variance. (b) PC elbow plot. (c) Cell cluster analysis.

Supplementary Table 1: List of phagocytosis regulator-related genes (PRGs).

Supplementary Table 2: List of differentially expressed genes (DEGs).

Supplementary Table 3: List of enriched Gene Ontology (GO) terms.

Supplementary Table 4: List of Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment pathways.

Supplementary Table 5: Primer sequences for quantitative real-time PCR.

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Statements & Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Hongling Wen, Rui Zhang, Fang Zhou, Xiaodi Tan, Shengjiao Lan, Longjiu Zhang and Jiangquan Fu. The first draft of the manuscript was written by Hongling Wen and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data availability

All data used in this study are derived from publicly available databases (GEO: GSE223245, GSE89866, GSE182398; MSigDB). No new raw sequencing or microarray data were generated. The processed data and analysis scripts are available from the corresponding author upon reasonable request. Data will be made available on request.

Ethics Approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Guizhou Medical University (Guizhou 2,602,001, China).

Abbreviations

Abbreviation	Full term
ANOVA	Analysis of variance
BP	Biological process
CC	Cellular component

CCC	Cell–cell communication
DEG	Differentially expressed gene
FC	Fold change
GEO	Gene Expression Omnibus
GO	Gene Ontology
H&E	Hematoxylin and eosin
HVG	Highly variable gene
KEGG	Kyoto Encyclopedia of Genes and Genomes
mNSS	Modified neurological severity score
NKT	Natural killer T
PCA	Principal component analysis
PLD4	Phospholipase D family member 4
PR	Phagocytosis regulator
PRG	Phagocytosis-related gene
qPCR	Quantitative real-time polymerase chain reaction
scRNA-seq	Single-cell RNA sequencing
TBI	Traumatic brain injury
TCA	Tricarboxylic acid
TF	Transcription factor

TREM2	Triggering receptor expressed on myeloid cells 2
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick-end labeling
UMAP	Uniform manifold approximation and projection
UMI	Unique molecular identifier

786

787 **Table 1:** Cell types and their corresponding marker genes.

Cluster	Cell_name	Marker_gene
5/8	Oligodendrocyte	PLP1, MOG, MOBP, MBP
12	Fibroblast	PDGFRB, MYL9
9	Microglial cell	GYPC, KCNMA1
3	Epithelial celll	ADGRG6, ACKR1
1/2/11	Natural killer T cell	COMMD6, ARHGDIB, PLCG2
0/4/6/7/10	B cell	SNHG8, SWAP70

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