

LAYN Serves as a Prognostic Biomarker and Downregulates Tumor-Infiltrating CD8⁺ T Cell Function in Hepatocellular Carcinoma

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Background: Layilin (LAYN) represents a valuable prognostic biomarker across various tumor types, while also serving as an innovative indicator of dysfunctional or exhausted CD8⁺ T cells and exhibiting correlation with immune context. However, the immune function and prognostic significance of LAYN in hepatocellular carcinoma (HCC) remain unexplored. Therefore, our objective is to investigate the role of LAYN in CD8⁺ T cell exhaustion, clinical prognosis, and the tumor microenvironment within HCC.

Methods: TIMER or GEPIA databases were used to analyze LAYN expression level and its correlation with immune infiltration in HCC. Bioinformatics analysis was conducted on TCGA and scRNA-seq cohorts. The evaluation of LAYN expression level in fresh specimens was performed through IF, IHC, and ELISA assays. Flow cytometry and mRNA-seq were employed to investigate co-expressed genes of LAYN, the LAYN⁺CD8⁺ T cell exhaustion signature and immune function. Cell proliferation ability and killing activity were assessed using CCK8 and CFSE/PI.

Results: The expression level of LAYN in HCC tumors was significantly higher compared to peri-tumors. Patients with high levels of LAYN exhibited poorer OS. GO or KEGG analysis confirmed that LAYN was involved in immune response and was positively associated with CD8⁺ T cell immune infiltration levels. Furthermore, LAYN negatively regulated the immune function of CD8⁺ T cells, leading to dysfunctional phenotypes characterized by elevated levels of CD39, TIM3 and reduced levels of perforin, TNF- α , Ki-67. CFSE/PI assays demonstrated that LAYN⁺CD8⁺ T cells displayed decreased cytotoxic activity. Additionally, there was a positive correlation between LAYN and CD146 levels, which are involved in adhesion and localization processes of CD8⁺ T cells. Interestingly, blocking LAYN partially restored the exhaustion properties of CD8⁺ T cells.

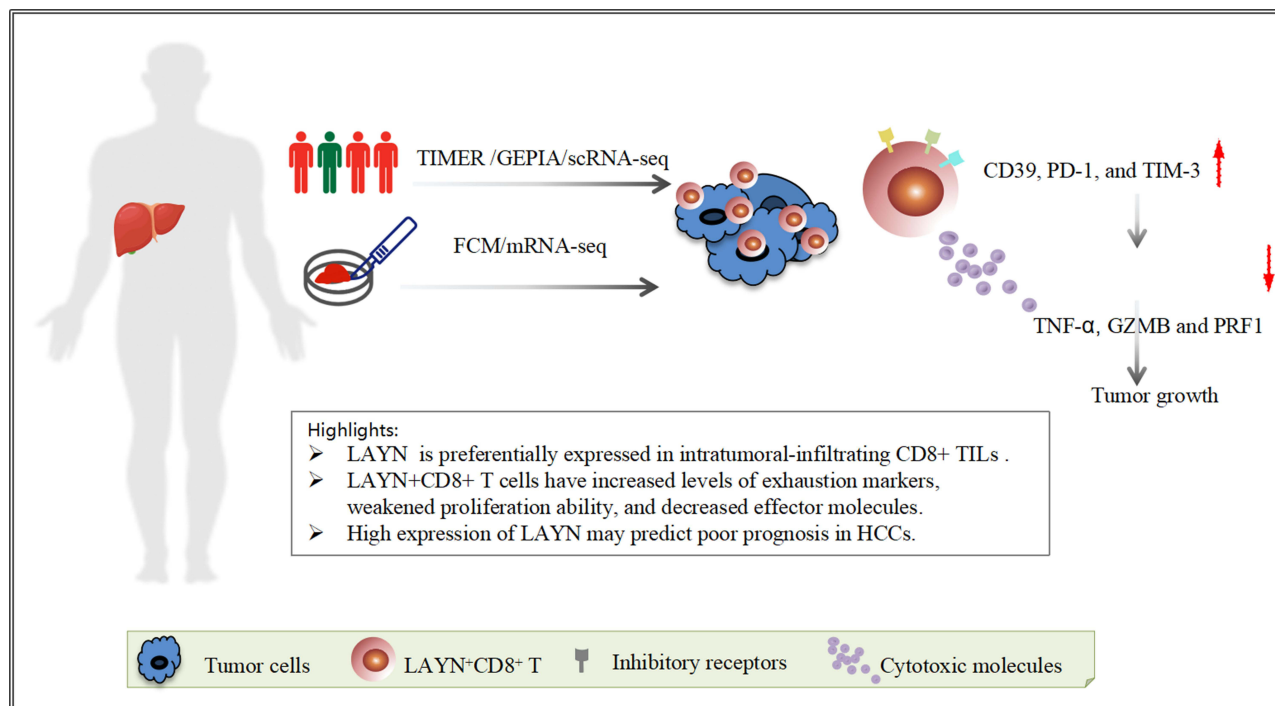
Conclusion: LAYN exhibits a strong correlation with immune infiltration in the TME and represents a novel biomarker for predicting clinical prognosis in HCC. Moreover, targeting LAYN may hold promise as an effective strategy for HCC immunotherapy.

Keywords: hepatocellular carcinoma, layilin, CD8⁺ T cell exhaustion, prognosis

Introduction

Liver cancer is currently one of the cancers with high incidence rates, ranking as the fifth most malignant cancer in China and having the second highest mortality rate (0.91) worldwide.^{1,2} Annually, there are approximately 854,000 new cases and 695,000 deaths, with 85–90% of them being individuals diagnosed with HCCs.³ Malignancy typically develops in patients suffering from chronic liver disease caused by hepatitis B virus (HBV) or hepatitis C virus (HCV) infection or metabolic diseases.^{4,5} Until recently, treatment options for HCC include liver transplantation, surgical resection, and

Graphical Abstract



molecular targeted therapy.⁶ Unfortunately, available therapeutic approaches for HCC were limited and ineffective resulting in a recurrence and metastasis rate of 70% within five years.³

Cancer immunotherapy has significantly revolutionized the prospects for oncology treatment in the past decade.⁷ The recent success of immune checkpoint inhibitors (ICIs) like pembrolizumab and nivolumab, which obstruct the programmed cell death 1 (PD1)-PD-L1 pathway, has stimulated research on immune therapy in HCC.⁸ This indicates a shift in focus from solely targeting the tumor to considering the tumor microenvironment (TME). However, it is worth noting that the objective response rate (ORR) of ICI monotherapy among HCC patients remains at only 15–20% and combination therapy at about 30%, underscoring an urgent need to explore novel and potent strategies for HCC immunotherapy.⁸

Cytotoxic CD8⁺ T cells are highly enriched in the tumor microenvironment (TME) and play a crucial role in antitumor immunity. However, persistent antigen stimulation and an immunosuppressive microenvironment can lead to effector CD8⁺ T cell dysfunction, known as T cell exhaustion.^{9–11} The hallmarks of exhausted CD8⁺ T cells are as follows: (a) gradual loss of TNF- α and IFN- γ ;¹² (b) sustained overexpression of multiple inhibitory receptors (IRs) (PD1, cytotoxic T lymphocyte-associated antigen-4 [CTLA-4], T-cell immunoglobulin domain and mucin domain-containing protein 3 [TIM3], CD39, T-cell immunoreceptor with Ig and ITIM domains [TIGIT], lymphocyte activation gene 3 [LAG-3]);^{13,14} and (c) unique gene expression profiles (low expression of T-bet and high expression of TOX).^{12,14,15} Consistently, increased expression levels of IRs including CD39, PD1 and TIM3 have been observed on infiltrating CD8⁺ T cells in HCC.^{16–19} Nevertheless, the functional significance and clinical implications associated with IR expression on HCC-infiltrating CD8⁺ T cells remain largely unexplored.

Layilin (LAYN) is a 55-kDa transmembrane protein harboring a C-type lectin domain. Previous studies have indicated that LAYN plays a crucial role in cancer cell invasion and can serve as a prognostic marker for assessing clinical outcomes.^{20–23} In patients with high expression levels of LAYN, such as those with Colorectal Cancer (CRC) or Non-Small Cell Lung Carcinoma (NSCLC), the overall survival (OS), progression-free survival (PFS), disease-free survival (DFS), and disease-specific survival (DSS) rates are significantly poorer.²² Previous studies had found that

LAYN was a key gene involved in tumor-infiltrating lymphocytes.^{22,24} Single-cell RNA sequencing of T cells reported that LAYN was up-regulated in intratumoral-infiltrating CD8⁺ T and Treg cells and inhibited the function of CD8⁺ T cells in vitro.⁷

However, inconsistencies have been observed in the specific impacts of LAYN on immune cells. A report has suggested that LAYN may enhance the antitumor effects of CD8⁺ T cells through activation of the integrin signaling pathway.²⁴ Furthermore, the clinical significance of LAYN in HCC and its correlation with tumor-infiltrating CD8⁺ T cell function in immune hot tumor remain largely unclear.

Here, we identified LAYN as a potential novel prognostic biomarker for predicting poor prognosis in HCC. Our findings demonstrated that LAYN exhibited high expression levels specifically in HCC specimens with immune hot tumor tissues, highly on infiltrating CD8⁺ T cells. Moreover, CD8⁺ T cells overexpressing LAYN exhibited characteristic signs of exhaustion and diminished antitumor effects. However, treatment with a LAYN antagonist partially restored the exhausted phenotypes and immune function of CD8⁺ T cells. Collectively, these results highlight the therapeutic potential of targeting LAYN to recover the antitumor immune response mediated by CD8⁺ T cells in HCC.

Materials and Methods

Patients and Samples Collection

Fresh tumor samples, peri-tumor tissues, and peripheral blood were collected from patients diagnosed with HCC. Primary tumors were obtained from 15 patients who underwent curative resection without prior anticancer therapy for immunofluorescence and flow cytometry analysis. This study was approved by the Research Ethics Committee of Zhongshan Hospital, and written informed consents were obtained from every participant. An external validation cohort was also included, consisting of patients from TCGA cohort (n=368). Patient characteristics of TCGA data set were obtained from UCSC Xena (<https://xenabrowser.net/datapages/>) in January 2022. We use the average of LAYN and CD8A as the LAYN⁺CD8⁺ T cell score. A total of 368 patients with HCC were feasible for the statistical analysis with gene expression, clinical data and follow-up information.

Flow Cytometry

Flow cytometry data were acquired via a BD FACS CantoTM II and were analyzed via Flowjo V.10.0 software (Tree Star). And the antibodies information was in the [Supplementary Materials](#) and [Methods](#).

Bioinformatics Analysis

The Tumor Immune Estimation Resource (TIMER) algorithm database (<https://cistrome.shinyapps.io/timer/>) and the Gene Expression Profiling Interactive Analysis (GEPIA) database (<http://gepia2.cancer-pku.cn>) were used to estimate LAYN gene expression levels in multiple cancer types. Then, the correlations between LAYN and tumor-infiltrating CD8⁺ T cells, as well as other functional immune cells like CD4⁺ T cells, Macrophages, and Dendritic cells in HCC were analyzed using the TIMER database. The relationships between LAYN expression and immune checkpoint receptors such as CD39, PD1 and TIM3, were also analyzed. In the TCGA cohort, transcriptomic and clinical data of HCCs were downloaded from UCSC Xena (<https://xenabrowser.net/datapages/>). The normalized expression of LAYN and other relevant genes were presented in the form of $[\log_2(\text{TPM} + 1)]$.

Single-Cell Data Analysis

Single-cell raw data were obtained from the Gene Expression Omnibus (GEO) database,⁷ containing data from 5 HCC patients generated with the smart-seq2 and 10X Genomics platforms. These data sets were integrated using Seurat's CCA to remove potential batch effects. And, the normalized expression of LAYN and other related genes were presented in the form of $[\log_2(\text{TPM} + 1)]$.⁷ Data represent five individual patients, with 5 points showing the mean data per group.

Statistical Analysis

Statistical analysis was performed with GraphPad Prism 8.0 and data was presented as mean \pm SEM. The correlation analysis was determined through unpaired *t*-test (Pearson's test). Kaplan-Meier survival curves were compared between different groups and P values were determined using Log rank test. All the statistical tests were two-tailed and performed at a significant level of 5% ($*p < 0.05$), 1% ($**p < 0.01$) or 1‰ ($***P < 0.001$). ns, no significance.

Results

LAYN is Highly Expressed in Tumor Tissues of HCC Patients

To preliminarily assess the involvement of LAYN in cancer, we conducted a comprehensive analysis of its expression at the transcriptional level in HCC, breast invasive carcinoma (BRCA), cholangiocarcinoma (CHOL), and other tumor tissues, as well as their corresponding adjacent tissues. Utilizing the TIMER database for public database analysis, we observed upregulation of LAYN across various human cancers, including HCC (Figure 1A). Furthermore, confirmation of significantly elevated mRNA expression levels of LAYN in HCC tumor tissues was achieved through GEPIA database analysis and TCGA RNA-sequencing data examination, respectively (Figure 1B and C). Subsequently, the real-time PCR analysis (Figure 1D) along with IHC (Figure 1E) and ELISA measurements (Figure S1A) confirmed higher transcriptional expression levels and protein abundance of LAYN in HCC tumor tissues compared to peri-tissues, thereby suggesting its potential role in the development and progression of HCC.

High Expression of LAYN May Predict Poor Prognosis in HCC Patients

Next, we investigated the potential clinical significance of LAYN expression in predicting prognosis for patients with HCC. The Kaplan-Meier curves demonstrated a negative correlation between LAYN expression level and patient prognosis among nonmetastatic HCC cases in the TCGA cohort. Patients with higher levels of LAYN expression exhibited significantly worse OS ($P = 0.0473$) (Figure 2A), DFS ($P = 0.0371$) (Figure 2B), and DSS ($P = 0.0436$) (Figure 2C) rates compared to those with low expression levels. Moreover, patients with a higher frequency of LAYN⁺CD8⁺ T cells displayed poorer OS rates as well ($P = 0.037$) (Figure 2E). No significant differences were observed in PFS between the low and high LAYN groups (Figure 2D and F). Additionally, there was a significantly higher level of LAYN expression in female patients compared to male patients, while no association was found between LAYN expression level and TNM stage classification (Figure 2G and H). These findings suggest that LAYN may serve as an independent predictive factor for unfavorable clinical outcomes among HCC patients.

LAYN is Highly Expressed in CD8⁺ TILs, and the Population of LAYN⁺CD8⁺ TILs is Highly Abundant Within HCC Tumor Tissues

Cancer cells are infiltrated by abundant noncancer cells, including a large number of immune cells that are recruited in the TME of immune hot tumor and exert significant influences on cancer progression and survival.²⁵ Subsequently, we utilized the TIMER database to investigate the correlation between LAYN expression and immune cell infiltration in the TME of HCC. As depicted in Figure 3A and B, LAYN expression was significantly associated with tumor purity ($R = -0.302$, $P < 0.001$) as well as infiltration of CD8⁺ T cells ($R = 0.482$, $P < 0.001$), and were also closely associated with other cell types such as CD4⁺ T cells ($R = 0.379$, $P < 0.001$), dendritic cells ($R = 0.567$, $P < 0.001$), and macrophages ($R = 0.435$, $P < 0.001$) within HCC tissues (Figures 3A and S1B). Furthermore, LAYN expression was significantly correlated with multiple cellular marker genes representing different types of cells including macrophages, neutrophils, Th1, Th2, Th17 and Treg cells (Table 1). Immunofluorescence results demonstrated colocalization of LAYN and CD8⁺ T cells within HCC tissues while showing higher infiltration levels of LAYN⁺CD8⁺ T cells in tumor tissues compared to peritumoral tissues (Figure 3C). To determine whether LAYN is expressed in tumor-infiltrating CD8⁺ TILs, mRNA-seq (Figure 3D) and flow cytometric quantification (Figure 3E and F) were performed on CD8⁺ T cells isolated from peripheral blood samples as well as primary tumors and peritumoral tissues obtained from HCC patients. The findings indicated that LAYN was highly expressed within CD8⁺ TILs derived from tumor tissues while being nearly absent from resting stage peripheral blood samples. Notably, LAYN expression was highly upregulated on activated CD8⁺ T cells

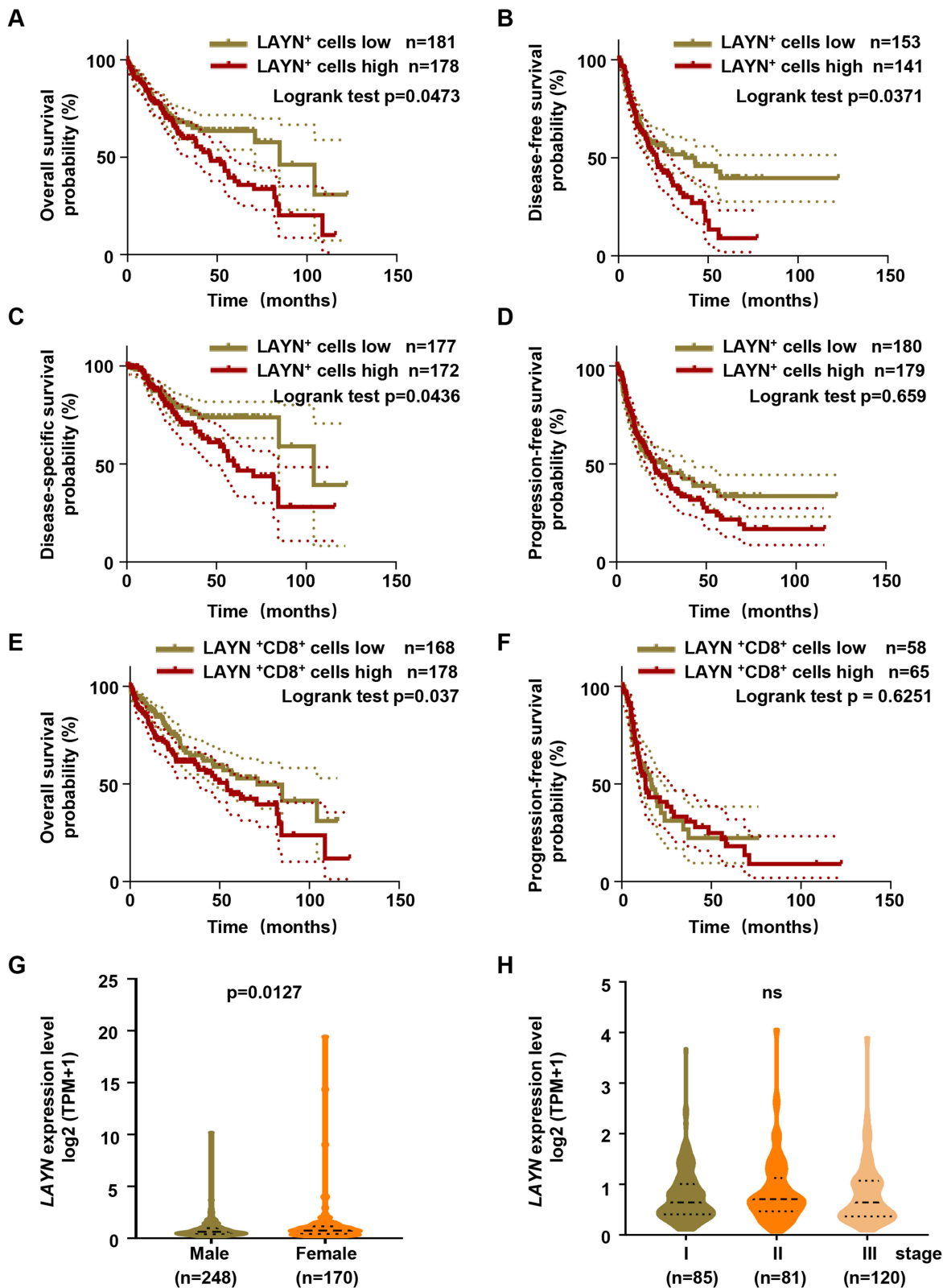


Figure 2 The prognostic value analysis of LAYN in HCC patients using the TCGA database. (A–D) Kaplan-Meier curves of overall survival (OS), disease-free survival (DFS), Disease-specific survival (DSS) and Progression-free survival (PFS) based on LAYN expression in TCGA cohort. (E and F) Kaplan-Meier curves of OS and PFS based on LAYN⁺CD8⁺ T cell frequency. (G and H) The violin chart displayed the association between LAYN expression and gender (G), or the correlation of LAYN expression with TNM stage (H). Log rank tests was performed for Kaplan-Meier curves and Log-rank p value were shown (A–F). Student's t-test (G and H) was performed, and the data were presented as the mean ± SEM. ns, not significant.

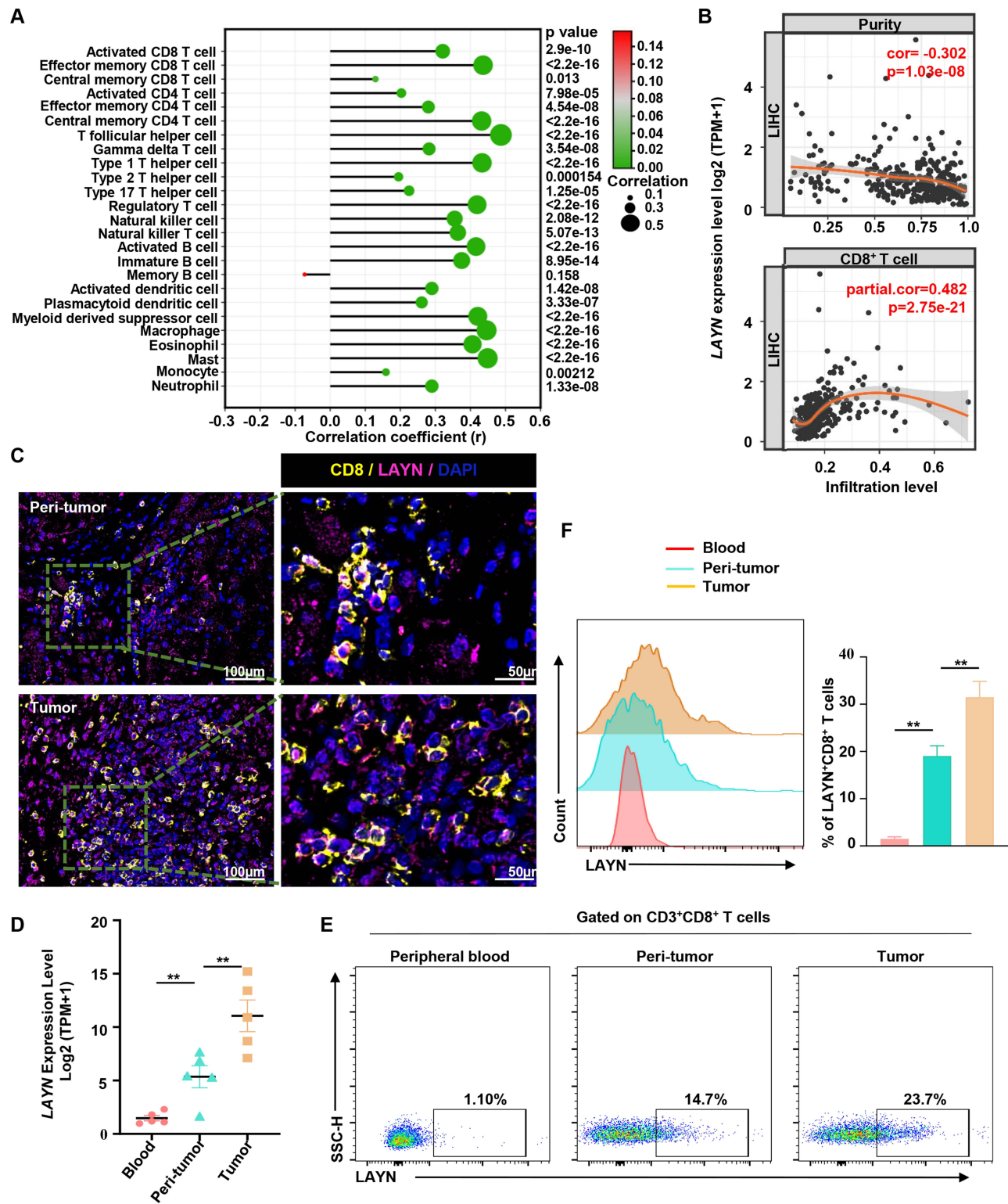


Figure 3 LAYN is highly expressed in CD8⁺ TILs and intratumoral LAYN⁺CD8⁺ T cells accumulate in HCC tumor tissues. (A and B) Correlation analysis between LAYN expression and immune cells including CD8⁺ T cells in HCCs using the TIMER database. (C) Representative images demonstrating double-stained LAYN⁺CD8⁺ T cells in HCC tumor tissue and peri-tumor tissue, captured at magnifications of 200× and 400×. (D) mRNA expression of LAYN in peripheral blood, peri-tumor and tumor tissues. Data represent five individual patients. (E and F) Representative flow cytometric images of the proportion/median fluorescence intensity (MFI) level of LAYN⁺CD8⁺ T cells in peripheral blood, peri-tumor and tumor tissues (n=8). Cells were pregated on CD45, CD3 and CD8. Student's t-test (D and F) was performed, and the data were presented as the mean ± SEM. Results were replicated (n = 3 experiments) (C–F). **P < 0.01.

Table I Correlation Analysis Between LAYN and Markers of Immune Cells in HCC via the TIMER Database

Description	Gene Markers	HCC			
		None		Purity	
		Cor	P	Cor	P
T cell exhaustion	PD1	0.424	***	0.360	***
	CTLA4	0.382	***	0.305	***
	LAG3	0.253	***	0.206	**
	TIM-3	0.534	***	0.479	***
	GZMB	0.379	***	0.303	***
Treg	FOXP3	0.271	***	0.219	***
	CCR8	0.448	***	0.397	***
	TGFB1	0.541	***	0.467	***
	IL10	0.439	***	0.354	***
CD8⁺ T cell	CD8A	0.42	***	0.35	***
	CD8B	0.37	***	0.29	***
TAM	CCL2	0.502	***	0.408	***
	CD68	0.368	***	0.282	***
Monocyte	CD86	0.55	***	0.495	***
	CD115	0.523	***	0.449	***
	CCR7	0.448	***	0.350	***
Neutrophils	CD66b	-0.007	0.899	-0.036	0.509
	CD11b	0.396	***	0.319	***
	CD19	0.341	***	0.255	***
B cell	CD79A	0.395	***	0.306	***
	STAT4	0.323	***	0.270	***
Th1	IFNG	0.296	***	0.221	***
	TNF	0.379	***	0.283	***
	STAT6	0.244	***	0.255	***
Th2	IL13	0.098	0.58	0.062	0.254
	STAT3	0.368	***	0.338	***
Th17	IL17A	0.122	0.019	0.113	0.036

Notes: Correlation analysis between LAYN and markers of immune cells in HCC, together with the Spearman's rho value and estimated statistical significance. *** $P < 0.01$; **** $P < 0.001$.

LAYN⁺CD8⁺ TILs and LAYN⁻CD8⁺ TILs isolated from HCC tumor tissues. The transcriptome-level standardized data revealed that LAYN⁺CD8⁺ TILs expressed higher levels of IRs such as *ENTPD1*, *PDCD-1*, and *HAVCR2* (identified as exhaustion markers)^{26–28} than LAYN⁻CD8⁺ TILs (Figure 4A). Next, we employed flow cytometry to comprehensively characterize these two subgroups by examining differentiation (CD) surface markers, IRs, adhesion molecules, chemokines, and cytokines. The differentially expressed markers between LAYN⁺ and LAYN⁻CD8⁺ TILs are visually represented in the heatmap (Figure 4B), and several representative markers are depicted as overlay histograms (Figure 4C–F) along with their corresponding cell frequency statistical charts (Figure S2A).

LAYN⁺CD8⁺ TILs within HCC tumor tissues exhibited elevated protein levels of the well-known IRs, including CD39, PD1, TIM3, and TIGIT compared to LAYN⁻CD8⁺ TILs (Figure 4B and C). Furthermore, the analysis revealed a significant increase in the activation markers CD137 and CD25 in LAYN⁺CD8⁺ TILs while other costimulatory markers such as CD38 and CD69 showed no significant differences between the two subsets (Figure S2B and Figure 4D), indicating an unbalanced activation status of these T cells. Additionally, LAYN⁺CD8⁺ TILs demonstrated high expression levels of cell adhesion and tissue positioning markers including CD106, CD146, and CD54,^{29,30} suggesting potential involvement of LAYN in T cell adhesion and migration processes (Figure 4E and Figure S2C). Moreover, CXCR6 expression was found to be lower in LAYN⁺CD8⁺ TILs (Figure 4F). Furthermore, in LAYN⁺CD8⁺ TILs, the frequency

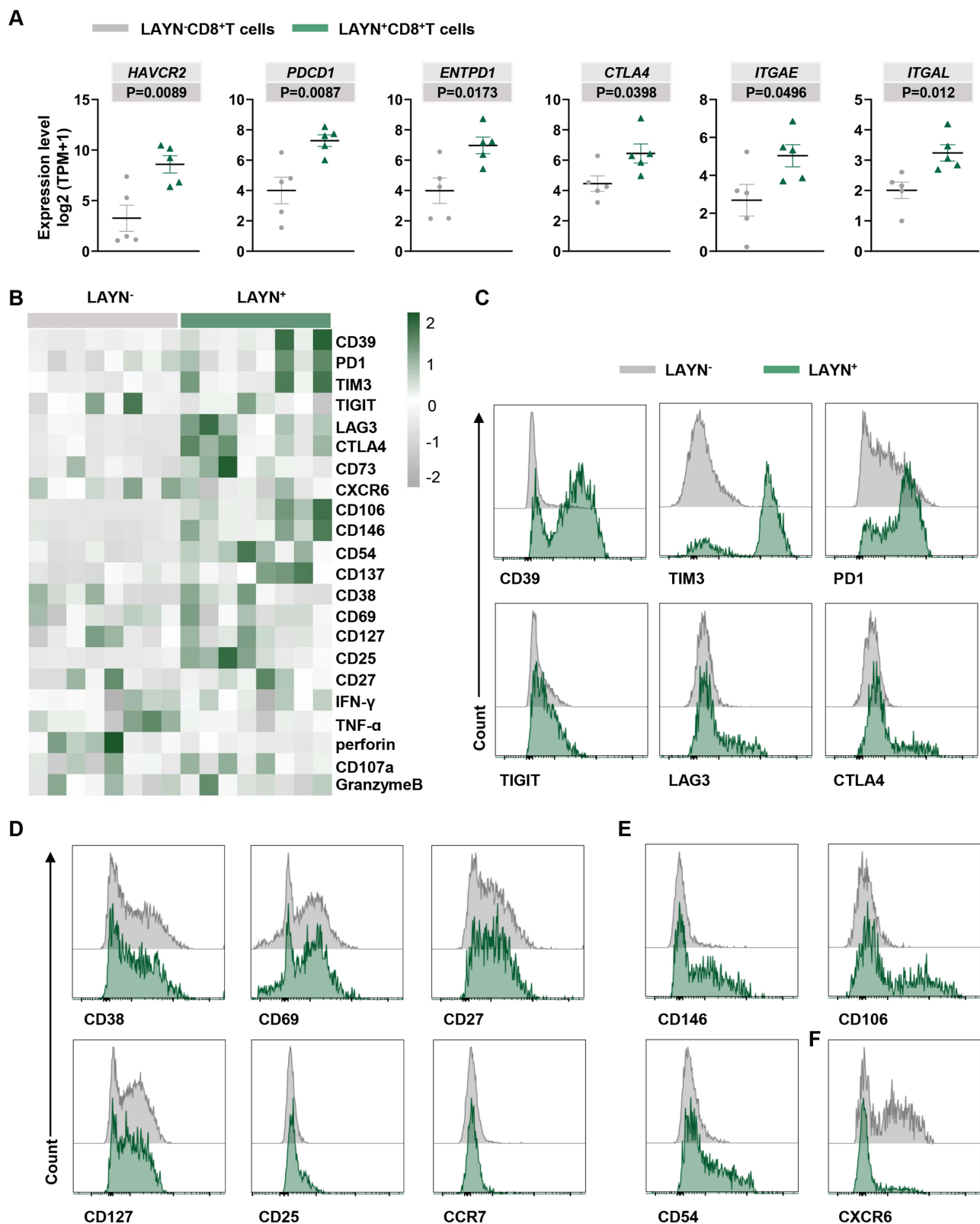


Figure 4 Dysfunctional phenotypic characteristics of tumor infiltrating LAYN⁺CD8⁺ T cells in HCC. **(A)** The scatter plots illustrated the gene expression of LAYN-related genes in freshly isolated LAYN⁺CD8⁺ T cells and LAYN⁻CD8⁺ T cells from HCC tumor tissues. Data represent five individual patients. **(B)** A heatmap to show the global phenotypic characteristics of LAYN⁺CD8⁺ T cells and LAYN⁻CD8⁺ T cells from HCC tissues detected by flow cytometry. Cells were pre-gated on CD45, CD3 and CD8. Data represent eight individual patients. **(C–F)** Representative flow cytometric overlays of different markers expressed by LAYN⁺ (green line) and LAYN⁻ (grey line) CD8⁺ T cells, including exhaustion associated immune checkpoint inhibitors **(C)**, differentiation and activation markers **(D)**, adhesion and localization related molecules **(E and F)**. Student's *t*-test **(A)** was performed, and the data were presented as the mean ± SEM. Results were replicated (*n* = 3 experiments) **(B–F)**.

of PD1⁺TIM3⁻CD39⁻CD8⁺ TILs identified as stem-like T cells^{31,32} was significantly lower while the proportion of PD1⁺TIM3⁺CD39⁺CD8⁺ TILs identified as terminally differentiated cells (TDEs)³¹ was higher (Figure S2D). In addition, the proportion of LAYN⁺CD8⁺ TILs was positively correlated with the proportions of CD39⁺CD8⁺ T cells, CTLA-4⁺CD8⁺ T cells, CD73⁺CD8⁺ T cells, CD146⁺CD8⁺ T cells and CD106⁺CD8⁺ T cells (Figure S4A and B). These findings collectively suggest that LAYN⁺CD8⁺ TILs represent a specific subset exhibiting exhaustion features similar to classical exhausted cell population.

Genes Enriched in LAYN⁺CD8⁺ T Cells are Implicated in the Immune Response Process as Well as the Signaling Molecules and Interaction Pathway Within the TME

To further investigate LAYN⁺CD8⁺ T cells, we performed mRNA-seq analysis and compared their transcriptional profile with that of non-exhausted cells like naïve CD8⁺ T cells (CD8-LEF1) and effector memory CD8⁺ T cells (CD8-CX3CR1). Unsupervised hierarchical clustering analysis showed that LAYN⁺CD8⁺ T cells were preferentially enriched for genes associated with exhaustion (HAVCR2, PDCD1, ENTPD1, TOX, MYO7A, TIGIT and CTLA4) (Figure S3A). Furthermore, gene ontology (GO) analysis revealed that these differentially expressed genes were enriched in the GO_biological process (BP) terms including inflammatory response, immune response and negative regulation of gene expression, GO_cellular component (CC) terms including plasma membrane and integral component of membrane, and GO_molecular function (MF) terms protein binding (Figure 5A and Figure S3B and C). The top 9 GO term-associated genes are shown in Figure 5B with LAYN being preferentially involved in the integral component of the membrane. Besides, the Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis indicated that highly expressed genes in LAYN⁺CD8⁺ T cells were enriched in the cytokine-cytokine receptor interaction and cell adhesion molecules pathways (Figure 5C and Figure S3D).

LAYN⁺CD8⁺ TILs Exhibit Diminished Capacity for Proinflammatory Cytokine Production in HCC

Next, we investigated the cytokine-producing capacity of CD8⁺ TILs based on the LAYN expression. Initially, mRNA-seq analysis was performed on CD8⁺ TILs isolated from fresh HCC tumor tissues. Our findings revealed that LAYN⁺CD8⁺ TILs expressed fewer cytotoxic molecules, including *GZMB* and *PRF1*. However, there was no significant difference in the expression levels of effective molecules (*IFNG*, *TNFA*) compared with LAYN⁻CD8⁺ TILs (Figure 6A). Subsequently, flow cytometry was applied to assess protein-level production of these effective molecules. The data obtained demonstrated that LAYN⁺CD8⁺ TILs displayed defective production of TNF- α to some extent but had IFN- γ expression levels comparable to those observed in LAYN⁻CD8⁺ TILs (Figure 6B and C). Furthermore, a negative correlation between the proportion of LAYN⁺CD8⁺ T cells and TNF- α ⁺CD8⁺ T cells (Figure S4C).

Additionally, flow cytometry-based measurements were conducted to evaluate the killing capacity of LAYN⁺CD8⁺ TILs. Notably, we found a considerable decrease in the frequency of perforin-producing cells among LAYN⁺CD8⁺ TILs; however, the frequency of granzyme B-producing or CD107a-producing cells remained comparable between these cell populations (Figure 6D and E).

To gain further insights into the role of LAYN in CD8⁺ T cells, PBMC-derived CD8⁺ T cells were sorted with a purity of 95.8% using microbead (Figure S5A), followed by retroviral-mediated overexpression of LAYN aimed at mimicking high levels seen in tumor-infiltrating CD8⁺ T cells. After retroviral infection, the upregulation of LAYN on CD8⁺ T cells was quantified and validated using qPCR and flow cytometry on day 5 (Fig. S5B and C). Firstly, assessment of Ki-67 or CCK8 proliferation revealed that LAYN^{OE} T cells exhibited a relatively diminished proliferative capacity (Figure 6F and Figure S5D). Additionally, LAYN-overexpressing CD8⁺ T cells expressed more IRs, including CD39, TIM3, and CTLA-4, than control virus-infected cells (Figure S5E), consistent with the findings presented in Figure 4. Furthermore, we evaluated the cytotoxicity of LAYN^{OE} T cells by analyzing CFSE/PI staining results which demonstrated that 97H cell lines co-cultured with control virus-infected cells were more susceptible to apoptosis than those co-cultured with LAYN^{OE} T cells at different effector-to-target (E/T) ratios

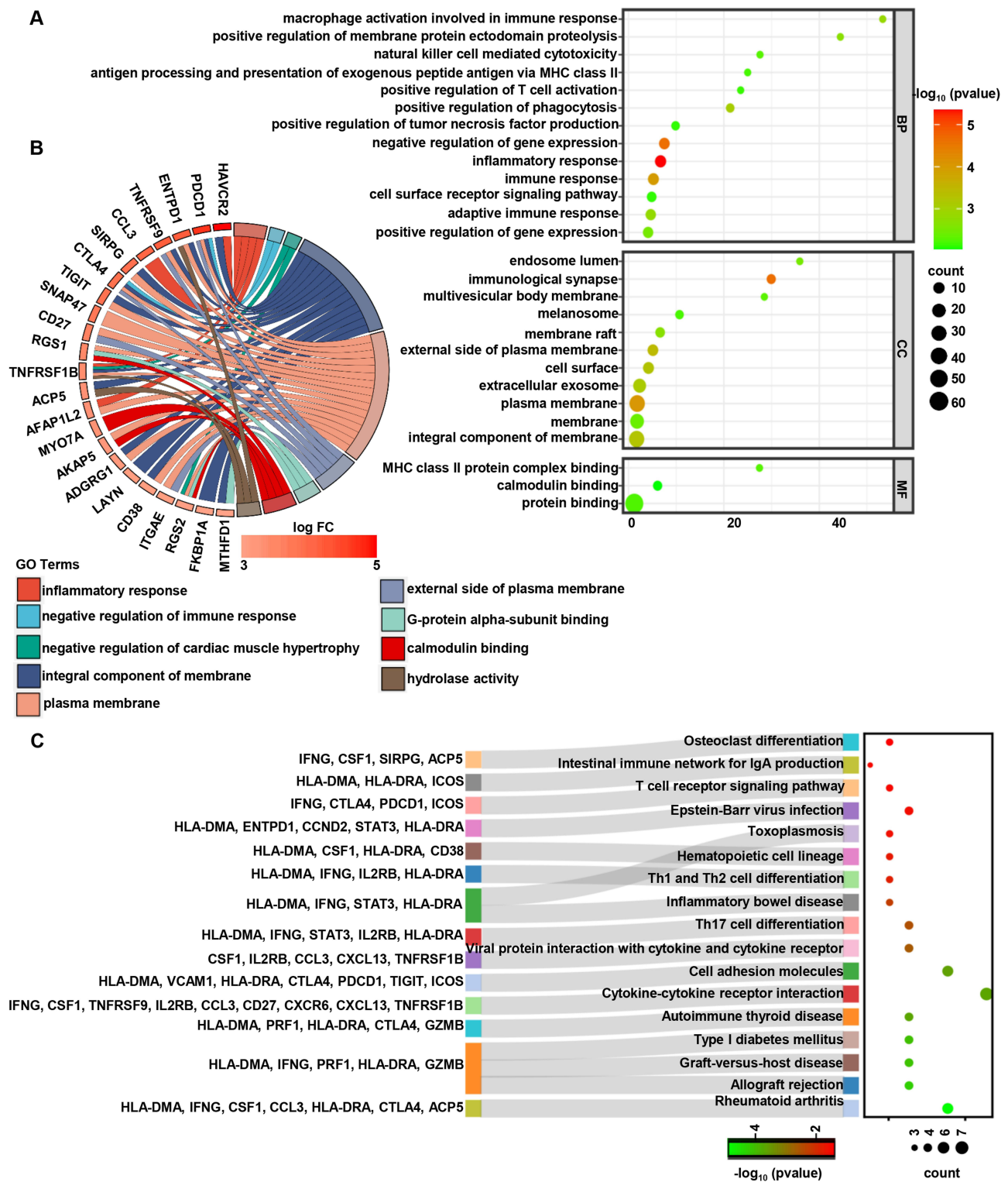


Figure 5 The highly expressed genes in LAYN⁺ CD8⁺ T cells have a specific enrichment pathway. **(A)** GO function analysis based on differentially up-regulated genes within LAYN⁺ CD8⁺ T cells detected by mRNA-seq compared to non-exhausted cells. **(B)** Up-regulated genes within LAYN⁺ CD8⁺ T cells enriched in the top 9 GO terms. **(C)** Enriched pathways within the Kyoto Encyclopedia of Genes and Genomes database based on differentially up-regulated genes within LAYN⁺ CD8⁺ T cells. The p value was set to < 0.01 **(A)**. The p value was set to < 0.05 **(C)**.

Abbreviations: GO, Gene Ontology; BP, biological process; CC, cell component; MF, molecular function; KEGG, Kyoto Encyclopedia of Genes and Genomes.

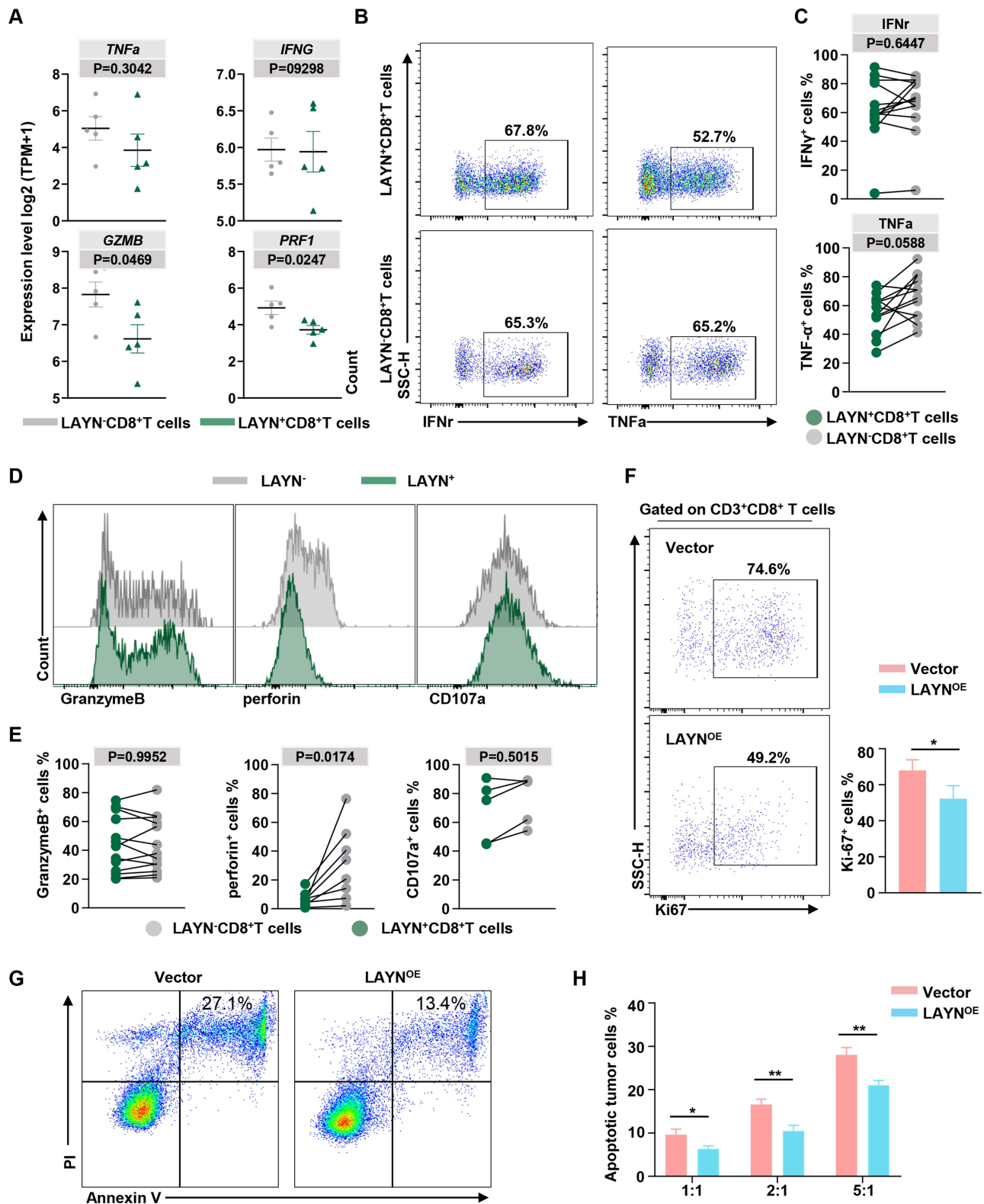


Figure 6 Assessment of anti-inflammatory cytokines profiling and cytotoxic potential of tumor infiltrating LAYN⁺ CD8⁺ T cells. **(A)** The scatter plots displayed the gene expression of cytokines and cytotoxic molecules in freshly isolated LAYN⁺ CD8⁺ T cells and LAYN⁻ CD8⁺ T cells from HCC tumor tissues. Data represent five individual patients. **(B and C)** Representative flow cytometric plots **(B)** and statistical results **(C)** to show the pro-inflammatory cytokines IFN- γ and TNF- α secreting profile of intra-tumoral LAYN⁺ and LAYN⁻ CD8⁺ T cells following the stimulation of PMA, ionomycin and Brefeldin for 6 h ($n \geq 8$). **(D and E)** Representative flow cytometric overlays **(D)** and statistical data **(E)** of intracellular Granzyme B, CD107a and perforin expression in freshly isolated LAYN⁺ and LAYN⁻ CD8⁺ T cells from HCC tumor tissues ($n \geq 5$). **(F)** Flow cytometry analysis of proliferation molecules (Ki-67) expression in LAYN^{OE}-CD8⁺ T cells and control virus-infected cells. **(G and H)** The apoptosis assay (using CFSE/PI staining) of 97H tumor cell line after co-culture with LAYN^{OE}- CD8⁺ T cells or control virus-infected cells at different **(E)**T ratio of 1:1, 2:1 or 5:1 for 20h ($n = 4$). Student's *t*-test **(A, C, E, F and H)** was performed, and the data were presented as the mean \pm SEM. Results were replicated ($n = 3$ experiments) **(B-H)**. * $P < 0.05$; ** $P < 0.01$.

(Figure 6G and H). In summary, these results indicate that the subset of CD8⁺ TILs characterized by high expression of LAYN exhibits impaired ability to produce proinflammatory cytokines and exert antitumor activity.

The Abundance of Intratumoral LAYN⁺CD8⁺ TIL is Associated with Impaired Infiltrating CD8⁺ T Cell Functionality with HCC, and Targeting LAYN Can Partially Restore the Functions of CD8⁺ T Cells

The phenotypic characteristics and immune function of infiltrating LAYN⁺CD8⁺ TILs have been discussed above. However, it is important to note that LAYN⁺CD8⁺ TILs represent only a specific population and may not fully reflect the overall immune status. Given the crucial role of CD8⁺ T cells in antitumor immunity, we aimed to investigate whether CD8⁺ T cell function was inhibited within the HCC microenvironment. Therefore, we examined the overall features of CD8⁺ T cells based on their infiltration level with LAYN expression.

Interestingly, there was no significant difference in the abundance of total CD8⁺ T cells between the low infiltration group and high infiltration group (Figure 7A). However, elevated expression levels of immune checkpoint molecules such as CD39 and LAG-3 were observed on CD8⁺ T cells within the LAYN^{high}CD8⁺ T cell group (Figure 7B). Additionally, CD8⁺ T cells in the LAYN^{high}CD8⁺ T cell group had lower levels of effective molecules like TNF- α (Figure 7C). Despite increased exhaustion markers and decreased activation molecule TNF- α levels in these CD8⁺ T cells, proportions of IFN- γ , granzyme B, and perforin within the total CD8⁺ T cell population were not significantly different between the LAYN^{high}CD8⁺ T and LAYN^{low}CD8⁺ T cell groups (Figure 7C).

Based on the aforementioned findings regarding the potential role of LAYN⁺CD8⁺ T cells in HCC progression, we further explored potential therapeutic interventions by antagonizing LAYN using an anti-human LAYN antibody. Single cell suspensions isolated from HCC tumor tissues were incubated with either a LAYN antagonist or vehicle for 36 h. Then, the immunoregulatory signals and functional capabilities were assessed using flow cytometry. Results showed that after treatment with LAYN antagonist, the percentage of CD39⁺, PD1⁺, and TIGIT⁺CD8⁺ T cells slightly decreased (Figure 7D). Notably, there was a significant upregulation in the frequency of CXCR6⁺CD8⁺ T cells observed in the anti-LAYN group (Figure 7E). Furthermore, the proportions of IFN- γ ⁺, TNF- α ⁺, granzyme B⁺, and perforin⁺ CD8⁺ T cells were also partially restored after the LAYN antagonist action (Figure 7F). These data suggest that therapeutically targeting LAYN holds promise for HCC treatment.

Discussion

The adaptive immune response initiated by immune cells within the TME plays a pivotal role in generating an antigen-specific immune response against tumors, thereby impacting survival outcomes and disease progression.²⁵ CD8⁺ T cells are widely recognized as crucial players in antitumor activity across various solid tumors.³³ Despite their infiltration into HCC, the suppressive TME induces T cell exhaustion characterized by heterogeneous subtypes and dysfunctional status.¹⁰ Investigating the characteristics of partially dysfunctional CD8⁺ T cells and identifying novel biomarkers or targets to reverse their function and activity represents a potentially relevant therapeutic approach in antitumor immunotherapy.

LAYN, a transmembrane protein, is involved in cell adhesion, immune cell activation, and the regulation of various subsets of T cell differentiation.^{21,23,34} Recently reports have identified preferential expression of the LAYN in TILs isolated from different human cancers.^{7,24} However, limited research has been conducted on the impact of this molecule on immune cells. One study demonstrated that overexpression of LAYN could decrease the ability of CD8⁺ T cells to secrete IFN- γ .⁷ Nevertheless, another study showed that LAYN augmentation enhanced integrin activation through LFA-1-dependent cellular adhesiveness to promote CD8⁺ T cell antitumor capability.²⁴

To better understanding the potential function and mechanism of LAYN in HCC carcinogenesis, our study employed various methods to validate its role in CD8⁺ T cells and its clinical prognostic relevance. Firstly, we observed high expression levels of both transcriptional and protein forms of LAYN in tumor tissues using public databases and clinical specimens, consistent with previous reports.²² Subsequently, we analyzed the correlation between LAYN expression and survival outcomes for HCC patients within the TCGA database; results indicated that high levels of LAYN expression

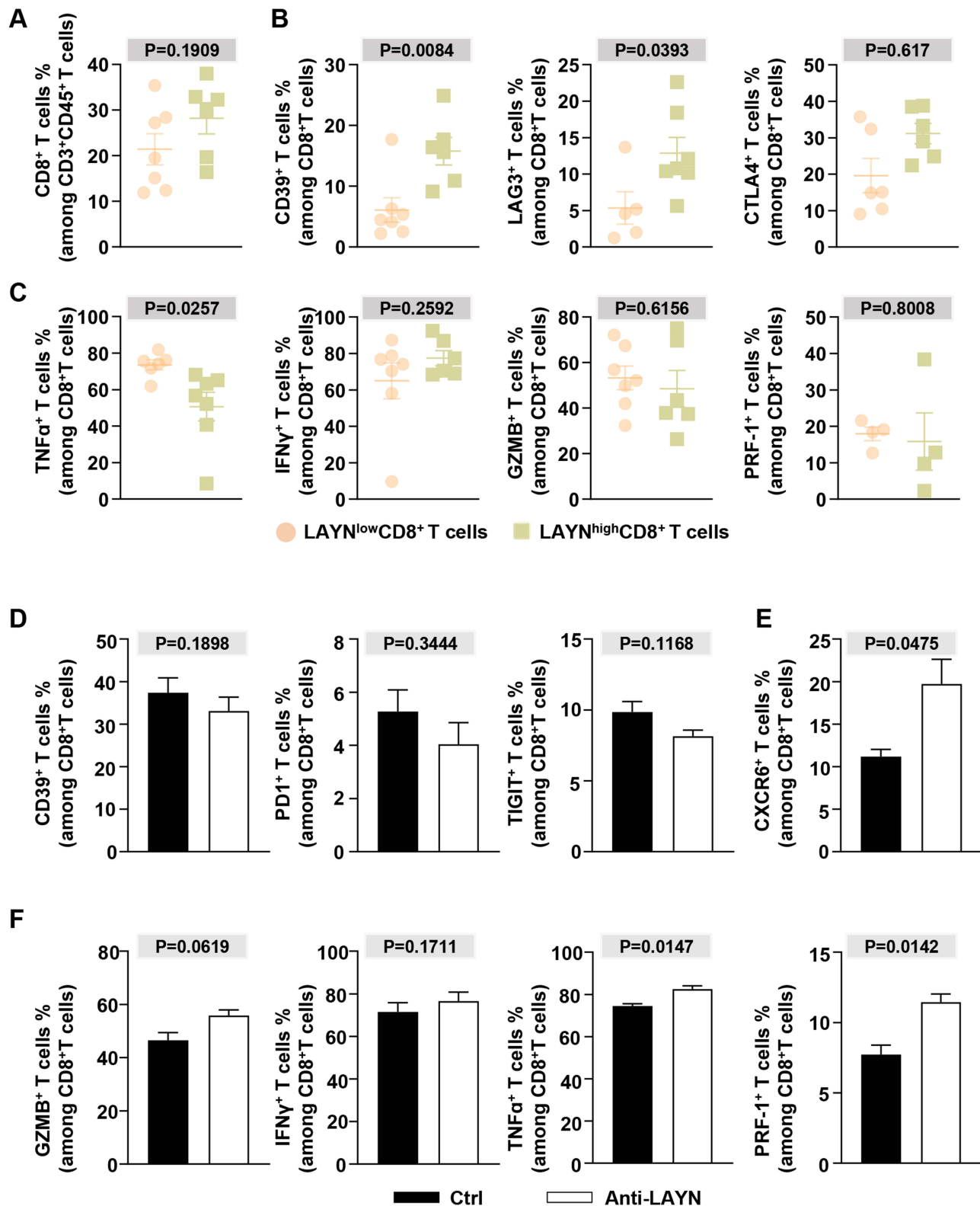


Figure 7 Intratumoral LAYN⁺ CD8⁺ T cell high infiltration impairs total CD8⁺ T cell partial immune function in HCC patients and antagonizing LAYN can partially reverse CD8⁺ T cell functions. (A) CD8⁺ T cell infiltration level in LAYN⁺CD8⁺ T cell high or low subgroup. (B and C) The charts showed the expression of co-inhibitory receptors (B) and effector markers, cytotoxic molecules (C) for total CD8⁺ T cells in LAYN⁺CD8⁺ T cell high or low subgroup. (D-F) Single cells isolated from HCC tumor tissues were incubated with LAYN antagonist (ANT-518) or vehicle for 36 h. The percentage of immune checkpoint molecules (D), receptor molecule (E) and effector markers, cytotoxic molecules (F) were analyzed by flow cytometry (n ≥ 3). Cells were pregated on CD45, CD3 and CD8. Student's t-test (A-F) was performed, and the data were presented as the mean ± SEM. Results were replicated (n = 3 experiments) (A-F).

were associated with poorer OS, DFS, and DSS among HCC patients. Our findings suggest a potential protumor effect for LAYN in HCC.

Lymphocytes infiltrating the TME are associated with cancer progression and survival. And, in our study, we chose the hot tumor tissues with high infiltration of immune cells. Our findings demonstrate a close correlation between LAYN expression and major immune cells within the TME, including CD8⁺ T cells, CD4⁺ T cells, macrophages, as well as multiple immune cell markers. These results indicate that LAYN may play a regulatory role in immune cell infiltration and responses within the HCC TME. We have discovered a specific subset of LAYN⁺CD8⁺ T cells in HCC tumor exhibiting a classical dysfunctional phenotype featuring overexpression of IRs, decreased proliferation molecules, and reduced effector functions. Transcriptome data and flow cytometry-based protein marker profiling confirmed co-expression of indicators for exhausted CD8⁺ T cells involved in tumor immune scape (CD39, TIM3 and PD1^{26,27} with LAYN on these specific CD8⁺ T cells. GO analysis reveals enrichment of highly expressed genes in inflammation-related processes while KEGG analysis highlights involvement in signaling molecules and interaction. Furthermore, our cytokine measurements demonstrated impaired antitumor function of LAYN⁺CD8⁺ T cells through downregulation of effector cytokines such as TNF- α along with decreased levels of the cytotoxic molecule perforin. These findings differ from those reported by Mahuron,²⁴ possibly due to differences in experimental methods or heterogeneity within the LAYN⁺CD8⁺ T cell population leading to distinct phenotypes or functions. Additionally, CFSE/PI results confirmed restricted killing capacity of LAYN⁺CD8⁺ T cells against 97H cells. All the findings indicated that LAYN⁺CD8⁺ T cells exhibited impaired cytokine production and antitumor activity. The compromised cytotoxicity and elevated inhibitory molecules may contribute to tumor growth and evasion, consequently leading to unfavorable outcomes in HCC patients.

Moreover, we observed a paradoxical activation state of LAYN⁺CD8⁺ T cells. On one hand, the frequency of PD1⁺TIM3⁺CD39⁺CD8⁺ TDEs was higher within the LAYN⁺CD8⁺ T cell population. On the other hand, there was an increase in CD137 and CD25 levels on LAYN⁺CD8⁺ T cells. These results suggest that LAYN⁺CD8⁺ T cells might be partially activated and potentially capable of restoring antitumor immunity.

Additionally, the accumulation and persistence of LAYN⁺CD8⁺ T cells in HCC tumors raise questions about their recruitment and localization mechanisms. Our flow cytometry analysis revealed that LAYN⁺CD8⁺ T cells express high levels of CD146, CD106, which facilitate lymphocytes extravasation from blood vessels to inflammation sites.³⁰ These results demonstrate apart from affecting proliferation, activation or partial effector molecule production by CD8⁺ T cells, LAYN also influence their localization and accumulation within tumor tissues.

Recently, the use of ICIs to reverse exhausted CD8⁺ T cells has garnered significant attention in the field. Nivolumab, ipilimumab, or Trelizumab have already been approved as second-line treatments for advanced HCC.^{35,36} Despite the improved efficacy observed with ICIs in advanced HCC patients, a majority of them do not respond to ICI monotherapy. This could be attributed to the complex TME of HCC compared to other solid tumors and the influence of multiple inhibitory mechanisms on ICIs. Therefore, exploring combinatorial strategies that simultaneously target several immune checkpoints such as PD1, CD39, and TIM3 is a valuable research direction.^{16,37,38} Consequently, there is an urgent need to identify new immune checkpoints that can enhance therapeutic approaches. As demonstrated above in our study findings indicate that LAYN⁺CD8⁺ T cells exhibit impaired antitumor function. However, upon administration of a LAYN antagonist treatment approach was able to partially restore effector function in LAYN⁺CD8⁺ T cells while also reducing expression levels of IRs like CD39 and PD1. Furthermore, CXCR6 expression which plays a crucial role in the antitumor effect of CD8⁺ T cells,³⁹ was found to be upregulated following LAYN antagonist treatment. In summary, this study provides evidence supporting LAYN as a prognostic marker for survival in HCCs. And, LAYN shows promise as a potential candidate for clinical intervention in HCC.

However, there are certain limitations within our current study. Firstly, as our experimental specimens were obtained from the tissues of viral HCC patients, there is a lack of research on those from NASH patients. Further studies can be warranted to investigate the differential expression of LAYN in NASH and viral HCC, aiming to better elucidate the expression characteristics and functions of LAYN. Then, further investigations into specific biological mechanisms underlying formation and differentiation of LAYN⁺CD8⁺ T cell using mouse or human systems are required. Moreover, there is a need for further exploration the correlation between LAYN⁺CD8⁺ T cells and the TME. The impact of LAYN expression on other tissue-infiltrating macrophages or T cell subsets, such as Treg cells, remains to be determined. Furthermore, additional trials are required to study the efficacy and safety of combining LAYN antagonists with other ICIs.

Abbreviations

HCC, Hepatocellular carcinoma; HBV, Hepatitis B virus; HCV, Hepatitis C virus; LAYN, Layilin; IL, Interleukin; IFN, Interferon; TNF, Tumor necrosis factor; GZMB, GranzymeB; PRF-1, Perforin; PD1, Programmed cell death 1; PD-L1, Programmed cell death-ligand 1; CTLA4, Cytotoxic T-lymphocyte antigen; TIM3, T cell immunoglobulin domain and mucin domain-3; LAG3, Lymphocyte activation gene 3; TIGIT, T cell immunoreceptor with Ig and ITIM domains; TME, Tumor microenvironment; MDSCs, Myeloid-derived suppressor cells; CTL, Cytotoxic T cell; T_{reg}, Regulatory T-cells; T_{DE}, Terminally differentiated T cells; DCs, Dendritic cells; PBMC, Peripheral blood mononuclear cell; PB, Peripheral blood; TILs, Tumor-infiltrating lymphocytes; OS, Overall survival; PFS, Progression-free survival; DFS, Disease-free survival; DSS, Disease-specific survival; ORR, Objective response rate; ICIs, Immune checkpoint inhibitors; IRs, Inhibitory receptors; E/T, Effector-to-target; CRC, Colorectal cancer; NSCLC, Non-small cell Lung carcinoma; COAD, Colon adenocarcinoma; BRCA, Breast invasive carcinoma; CHOL, Cholangiocarcinoma; LUAD, Lung adenocarcinoma; GO, Gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; BP, Biological process; CC, Cell component; MF, Molecular function.

Data Sharing Statement

Data are available upon reasonable request. All data generated that are relevant to the results presented in this article are included in this article or [Supplementary Materials](#).

Ethics Approval and Consent to Participate

The study was approved by the Clinical Research Ethics Committee of Zhongshan Hospital of Fudan University (B2020-262). Written informed consent was obtained from each patient included and this study was performed in accordance with the Declaration of Helsinki.

Consent for Publish

All authors agree to publish this manuscript.

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Disclosure

All authors declared no competing interests in this work.

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