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ORIGINAL RESEARCH

Comprehensive Explorations of CCL28 in Lung Adenocarcinoma Immunotherapy and Experimental Validation

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Background: Chemokines have been reported to play an important role in cancer immunotherapy. This study aimed to explore the chemokines involved in lung cancer immunotherapy.

Methods: All the public data were downloaded from The Cancer Genome Atlas Program database. Quantitative real time-PCR was used to detect the mRNA level of specific molecules and Western blot was used for the protein level. Other experiments used include luciferase reporter experiments, flow cytometric analysis, Chromatin immunoprecipitation assay, ELISA and co-cultured system.

Results: We found that the CCL7, CCL11, CCL14, CCL24, CCL25, CCL26, CCL28 had a higher level, while the CCL17, CCL23 had a lower level in immunotherapy non-responders. Also, we found that immunotherapy non-responders had a higher level of CD56dim NK cells, NK cells, Th1 cells, Th2 cells and Treg, yet a lower level of iDC and Th17 cells. Biological enrichment analysis indicated that in the patients with high Treg infiltration, the pathways of pancreas beta cells, KRAS signaling, coagulation, WNT BETA catenin signaling, bile acid metabolism, interferon alpha response, hedgehog signaling, PI3K/AKT/mTOR signaling, apical surface, myogenesis were significantly enriched in. CCL7, CCL11, CCL26 and CCL28 were selected for further analysis. Compared with the patients with high CCL7, CCL11, CCL26 and CCL28 expression, the patients with low CCL7, CCL11, CCL26 and CCL28 expression had a better performance of immunotherapy response and this effect might partly be due to Treg cells. Furthermore, biological exploration and clinical correlation of CCL7, CCL11, CCL26 and CCL28 were conducted, Finally, CCL28 was selected for validation. Experiments showed that under the hypoxia condition, HIF-1α was upregulated, which can directly bind to the promoter region of CCL28 and lead to its higher level. Also, CCL28 secreted by lung cancer cells could induce Tregs infiltration.

Conclusion: Our study provides a novel insight focused on the chemokines in lung cancer immunotherapy. Also, CCL28 was identified as an underlying biomarker for lung cancer immunotherapy.

Keywords: chemokines, lung adenocarcinoma, immunotherapy, Tregs, HIF-1α

Introduction

Globally, lung cancer is considered to be one of the major health concerns, responsible for almost 2.1 million new cases annually.[1] Lung cancer is generally a multifactorial disease that is influenced by many factors, like race, smoking, genetic susceptibility, and so on.[1] Among all subtypes, lung adenocarcinoma is a major one and accounts for approximately 40% of lung cancer.[2] Generally, surgery can provide satisfactory survival benefits to patients with early disease and is still the standard option.[3] Nevertheless, advanced lung cancer is often accompanied by local or distant metastases, making treatment more difficult.[3] Typically, chemotherapy and targeted therapy are used to treat advanced lung cancer, yet toxic side effect is still intractable. Additionally, the sensitivity rate of chemotherapy was only 30%.[4] Immunotherapy is an emerging therapy for lung cancer and has achieved promising results, especially in lung cancer. In the same way, there are still some people who are insensitive to immunotherapy. Gradually, researchers began to notice those indicators that

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indicated immunotherapy effectiveness. For instance, Zhang et al noticed the signature of CD8+ T cells related genes could indicate the immunotherapy response of lung cancer.[5] Zhou et al found that low-dose carboplatin synergizes with PD-1 inhibitors in lung cancer through the STING signaling pathway.[6] Consequently, it is important and meaningful to explore the underlying factors affecting immunotherapy sensibility.

Chemokines are small cytokines or signal proteins secreted by cells, which can induce directional chemotaxis of nearby reactive cells.[7] Also, chemokines can coordinate the migration and localization of immune cells in tissues and therefore exert an important role in the tumor microenvironment.[7] Depending on the arrangement of amino-terminal cysteines (N-terminals), cytokines can be divided into four subfamilies, including CXC, CC, XC, and CX3C.[8] Previous studies have noticed the role of the CC chemokines family in cancers. Wunderlich et al found that obesity can induce M2 macrophage polarization by regulating IL6 and the polarized macrophages can facilitate colitis-associated cancer progression through CCL-20/CCR-6-mediated lymphocyte recruitment.[9] Tas et al indicated that the increased level of MAP-1 and CCL-2 can indicate a poor prognosis of gastric cancer patients treated with platinum- and taxane-based combination chemotherapy.[10] Facciabene et al found that in ovarian cancer, local hypoxia can increase the expression of CCL28 in tumor cells, which can recruit the Treg cells expressing CCR10, leading to poor survival.[11] Ren et al found that the increased HIF-1α induced by hypoxia leads to the overexpression of CCL28, which can directly interact with the CCR10 on the surface of Tregs.[12] However, the role of CC chemokines in lung cancer immunotherapy has not been fully investigated.

In our study, we comprehensively explored the underlying role of the CC chemokines family in lung cancer immunotherapy. We found that many CC chemokines family molecules can significantly affect the immunotherapy response of lung cancer, especially CCL7, CCL11, CCL26 and CCL28. Moreover, we noticed that Treg cells in the tumor microenvironment can also affect the performance of lung cancer immunotherapy and were recruited by CCL7, CCL11, CCL26 and CCL28. Next, CCL28 was selected for further validation. Results indicated that under the hypoxia condition, HIF-1α was upregulated, which can directly bind to the promoter region of CCL28 and lead to its higher level. Also, CCL28 was associated with Treg cell infiltration.

Methods

Data Collection

The open-accessed next-sequence, mutation data, and corresponding clinical information of lung adenocarcinoma were downloaded from The Cancer Genome Atlas database (TCGA-LUAD project). The initial expression profile files were in the "STAR-Counts" form and the clinical data files were "bcr-xml" form. All the data were collated using the R software. Reference genomic file GRCh38.gtf was used for probe annotation. The probes with mean expression > 0.05 were selected for further analysis. Limma package was used to perform the differentially expressed genes (DEGs) analysis with the threshold of $|logFC| > 1$ and adj. $P < 0.05$. Before analysis, all the data were preprocessed, including missing value completion, probe annotation, and data Standardization. The gene list of IFN-γ and Expanded Immune Gene signatures was obtained from the study conducted by Ayers et al, which could indicate the immunotherapy response.[13] The makers of IFN-α/β, STING and innate immunity markers were obtained from the https://www.gsea-msigdb.org/gsea/index.jsp (REACTOME_INTERFERON_ALPHA_BETA_SIGNALING.v2022.1.Hs, GOBP_ACTIVATION_OF_INNATE_IMMUNE_RESPONSE.v2022.1.Hs, REACTOME_STING_MEDIATED_INDUCTION_OF_HOST_IMMUNE_RESPONSES.v2022.1.Hs).

Evaluation of the Immunotherapy Response

The assessment of patients on immunotherapy response was conducted using the Tumor Immune Dysfunction and Exclusion (TIDE) analysis.[14] The "Cancer type" was set as "NSCLC". The "Previous immunotherapy" was "No". All the patients were assigned a TIDE score based on the TIDE analysis, in which TIDE score > 0 was regarded as immunotherapy non-responders, while < 0 were responders.

Immune Microenvironment Quantification

Quantification of the immune microenvironment was performed using the single sample Gene Set Enrichment Analysis (ssGSEA) algorithm.[15,16] The microenvironment terms include Tem, pDC, neutrophils, mast cells, eosinophils, macrophages, DC, iDC, aDC, Th17 cells, NK cells, CD8+ T cells, CD56 bright NK cells, TFH, B cells, TReg, Th2 cells, Cytotoxic cells, T cells, Th1 cells, CD56 dim NK cells, Tcm and T helper cells.

Biological Enrichment

Pathway enrichment analysis of two specific groups was conducted using the Gene Set Enrichment Analysis (GSEA) and Gene Set Variation Analysis (GSVA).[17] Gene Ontology (GO) analysis was performed using the Clusterprofiler package.

Cell Lines

A549 (1101HUM-PUMC000002, RRID: CVCL_0023) and H1299 (1101HUM-PUMC000469, RRID: CVCL_0060) were purchased from CCRID. Cells were cultured at 37°C under 5% CO_2. Cells were passaged every four days. CoCl2 was used to induce hypoxia in the hypoxia group.

Cell Transfection

Lipofectamine 2000 was used for transient transfection according to the standardized process. HIF-1α siRNA (sc-35561) and control shRNA (sc-37007) was bought from Santa Cruz Biotechnology. The constructed wild-type colon of HIF-1α is nearly 250 bp and directly synthesized onto the pGL3 basic vector. The mutant bases are ACACCTGC.

Western Blot

Western blot was used to detect the protein level of molecules. The BCA method was used to determine the protein content of cell lysates. According to the protocol, protein extraction kits were used to extract total protein. Western blot was performed following the standard process with 10% SDS-PAGE gel. The primary antibody of HIF-1α (1:2000), CCL28 (1:3000) and GAPDH (1:5000) were obtained from Protentech. After blocking with the primary antibody (4°C and overnight), the membrane was reacted with the secondary antibody for 2 hours at room temperature. Visualization was conducted using the enhanced chemiluminescence system.

Luciferase Reporter Experiments

Promega's Renilla luciferase reporter vector phRL-null was co-transfected with promoter constructs. To assess promoter activity, dual Luciferase reporter assays were performed on transfected cells. The luciferase activity from individual constructs was normalized by Renilla-driven luciferase activity in each experiment.

Quantitative Real Time-PCR

Total RNA was extracted by an RNA extraction kit and then reverse transcribed to cDNA. The primers used were designed and synthesized from by Shanghai GenePharma Co., Ltd. SyBr Green PCR system was used for PCR detection. The primers used were as follows: CCL2, forward, 5'-CAGCCAGATGCAATCAATGCC-3', reverse, 5'-TGGAATCCTGAACCCACTTCT-3'; CCL5, forward, 5'-CCAGCAGTCGTCTTTGTCAC-3', reverse, 5'-CTCTGGGTTGGCACACACTT-3'; CCL17, forward, 5'-GGCTTCTCTGCAGCACATC-3', reverse, 5'-GGAATGGCTCCCTTGAAGTA-3'; CCL18, forward, 5'-ACAAAGAGCTCTGCTGCCTC-3', reverse, 5'-CCCACTTCTTATGGGGTCA-3'; CCL12, forward, 5'-TGAGAGCTCGCTTTGAGTGA-3', reverse, 5'-CACCAGGACCTTCTGTGGAT-3'; CCL5, forward, 5'-GCTGTCATCCTCATTGCTACTG-3', reverse, 5'-TGGTGTAGAAATACTCCTTGATGTG-3'; CCL22, forward, 5'- CGTGGTGAAACACTTCTACTGG-3', reverse, 5'-CCTTATCCCTGAAGGTTAGCAA-3'; HIF-1α, forward, 5'-TATGAGCCAGAAGAACTTTTAGGC-3', reverse, 5'-CACCTCTTTTGGCAAGCATCCTG-3'; CCL28, forward, 5'-CAGAGAGGACTCGCCATCGT-3', reverse, 5'-TGTGAAACCTCCGTGCAACA-3'.

Sample Collection and Separation

The morning venous blood of all subjects was collected with vacuum anticoagulation blood collection vessels (5 mL × 3 pipes) and then made into single-cell suspension. Then, 15 μL FITC-labeled CD4 monoclonal antibody and PE-labeled CD25 monoclonal antibody were added into 150 μL single cell suspension and then were kept away from light for 30 min at room temperature. After that, 2 mL precooling PBS was added to the blood samples and were then centrifuged at 1, 000 r/min for 5 min. Then, a 1 mL film breaker was added to the blood samples. After standing for 1 hour, membrane permeation buffer was added. After that for 10 min, the samples were resuspended by centrifugation and added with 10 mL pe-cy5 labeled Foxp3 antibody. After the sample is kept away from light for 30 min at room temperature, 2 mL of precooled PBS was added. Then, samples were shaken with a centrifuge at 1, 000 r/ min for 5 min, and then was added 500 μL of PBS. This study design was reviewed and approved by the Medical Ethics Committee of the Zhongda Hospital, Southeast University (No. 2020ZDSYLL043-P01). Following the principles of the Declaration of Helsinki. All patients provided and signed the informed consent.

Flow Cytometric Analysis

$CD8^+$ T cells were eliminated by negative selection method combining anti-$CD8^+$-FITC antibody and FITC antibody labeled magnetic beads. $CD25^+$ T cells were selected by a positive selection method combining anti-$CD25^+$-FITC and FITC-labeled magnetic beads. Subsequently, the concentration of analyzed cells was adjusted to 1×10^6/mL, and anti-$CD4^+$-PE antibody, anti-$CD8^+$-FITC antibody and $Foxp3^+$-APC antibody were added for labeling for 30 min. A FACS detection tube was used for computer detection, and FACS flow cytometry was used to detect the proportion of sorted $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells.

Chromatin Immunoprecipitation (ChIP) Assay

The ChIP assay kit (Pierce Agarose ChIP Kit) was used for the chromatin immunoprecipitation experiment. In brief, A549 cells were cross-linked and sonicated. Then, the sonicated DNA was immunoprecipitated with HIF-1α and isotype-matched control IgG. The primer used were as follows: Primer1, forward, 5'-TGCTACCCCATGCTTTTATTCAC-3', reverse, 5'-CAATGCCTTGTAGAAAAGAGCCCA-3'; Primer2, forward, 5'-TGCACTAGCGCTCATGCCTC-3', reverse, 5'-ACCCCACTTTAGTCTTGGTTCAC-3'.

Co-Cultured System and Transwell Assay

For the co-cultured system, the Treg cells were resuspended at a density of 3×10^4 cells per hole and were seeded in the upper chamber with a serum-free medium. Subsequently, 5×10^4 A549 cells and culture supernatant (800 μL, control or hypoxia group) were added into the lower chamber. After incubation for 24 hours, the cells on the lower surface of the Transwell membrane were fixed and stained.

Elisa

For the ELISA detection, 0.5 mL culture medium supernatant was firstly taken out and then centrifuged at 3500 r/min for 10 min. ELISA detection was performed using the ELISA kit following the standard process.

Statistical Analysis

The analysis of public data was conducted using the R software. SPSS 22.0 software was used for data analysis, and the measurement data conforming to normal distribution were expressed as X ± s, and a *t*-test was used for comparison between groups. Spearman or Pearson test was used for correlation analysis. $P < 0.05$ indicates that the difference is statistically significant.

Results

Evaluation of Immunotherapy Response

Firstly, the open-accessed data of the TCGA-LUAD project was downloaded for further analysis (Figure 1A). TIDE analysis was performed to evaluate the immunotherapy response of patients by calculating the TIDE score, in which (Figure 1B). CC chemokines family were extracted, including CCL1, CCL2, CCL3, CCL4, CCL5, CCL7, CCL8, CCL11, CCL13, CCL14, CCL15, CCL16, CCL17, CCL18, CCL19, CCL20, CCL21, CCL22, CCL23, CCL24, CCL25, CCL26 and CC28 (Figure 1C). The correlation analysis was then performed between the TIDE score and the CC chemokines family (Figure 1D). Results showed that the CCL1, CCL2, CCL7, CCL11, CCL24, CCL25, CCL26, CCL28 were positively correlated with the TIDE scores, while CCL14, CCL15, CCL17, CCL21 and CCL23 were

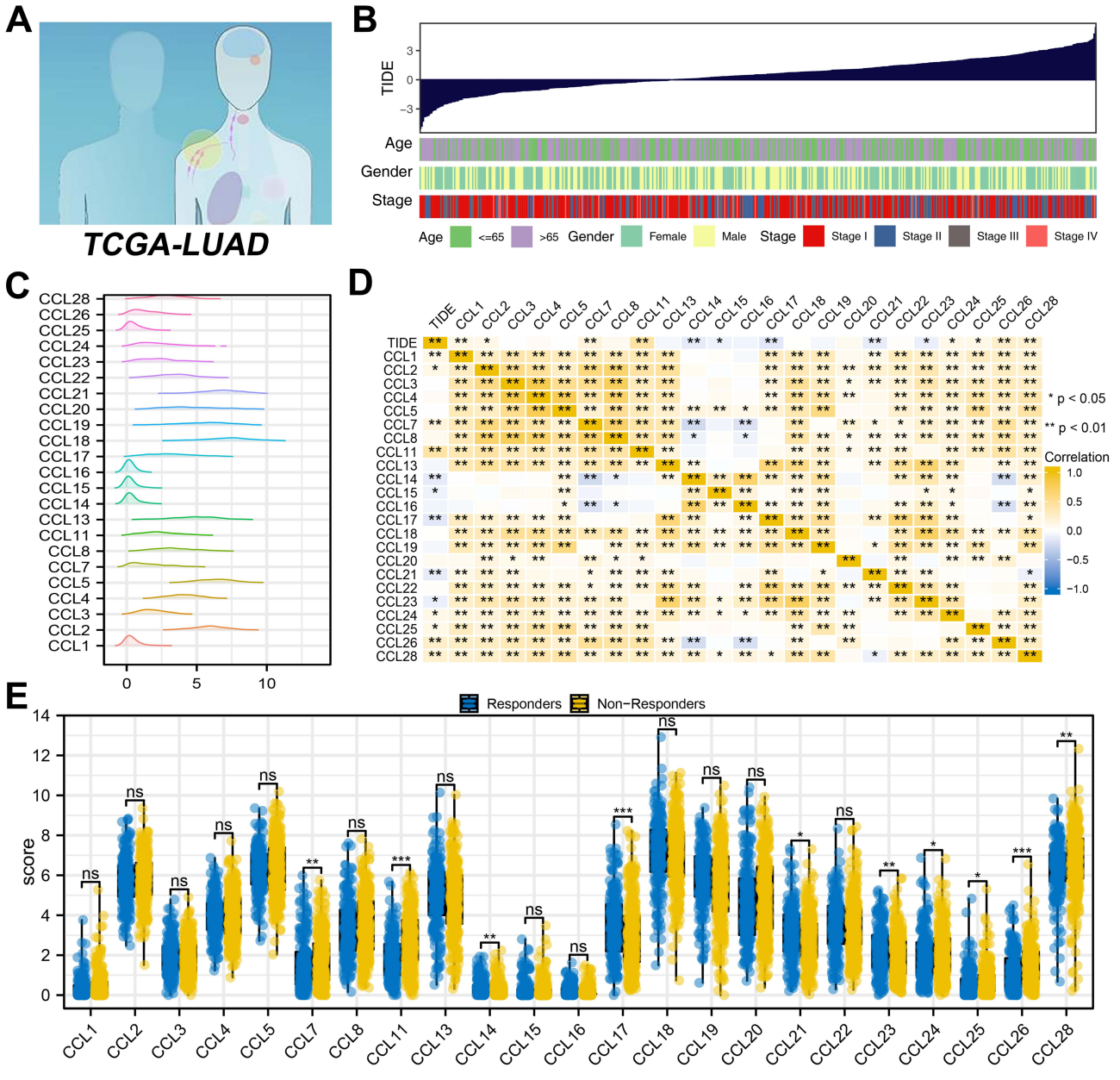

Figure 1 Exploration of CC chemokines family in lung cancer.

Notes: (**A**) TCGA-LUAD; (**B**) TIDE analysis was performed to evaluate the immunotherapy response; (**C**) CC chemokines family was extracted; (**D**) Correlation analysis of CC chemokines family and TIDE score, *P < 0.05, **P < 0.01; (**E**) The expression level of CC chemokines family in immunotherapy responders and non-responders, ns = P > 0.05, *P < 0.05, **P < 0.01, ***P <0.001.

negatively correlated with the TIDE score. In addition, we found that the CCL7, CCL11, CCL14, CCL24, CCL25, CCL26, CCL28 had a higher level, while the CCL17, CCL23 had a lower level in immunotherapy non-responders (Figure 1E). We next explored the correlation between the CC chemokines family and the immune score raised by Aysers et al, including expanded immune gene signature score and IFN-γ score, which could indicate the immunotherapy response.[13] The result showed that all these CC chemokines family genes were positively correlated with expanded immune gene signature score, and most of all also positively correlated with IFN-γ score (Figure S1). Then, we evaluated the correlation between the CC chemokines family and markers of IFN-α/β signaling, the results showed that the genes in the CC chemokines family might be positively correlated with most markers of IFN-α/β signaling, especially IRF1, IRF4, IRF5, IRF8, OAS1, OAS2, OAS3, PSMB8, PTPN6, RSAD2, SAMHD1, SOCS1, SOCS3, STAT1, XAF1 (Figure S2). For the markers of STING signaling, we found that the markers PYCARD, PYHIN1, SYK, TLR4, TYROBP, ZBP1 and ZNFX1 were positively correlated with most genes in the CC chemokines family, while the SFPQ and XRCC5 were contrary (Figure S3). For the markers of innate immunity, we noticed that the markers of KLRD1, KLRK1, LILRA2, MNDA, NLRC4, PLCG2, PYCARD, PYHIN1, SYL, TLR4, TYROBP, ZBP1, AIM2, CLEC6A, CLEC7A, FCN1, HCK, FYN and IFI16 were positively correlated with most genes in CC chemokines family (Figure S4). KRAS mutation is a common mutation in lung cancer. Therefore, we explored the expression of the CC chemokines family in KRAS mutant and wild-type patients. The result indicated a lower CCL2, CCL3, CCL4, CCL5, CCL8, CCL11, CCL25 and CCL28, while a higher CCL20 level in KRAS mutant LUAD patients (Figure S5).

Treg Cells Affect the Immunotherapy Response of Lung Cancer

Further, we quantified the immune microenvironment of lung cancer using the ssGSEA algorithm (Figure 2A). Correlation analysis indicated that the TIDE analysis was positively correlated with CD56 dim NK cells, NK cells, Tem, TFH, Th1 cells, Th2 cells and Treg cells, while negatively correlated with iDC and Th17 cells (Figure 2B). Also, we found that immunotherapy non-responders had a higher level of CD56dim NK cells, NK cells, Th1 cells, Th2 cells and Treg, yet a lower level of iDC and Th17 cells (Figure 2C). A positive correlation was found between TIDE score and TReg cells (Figure 2D, Correlation = 0.144, P = 0.001). Moreover, a higher TReg level was observed in the immunotherapy non-responders patients (Figure 2E). Moreover, in the patients with high TReg infiltration, we noticed a lower immunotherapy responder rate (Figure 2F, 33.2% vs 44.1%).

Biological Exploration of TReg Cells

The overview of Treg infiltration in TCGA-LUAD was shown in Figure 3A. Then, DEGs analysis was conducted in the patients with high and low TReg infiltration, in which 107 upregulated and 3 downregulated genes were identified (Figure 3B). GSEA analysis indicated that in the patients with high Treg infiltration, the pathways of pancreas beta cells, KRAS signaling, coagulation, WNT BETA catenin signaling, bile acid metabolism, interferon alpha response, hedgehog signaling, PI3K/AKT/mTOR signaling, apical surface, myogenesis were significantly enriched in (Figure 3C). GO analysis showed that the terms of vitamin metabolic process (GO:0006766), water-soluble vitamin metabolic process (GO:0006767), cobalamin metabolic process (GO:0009235) and tetrapyrrole metabolic process (GO:0033013) (Figure 3D). Interestingly, we found that Treg cells were positively correlated with all the key immune checkpoints, including PD-1 (CD274), PD-L1 (PDCD1), PD-L2 (PDCD1LG2) and CTLA4 (Figure 3E–H, PD-1: Correlation = 0.198, $P < 0.001$; PD-L1: Correlation = 0.680, $P < 0.001$; PD-L2: Correlation = 0.250, $P < 0.001$; CTLA4: Correlation = 0.623, $P < 0.001$).

CCL7, CCL11, CCL26 and CCL28 May Affect Lung Cancer Immunotherapy by Recruiting Treg Cells

Previous studies have shown that the CC cytokines family can affect Treg cells in the tumor microenvironment.[11] Next, we found that the CCL7, CCL11, CCL26 and CCL28 had the same trend as TReg in immunotherapy response. Therefore, CCL7, CCL11, CCL26 and CCL28 were selected for further analysis (Figure 4A–E; CCL7: Correlation = 0.323, $P < 0.001$; CCL11: Correlation = 0.346, $P < 0.001$; CCL26: Correlation = 0.336, $P < 0.001$; CCL28: Correlation = 0.510, $P < 0.001$).

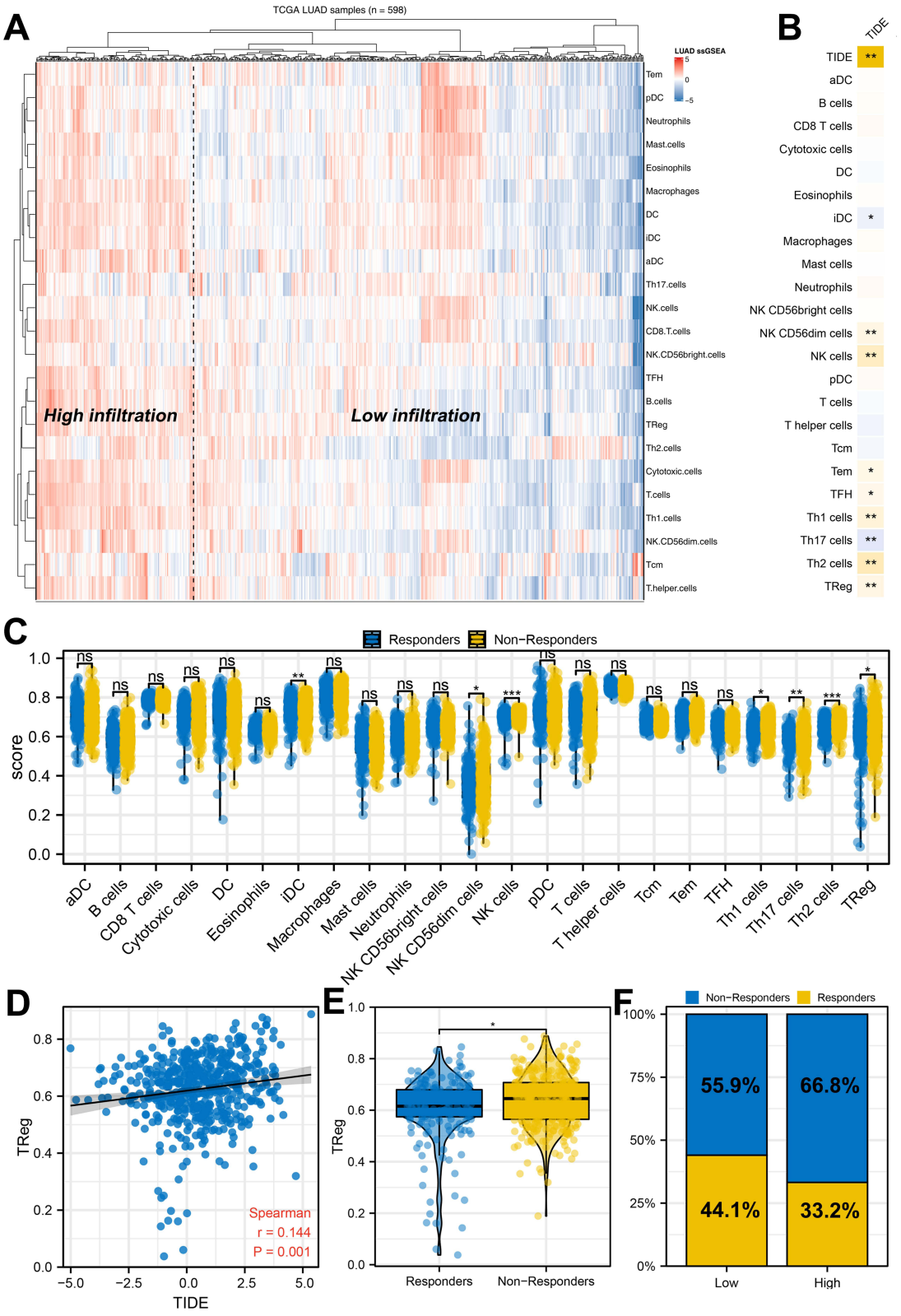

Figure 2 Treg affects immunotherapy of lung cancer.

Notes: (**A**) ssGSEA algorithm was performed to quantify the immune microenvironment of lung cancer patients; (**B**) Correlation between TIDE score and immune terms, *P < 0.05, **P < 0.01; (**C**) The level of immune terms between immunotherapy responders and non-responders, ns = P > 0.05, *P < 0.05, **P < 0.01, ***P <0.001.; (**D**) TIDE was positively correlated with Treg; (**E**) Immunotherapy non-responders had a higher Treg, *P < 0.05; (**F**) The percentage of immunotherapy responders and non-responders in patients with high and low Treg infiltration.

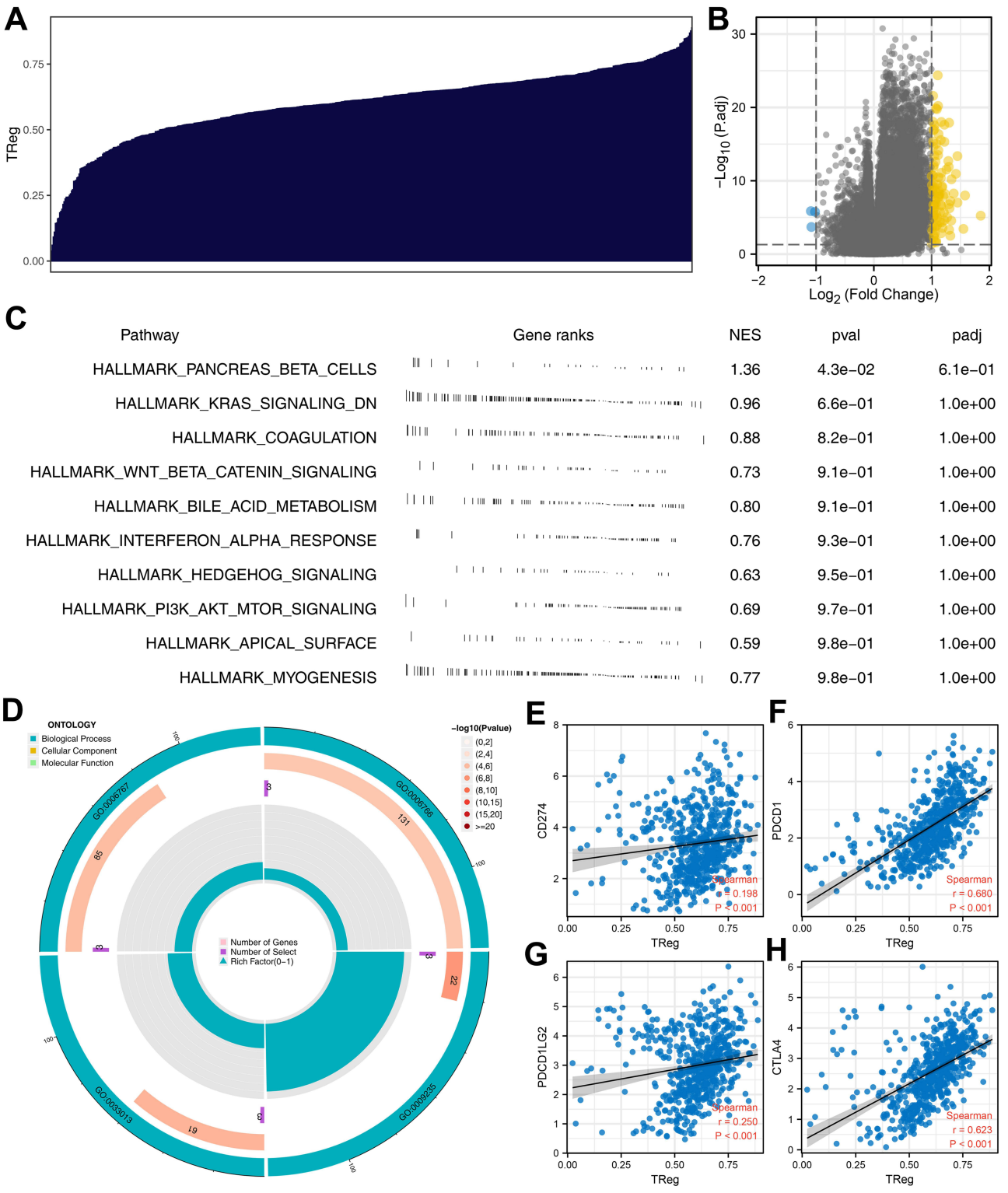

Figure 3 Biological exploration of Treg.
Notes: (**A**) Overview of the Treg in TCGA-LUAD patients; (**B**) DEGs analysis between patients with high and low Treg infiltration; (**C**) GSEA analysis of the Treg; (**D**) GO analysis of the Treg; (**E**–**H**) Correlation between Treg and immune checkpoint inhibitors.

Compared with the patients with high CCL7, CCL11, CCL26 and CCL28 expression, the patients with low CCL7, CCL11, CCL26 and CCL28 expression had a better performance of immunotherapy response (Figure 4F–I, CCL7: 31.8% vs 43.4%; CCL11: 23.6% vs 51.6%; CCL26: 28.7% vs 46.5%; CCL28: 33.3% vs 44.1%). Also, we found that the level of Treg

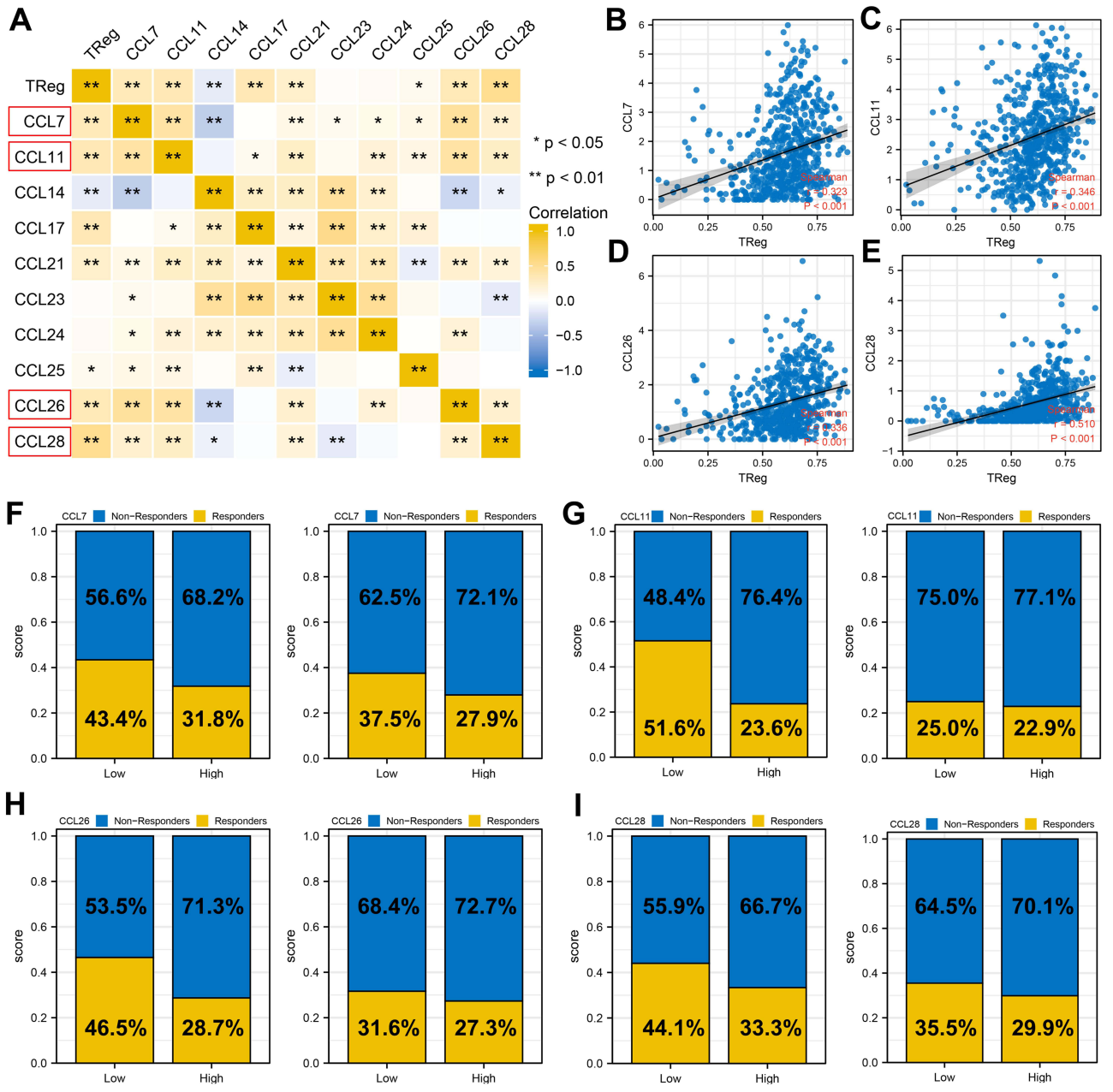

Figure 4 Further exploration of CCL7, CCL11, CCL26 and CCL28.

Notes: (**A**) Correlation between Treg and CC chemokines family, *P < 0.05, **P < 0.01; (**B**–**E**) Correlation between Treg and CCL7, CCL11, CCL26 and CCL28; (**F**) The effect of CCL7 on lung cancer immunotherapy; (**G**) The effect of CCL11 on lung cancer immunotherapy; (**H**) The effect of CCL26 on lung cancer immunotherapy; (**I**) The effect of CCL28 on lung cancer immunotherapy.

infiltration can affect the immunotherapy response in patients with high CCL7, CCL11, CCL26 and CCL28 expression (Figure 4F–I, CCL7: 27.9% vs 37.5%; CCL11: 22.9% vs 25.0%; CCL26: 27.3% vs 31.6%; CCL28: 39.6% vs 42.7%). These results indicated that CCL7, CCL11, CCL26 and CCL28 may affect lung cancer immunotherapy by recruiting Treg cells.

Biological Exploration of CCL7, CCL11, CCL26 and CCL28

Based on the Hallmark gene set, we found that the hypoxia pathway was the most significant difference between patients with high and low TReg infiltration, indicating that hypoxia can induce the recruitment of Treg cells (Figure 5A). The activity of hypoxia of TCGA-LUAD patients was shown in Figure 5B. Moreover, we found that the CCL7, CCL11 and CCL28 were significantly upregulated in the patients with high hypoxia activity (Figure 5C). Positive correlations was found between

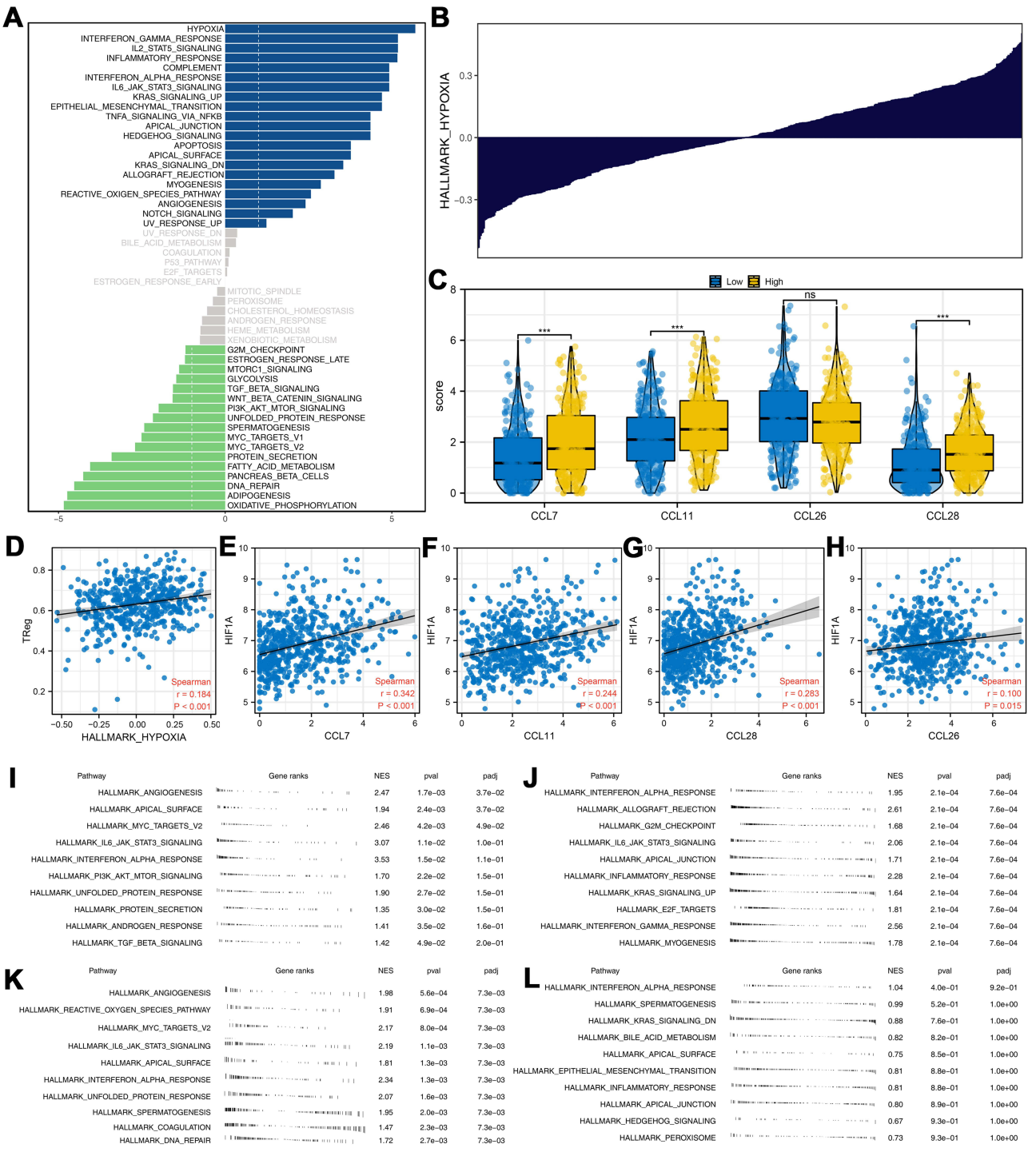

Figure 5 Hypoxia was associated with the Treg infiltration and CCL7, CCL11, CCL26, CCL28.

Notes: (**A**) GSVA analysis in patients with high and low Treg infiltration; (**B**) The quantified hypoxia activity of TCGA-LUAD patients; (**C**) The CCL7, CCL11, CCL26 and CCL28 expression in patients with high and low Treg infiltration, ns=P>0.05, ***P<0.001; (**D**) Correlation between Treg and hypoxia; (**E–H**) Correlation between HIF-1α and CCL7, CCL11, CCL26 and CCL28; (**I**) GSEA analysis of CCL7; (**J**) GSEA analysis of CCL11; (**K**) GSEA analysis of CCL26; (**L**) GSEA analysis of CCL28.

hypoxia and TReg, CCL7, CCL11, CCL26 and CCL28 (Figure 5D–H, TReg, Correlation = 0.184, P < 0.001; CCL7: Correlation = 0.342, P < 0.001; CCL11: Correlation = 0.244, P < 0.001; CCL28, Correlation = 0.283, P < 0.001; CCL26, Correlation = 0.100, P < 0.001). Pathway enrichment analysis indicated that in patients with high CCL7 expression, the pathways of angiogenesis, apical surface, MYC targets, IL6/JAK/STAT3 signaling, interferon alpha response, PI3K/AKT/

mTOR signaling were significantly enriched (Figure 5I). For the patients with high CCL11 expression, the pathways of interferon-alpha response, allograft rejection, G2M checkpoints, IL6/JAK/STAT3 signaling, apical junction and inflammatory response were significantly enriched in (Figure 5J). For the patients with high CCL26 expression, the pathways of angiogenesis, reactive oxygen species pathway, MYC targets, IL6/JAK/STAT3 signaling, apical surface and interferon alpha response were significantly enriched in (Figure 5K). For the patients with high CCL28 expression, the pathways of interferon-alpha response, spermatogenesis, KRAS signaling, and bile acid metabolism were significantly enriched (Figure 5L).

Clinical Correlation of CCL7, CCL11, CCL26 and CCL28

Univariate Cox regression was used to evaluate the prognosis value of CCL7, CCL11, CCL26 and CCL28. However, no significant prognosis correlation was observed in overall survival, disease-free survival and progression-free survival (Figure 6A–C). Clinical correlation analysis showed that the T3-4 patients had a lower CCL11 expression compared to T1-2 patients (Figure 6D); the N1-3 patients had a higher CCL11 expression compared to N0 patients (Figure 6E); no significant difference was found between M0 and M1 patients (Figure 6F); no significant difference was found in patients with the different clinical stage (Figure 6G); the male patients had a higher CCL11 and CCL28 expression compared to female patients (Figure 6H); no significant difference was found in patients with different age (Figure 6I). Furthermore, we found that CCL7, CCL11 and CCL28 were positively correlated with TMB (Figure 6J); CCL7 and CCL11 were negatively correlated with MSI (Figure 6K); TReg, CCL11 and CCL28 were negatively correlated with mRNAsi, while CCL7 and CCL26 were positively correlated with mRNAsi (Figure 6L); TReg was negatively correlated with the EREG-mRNAsi, while CCL26 was positively correlated with EREG-mRNAsi (Figure 6M).

Hypoxia Induces HIF-1α and CCL28 Elevation

Based on our results, we suggested that hypoxia can induce the elevation of HIF-1α, which was responsible for the upregulation of CCL7, CCL11, and CCL28. The increased CCL7, CCL11 and CCL28 can recruit Treg cells and therefore affect lung cancer immunotherapy (Figure 7A). We took two lung adenocarcinoma cell lines, H1299 and A549, for further experiments (Figure 7B). By inducing hypoxia in A549 and H1299 cells, we detected the RNA expression level of CCL14, CCL24, CCL7, CCL11, CCL25, CCL17, CCL23, CCL26 and CCL28 in control and hypoxia groups (Figure 7C and D). The result indicated that only CCL28 was significantly upregulated in the hypoxia cells. Moreover, we noticed that in hypoxia cells, the mRNA and protein levels of HIF-1α and CCL28 were all increased (Figure 7E–I). Then, we knock down the HIF-1α in lung cancer cells (Figure S6). The result of the Western blot also showed that the inhibition of HIF-1α can remarkably decrease the protein level of CCL28 (Figure 8A). Also, ELISA experiments showed that the knockdown of HIF-1α can significantly reduce the secretion of CCL28 (Figure 8B). After that, we cloned and sequenced the CCL28 promoter fragment and its mutants (Figure 8C and Figure S7). Based on the luciferase reporter assay, wild-type CCL28-luciferase but not mutant CCL28-luciferase were able to respond to HIF-1α (Figure 8D). CHIP assay showed that HIF-1α can bind to the promotor of CCL28 (Figure 8E).

CCL28 Was Associated with the TReg Cells Infiltration

We tried to isolate the Treg cells from the blood based on the methods mentioned above. The experimental results showed that the content of Treg cells identified by FCS in peripheral blood was 3.87%, and the purity after purification was 89.14–94.38% (Figure 9A). Moreover, we confirmed the morphology of Treg cells under light microscopy (Figure 9B and C). We took the lung adenocarcinoma cell line, A549 (control and hypoxia group), and co-cultured them with Treg cells in the Transwell system, as shown in Figure 9D. After that for 24 hours, we found that the Tregs co-cultured with hypoxia A549 cells had more access to the lower chamber (Figure 9E). Considering the higher level of CCL28 in hypoxia A549 cells compared to the control cells (Figure 7F), we think the CCL28 might be associated with the local recruitment of Tregs, which could be induced by the hypoxia condition.

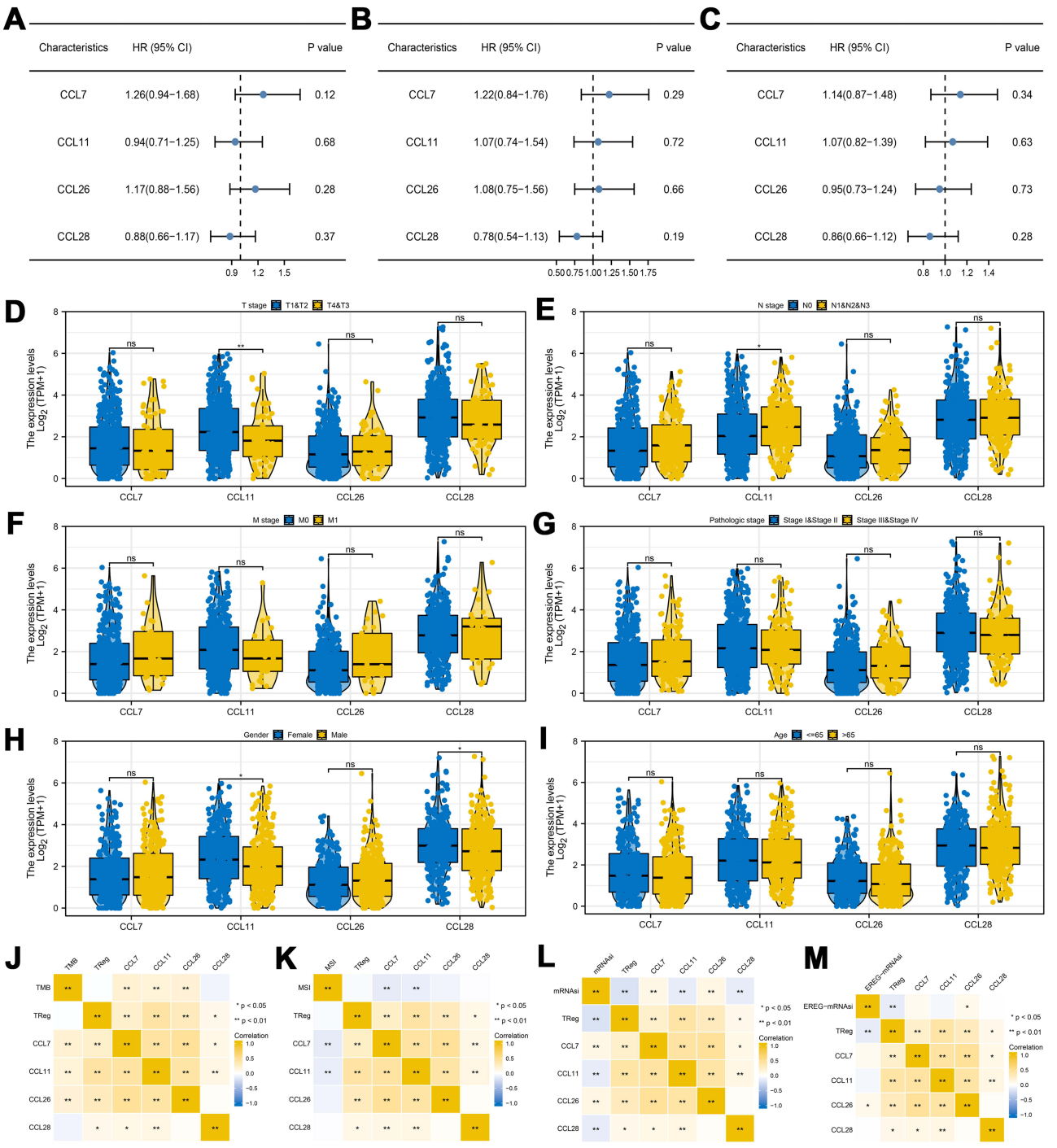

Figure 6 Clinical correlation of CCL7, CCL11, CCL26, CCL28.

Notes: (**A**) Univariate Cox regression analysis (overall survival) of CCL7, CCL11, CCL26, CCL28; (**B**) Univariate Cox regression analysis (disease-free survival) of CCL7, CCL11, CCL26, CCL28; (**C**) Univariate Cox regression analysis (progression-free survival) of CCL7, CCL11, CCL26, CCL28; (**D–I**) Expression level of CCL7, CCL11, CCL26, CCL28 in patients with different clinical features, ns = P > 0.05, *P < 0.05, **P < 0.01.; (**J–M**) Correlation between TIDE and Treg, CCL7, CCL11, CCL26, CCL28, *P < 0.05, **P < 0.01.

Discussion

Despite substantial technical advances in medicine, lung cancer is still a serious public health concern globally.[18] Recently, treatment patterns for lung cancer are gradually being changed by immunotherapy.

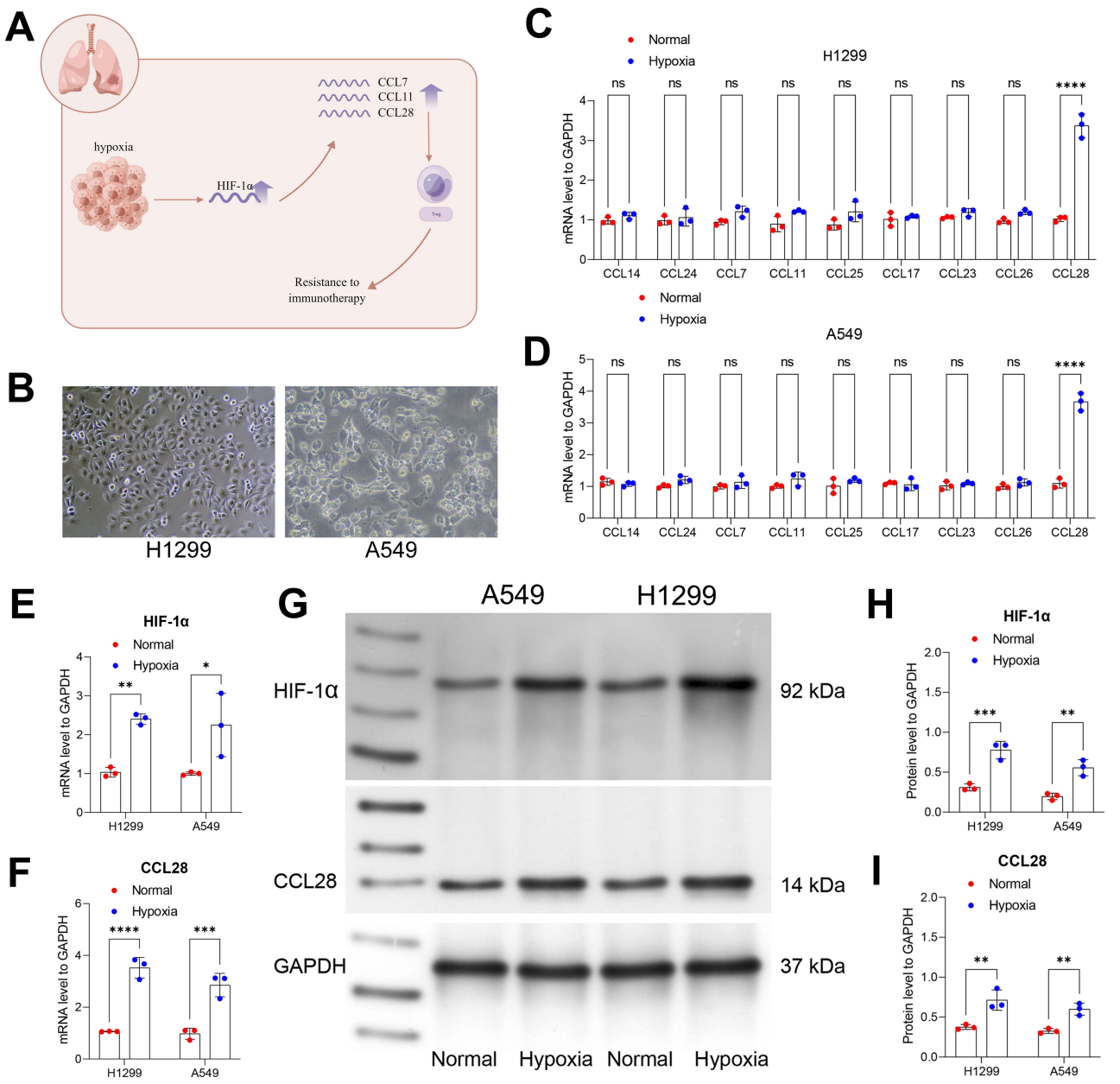

Figure 7 Hypoxia induces HIF-1α and CCL28 elevation.
Notes: (**A**) Hypothesis plot; (**B**) The morphology of H1299 and A549 cells was observed under the light microscope; (**C** and **D**) The level of CCL14, CCL24, CCL7, CCL11, CCL25, CCL17, CCL23, CCL26 and CCL28 in control and hypoxia cells, ns = P < 0.05, ****P < 0.0001; (**E**) The level of HIF-1α in control and hypoxia cells, *P < 0.05, **P < 0.01; (**F**) The level of CCL28 in control and hypoxia cells, ***P < 0.001, ****P < 0.0001; (**G**–**I**) The protein level of HIF-1α and CCL28 in control and hypoxia cells, **P < 0.01, ***P < 0.001.

Currently, researchers can conduct in-depth analyses based on public data and provide valuable research clues.[19–21] Here, we found that the CCL7, CCL11, CCL14, CCL24, CCL25, CCL26, and CCL28 had a higher level, while the CCL17 and CCL23 had a lower level in immunotherapy non-responders. Also, we found that immunotherapy non-responders had a higher level of CD56dim NK cells, NK cells, Th1 cells, Th2 cells and Treg, yet a lower level of iDC and Th17 cells. Biological enrichment analysis indicated that in the patients with high Treg infiltration, the pathways of pancreas beta cells, KRAS signaling, coagulation, WNT BETA catenin signaling, bile acid metabolism, interferon alpha response, hedgehog signaling, PI3K/AKT/mTOR signaling, apical surface, myogenesis were significantly enriched in. CCL7, CCL11, CCL26 and CCL28 were selected for further analysis. Compared with the patients with high CCL7,

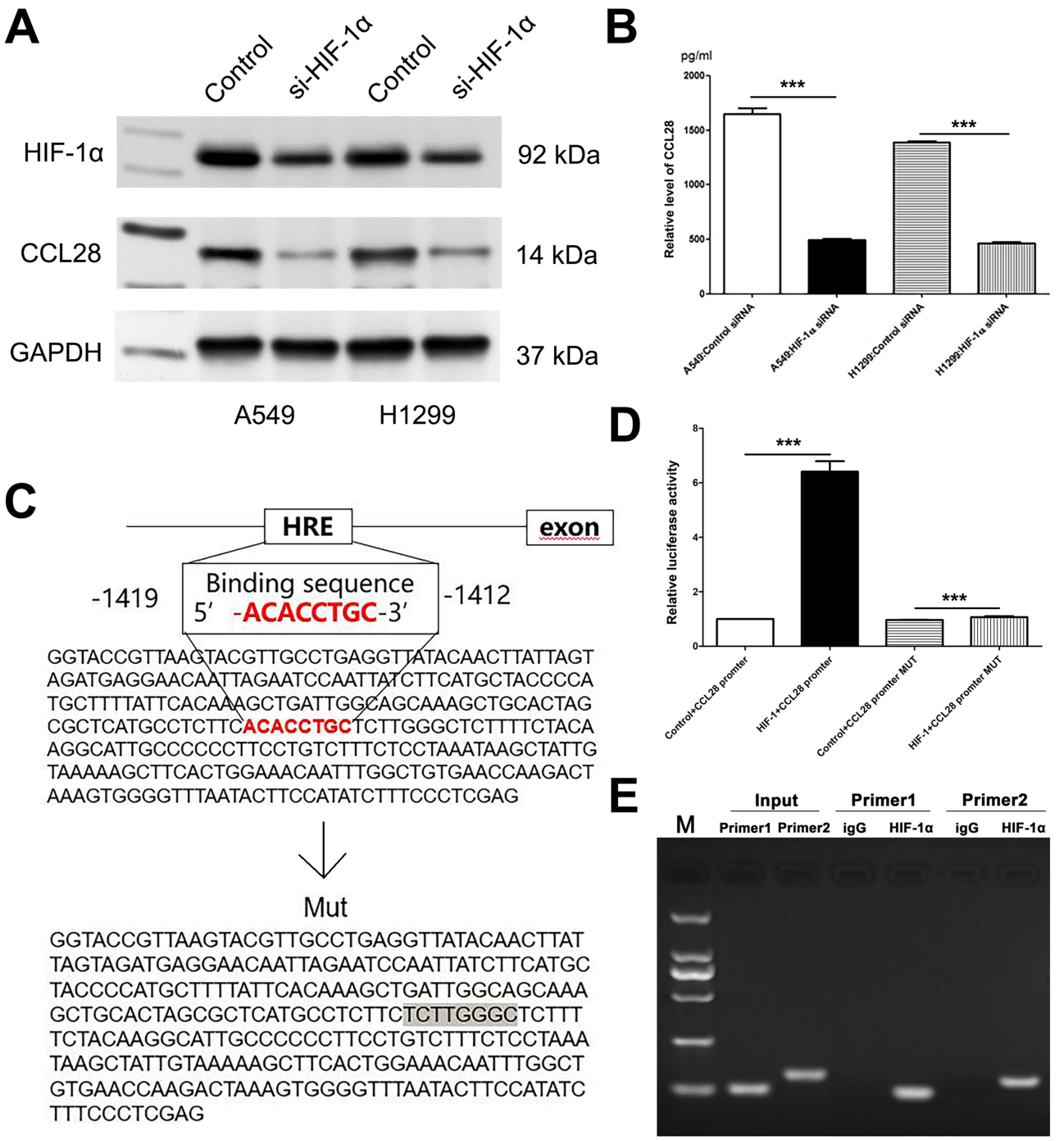

Figure 8 HIF-1α can regulate the CCL28 expression.
Notes: (**A**) Knockdown of HIF-1α can significantly decrease the protein level of CCL28; (**B**) ELISA was used to detect CCL28 secretion of lung gland cells before and after interference, ***P < 0.001; (**C**) Cloning and sequencing identification of CCL28 promoter fragment and its mutants; (**D**) Wild-type CCL28-luciferase but not mutant CCL28-luciferase were able to respond to HIF-1α, ***P < 0.001; (**E**) ChIP assay showed that HIF-1α can bind to the promotor of CCL28.

CCL11, CCL26 and CCL28 expression, the patients with low CCL7, CCL11, CCL26 and CCL28 expression had a better performance of immunotherapy response and this effect might partly be due to Treg cells. Furthermore, biological exploration and clinical correlation of CCL7, CCL11, CCL26 and CCL28 were conducted, Finally, CCL28 was selected for validation. Experiments showed that under the hypoxia condition, HIF-1α was upregulated, which can directly bind to the promoter region of CCL28 and lead to its higher level. Also, CCL28 was associated with Treg cell infiltration.

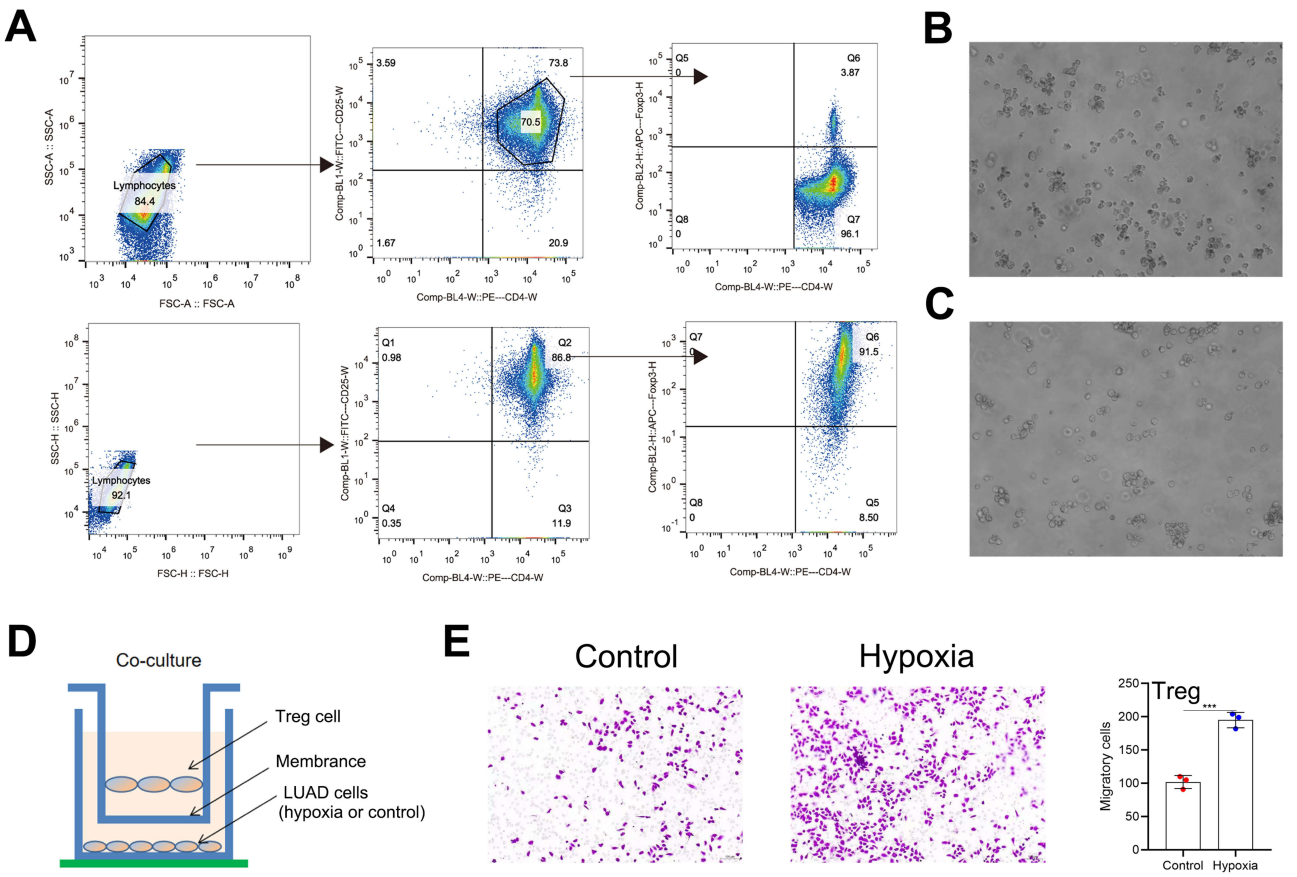

Figure 9 Isolation and identification of Treg cells.
Notes: (**A**) FCS identifies the proportion of $CD4^+CD25^+Foxp3^+$ Treg cells and purified $CD4^+CD25^+Foxp3^+$ Treg cells in peripheral blood; (**B** and **C**) Treg cell morphology under the light microscope; (**D**) Co-cultured system; (**E**) Tregs co-cultured with hypoxia A549 cells had more access to the lower chamber, ***P < 0.001.

The result showed that the CCL1, CCL2, CCL7, CCL11, CCL24, CCL25, CCL26, CCL28, CCL14, CCL15, CCL17, CCL21 and CCL23 were significantly associated with the immunotherapy response of lung cancer according to the TIDE analysis. Previous studies have focused on their role in cancer immunotherapy. Zhang et al found that CCL7 can affect lung cancer immunotherapy through recruiting cDC1 cells, indicating that CCL7 might be an underlying biomarker and adjuvant for lung cancer immunotherapy.[22] Hoelzinger et al found that CCL1 can neutralize the immunosuppressive effect of Treg cells, but not affect the function of T effector cells, making it a mean for cancer immunotherapy.[23] Liu et al revealed that mice exposed to environmental stress have increased anti-tumor immunity and become more sensitive to immunotherapy against liver cancer, which was dependent on the β-ARs/CCL2 axis.[24] Also, Yang et al found that the CCL2/CCR2 axis can recruit tumor-associated macrophages to induce immune evasion through PD-1 signaling in esophageal cancer.[25] Our result identified these CC chemokines family molecules with the potential to affect the immunotherapy response of lung cancer, which can guide the direction of future studies.

Our result also found that Treg cells can significantly affect lung cancer immunotherapy. The immune-suppressive microenvironment of tumors is strongly influenced by Treg cells.[26] Generally, in the tumor microenvironment, Treg cells are numerous and highly activated, mainly responsible for tumor-induced immunosuppression.[26] Li et al found that the TLR8 can specifically inhibit the glucose metabolism of Treg cells and reversibly hamper the immunosuppressive function of Treg cells, further affecting immunotherapy.[27] Bai et al indicated that the ANXA1 blocker Boc1 can decrease granzyme A mRNA expression in Treg cells, therefore antagonizing Treg cell-mediated immunosuppression.[28] In lung cancer, macrophage-related molecules MARCO and IL37R can block Treg cells and support cytotoxic lymphocyte function.[29] Redin et al found that the SRC family kinase inhibitor dasatinib can enhance the anti-PD-1 activity in lung cancer by inhibiting Treg cell conversion and proliferation.[30] Our results

provide evidence for the role of Tregs in lung cancer immunotherapy and might be a clue for relevant studies in the future.

Moreover, we found that the hypoxia microenvironment can induce Tregs recruitment and was positively correlated with the expression of CCL7, CCL11, CCL26 and CCL28. Generally, most bioenergetic processes are based on oxygen. However, hypoxia status is prevalent in the tumor microenvironment and contributes to wide reprogramming in cancers.[31] In liver cancer, Suthen et al found that Tregs was significantly enriched in the local hypoxia area. Also, this recruitment effect was dependent on the CCL20 and CXCL5. In the hypoxia tumor microenvironment, the interaction between Tregs and cDC2 results in the loss of antigen-presenting HLA-DR on cDC2, further affecting the immunosuppression effect of Treg.[32] In addition, Liu et al revealed that hypoxia leads to the overexpression of CCL28, which could recruit Treg cells to enhance angiogenesis in lung cancer.[33] Shi et al indicated that the HIF-1α could regulate the metabolic checkpoints of Th17 and Treg cells in a glycolytic manner.[34] These results indicated the underlying crosstalk between hypoxia, CC chemokines family and Treg cells, which might provide direction for future studies or researchers in this field.

We finally selected CCL28 for experiment validation due to its strong correlation with Treg cells. Our result showed that under hypoxia induction, HIF-1α and CCL28 were significantly upregulated compared to the normal conditions. Huang et al found that in lung adenocarcinoma, hypoxia-induced CCL28 targets CCR3 on endothelial cells to promote angiogenesis.[35] Moreover, the luciferase reporter and ChIP assay showed that HIF-1α can directly bind to the promoter region of CCL28. Meanwhile, we found that CCL28 was associated with Treg cell infiltration, which might partly explain its effect on immunotherapy response. Nowadays, immunotherapy has only succeeded in a small number of patients with lung cancer and most lung cancer patients suffer from insensitivity. Therefore, it is extremely important to find molecules that may affect the response rate of lung cancer immunotherapy. Our result indicated that CCL28 could influence lung cancer immunotherapy, making it a potential biomarker for clinical applications.

There are some limitations to be aware of. Firstly, the public sample included in our analysis was mainly the Western population. Therefore, potential race bias is inevitable. Secondly, the experimental validation in our study was only limited to the cell level. Subsequently, in vivo experiments should be conducted to improve the reliability of our conclusions. Thirdly, bioinformatics analysis can not completely reflect the real biological situation, which might cause some underlying bias.

Publication Permission

All authors have agreed to publish this paper.

Data Sharing Statement

The raw data mentioned in this study can be downloaded from online databases. More detailed information can be provided by the corresponding author upon reasonable request.

Ethics and Consent Statement

This study design was reviewed and approved by the Medical Ethics Committee of the Zhongda Hospital, Southeast University (No. 2022ZDSYLL303-P01). In accordance with the principles of the Declaration of Helsinki. All patients provided and signed the informed consent.

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Disclosure

All authors declare no potential conflict of interests.

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