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SMAD4 Regulates the Expression of LCK Affecting Chimeric Antigen Receptor-T Cells Proliferation Through PI3K/Akt Signaling Pathway

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ABSTRACT

The proliferation of CAR-T cells was hindered and cannot play its killing function well in solid tumors. And yet the regulatory mechanism of CAR-T cell proliferation is not fully understood. Here, we showed that recombinant expression of CD19CAR in T cells significantly increased the basal activation level of CAR-T cells and LCK activation. Both LCK and SMAD4 were essential for CAR-T cells proliferation since over-express LCK or SMAD4 significantly promotes CAR-T cells proliferation, while knock-down LCK or SMAD4 expression inhibited the proliferation of CAR-T cells seriously. More cells go into apoptosis when knock-down LCK or SMAD4 expression, and the cell cycle was arrested in G2/M or S phase, respectively. Over-express LCK or SMAD4 significantly promotes phosphorylation of PI3K and Akt, while it was inhibited when cells were treated with PI3K and Akt inhibitors (LY294002 or MK2206). Further mechanism exploration experiments showed that SMAD4 bound on the promoter region of LCK regulating its expression. Taken together, we reported that the transcription factor SMAD4 regulated the expression of LCK and further involved in the PI3K/Akt signaling pathway to affect the proliferation of CAR-T cells.

1 | Introduction

Chimeric antigen receptor (CAR) T cell therapy was a new-type strategy by arming T cells to recognize and kill target tumor cells. T cells were engineered with CAR commonly consisting of single chain variable fragment (scFv) recognizing target antigen on the surface of tumor cells, hinge region, transmembrane domain, and intracellular domain including co-stimulatory domain and CD3-ζ domain (Yoo and Harapan 2021). Many types of CAR-T cells were developed to treat hematological malignances and showed promising results (Da Vià et al. 2021). For instance, CD19CAR-T cells targeting CD19 were used to treat patients with relapsed and/or refractory pre-B cell acute lymphoblastic leukemia (B-ALL) (Elsallab et al. 2023), CD22-targeted CAR-T cells also used to treat B-ALL, especially for patients resistant to CD19CAR-T therapy (Fry et al. 2018).

CD19CD20CAR-T cells, dual antigen targeting of CD19 and CD20, were used to treat relapsed/refractory non-Hodgkin lymphoma (r/rNHL) (Tong et al. 2020). CD5CD7CAR-T cells were developed to treat T-cell leukemia/lymphoma (Dai et al. 2022). Otherwise, CD19CAR-T cells also used to treat autoimmune diseases including refractory systemic lupus erythematosus (Mackensen et al. 2022). CAR-T cell therapy also applied in solid tumors treatment. For example, PSMA-targeting CAR-T cells treated metastatic castration-resistant prostate cancer (Narayan et al. 2022), mesothelin (MSLN)-targeting CAR-T cells treated ovarian cancer (Schoutrop et al. 2023, 2021).

Although CAR-T cell therapy showed promising results in combating cancers, there were disadvantages or limitations for CAR-T cell therapy, including relapse caused by antigen lose, lacking tumor specific antigens, poor persistence and infiltration, high exhaustion and so on. To overcome these limitations, some complement strategies were applied. Antigen lose was one of the reasons for tumor recurrence after CAR-T cell treatment (Gazeau et al. 2021; Chong, Ruella, and Schuster 2021). Benefiting from the complementary antitumor functions of T cells and natural killer cells, Changqing Pan et al. developed poliovirus receptor-based CAR-T cells, and significantly suppressed tumor recurrence due to antigen lose in glioblastoma when combining with NK-92 cells therapy (Pan et al. 2024). By integrating multiple imperfect but complementary antigens through introducing synNotch system in CAR-T cells improved CAR-T cells antitumor specificity, completeness and persistence in glioblastoma (Choe et al. 2021). CAR-T cell therapy applied in allogeneic infusion needs to concern human leukocyte antigen (HLA) compatibility, CAR-natural killer (CAR-NK) cell complements CAR-T cell therapy do not require HLA compatibility (Peng et al. 2024). Additionally, arming CAR-T cells with cytokines and/or chemokines, such as IL-12, IL-7, CCL19, IL-18, and so on, could improve CAR-T cells infiltration, survival and against exhaustion (Adachi et al. 2018; Li et al. 2022; Lee et al. 2023; Chmielewski and Abken 2017).

Cell proliferation is a fundamental process for cell survival. The proliferation was important for keeping functional capacity of CAR-T cells. Inhibited tumor microenvironment hindered the proliferation of CAR-T cells. Some strategies were designed to improve CAR-T cells proliferation and overcome T cells dysfunction, including incorporating IL-15 or IL-10, deleting checkpoint cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) (Yu et al. 2024; Zhao et al. 2024; Agarwal et al. 2023). And yet the mechanism of regulating CAR-T cells proliferation still not fully understood.

Lymphocyte-specific protein tyrosine kinase (LCK) was reported important for T cells activation, proliferation, differentiation, survival, and immune responses (Salmond et al. 2009; Zamoyska et al. 2003). The expression of LCK affected T cells immune functions. Overexpression of LCK promoted the co-receptor CD4, binding with LCK during T cell receptor (TCR) signal transduction, expression in Th2 cells (Shebzukhov et al. 2017). Enhanced LCK signaling facilitated T cells activation and antitumor responses (Wu et al. 2021). However, abnormal expression of LCK was related with serious immune deficiency of T cells (Lanz et al. 2023; Keller et al. 2023; Lui et al. 2024). It was also reported that in some malignancies, LCK showed higher expression level. The survival of patients suffered from ovarian cancer was reported benefited from high LCK expression (Hinchcliff et al. 2019). In some malignancy treatment, the inhibitors of LCK was used to treat patients, for example, LCK inhibitor dasatinib showed efficacy against T cell acute lymphoblastic leukemia (T-ALL) (Salmond et al. 2009). A small molecular inhibitor of LCK was used to inhibit human glioma cells growth and migration (Zepecki et al. 2019). In chronic lymphocytic leukemia with aberrant expression of LCK, LCK was related with the resistance to dexamethasone (Harr et al. 2010). However, the mechanism of the expression of LCK affecting CAR-T cells proliferation and activation was unclear.

Transcription factor SMAD family member 4 (SMAD4) regulates T cells growth, differentiation and immune response in transforming growth factor β (TGF-β) dependent or independent way (Wan, Feng, and Tang 2021). SMAD4 was reported as an important regulator of activated T cells proliferation, and the deficiency of SMAD4 impaired the proliferation and growth of T cells (Gu et al. 2015; Kim, Lee, and Jun 2017). Chandiran et al. reported that SMAD4 affects the $CD8^+$ T cell differentiation in TGF-β independent way (Chandiran et al. 2022). In mice, the deletion of SMAD4 of T cells increased the cytokine interferon-gamma (IFN-γ) expression (Choi et al. 2022). SMAD4 promoted the cytotoxic function of $CD8^+$ T cells in TCR signaling dependent way rather than TGF-β dependent way (Liu et al. 2022). Choi et al. showed that T cell restricted deletion of SMAD4 in mice leaded to the decreased expression of tumor suppressor p27 resulting in the expansion of $CD4^+$ T cells of colon tumor (Choi et al. 2020).

In many tumors, TGF-β was accumulated in tumor sites. And it was reported that TGF-β suppressed the activation of LCK (Choudhry, Sir, and Sayeed 2001). During T cell activation, the signaling through TGF-β activated protein kinase A (PKA) and c-Src tyrosine kinase (CSK) through SMAD3/4 inhibiting the activation of LCK kinase and Zap70 (Cattley et al. 2020). Activated LCK regulated the cytokine IL-2 secretion, mediating TGF-β resistance (Golumba-Nagy et al. 2018). To overcome TGF-β induced resistance of CAR-T cells in $TGF\text{-}\beta^+$ tumors, researchers deleted the LCK binding motif on the CD28 domain of CAR construct, resulting in improved antitumor efficiency in $TGF\text{-}\beta^+$ tumors (Golumba-Nagy et al. 2018). During CAR-T cell therapy process, whether SMAD4 and LCK keep direct or indirect regulatory relationship still need to answer.

In this study, we focused on the CD19CAR-T cells proliferation, mainly explored the effects of SMAD4 and LCK on CAR-T cells proliferation, and the mechanism that SMAD4 and LCK involving in regulating CAR-T cell proliferation.

2 | Methods

2.1 | Cell Culture

Jurkat E6-1 cells and 293T cells were purchased from cell bank of Chinese Academy of Sciences. Human peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood samples donating by healthy volunteers using Ficoll gradients (Cytiva, Cat#17144002). Jurkat and PBMC cells were cultured in Roswell Park Memorial Institute 1640 (RPMI1640)

medium containing 10% fetal bovine serum (FBS), 100 Unit penicillin and 100 Unit streptomycin in 25 cm^2 flasks (Corning, Cat#3815). And for PBMC culturing, adding 10 ng/mL interleukin-2 (IL-2) in culture medium. 293T cells were cultured in Dulbecco's Modified Eagle Medium/High Glucose (DMEM/High Glucose) medium containing 10% FBS 100 Unit penicillin and 100 Unit streptomycin in 10 cm dishes. Cells were incubated in a humidified cell culture incubator with 5% CO_2 at 37°C.

2.2 | DNA Constructs

To induce lentivirus, antiCD19-CAR, LCK, and SMAD4 gene fragments were inserted into pLenti-CMV vectors, respectively. The single chain fragment variable (scFv) of antiCD19-CAR was synthesized by Sangon company (Morsut et al. 2016). The CD28 and CD3-ζ fragments were amplified through reverse transcription-polymerase chain reaction (RT-PCR) using cDNA of Jurkat cells as template. The scFv, CD28, and CD3-ζ gene fragments were then ligated and inserted into pLenti-CMV vectors through 2 × Seamless Cloning Mix (Biomed, Cat#CL117-01). The LCK fragments were amplified through RT-PCR using cDNA of Jurkat cells as template and then inserted into pLenti-CMV vectors. The pLenti-CMV-SMAD4 plasmids were gift from Prof. Liling Tang. The primers that used to amplify gene fragments were listed in Table 1.

To knockdown LCK and SMAD4, we inserted the short hairpin RNA (shRNA) of LCK and shRNA of SMAD4 into pLKO.1 vectors. The sequences of shRNA of LCK were: forward:5′- CCGG GCATGAACTGGTCCGCCATTACTCGAGTAATGGCGGACCAG TTCATGCTTTTTG-3′, reverse:5′- AATTCAAAAAGCATGAACT GGTCCGCCATTACTCGAGTAATGGCGGACCAGTTCATGC-3′. The sequences of shRNA of SMAD4 were: forward:5′- CCGGG CCAGCTACTTACCATCATAACTCGAGTTATGATGGTAAGTA GCTGGCTTTTTTG-3′, reverse: 5′- AATT*CAAAAAA*GCCAGC TACTTACCATCATAACTCGAGTTATGATGGTAAGTAGCTGG C-3′.

All sequences of constructed plasmids were confirmed by sanger sequencing.

2.3 | Lentivirus Production and Infection

To produce lentiviruses, 293T cells were co-transfected pLenti-CMV plasmids or pLKO.1 plasmids containing the target gene fragments (13 μg), with pCMV-dR8.2-dvpr (13 μg) and pCMV-VSV-G (6.5 μg) using lipofectamine 3000 transfection reagent (Invitrogen, Cat#L3000015) in 10 cm dishes. Seventy-two hours after transfection, the viruses were collected and filtered with a 0.45 μm filter. And then concentrated the viruses through lentivirus concentration solution (Yeasen, Cat#41101ES) following the protocol of manufacturer.

For infection, Jurkat cells were cultured in a 6-well plate. In the following day, cells were changed with 1 mL fresh RPMI1640 culture medium and added 1 mL unconcentrated virus supernatant. Seventy-two hours after infection, Jurkat cells were cultured with RPMI1640 culture medium containing 1 μg/mL puromycin to select infected cells lasting for no less than 1 week. Then the quantitative polymerase chain reaction (qPCR), western blot or flow cytometry assay were performed to confirm the expression of target genes.

For PBMC cells infection, 24 h before infection, the PBMC cells were activated by dynabeads human T-activator CD3/CD28 (ThermoFisher, Cat#11131D). 24-well plates were coated with 20 μg/mL retronectin (Takara, T100A) at 4°C overnight. The next day, diluted the concentrated virus in 300 μL RPMI1640 culture medium and added in 24-well plates (the multiple of infection was two to three). Centrifuging the plate at 1500*g* for 30 min, and then added the PBMC cells into the plates. Centrifuging the plate at 1500*g* for 2 h. Then put the plate into the incubator. Seventy-two hours later, using flow cytometry to check the infection efficiency.

2.4 | RT-PCR and qPCR Assay

Cells were treated as indicated, and then collected the cells. Extracted the total RNA by using molpure cell RNA kit (Yeasen, Cat#19231ES50). Hifair IIII 1st strand cDNA synthesis supermix for qPCR (Yeasen, Cat#11141ES60) was used to get cDNA and Hieff qPCR SYBR green master mix (High Rox plus)

TABLE 1 | Primers used for plasmids construction.

Gene name	Primers	Primer sequences
antiCD19 scFv	Forward	5-CGCGGATCCTCATCTCCGGGCCTTTCGAATTCTCA-3′
antiCD19 scFv	Reverse	5′-CGGCCGCCCCAGTAATCCATTGCATAA-3′
CD28	Forward	5′-GATTACTGGG CGGCCGCAATTGAAGTTATGTATCCTCCTC-3′
CD28	Reverse	5′-ACTCTGTGGTTGCAGTAAAGGGAGCGATAGGCTGCGAAGT-3′
CD3-ζ	Forward	5′-AGAGTGAAGTTCAGCAGGAGCGCAGA-3′
CD3-ζ	Reverse	5′-ACGCGTCGACCTATTAGCGAGGGGGCAGGGCCTGCATGTGAAGGGCGTCGTA-3′
LCK	Forward	5′-CTCCATAGAAGACACCGACTCTAGAGCCACCATGGGCTGTGGCTGCAGC TCACACCCGGA-3′
LCK	Reverse	5′-TTTGTAATCCAGAGGTTGATTGTCGACTCAAGGCTGAGGCTGGTACTGGCCCTCTGT-3′

TABLE 2 | Primers used for qPCR.

Gene name	Primers	Primer sequences
GAPDH	Forward	5′-GGAGCGAGATCCCTCCAAAAT-3′
GAPDH	Reverse	5′-GGCTGTTGTCATACTTCTCATGG-3′
LCK	Forward	5′-TTCTGTGGCTGGTGAATGGG-3′
LCK	Reverse	5′-CGTGAGAGGGCTCATAGCTG-3′
SMAD4	Forward	5′-GCTCCAGCTATCAGTCTGTCA-3′
SMAD4	Reverse	5′-GTCTTGGGTAATCCGGTCCC-3′

(Yeasen, Cat#11203ES08) was used for qPCR. And the primers used for qPCR were listed in Table 2.

2.5 | Western Blot

Cells were treated as indicated and lysed using radio immunoprecipitation assay (RIPA) lysis buffer (CST, CAT#9806). The total protein concentration was measured by bicinchoninic acid (BCA) protein quantification Kit (Yeasen, CAT#20200ES). Thirty micrograms total protein were used for sample loading. The phospho-Lck(Tyr505) (CAT#2751), anti-SMAD4 (CAT#46535S), anti-PI3K (CAT#4249), anti-phospho-PI3K (CAT#13857), anti-Akt (CAT#4685), anti-phospho-Akt (CAT#4060), anti-Bax (CAT#41162), anti-Bcl-2 (CAT#15071), anti-cyclin A (CAT#67955), anti-cyclin B (CAT#4138), anti-CDK2 (CAT#2546) were purchased from cell signaling technology. Anti-CDK1 (CAT#ab133327) were purchased from abcam. Anti-GAPDH antibody (CAT#AC033) were purchased from abclonal. All antibodies were diluted according to manufactural instruction.

2.6 | Basal Activation Assay

Counting $2 \times 10\text{^}6$ Jurkat-GFP or Jurkat-CAR cells and seeded in 6-well plate, cultured with RPMI1640 medium for 48 h, and then stained with pacific Blue-CD69 antibodies (CAT#310919, purchased from biolegend). Analyzed by flow cytometry to show the basal activation.

2.7 | Killing Assay

FACS based killing assay: Nalm-6 cells were infected with lentiviral GFP and Nalm-6-GFP cells were sorted by flow cytometry. Nalm-6-GFP cells were co-cultured with CAR-T cells as indicated effector: target (E:T) ratio for 6 h, and then using flow cytometry to analyze the viability of Nalm-6-GFP cells.

2.8 | Cell Counting kit-8 (CCK-8) Assay

To measure the proliferation of cells, the CCK-8 assay was performed. Counting $1 \times 10\text{^}4$ cells in 100 μL culture medium and seeded into 96-well plate keeping triple replicates at each time point as indicated. Added 10 μL CCK-8 to each well and incubated for 2 h in incubator at each time point. Then measure the OD value at 450 nm.

2.9 | Annexin-V-FITC/PI Staining Assay

Jurkat cells were infected with shRNA as indicated for 72 h. Cells were collected and prepared following the manufacture instruction of Annexin-V-FITC/Propidium Iodide (PI) cell apoptosis kit (Yeasen, CAT#40302ES50). Then using flow cytometer to measure the Annexin-V-FITC/PI signal. The data was analyzed using flowJo software.

2.10 | Trypan Blue Staining Assay

Jurkat cells were infected with shRNA as indicated for 72 h. 0.5 mL cell solution was added with 0.5 mL 0.4% trypan blue solution (Yeason, CAT#40207ES60). Incubating for 5 min at room temperature. Then taking images for stained cells through microscope.

2.11 | Cell Cycle Detection Assay

Jurkat cells were infected with shRNA as indicated for 72 h. Collect the cells and washed with cold dulbecco's phosphate buffered saline (DPBS) once, and then fixed the cells with 70% ethanol at 4°C overnight. Centrifuged and discarded the supernatant, and then washed with cold DPBS once. Stained the cells with PI containing RNase (Yeasen, CAT#40301ES50) and incubated at 37°C for 30 min. Using flow cytometer to analyze the PI signal and cell cycle was analyzed by using Modfit software.

2.12 | Dual Luciferase Reporter Gene Assay

To explore whether the transcription factor SMAD4 regulate the expression of LCK, the dual luciferase reporter gene assay was performed. The promoter fragments of LCK was inserted into pGL3-basic plasmids (pGL3-LP). pGL3-control plasmids were constitutively express firefly luciferase which was used as positive control in this assay. 293T cells co-transfected with phRL-TK constitutively express Rinilla luciferase. pGL3-LP plasmids were co-transfected with phRL-TK with or without pLenti-CMV-SMAD4 in 293T cells. Forty-eight hours later, cells were

collected and prepared for luciferase assay according to the instruction of Dual Glo Luciferase Reporter Gene Assay Kit (Yeason, CAT#11405ES).

The potential binding sites of SMAD4 on promoter of LCK was analyzed according to the Human Transcription Factor Database (http://bioinfo.life.hust.edu.cn). According to the results, we inserted different truncated promoters of LCK into pGL3-basic plasmids, and then co-transfected these truncated plasmids into 293T cells with pLenti-CMV-SMAD4 and phRL-TK. Forty-eight hours later, Dual luciferase assay was performed.

2.13 | Statistical Analysis

Two-tailed student's *t*-tests with unequal variants were used for statistical analysis. All results shown in figures had at least triple independent repeats.

3 | Results

3.1 | CAR Expression Affects the Basal Activation of CAR-T Cells and LCK Kinase

The CD19CAR used in this study composed of scFv that binding to CD19 antigen containing c-Myc, tandemed with CD28 hinge region and transmembrane region, and followed by CD28/CD3-ζ cytoplasmic signaling domain (Supporting Information S7: Figure 1A). We used this CD19CAR construct to produce CD19CAR-T cells and the killing ability was measured by flow cytometer. As shown in Supporting Information S7: Figure 1B,C, when E:T ratio was 1:1, about 42% target cells were lysed by CD19CAR-T cells specifically after 6 h co-culture, indicating the CD19CAR-T cells we constructed here could recognize $CD19^+$ cells and efficiently lyse them.

To explore the effects of CAR expression on basal activation of T cells, Jurkat cells were infected with lenti-viral CD19CAR to generate Jurkat-CD19CAR (JC) cells. The expression of CAR on the surface of Jurkat cells was measured by flow cytometer stained with c-Myc antibody. As shown in Figure 1A, about 98.8% Jurkat cells successfully expressed CAR on the surface. Furthermore, the flow cytometry results showed that the CD69 expression level was significantly higher in JC cells than in Jurkat-vector cells (Figure 1B–D), indicating that the CAR gene expression affected the basal activation level of CAR-T cells.

LCK as an important regulator in TCR signaling, affected the activation, proliferation of T cells. In previous results we know that CAR expression affected the basal activation of T cells, whether basal activation level of LCK was affected as well. To explore this concern, we performed western blot experiment to measure the phosphorylation level of Try505 of LCK, which was negatively regulating LCK activation level (Nyakeriga, Garg, and Joshi 2012). As shown in Figure 1E,F, compared with Jurkat-GFP (JG) cells, the phosphorylation level of LCK(Y505) in JC cells was significantly decreased, which means higher basal activation level of LCK kinase in JC cells. These results showed that the CAR expressed in T cells affected CAR-T cells basal activation and basal phosphorylation level of LCK.

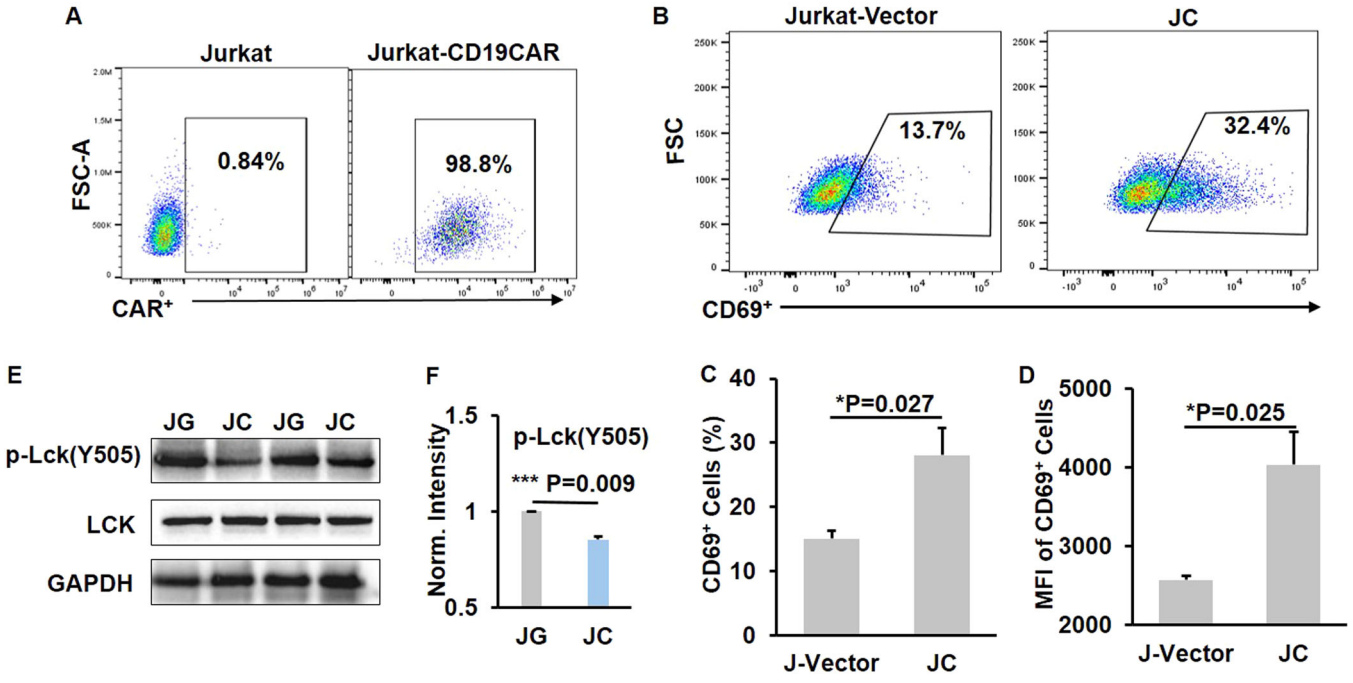

FIGURE 1 | CAR expression in T cells affected cells basal activation and LCK basal phosphorylation. (A) The representative FACS results to show the expression of CAR in Jurkat-CAR cells (JC). (B) The expression of CD69 in Jurkat or JC cells at basal state. (C and D) Bar graph to show the percentage (C) and MFI (D) of $CD69^+$ cells as indicated in (B). (E) Representative images to show the phosphorylation level of LCK (Y505) in Jurkat or JC cells at basal state. (F) Bar graph to show the normalized intensity of phosphorylation level of LCK (Y505) as indicated in (E). Error bar: mean ± SEM. $^*p < 0.05$, $^{***}p < 0.001$.

3.2 | Expression of LCK and SMAD4 Affected the Proliferation of CAR-T Cells

In previous results we know that CAR-T cells are not exactly the same as T cells. LCK regulates the proliferation of T cells, whether it has the same effects on CAR-T cell proliferation. Then we using lenti-virus to over-express or knock-down the expression of LCK in JC cells. The qPCR and western blot results showed that LCK was successfully overexpressed or knock-down in JC cells, respectively (Supporting Information S7: Figure 2A–F). To explore whether different expression level of LCK has effects on the proliferation of CAR-T cells, CCK-8 assay was performed. The results showed that overexpression of LCK in JC cells significantly increased the proliferation rate of JC cells (Figure 2A). However, the proliferation of JC cells was almost inhibited when knock-down LCK expression (Figure 2B), indicating that LCK was essential for CAR-T cells proliferation.

The transcription factor SMAD4 was reported regulating the proliferation, activation and growth of T cells (Wan, Feng, and Tang 2021). To further explore its effect on CAR-T cells proliferation, we recombinant expressed SMAD4 in JC cells or knock-down its expression. As shown in Supporting Information S7: Figure 3A–F, qPCR and western blot results indicated the successfully over-expression or knock-down expression of SMAD4 in JC cells. Furthermore, the CCK-8 assay results showed that over express SMAD4 increased the proliferation rate of JC cells, however, knock-down SMAD4 expression significantly inhibited the proliferation of JC cells (Figure 2C,D). These data showed that SMAD4 was indispensable for CAR-T cells proliferation as well.

3.3 | Knock-Down the Expression of SMAD4 or LCK Affect the Apoptosis and Cell Cycle of CAR-T Cells

Knock-down the expression of LCK kinase or SMAD4 showed seriously inhibition on the proliferation of CAR-T cells. And under microscope, we saw much debris in cell culture medium of JC-shLCK or JC-shSMAD4 cells, as trypan blue staining images showed in Supporting Information S7: Figure 4A, and the flow cytometry results also showed much more debris population in JC-shLCK and JC-shSMAD4 cells group (Supporting Information S7: Figure 4B,C). Then we used the Annexin-v-FITC and PI to stain the cells to identify the apoptosis cells. As shown in Supporting Information S7: Figure 5, the representative images showed that more cells were stained with Annexin-v-FITC and PI in JC-shLCK and JC-shSMAD4 cells, indicating that more cells were apoptotic in these two groups. The Annexin-v-FITC and PI stained cells were also analyzed by flow cytometer, and the results showed that knock-down the expression of LCK or SMAD4 in JC cells increased the percentage of apoptotic cells compared with Jurkat cells (Figure 3A,B). Western blot results also showed that the protein expression of Bax, a proapoptotic protein, was significantly increased in JC-shLCK and JC-shSMAD4 cells, and the protein expression of Bcl-2, an apoptosis inhibitory protein, was significantly decreased in JC-shLCK and JC-shSMAD4 cells (Supporting Information S7: Figure 6A,B). These results suggest that knock-down the expression of LCK or SMAD4 significantly affected the apoptosis of CAR-T cells.

Furthermore, the cell cycle analysis results showed that knock-down the expression of LCK kinase, the G2/M phase

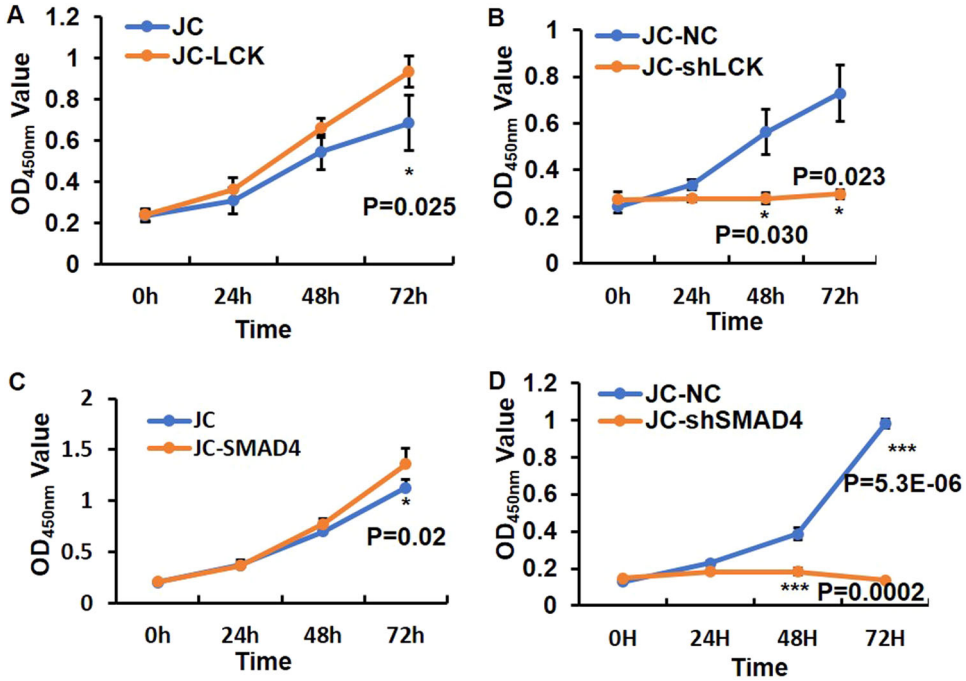

FIGURE 2 | The expression level of LCK and SMAD4 affects CAR-T cells proliferation. (A) The proliferation ability of JC cells overexpressed with LCK. (B) The proliferation ability of JC cells knock-down with LCK. (C) The proliferation ability of JC cells overexpressed with SMAD4. (D) The proliferation ability of JC cells knock-down with SMAD4. Error bars: mean ± SEM. Error bars: mean ± SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

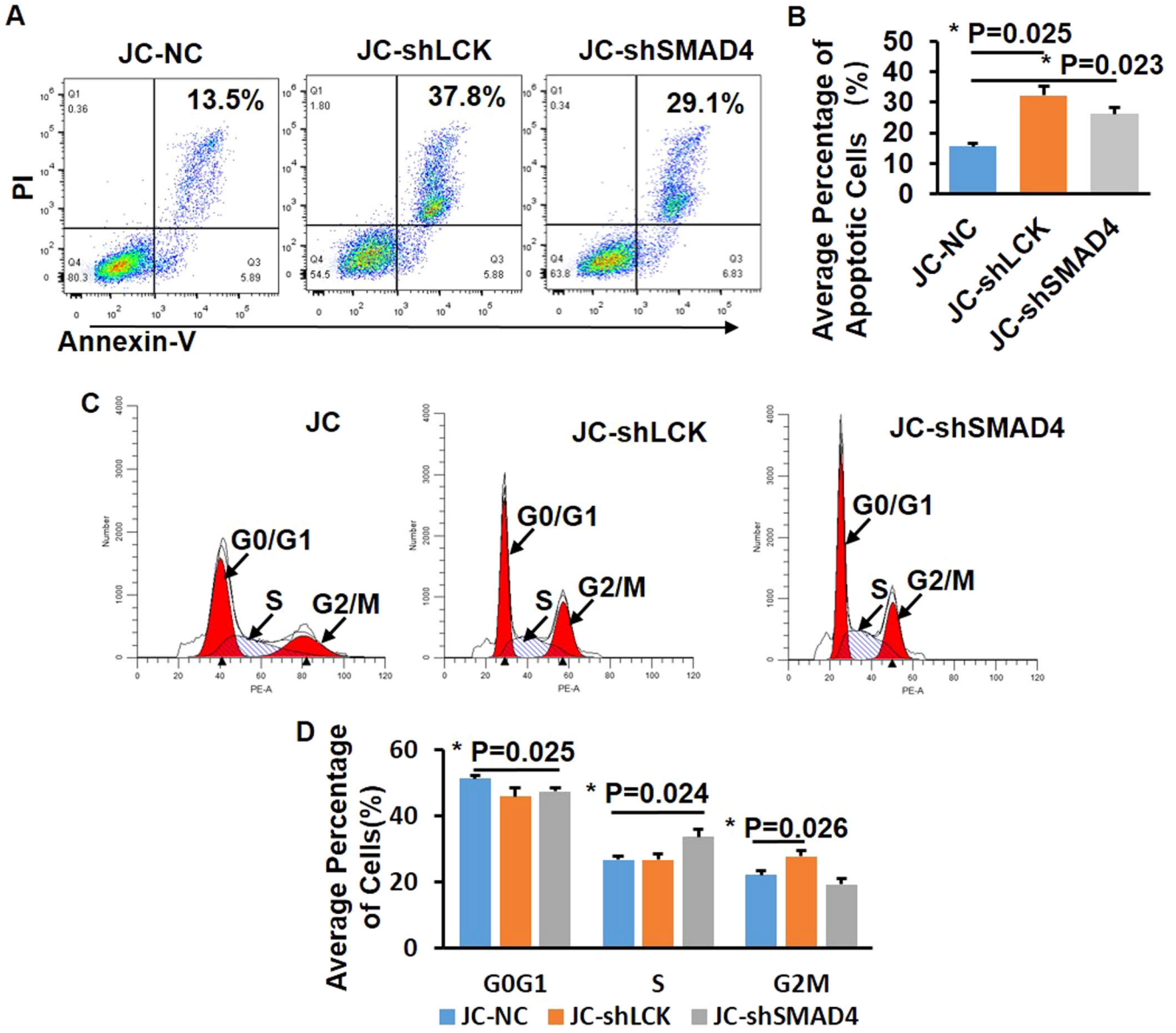

FIGURE 3 | Knocking down expression of LCK or SMAD4 affects the apoptosis and cell cycle of CAR-T cells. (A) Representative flow cytometry results to show the effects of knocking down expression of LCK or SMAD4 on the apoptosis of Jurkat-CAR cells. (B) Bar graph to show the percentage of apoptotic JC cells as indicated in (A). (C) The effects of knocking down expression of LCK or SMAD4 on the cell cycle of Jurkat-CAR cells. (D) Bar graph to show the average percentage of JC cells in different cell cycle phases as indicated in (C). Error bars: mean ± SEM. $*p < 0.05$.

cells were increased (Figure 3C,D). However, knock-down the expression of SMAD4, the S phase cells were increased (Figure 3C,D). To further explore the cell cycle of JC-shLCK and JC-shSMAD4 cells, we performed western blot experiment to measure the expression of cell cycle related proteins. As shown in Supporting Information S7: Figure 6C,D, the cyclin B, a cell cycle protein regulating the G2 phase to M phase, was significantly accumulated in JC-shLCK cells, and cyclin A, a cell cycle protein regulating the S phase to G2 phase, was significantly increased in both JC-shLCK and JC-shSMAD4 cells, consistent with the flow cytometry analyzed results as shown in Figure 3C,D. These data indicated that knock-down LCK expression, the cell cycle of CAR-T cells was arrested at G2/M phase, while knock-down SMAD4 expression, the cell cycle of CAR-T cells were arrested at S phase. But finally, cells go into apoptosis.

3.4 | LCK and SMAD4 Affects CAR-T Cells Proliferation Through PI3K/Akt Signaling Pathway

In previous results we know that LCK and SMAD4 are essential for the proliferation of CAR-T cells, however, the mechanism of LCK and SMAD4 regulate CAR-T cells proliferation was unclear. PI3K/Akt signaling pathway related to T cells proliferation. To explore whether LCK and SMAD4 regulate CAR-T cells proliferation by PI3K/Akt signaling, we measured the phosphorylation level of PI3K and Akt in JC cells overexpressed

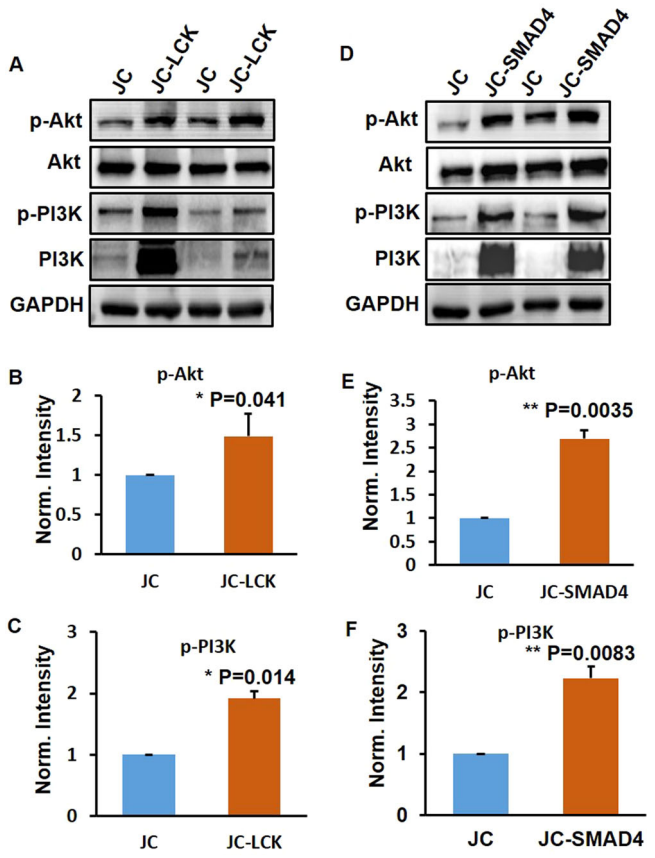

FIGURE 4 | Overexpression LCK or SMAD4 in Jurkat-CAR cells affected the basal phosphorylation level of PI3K and Akt. (A) Representative blot images to show the phosphorylation level of PI3K and Akt in JC cells overexpressed with LCK. (B and C), bar graphs to show the normalized intensity of p-Akt and p-PI3K as indicated in (A). (D) Representative blot images to show the phosphorylation level of PI3K and Akt in JC cells overexpressed with SMAD4. (E and F) Bar graphs to show the normalized intensity of p-Akt and p-PI3K as indicated in (D). Error bars: mean ± SEM. $*p < 0.05$, $**p < 0.01$.

with LCK or SMAD4. As shown in Figure 4, the phosphorylation levels of PI3K and Akt were significantly higher in JC-LCK cells and JC-SMAD4 cells compared with JC cells, indicating that overexpression of LCK and SMAD4 in JC cells activated the PI3K/Akt signaling pathway.

To further confirm that LCK and SMAD4 regulate JC cells proliferation through PI3K/Akt signaling pathway, we first used LY294002, an inhibitor of PI3K and Akt, to treat cells as indicated, and the phosphorylation level of PI3K and Akt in JC cells was significantly inhibited (Figure 5A). And we also used MK2206, which was reported as an inhibitor of Akt, to treat cells. In our experiment, MK2206 treatment decreased the phosphorylation of both PI3K and Akt in JC cells as well (Figure 5B). Then, we measured the proliferation of JC-LCK and JC-SMAD4 cells treated with LY294002 or MK2206 by CCK-8 assay. As shown in Figure 5C–F, when treated with LY294002 or MK2206, the proliferation of JC-LCK and JC-SMAD4 cells were significantly inhibited. These data suggested that LY294002 and MK2206 inhibited the phosphorylation of PI3K and Akt, leading to the proliferation inhibition of JC-LCK and JC-SMAD4 cells, indicating that LCK and SMAD4 affected the proliferation of CAR-T cells probably through regulating PI3K/Akt signaling transduction.

3.5 | SMAD4 Regulates LCK Expression by Binding on LCK Promoter in CAR-T Cells

Till now, our results showed that LCK and SMAD4 have similar effects on the proliferation of CAR-T cells, hinting us that there may be some kind of regulatory relationship between transcription factor SMAD4 and LCK kinase. To explore this, we measured the gene and protein expression of SMAD4 in JC-shLCK cells by qPCR and western blot, respectively. As shown in Figure 6A–C, in JC cells, knock-down the expression of LCK has no significant effects on the expression of SMAD4 in both mRNA and protein levels. However, knock-down the expression of SMAD4 in JC cells significantly inhibited the expression of LCK in both mRNA and protein levels (Figure 6D–F). These data point out that transcription factor SMAD4 was an upstream regulator of LCK.

To further explore the regulation relationship between SMAD4 and LCK, we performed dual luciferase assay to measure whether transcription factor SMAD4 binds to the promoter region of LCK to regulate its expression. As shown in Figure 7A, only co-transfected SMAD4 with LCK-promoter (transcription starting site ATG upstream 2000bp) reporter gene, the luciferase signal was positive, indicating SMAD4 binding on the promoter of LCK to trigger reporter gene expression. Furthermore, we analyzed the possible binding sites of SMAD4 on LCK promoter through *human transcription factor database* (http://bioinfo.life.hust.edu.cn). The analyzed results showed that there were 10 possible sites for SMAD4 binding on the promoter region of LCK (Figure 7B). To confirm which site was SMAD4 binding on, we constructed luciferase reporter plasmids inserted with nine different truncated promoter region of LCK, respectively. And named them a, b, c, d, e, f, g, h, and i (Figure 7B). As shown in Figure 7C, the luciferase signal of JC cells co-transfected with SMAD4 and f, g, h, or i were significantly decreased, suggested that e region was essential for SMAD4 binding to regulate reporter gene expression, indicating that SMAD4 probably binds to the promoter region of LCK between 1169 and 1388 bp (Figure 7B, fragment between two blue lines). And the sequences of possible binding sites were *CTGTCTTCCACCT* and/or *CAGCAGG*.

In our study, we reported that CAR gene expression in T cells affected the basal activation of CAR-T cells and the basal activation of LCK kinase. Furthermore, the LCK and transcription factor SMAD4 were essential for CAR-T cells proliferation, apoptosis and cell cycle regulation. The mechanism exploring results showed that transcription factor SMAD4 binding on the promoter region of LCK to regulate its expression, and affecting the activation of PI3K/Akt signaling pathway, regulating CAR-T cells proliferation, apoptosis and cell cycle process (Figure 7D).

4 | Discussion

Continuous Stimulation of CAR-T cells with CD3/CD28 antibody or other cytokines during the process of CAR-T cells

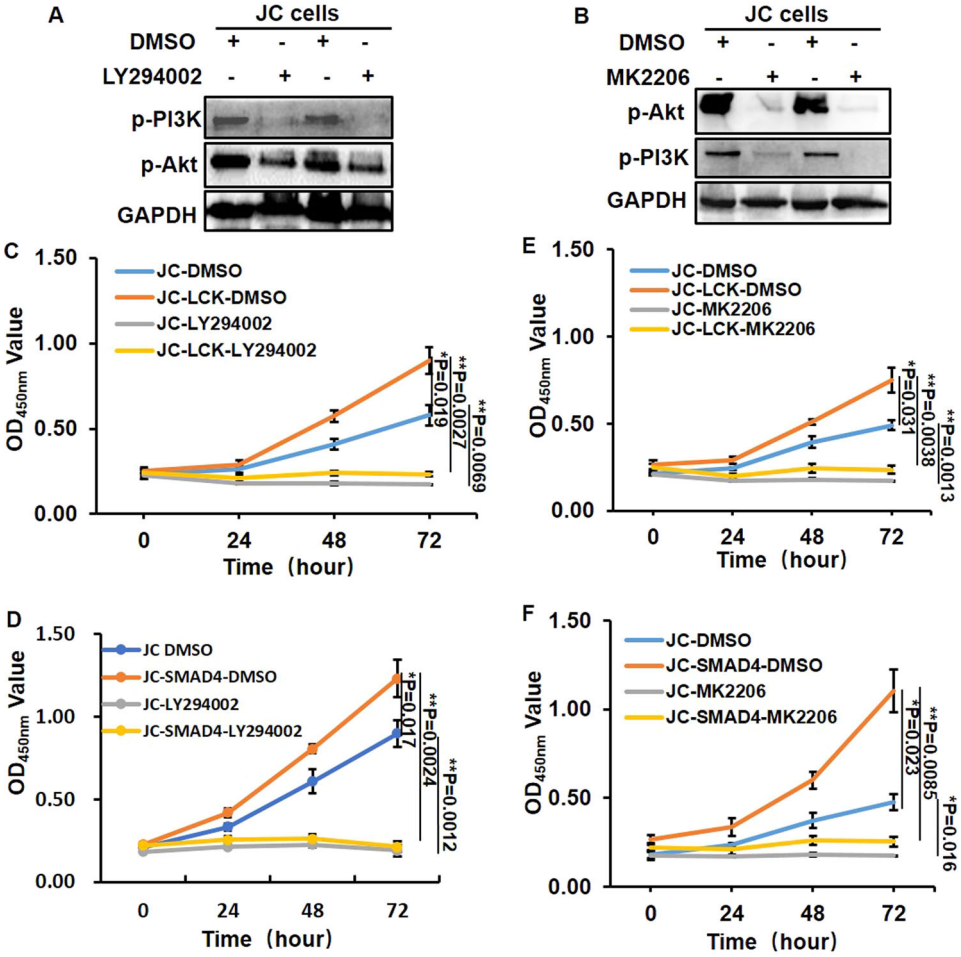

FIGURE 5 | Inhibitor of p-PI3K or p-Akt hindered the high proliferation rate in JC cells overexpressed with LCK or SMAD4. (A and B) Representative blot images to show the phosphorylation level of p-PI3K and p-Akt in JC cells treated with LY234002 or MK2206, respectively. (C and D) The effects of LY294002 treatment on the proliferation of JC cells overexpressed with LCK or SMAD4, respectively. (E and F) The effects of MK2206 treatment on the proliferation of JC cells overexpressed with LCK or SMAD4, respectively. Error bars: mean ± SEM. $*p < 0.05$, $**p < 0.01$.

production leading to CAR-T cells continuous activation and early exhaustion (Wang, Qin, and Li 2023; Scharping et al. 2021). To against exhaustion of CAR-T cells, researchers used 4-1BB co-stimulatory domain to construct CD19CAR-T cells rather than CD28 reduced the exhaustion and improved the persistent of CAR-T cells (Long et al. 2015). Other strategies including blockade of PD-1 signaling, deplete TOX/NR4A, SMAD3 inhibitor delivered into the tumor microenvironment were used to against CAR-T cells exhaustion (Gumber and Wang 2022; Seo et al. 2019). In our study, we saw that recombinant expression of CAR in T cells significantly affected the basal activation of CAR-T cells, which was related to the exhaustion of CAR-T cells. And we found that CAR expression in T cells leads to the high basal activation level of LCK kinase, which was important for T cell activation and proliferation. These results gave us new insights on why CAR-T cells are more easily goes into exhausted stage than T cells. And suppressing the basal activation level of CAR-T cells caused by CAR gene recombinant expression during CAR-T cell producing probably was a strategy to produce low exhausted CAR-T cells.

Transcription factor SMAD4 is an important regulator in TGF-β signaling transduction. However, the effects of TGF-β and SMAD4 on the proliferation of T cells are different. TGF-β signaling significantly inhibited T cells proliferation, immune response and so on (Wang et al. 2020). At the same time, TGF-β could help tumor cells immune escape. SMAD4 was reported promote the proliferation of activated T cells in an TGF-β independent pathway (Gu et al. 2015; Wang et al. 2020). Loss expression of SMAD4 have some effects on T cells proliferation, growth and TGF-β induced differentiation (Kim, Lee, and Jun 2017). In our study, we found that SMAD4 was important for the proliferation of CAR-T cells since overexpression of SMAD4 promoted the proliferation of CAR-T cells and knock-down SMAD4 expression inhibited the proliferation of CAR-T cells seriously, which was consistent with others reports (Kim, Lee, and Jun 2017). Further study showed that knock-down SMAD4 expression arrested the cell cycle of CAR-T cells mainly in S phase and more cells became apoptotic. These results indicated that SMAD4 was essential for CAR-T cells proliferation and survival and need to be considered in CAR-T cell production. However, the mechanism of SMAD4 regulating the cell cycle and cell apoptosis of CAR-T cells was still unclear.

In TCR signal transduction, LCK was reported as the first phosphorylated kinase, regulating the activation, development and adaptive immune response of T cells (Chakraborty and

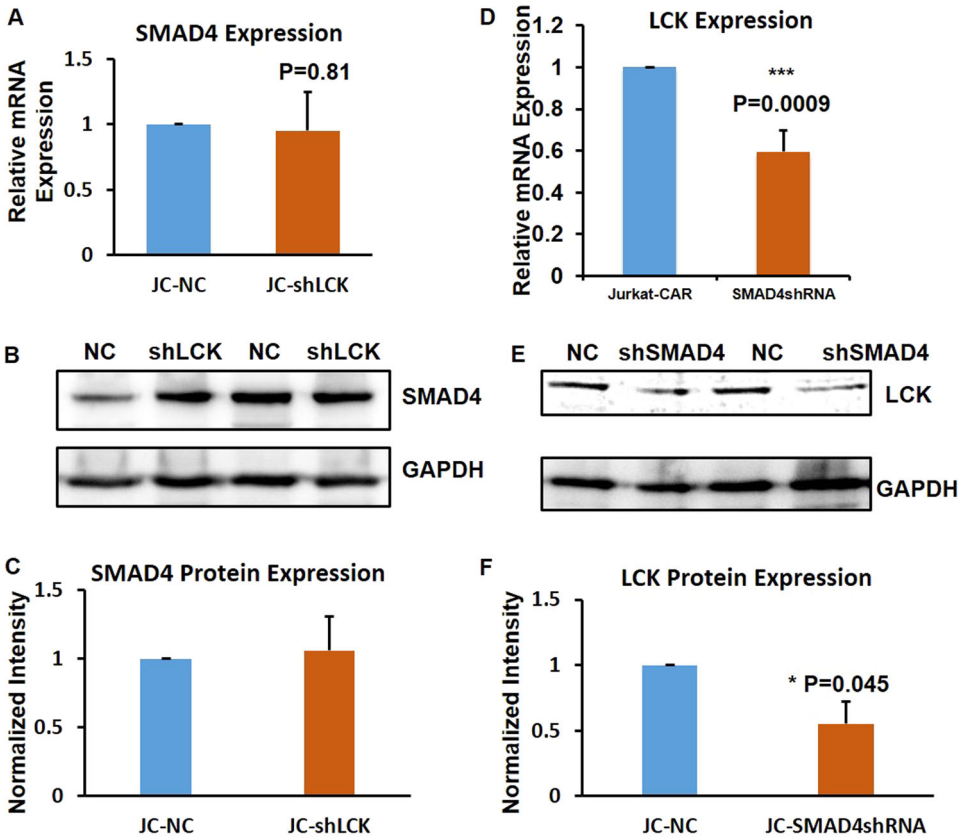

FIGURE 6 | The expression level of SMAD4 affected LCK expression in JC cells. (A) qPCR results to show the SMAD4 expression level in JC-shLCK cells. (B) Representative blot images to show SMAD4 protein expression level in JC-shLCK cells. (C) Bar graphs to show the normalized intensity of SMAD4 protein expression as indicated in (B). (D) qPCR results to show the LCK expression level of JC-shSMAD4 cells. (E) Representative blot images to show LCK protein expression level in JC-shSMAD4 cells. (F) Bar graphs to show the normalized intensity of LCK protein expression as indicated in (E). Error bars: mean ± SEM. $^{*}p < 0.05$, $^{***}p < 0.001$.

Weiss 2014; Van Laethem et al. 2013). The abnormal expression, activation or transport of LCK could lead to serious immunodeficiency diseases (Gorska and Alam 2012). Peripheral T cells lacking LCK expression cannot be activated through TCR, and T cell development was severely affected in the absence of LCK (Molina et al. 1992). Here, we reported that LCK was important for CAR-T cells proliferation, and when over-express LCK in CD19CAR-T cells, the proliferation of cells was significantly improved, however, when knock-down LCK expression severely inhibited the proliferation of CAR-T cells, and more cells became apoptotic. Further analysis results showed that the cell cycle of CAR-T cells lacking LCK expression was arrested mainly at G2/M phase. These results indicated that LCK was essential for CAR-T cells proliferation and survival.

LCK kinase is important for T cell proliferation, however, the mechanism of the expression and activation of LCK kinase in T cell was still controversial, and the abnormal expression of LCK affected T cells functions. It was reported that, TGF-β altered the overall phosphotyrosine signaling through SMAD3/SMAD4 during T cell activation, including the phosphorylation of Zap70 and PI3K (Cattley et al. 2020). After TCR signal triggering, LCK kinase was activated and then activated LCK phosphorylated the Zap70 to regulate TCR signal transduction (Gaud, Lesourne, and Love 2018). These reported results suggested that there is probably some regulatory relationship between SMAD4 and LCK. To explore this, in this study, we showed that knock-down the expression of SMAD4 significantly inhibited LCK expression both in gene and protein levels, however, when knock-down the LCK expression showed no much influence on the SMAD4 expression, indicating that SMAD4 was an upstream regulator of LCK. Furthermore, dual luciferase analysis results showed that transcription factor SMAD4 binds to the promoter region of LCK to regulate its expression, which others was not reported elsewhere. And over-express SMAD4 or LCK in CAR-T cells significantly improved the phosphorylation level of Akt and PI3K, and the cells proliferation, which was inhibited when treated with inhibitors of Akt and PI3K. These results implied that transcription factor SMAD4 regulate LCK expression.

In conclusion, CAR-T cells were not exactly same as T cells. And we reported that both LCK and SMAD4 are important for CAR-T cells proliferation and survival. SMAD4 regulate the expression of LCK to affect the PI3K/Akt phosphorylation to regulate CAR-T cells proliferation. These results give us new insights in CAR-T cells proliferation regulation mechanism. And here we first reported the transcription factor SMAD4 binds on the promoter region of LCK to regulate its expression. Although our results showed that the possible binding sites were *CTGTCTTCCACCT* and/or *CAGCAGG* between "1169-1388" as shown in Figure 7B, more experiments need to do to

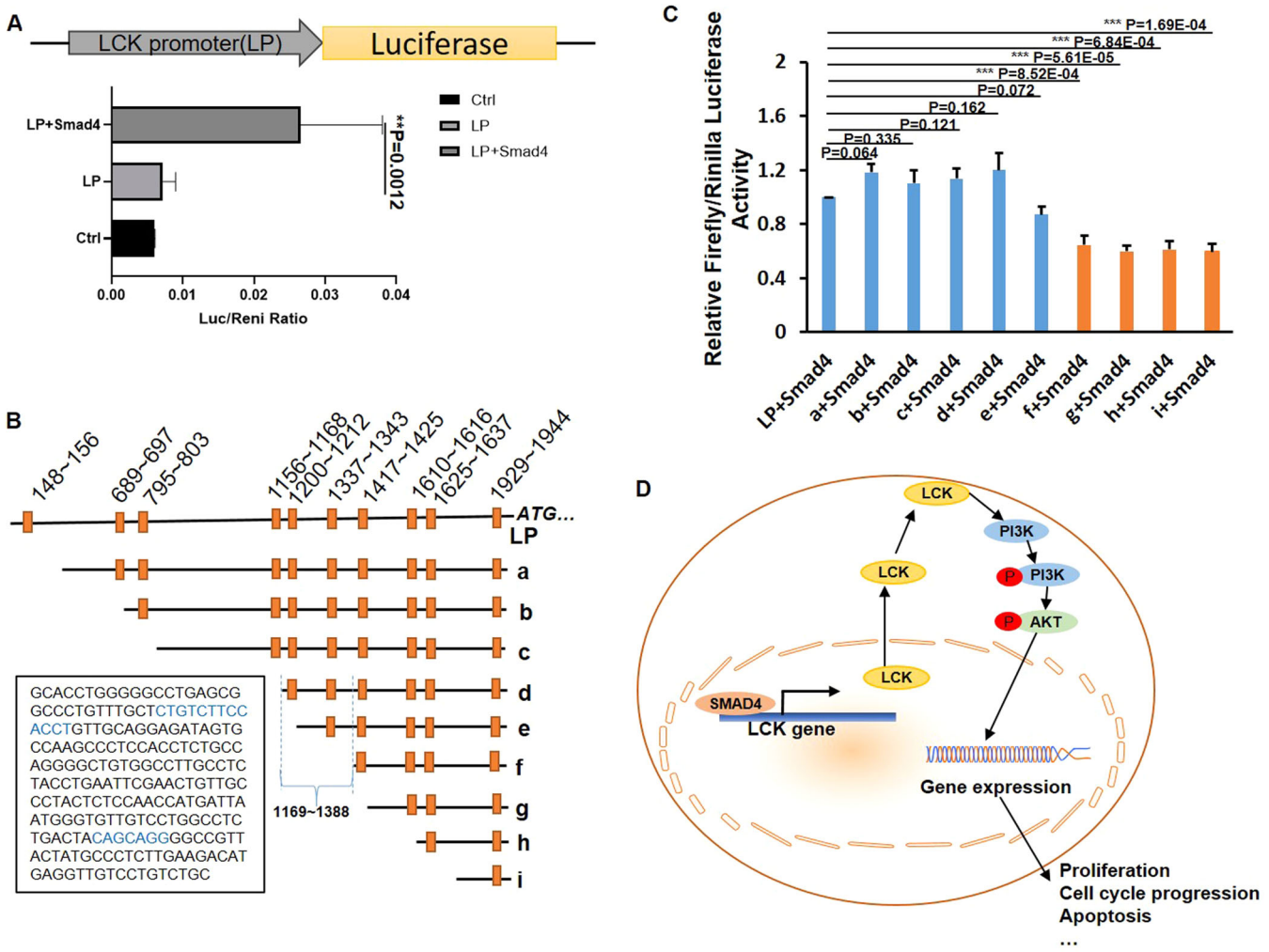

FIGURE 7 | Transcription factor SMAD4 bound on the promoter of LCK. (A) Dul luciferase assay results to show the SMAD4 bound on the promoter of LCK or not (Error bars: mean ± SD). (B) The schematic diagram to show the potential binding sites of SMAD4 on the promoter of LCK, and the truncated promoter fragments inserted into reporter plasmids. The DNA sequence showed in box was sequence of "1169-1388" region, and the possible binding sites were marked in blue font. (C) Bar graphs to show relative firefly/rinilla luciferase signal in JC cells co-transfected with SMAD4 and different truncated promoters of LCK (Error bars: mean ± SEM). (D) The schematic diagram to show SMAD4 and LCK regulate CAR-T cells biological processes through PI3K/Akt signaling. $^{**}p < 0.01$, $^{***}p < 0.001$.

confirm this. And SMAD4 regulates LCK expression is TGF-β dependent or independent still need more work to define.

Author Contributions

Rongxue Wan, Bowen Fu, Xiaokang Fu, Zengping Liu, Nafeisha Simayi, Yongshui Fu, Huaqin Liang, Chengyao Li, and Wenhua Huang conceived the project. Rongxue Wan, Bowen Fu, Xiaokang Fu, Zengping Liu, and Nafeisha Simayi performed the experiments. Rongxue Wan and Bowen Fu ananlyzed data. Rongxue Wan wrote the manuscript. Bowen Fu, Yongshui Fu, Huaqin Liang, Chengyao Li, and Wenhua Huang revised the manuscript. All authors contributed to the article and approved the submitted version.

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Ethic Statement

Human peripheral blood mononuclear cells (PBMCs) isolated from healthy peripheral blood samples was approved by the Guangzhou Blood Center Human Research Ethics committee (NO. 2024032).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Please contact Rongxue Wan through wrx1253071629@163.com to get raw data.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.