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Research Article

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PICK1 modulates proliferation and migration of gastric cancer cells via regulating TLR4

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Abstract: Protein interacting with C kinase 1 (PICK1) interacts with a variety of membrane proteins and receptors involved in nervous system diseases and multiple cancers. However, the role of PICK1 in gastric cancer remains unclear. In the present work, we explored the expression and interactions of PICK1 with Toll-like receptor 4 (TLR4) in gastric cancer. Clinical data analysis showed that PICK1 expression decreases and is predictive of worse outcomes in patients with gastric cancer. High PICK1 levels attenuated the proliferation and migration of gastric cancer cells, which is dependent on the TLR4/MYD88 signaling pathway. Furthermore, in vitro experiments demonstrated that PICK1 affects the trafficking and degradation of TLR4 and promotes TLR4 degradation via autophagy in gastric cancer cells. Molecular dynamics simulations highlighted the binding strength and stability of the TLR4-PICK1 complex. Our study provides new insights into the cellular and pathological functions of PICK1 in gastric cancer.

Key words: Protein interacting with C kinase 1; Gastric cancer; Toll-like receptor 4; MYD88 signalling pathway; Autophagy

1 Introduction

Gastric cancer, which occurs in the digestive tract, is the third leading cancer in the world in terms of mortality (Siegel et al., 2023). Therapeutic strategies have advanced significantly in the past few years, including chemotherapy, radiotherapy, immunotherapy, and surgical resection; however, invasion and metastasis

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are still frequent and lead to poor outcomes in many patients (Park and Chun, 2013; Lee et al., 2013; Stein et al., 2014; Paulo and Godoy, 2013). Therefore, it is necessary to investigate the mechanisms of gastric cancer, improve the diagnosis and prognosis of patients, and enhance prevention strategies.

Protein interacting with C kinase 1 (PICK1), as a peripheral membrane protein comprising the PSD-95/Discs-large/Zona Occludens-1 (PDZ) and Bin/Amphiphysin/Rvs (BAR) domains, can bind to a variety of lipid molecules and membrane proteins (Hanley, 2008; Inoue, 2007), such as acid-sensing ion channels, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors, and ErbB2/Her-2 (Baron et al., 2002; Hanley and Henley, 2005; Jaulin-Bastard et al., 2001; Lu and Ziff, 2005; Rocca et al., 2008; Xia et al., 1999). Abnormal expression of PICK1 in the brain has been shown to correlate with nervous system diseases, such as epilepsy, schizophrenia, and Parkinson's disease (Gainetdinov and Caron, 2003; "Giros B et al., 1996; Jones et al., 1998; Lorgen et al., 2017; Torres et al., 2001). Recent studies suggest the important role of PICK1 in cancer progression and metastasis. For instance, increased expression of PICK1 contributes to the growth and migratory potential of astrocytic tumour cells (Cockbill et al., 2015). PICK1 is overexpressed in breast cancer and correlates with lymph node infiltration, tumour grade and the expression of Her-2/neu protein, indicating that it may serve as a prognostic biomarker and therapeutic target in invasive breast cancer (Zhang et al., 2010). Our previous study showed that PICK1 is involved in epithelial-mesenchymal transformation (EMT) and inhibits the progression of gastric cancer (Zhou et al., 2021); however, the details of this effect remain to be discovered.

Toll-like receptor 4 (TLR4) is a transmembrane pattern recognition receptor with an important role in innate and adaptive immune response (Pasare and Medzhitov, 2005). Recently, several studies have demonstrated the vital role of TLR4 in the initiation, metastasis and progression of cancer (Chen et al., 2008; Rakoff-Nahoum and Medzhitov, 2009). TLR4 is overexpressed in various solid tumors and cancer cell lines, such as colorectal, cervical and breast cancer (Chen et al., 2015; Wang et al., 2010; Zhang and Zhang, 2017). A study by Yang et al. (Yang et al., 2016) has shown that TLR4/NF-κB signaling is associated with the survival of oral squamous cell carcinoma patients. Moreover, high expression levels of TLR4 were associated with gastric cancer progression (Meliț et al., 2019). TLR4, a membrane protein, may participate in the mechanism of action of PICK1 in gastric cancer.

In the present study, we aimed to explore whether PICK1 protein is involved in the proliferation and migration of gastric cancer cells and whether it has an interaction with TLR4 in gastric cancer.

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2 Materials and methods

2.1 Microarray data

The mRNA expression profiles and data were downloaded from the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo), with 300 gastric cancer samples from the GSE62254 dataset and 200 from the GSE15459 dataset. Among these, eight unavailable samples were excluded from the GSE15459 dataset, leaving a total of 492 gastric cancer samples for analysis (Zhou et al., 2021).

2.2 Clinical specimens

The clinical specimens were obtained from the Department of Gastroenterology, Zhejiang Provincial People's Hospital, and Shanghai Outdo Biotech Company (http://www.superchip.com.cn).

2.3 Immunohistochemical (IHC) analysis

Fifty-six human gastric samples derived from HStmA180Su16 tissue microarray were used to detect PICK1 expression by IHC. Specific antibodies against PICK1 (1:2000 (volume ratio), HuaBio, Hangzhou, China) were employed to determine the staining density. Scores were calculated based on the staining intensity and percentage of positive tumor cells in the whole tissues, which were evaluated according to the Fromowitz standard. All staining was assessed at 200× magnification, and at least three fields from each core were counted.

2.4 Cell culture and treatment

The human gastric cancer cell lines AGS (RRID: CVCL_0139), BGC-823 (RRID: CVCL_3360), SGC-7901 (RRID: CVCL_0520), Human Embryonic Kidney Cells 293 (HEK293T) (RRID: CVCL_0063), and GES-1 (RRID: CVCL_EQ22) were purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China). All cells were grown in DMEM (Hyclone, Shanghai, China) supplemented with 10% FBS (volume fraction) (FBS, Gibco, USA), penicillin (100 U/mL)-streptomycin (0.1 mg/mL) in a cell incubator at 37 °C with 5% CO_2 (volume fraction). To construct stable PICK1 overexpression gastric cells, AGS cells were infected with OV-PICK1 or control lentivirus at a multiplicity of infection (MOI) of 5, according to the company's instructions. After 24 h of infection, the cells were incubated with puromycin (5 μg/mL) for 10 d to select stably expressing cells. Lipopolysaccharide (LPS) extracted from *Escherichia coli* serotype O55:B5 (LPS 055:B5) (Sigma-Aldrich, Dorset, UK) was dispersed in phosphate-buffered saline (PBS) at 100 ng/mL. For the in vitro experiments, the cells were incubated with LPS for 2 h before further examination. Cells were incubated with FSC-231 (15 μmol/L) (Merck, Shanghai, China), MG132 (1 μmol/L) (Merck, Shanghai, China), and chloroquine (30 μmol/L) (Merck, Shanghai, China) for 24 h.

2.5 Cell growth and proliferation

The cell suspension was added to 96-well plates. After 24 h, the original culture medium in the well plate was replaced with 100 μL of CCK-8 solution (Vazyme, Nanjing, China) (culture medium: CCK-8 reagent=9:1 (volume ratio)). The cells were incubated for 2 h and the optical density (OD) value at 450 nm was measured by a microplate reader (Tecan, Männedorf, Switzerland).

The cell suspension was added to 6-well plates. After 10 d, the cells were washed with PBS. The cells were fixed with 4% paraformaldehyde (volume fraction) (Servicebio, Wuhan, China) for 15 min, then stained by crystal violet solution for 10 min and photographed.

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2.6 Cell cycle FACS analysis

The cell suspension was added to 12-well plates. After 24 h, serum-free medium was added for another 24 h to realize cell cycle synchronization. The treated cells were collected, fixed with pre-cooled 70% ethanol (volume fraction), and then stained with propidium iodide (PI). The cell cycle was analysed by flow chemistry (BD Biosciences, Franklin Lakes, NJ, USA).

2.7 Transwell migration assay

The treated AGS cell suspension was added to the upper chambers of 24-well plates (2×10^4 cells/well) with serum-free medium, while DMEM with 10% FBS (volume fraction) was added to the lower chambers. After 24 h, the cells on the upper chambers were removed and the cells passing through the membrane were fixed with 4% paraformaldehyde (volume fraction) and stained with crystal violet solution. Images of cells were taken using a microscope (Nikon).

2.8 Scratch assay

The cell suspension was added to 6-well plates. Until the cells reached 90%–100% confluency, a 1-mm straight scratch on the wells was made using a sterile pipette tip, and 10 μg/mL mitomycin C (Sigma-Aldrich Dorset, UK) was added for 1 h. The medium with low serum was added, and images of scratches were captured at 0 h, 24 h and 48 h. The images of cell migration were analyzed by ImageJ, and wound healing was calculated and compared with the initial wound area.

2.9 Western blotting

Briefly, equal amounts (20–30 μg) of proteins were separated by sodium dodecyl sulphate-polyacrylamide gel and transferred onto PVDF membrane (Millipore, Billerica, MA, USA). After blocking in 5% non-fat milk (mass fraction) for 1 h, the membranes were incubated with primary antibodies overnight at 4 °C, including

anti-CDK4 (1:1000 (volume ratio, the same below), HuaBio, Hangzhou, China), anti-CDK6 (1:1000, HuaBio, Hangzhou, China), anti-PICK1 (1:2000, HuaBio, Hangzhou, China), anti-TLR4 (1:1000, HuaBio, Hangzhou, China), anti-GAPDH (1:5000, HuaBio, Hangzhou, China), anti-MYD-88 (1:1000, CST, Massachusetts, USA), anti-phospho-NF-κB (1:1000, CST, Massachusetts, USA), and IL-12 (1:1000, CST, Massachusetts, USA). The membranes were subsequently incubated with HRP-labeled secondary antibodies (1:3000, HuaBio, Hangzhou, China) for 2 h. The protein bands were detected by ECL reagent (Beyotime, Shanghai, China) and the Bio-Rad image system (Bio-Rad, California, USA).

2.10 Immunofluorescence

Plasmids encoding PICK1 and TLR4 were purchased from Addgene. HEK293T cells were transfected with the PICK1 and TLR4 plasmids using Lipofectamine 3000 (Invitrogen) for 72 h, followed by immunofluorescence staining. Briefly, HEK293T cells were fixed with 4% paraformaldehyde (volume fraction) for 10 min and the cell membranes were permeabilized with 0.2% Triton X-100 (volume fraction) for 15 min. Then, cells were blocked with 10% BSA (mass fraction) and incubated with primary anti-PICK1 (1:500 (volume ratio, the same below), NeuroMab, Davis, CA) or anti-TLR4 (1:500, Abcam, Cambridge, UK) overnight at 4 °C, followed by incubation with solution of secondary antibodies (Thermo, Rockford, IL) for 1 h. DAPI was used for staining the cell nuclei. Finally, the fluorescent signals of cells were observed and captured using a confocal microscope (Leica SP8, Germany).

2.11 Co-immunoprecipitation

HEK293T cells were lysed in the IP-RIPA buffer (Beyotime, Shanghai, China) and centrifugated at 16,000×*g* at 4 °C for 12 min. The protein concentration was detected using a BCA kit assay (Thermo, Rockford, IL). To detect the interaction of PICK1 and TLR4, the cell lysates were incubated overnight at 4 °C with negative control IgG (Invitrogen, Carlsbad, CA, USA), or anti-PICK1, anti-TLR4 antibody and protein A-Sepharose 4B beads (GE Healthcare, Pittsburgh, PA). Proteins on the beads were collected using 2×SDS sample buffer, finally boiled at 100 °C for 5 min prior to a western blotting test.

2.12 Surface Plasmon Resonance (SPR)

SPR experiments were performed using a Biacore-8K (Cytiva, Massachusetts, USA). PICK1 proteins were immobilized on a CM5 chip (Cytiva, Massachusetts, USA) using the amino coupling method. TLR4 proteins were diluted from 100 to 50, 25, 12.5, 6.25, 3.12, 1.56 nM by 5% DMSO PBS (volume fraction) and injected into the flow cell at a flow rate of 30 μL/min. The contact time was 180 s and the dissociation time was 300 s. The results were analyzed using Biacore Evaluation Software.

2.13 Molecular dynamics simulations

Molecular dynamics (MD) simulations were conducted to evaluate the interactions between PICK1 and TLR4 at the atomic level. The 3D structures of PICK1 and TLR4 were obtained using UniProt codes Q9NRD5 and O00206, respectively. They were optimized using Alphafold2 (Vani et al., 2023), followed by UCSF chimaeras to remove water molecules and unrelated heteroatoms (Bedart et al., 2022). All MD simulations were performed using Gromacs 5.1.5. The temperature (298.15 K), PH (7), and pressure (1 bar per atmosphere) were selected to build the all-atom model system with the AMBEff14SB position parameters (Bedart et al., 2022). TIP3P was used for the water molecular simulations (Mohammadi et al., 2023). After 1,000 ps of energy minimisation, the system was balanced within the NVT and NPT procedures. We performed 50 ns MD simulations, with 2 fs per step. After all simulations were completed, the gmx module was used to calculate the radius of gyration (Rg), hydrophobic contact, and root-mean-square deviation (RMSD).

2.14 Binding energy analysis

The binding energies between PICK1 and TLR4 were calculated using molecular mechanics with gener-

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alized Born and surface area solvation (MM/GBSA) implemented in the MMPBSA package (Shoaib et al., 2023). In the MM/GBSA method, the binding-free energy (ΔG_{bind})=ΔH-$T_{\Delta S}$≈ΔG_{solv} +ΔG_{GAS}-$T_{\Delta S}$, where ΔG_{GAS} denotes the kinetic energy in vacuum before and after the binding, including E_{int}, E_{vdw}, and E_{elec}; ΔG_{solv} is a solvent effect term, divided into the polar term ΔE_{GB} and the non-polar term ΔE_{surf}; -$T_{\Delta S}$ denotes the entropy change, which reflects the energy loss caused by the flexibility or stability of the system, which was ignored in accordance with several studies (Yan et al., 2020).

2.15 Statistical analysis

All data were presented as mean±standard deviation (SD). Statistical analyses were calculated using unpaired Student's t-tests in GraphPad Prism 9.0. Log-rank test and Kaplan-Meier analysis were carried out to analyze the differences in survival rates. P<0.05 was considered as indicative of statistical significance.

3 Results

3.1 Expression of PICK1 in gastric cancer

We first detected PICK1 expression in several human gastric cells (SGC-7901, AGS, and BGC-823). As shown in Fig. 1a, human gastric cancer cells exhibited lower PICK1 levels than normal gastric cells (GES-1). Moreover, PICK1 was lowly expressed in human gastric tissues (Fig. 1b). The information of gastric cancer tissue samples is displayed in Table S1. Furthermore, we analyzed clinical data from 492 tumor cases taken from the GSE62254 and GSE15459 datasets (http://www.ncbi.nlm.nih.gov/geo) (Table S2). Patients were grouped according to the optimal cut-off (Youden Index) of PICK1 determined in our previous study (Zhou et al., 2021). Kaplan-Meier survival analysis demonstrated that patients in the PICK1 low-expression group had lower probability of survival than those in the high-expression group (Fig. 1c). The IHC data showed that PICK1 expression was significantly lower in gastric cancer tissue samples (Fig. 1d–f), and PICK1 expression was correlated with lymph node metastasis and higher clinical stage (Table 1 and S3).

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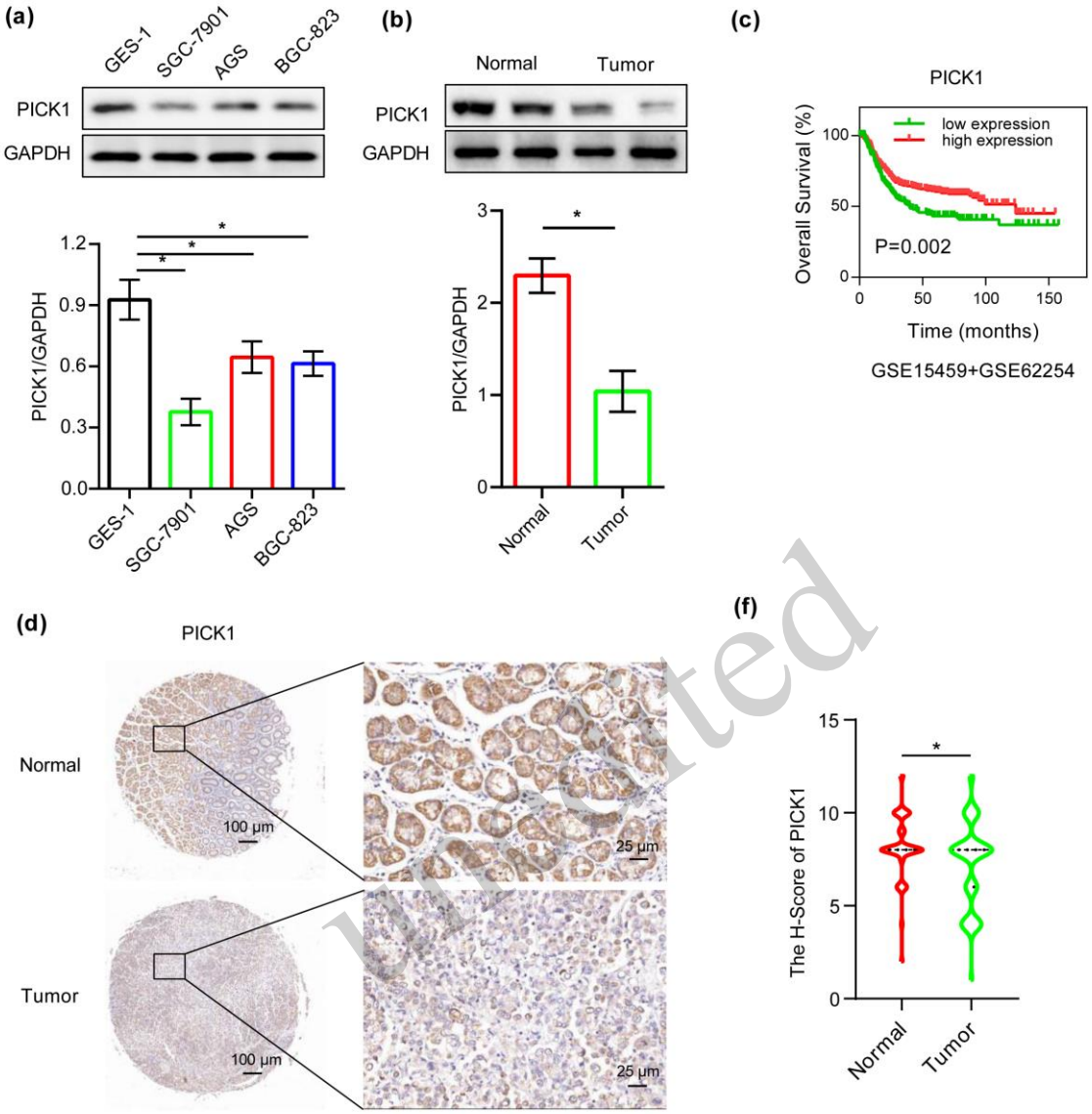

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Fig. 1 Expression and Kaplan-Meier survival analysis of protein interacting with C kinase 1 (PICK1). (a) Western blot showing the expression of PICK1 in human normal gastric cells (GES-1) and gastric cancer cells (SGC-7901, AGS, and BGC-823). (b) PICK1 expression in gastric tissues. (c) Kaplan-Meier survival analysis of high and low expression of PICK1 in gastric tissues using GSE62254 and GSE15459 datasets. (d–f) Immunohistochemical (IHC) analysis showing the expression of PICK1 in gastric tissue samples. Scale bar=100 μm (left). Scale bar=25 μm (right). Data are represented as mean±SD, n≥3. *P<0.05. SD: standard deviation; GES-1: gastric epithelial cells-1; SGC-7901: stomach gastric carcinoma cells-7901; AGS: adenocarcinoma gastric cells; BGC-823: bleeding gastric cancer-823; PICK1: protein interacting with C kinase 1; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

Table 1 Correlations between the PICK1 expression level in gastric tumor samples and clinical pathological characteristics of the corresponding patients

Characteristics	PICK1-low	PICK1-high	*P* value
n	30	26	
Gender, *n* (%)			0.678
Male	18 (32.1%)	17 (30.4%)	
Female	12 (21.4%)	9 (16.1%)	

Characteristics	PICK1-low	PICK1-high	*P* value
Age (years), *n* (%)			0.560
>60	21 (37.5%)	20 (35.7%)	
≤60	9 (16.1%)	6 (10.7%)	
Tumor invasion, *n* (%)			0.055
T1+T2	1 (2%)	6 (11.8%)	
T3+T4	27 (52.9%)	17 (33.3%)	
Lymph node metastasis, *n* (%)			0.019
N0+N1	8 (14.3%)	15 (26.8%)	
N2+N3	22 (39.3%)	11 (19.6%)	
Distant metastasis, *n* (%)			1.000
M0	27 (48.2%)	23 (41.1%)	
M1	3 (5.4%)	3 (5.4%)	
Clinical stage, *n* (%)			0.003
I+II	10 (17.9%)	19 (33.9%)	
III+IV	20 (35.7%)	7 (12.5%)	

PICK1: protein interacting with C kinase 1. Low expression of PICK1 in gastric cancer tissues of patients was correlated with lymph node metastasis and tumor malignant progression.

3.2 Effect of PICK1 overexpression on the growth and cell cycle of gastric cancer cells

We next examined the biological effects of PICK1 overexpression in gastric cancer cells. As shown in Fig. 2a and 2b, we overexpressed PICK1 in AGS cells and validated the efficiency using western blotting. Notably, PICK1 overexpression significantly suppressed gastric cell growth (Fig. 2c and 2d). Flow cytometry revealed that AGS cells arrested at the G_0–G_1 phase were significantly promoted by PICK1 (Fig. 2e and S1). Western blotting analysis revealed that the expression of CDK4 and CDK6 expression was inhibited by overexpressed PICK1 (Fig. 2f).

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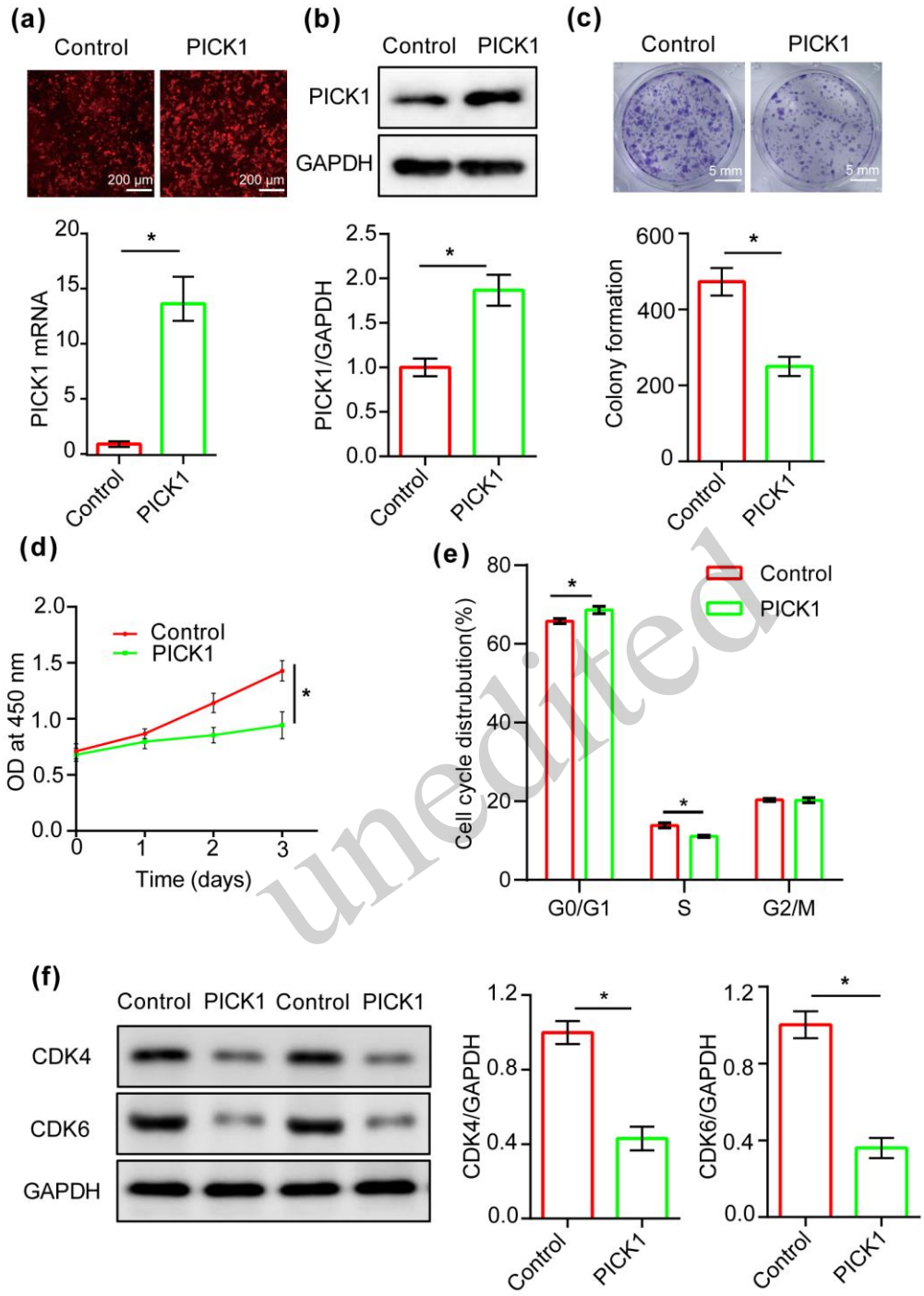
(a)
Control
PICK1
200 μm
200 μm
PICK1 mRNA
(b)
Control
PICK1
PICK1
GAPDH
PICK1/GAPDH
(c)
Control
PICK1
5 mm
5 mm
Colony formation
(d)
Control
PICK1
OD at 450 nm
Time (days)
(e)
Control
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Cell cycle distrubution(%)
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CDK4
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GAPDH
CDK4/GAPDH
CDK6/GAPDH

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Fig. 2 PICK1 overexpression inhibits AGS cell growth and induces cell cycle arrest. (a) After cells were infected with PICK1 and control lentivirus, fluorescence intensity was observed using confocal microscopy (Leica SP8, Germany). Scale bar=200 μm. (b) Efficiency was validated using western blotting. (c) Cell colony formation. Scale bar=5 mm. (d) The results of CCK-8 assay show the viability of stable PICK1-overexpressed or control cells. (e) Results of cycle analysis using flow cytometry. (f) Western blot showing the expression of CDK4 and CDK6 in each group. Data are presented as mean±SD, n⩾3, *P<0.05. SD: standard deviation; PICK1: protein interacting with C kinase 1; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; OD: optical density; CDK4: recombinant cyclin dependent kinase 4; CDK6: recombinant cyclin dependent kinase 4.

3.3 Effects of PICK1 overexpression on migration and TLR4 protein expression of gastric cancer cells

Next, to investigate the effects of PICK1 overexpression on cell migration, we performed transwell and scratch assays. The results indicated that PICK1 overexpression significantly reduced the migratory ability compared to the control group (Fig. 3a, b). To determine the mechanisms of functional changes in gastric cells, we used western blotting to detect chronic inflammation in gastric cells. As shown in Fig. 3c, we found that the IL-12 protein expression level was higher in the PICK1 group. The TLR4/MYD88 signaling pathway has been closely associated with chronic inflammation. TLR4, MYD88, and Phospho-NF-κB protein levels were significantly reduced by PICK1 overexpressed in AGS cells (Fig. 3c). Furthermore, we constructed the PCK1 PDZ truncated plasmid, PICK mutant (Mut) (Fig. S2), and found that the PICK1 PDZ mutation had no significant effect on AGS cell proliferation, cell cycle and TLR4 protein expression (Fig. S3). These results indicate that PICK1 plays an inhibitory role in the metastasis and inflammation of gastric cancer cells.

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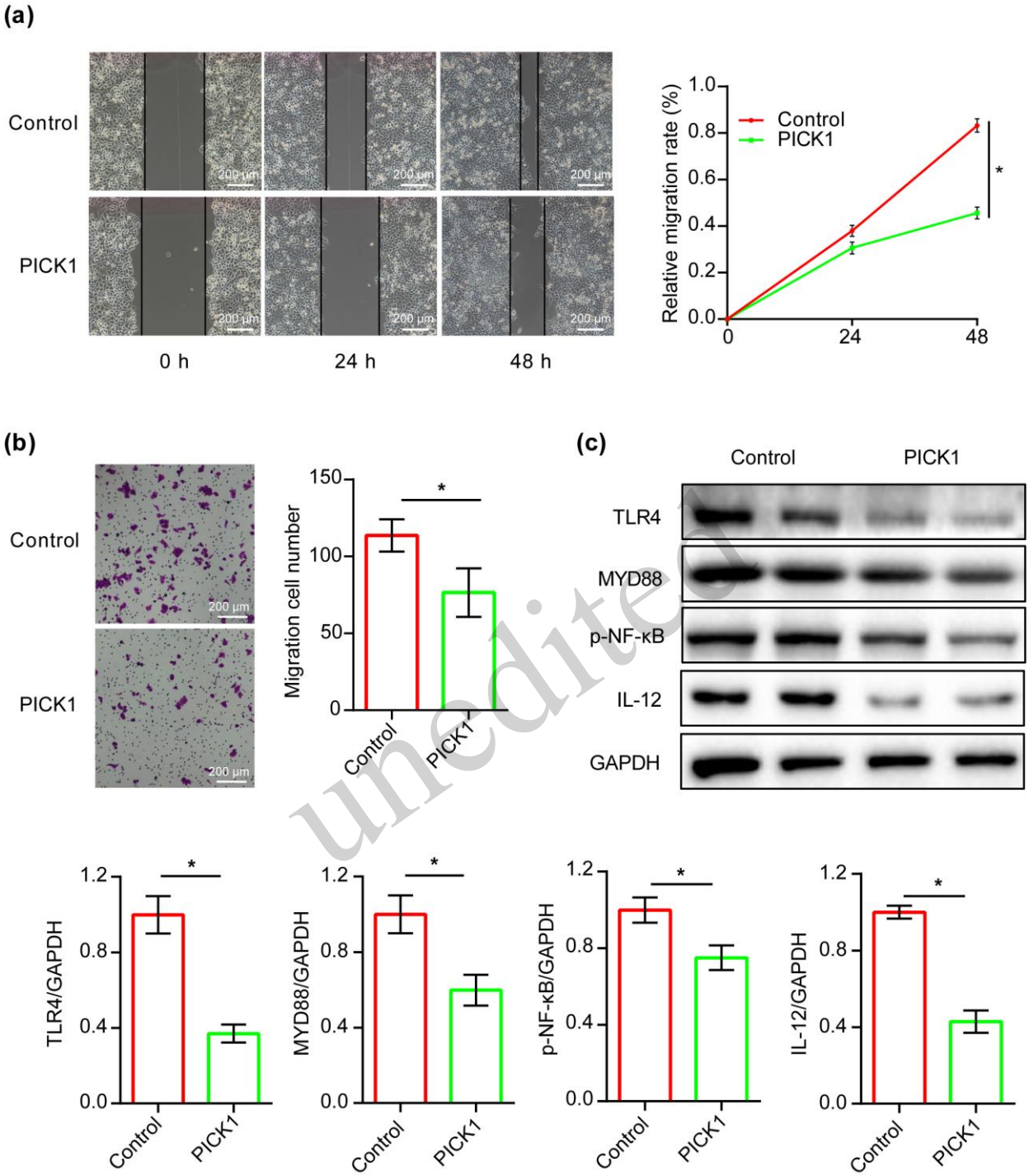

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Fig. 3 PICK1 overexpression inhibits cell migration and toll-like receptor 4 (TLR4) protein expression in gastric cells. (a) Representative images of wound healing assays in AGS cells infected with the PICK1 and control lentivirus. Images at 0, 24 and 48 h. Scale bar = 200 μm. (b) Transwell migration assay assessed the migratory capacities of AGS cells. Scale bar = 200 μm. (c) Relative protein levels of TLR4, MYD88, Phospho-NF-κB, and IL-12 in AGS cells. The loading mode in the figure was two multiple holes. Data are presented as mean±SD, n≥3, *P<0.05. SD: standard deviation; PICK1: protein interacting with C kinase 1; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; TLR4: toll-like receptor 4; MYD88: myeloid differential protein-88; p-NF-κB: phosho-nuclear factor kappa-B; IL-12: interleukin-12.

3.4 Effect of PICK1 down-regulation on proliferation and TLR4 protein expression in gastric cancer cells

Furthermore, we customized PICK1 siRNA for the downregulation of PICK1 gene expression in AGS cells. The siRNA information is shown in Table S4, whereas Fig. 4a demonstrates the efficiency of PICK1 knockdown in AGS cells infected with siRNA for 24 h. The CCK8 results indicated that AGS cell proliferation was enhanced after PICK1 gene downregulation (Fig. 4b). In addition, the flow cytometry results showed that the proportion of AGS cells blocked in the G0/G1 phase was reduced in the SA, SB, and SC groups with a knockdown of PICK1 compared with the NC group (Fig. 4c). The protein expressions of TLR4 and PICK1 were also examined. No significant changes in TLR4 protein levels occurred after knockdown of PICK1 expression, which may be due to the low expression of PICK1 protein in AGS cells, resulting in the failure of knockdown to significantly affect TLR4 protein expression (Fig. 4d).

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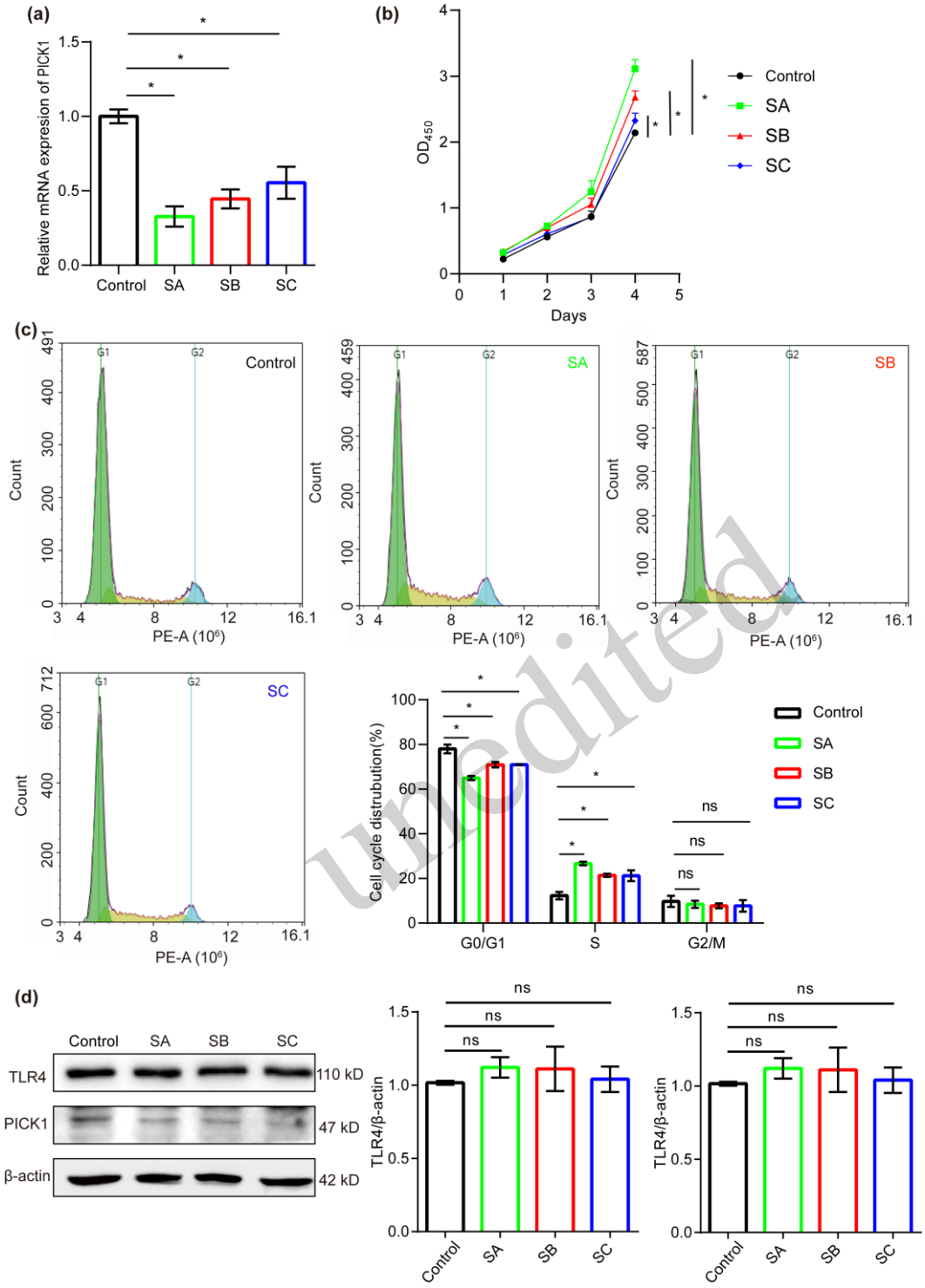

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Fig. 4 Effects of downregulation of PICK1 gene on AGS cells. (a) PICK1 gene expression in AGS cells infected with NC, SA, SB, and SC siRNA for 24 h. (b) Growth curves of AGS cells infected with NC, SA, SB, and SC siRNA for 48 h. (c) Cell cycle of AGS cells infected with NC, SA, SB, and SC siRNA for 48 h. (d) TLR4 and PICK1 protein expression in AGS cells infected with NC, SA, SB, and SC siRNA for 48h. Data are presented as mean±SD, n⩾3, *P<0.05, ns: no significant difference. SD: standard deviation; OD450: optical density at 450nm; PICK1: protein interacting with C kinase 1; TLR4: toll-like receptor 4; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; SA, SB, SC: PICK1 siRNA (three types).

3.5 Interaction between PICK1 and TLR4

The 3D structure of PICK1 bound to TLR4 is illustrated in Fig. 5a. It can be found that the folded area of PICK1 is combined with a semi-circular groove in TLR4, forming hydrogen bonds, pi-stacks and salt bridges (Fig. S4). PICK1 could bind with TLR4; the confidence of Rank1 was greater than 0.9, and the docking fraction was -261.85 kcal/mol (Table S5). The immunoprecipitation results revealed the interaction of PICK1 and TLR4 (Fig. 5b). Immunofluorescence using confocal microscopy (Fig. 5c) showed that PICK1 and TLR4 were both expressed in HEK293T cells and displayed a diffuse pattern in the cytoplasm. SPR assay was performed to detect the direct interaction between PICK1 and TLR4 (Fig. 5d), and the dissociation equilibrium constant (KD) was 4.07 μM. Furthermore, to investigate how PICK1 regulates TLR4 expression, PICK1-overexpressed AGS cells were treated with FSC-231 to suppress the PICK1 PDZ domain. As shown in Fig. 5e, FSC-231 treatment increased TLR4 expression. Moreover, when cells were treated with autophagy activator MG132 and autophagy inhibitor CQ, TLR4 expression was increased by CQ treatment (Fig. 5f). These results demonstrate that PICK1 binds to TLR4 and promotes TLR4 degradation by autophagy in gastric cells.

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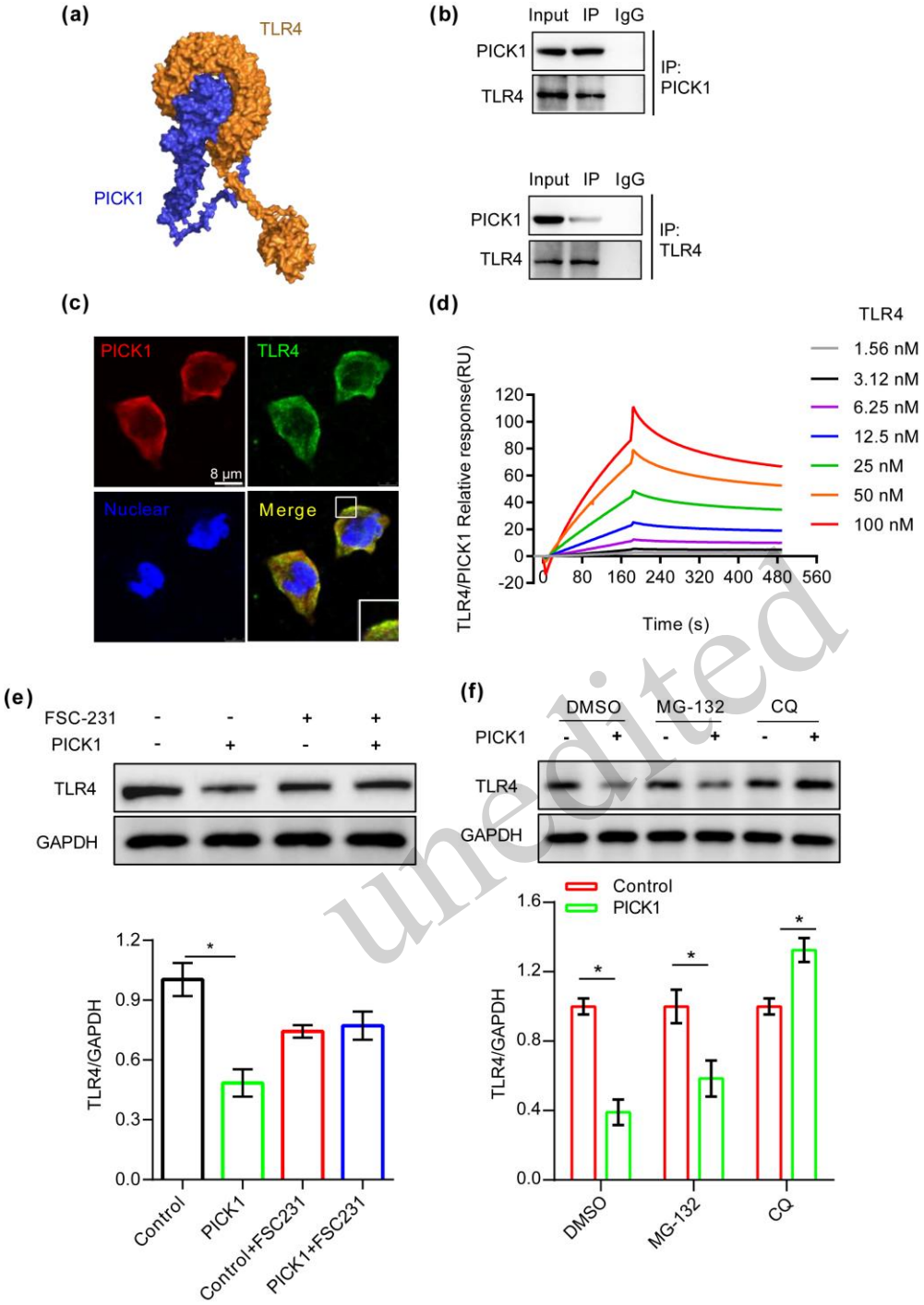

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Fig. 5 Interaction between PICK1 and TLR4. (a) 3D structure of PICK1 with TLR4. (b) PICK1 and TLR4 levels in HEK293T cell lysates were measured using western blotting after immunoprecipitation (IP) with mouse anti-PICK1 or rabbit anti-TLR4. (c) PICK1 and TLR4 are co-localised in the HEK293 cell, as demonstrated by immunofluorescence. Scale bar= 8 μm. (d) Direct interaction between PICK1 and TLR4 was confirmed using SPR analysis. (e) The protein expression level of TLR4 was detected with or without FSC-231 stimulation by western blotting. (f) Cells were preincubated with DMSO, MG-132, and CQ, and the expression of TLR4 was assessed by western blotting. Data are presented as mean±SD, n⩾3, *P<0.05. SD: standard deviation; PICK1: protein interacting with C kinase 1; TLR4: toll-like receptor 4; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; SPR: surface plasmon resonance; IP: immunoprecipitation; IgG: Immunoglobulin G; DMSO: dimethyl sulfoxide; CQ: Chloroquine.

3.6 Binding energy of PICK1 and TLR4

We further calculated the binding energy of PICK1 to TLR4 using MD simulations (Table 2). The structural characteristics of the complex in the 50 ns MD simulations are shown in Fig. 6a. It can be found that the number of hydrogen bonds increased most obviously from the original 2 to 22, and the number of salt bridges increased from 2 to 6 at the end of the MD simulations (Fig. 6b–d). The RMSD and Rg values indicated that the protein-binding mode was stable (Fig. 6e–f). The details of the binding amino acid pairs are shown in Table S6. The binding-free energy (ΔG_{bind}) changed from -122.42 kcal/mol to -197.22 kcal/mol in the MD simulations. The interaction of the protein PICK1 and TLR4 was promoted by Van der Waals energy (ΔE_{vdW}) and electrostatic interaction (ΔE_{elec}), and the polar free energy (ΔE_{GB}) and total free energy (ΔG_{solv}) generated energy losses in overcoming desolvation (Table 2). These results revealed the relative stability of the PICK1-TLR4 complex. The interaction network between them was gradually optimised during MD simulations, which network is crucial for maintaining the mechanical properties of PICK1.

Table 2 Correlations between the PICK1 expression level in gastric tumor samples and clinical pathological characteristics of the corresponding patients

	0 ns			50 ns		
	Average	SD	SEM	Average	SD	SEM
ΔE_{vdW}	-173.67	14.8953	1.0207	-209.1995	10.7279	2.2369
ΔE_{ele}	-62.4061	7.7085	5.7776	-86.1578	8.68	8.0653
ΔE_{GB}	138.3554	16.3787	5.5003	132.3733	13.3863	6.3360
ΔE_{surf}	-24.6977	1.6405	0.1336	-34.2337	1.7317	0.1526
ΔG_{gas}	-236.0761	18.6261	5.9690	-295.3573	14.4785	7.1893
ΔG_{solv}	113.6577	26.2400	5.4714	98.1396	3.3708	6.3327
ΔG_{bind}	-122.4184	7.0067	1.461	-197.2176	11.3316	2.3628

SD: standard deviation; *SEM*: standard error of measurement; ΔE_{vdW}: Van der Waals energy; ΔE_{ele}: electrostatic interaction; ΔE_{GB}: polar free energy; ΔE_{surf}: Nonpolar solvation free energy; ΔG_{gas}: gas-phase energy; ΔG_{solv}: solvation free energy; ΔG_{bind}: binding free energy.

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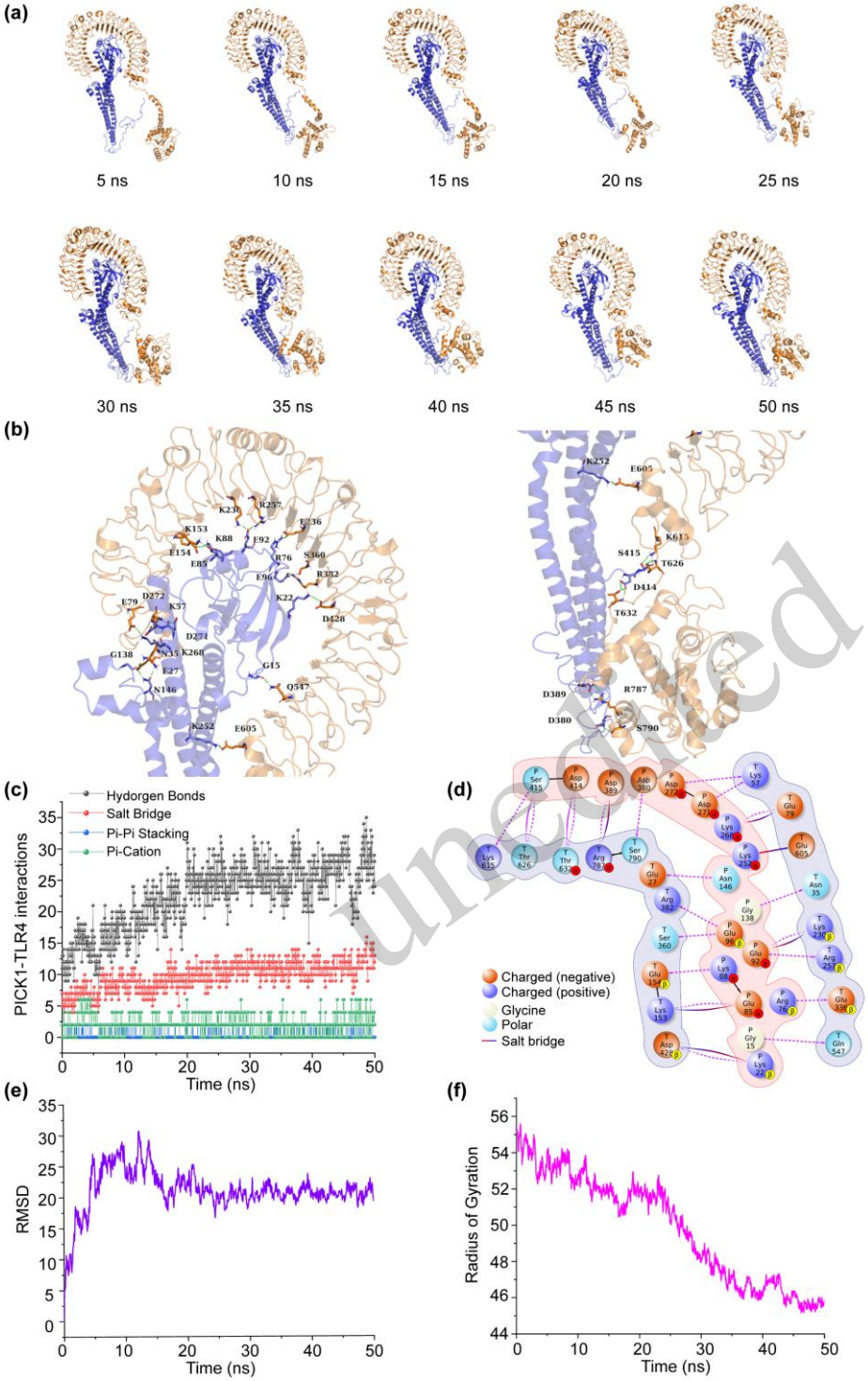

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Fig. 6 Binding energy of PICK1 and TLR4 by molecular dynamics simulations. (a) Structural characteristics of the complexes in 50 ns. (b) 3D structures. The hydrogen bond is represented by dotted green lines; the salt bridge is represented by dotted red lines; the π-stack is represented by dotted light green lines. (c) Molecular inter-atomic forces in the PICK1-TLR4 complex. (d) 2D structures. The hydrogen bond is represented by dotted lines and arrows; the salt bridge is represented by coloured lines; the π-stack is represented by straight green lines. Red=acidic amino acid; Blue=alkaline amino acid; Green=hydrophobic amino acid; Cyan=polar amino acid. (e–f) RMSD and Rg analysis. PICK1: protein interacting with C kinase 1; TLR4: toll-like receptor 4; RMSD: root mean square deviation.

4 Discussion

PICK1, widely expressed in various tissues, is involved in the transport of membrane receptors through a single PDZ domain (Ramsakha et al., 2023). Abnormal PICK1 expression has been associated with invasion and metastasis in some tumours, including gastric cancer (Dai et al., 2017; Zhang et al., 2010). In this study, PICK1 expression was found to be decreased in human gastric tissues, which was predictive of poorer outcomes in patients. PICK1 overexpression suppressed gastric cell growth and migration and caused cell cycle arrest at the G_0–G_1 phase. Mechanistically, we demonstrated that PICK1 directly binds to TLR4, thereby promoting TLR4 degradation and decreasing inflammation-related protein levels (Fig. 3). We also observed a complex interaction network at the binding interface between PICK1 and TLR4. Our results indicate that PICK1 is associated with the regulation of cell metastasis and inflammation and may be a useful therapeutic target for the treatment of human gastric cancer.

Inflammation has been linked to the activation of epithelial-mesenchymal transformation in many cancers (Ebrahimi et al., 2022). Changes in the inflammatory environment have been identified as key avenues for the treatment of human cancers. TLR4, an important molecule in inflammation-associated carcinogenesis, is a type I transmembrane protein that consists of three distinct structural domains: a leucine-rich repeat-containing ectodomain involved in pathogen-specific ligand recognition, a transmembrane domain, and a cytosolic domain that binds downstream adaptor proteins (Fukata and Abreu, 2008; Yuan et al., 2013). Studies have demonstrated that gastric cancer cell lines and cells express high levels of TLR4, which correlate with tumour stage and patient survival (Fernandez-Garcia et al., 2014; Taguchi and Mukai, 2019). Our research indicated that TLR4 expression was downregulated in PICK1-overexpressed human gastric cancer cells.

However, FSC-231 pretreatment in overexpressed PICK1 cells significantly upregulated TLR4 expression. TLR4 is mainly located in the endoplasmic reticulum (ER) and Golgi apparatus of quiescent cells. LPS stimulation causes the rapid migration of TLR4 from the Golgi apparatus to the cell membrane (Rocuts et al., 2010). However, recent evidence suggests that PICK1 has an important role in the transport of various vesicles from the Golgi complex to several cellular systems, including neurons (Xu et al., 2016). PICK1 also interacts with Golgi-related PDZ and may be involved in vesicular transport from the Golgi apparatus to the acrosome (Xiao et al., 2009). Colocalization analysis by immunofluorescence revealed that TLR4 mainly resides in the cytoplasm when present in PICK1-overexpressed cells. PICK1 and TLR4 interact with each other via co-immunoprecipitation and SPR. Autophagic inhibitors significantly suppressed PICK1-induced TLR4 downregulation (Fig. 5). Our study suggests that PICK1 overexpression may alternate the trafficking of TLR4 and enhance TLR4 degradation via autophagosomes. However, the specific degradation process is not yet clear and should be further explored in future research.

Further experiments explored the effect of PICK1 on TLR4 and the interaction network between the TLR4-PICK1 complex. It is known that clustering and the synaptic targeting of PICK1 is dependent on the PDZ domain-mediated direct interaction at lipid membranes (Pan et al., 2007). The PDZ domain binds to the Eph receptor tyrosine kinase family and their ephrin ligands, affecting the oncogenesis and migration of tumor cells (Liu et al., 2019; Son et al., 2014; Torres et al., 1998). We also observed that PDZ domain inhibitors significantly suppressed PICK1-induced TLR4. We further determined the binding energies of PICK1 and TLR4 using MD simulations, and the data showed that interactions in the complex were mainly reflected by hydrogen bonds and salt-bridge bonding (Fig. 6).

In summary, this study demonstrated that PICK1 modulates TLR4-mediated metastasis and inflammation in gastric cells, providing novel insights into the progression and treatment of human gastric cancer, and supplying additional information for a better understanding of PICK1 cellular and pathological functions.

5 Conclusions

In summary, our study demonstrated that high expression of PICK1 inhibited malignant progression of

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gastric cancer. And PICK1 inhibited gastric cancer cell proliferation and migration in vitro, which is dependent on the TLR4/MYD88 signaling pathway. These results provide new insights into gastric cancer treatment.

Data availability statement

The data presented in this study are available from the corresponding author upon reasonable request.

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Author contributions

Kaiqiang LI, Yimin YANG and Yaling WANG contributed equally to conceptualization, methodology, formal analysis, data curation, and writing – original draft. Jing JIN, Qianni Wang and Lina PENG contributed to sample collection, data curation, and supervision. Aibo XU and Xuling LUO contributed to data curation and visualization. Wei YANG and Peng XU contributed to investigation and writing – review & editing. Bingyu CHEN, Ke HAO and Zhen WANG contributed to funding acquisition and project administration. All authors have read and approved the final manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Kaiqiang LI, Yimin YANG, Yaling WANG, Jing JIN, Qianni Wang, Lina PENG, Aibo XU, Xuling LUO, Wei YANG, Peng XU, Bingyu CHEN, Ke HAO and Zhen WANG declare that they have no conflict of interest.

The clinical specimens were obtained from Zhejiang Provincial People's Hospital and the National Human Genetic Resources Sharing Service Platform. The protocol was approved by the Ethics Committee of the Zhejiang Provincial People's Hospital and Shanghai Outdo Biotech. The study was conducted in accordance with the guidelines of the Declaration of Helsinki (Approval code: 2020QT312, SHYJS-CP-1801015/YBM-05-02).

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Supplementary information
Table S1-S6; Figs. S1-S4