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IL-17A Promotes the Migration and Invasiveness of Colorectal Cancer Cells Through NF-κB-Mediated MMP Expression

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Interleukin-17A (IL-17A) plays a significant role in many inflammatory diseases and cancers. The aim of this study is to investigate the effect of IL-17A on the invasiveness of colorectal cancer. In the study, we found that IL-17A could promote the migration and invasion of colorectal cancer cells. Furthermore, after being treated with IL-17A, the expression and activity of matrix metalloproteinase 2 (MMP-2) and MMP-9 were upregulated. Moreover, the nuclear/overall fractions and DNA-binding activity of p65 and p50 were dramatically elevated by IL-17A. Pretreatment with a nuclear factor-κB (NF-κB) inhibitor (PDTC) or PI3K/AKT inhibitor (LY294002) was proven to abolish the promoting effect of IL-17A on the invasion ability of colorectal cancer cells and upregulation of MMP-2/9. In conclusion, our findings demonstrated that IL-17A could promote the invasion of colorectal cancer cells by activating the PI3K/AKT/NF-κB signaling pathway and subsequently upregulating the expression of MMP-2/9. Our results suggest that IL-17A could serve as a promising therapeutic target for colorectal cancer.

Key words: Interleukin-17A (IL-17A); Colorectal cancer; Migration; Invasion; Nuclear factor-κB (NF-κB)

INTRODUCTION

Colorectal cancer is the third most common type of cancer and the fourth leading cause of cancer-related death in the world (1). In China, colorectal cancer is the sixth most common cancer and the fifth leading cause of death (2). About 15–20% of colorectal cancer patients are diagnosed with synchronous distant metastases (3). The aggressive local invasion and metastasis of colorectal cancer are one of the important factors that lead to poor prognosis and make it recalcitrant to treatment (4). Therefore, it is significant that we clarify the underlying mechanisms and thus develop advanced treatments for colorectal cancer.

Interleukin-17A (IL-17A), which belongs to the IL-17 family, is a proinflammatory cytokine produced by several types of immune cells, including $CD4^+$ T cells, $CD8^+$ T cells, γδT cells, as well as NKT cells in different conditions (5). IL-17A has been found to play an essential role in many autoimmune diseases such as rheumatoid arthritis, inflammatory bowel disease, and multiple sclerosis (6). Recently, IL-17A has been frequently detected in many cancers, such as breast cancer (7), ovarian cancer (8), gastric cancer (9), hepatocellular carcinoma (10), and colorectal cancer (11). However, the role of IL-17A in cancer remains controversial. On one hand, IL-17 promotes an antitumor cytotoxic T-cell response, leading to tumor regression. On the other hand, by facilitating angiogenesis, metastasis, and egress of tumor cells from the primary focus, IL-17A promotes tumor growth (12). Thus, IL-17A plays multifactorial roles in tumor immunity.

A previous study found that IL-17A was associated with poor prognosis and promoted angiogenesis via stimulating VEGF production of cancer cells in colorectal carcinoma (13). IL-17A can cooperatively combine with TNF-α to stimulate glucose metabolism and growth factor production in human colorectal cancer cells and promote tumor growth (14). Intestinally, IL-17A can modulate circulating tumor cells in the tumor-draining vein of colorectal cancers and affect tumor metastases (15). Recently, Chin et al. reported that IL-17A can induce colorectal cancer migration through increasing nuclear factor-κB (NF-κB)-mediated CCR6 expression in tumor cells (16). However, the role of IL-17 in the development and progression of colorectal cancer still remains to be explored.

In the present study, we found that IL-17A could increase cell motility and invasion by the upregulation of matrix metalloproteinase 2 (MMP-2) and MMP-9 via activation of the PI3K/AKT/NF-κB signaling pathway.

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MATERIALS AND METHODS

Cell Culture

Human colon carcinoma cell lines HT29 and DLD1 were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). Cells were cultured in RPMI-1640 (Hyclone, Beijing, China) medium containing 10% FBS (Gibco BRL, Grand Island, NY, USA) at 37°C in a humidified incubator containing 5% CO_2.

Migration and Invasion Assay

The migration and invasion ability of the colorectal cancer cells was detected in vitro with Transwell chambers (Corning) as previously described. For migration assays, after treatment with or without IL-17A (50 ng/ml), 2×10^5 cells in serum-free medium were added into the upper chambers; FBS (10%) was added into the bottom chambers. For the invasion assay, the upper

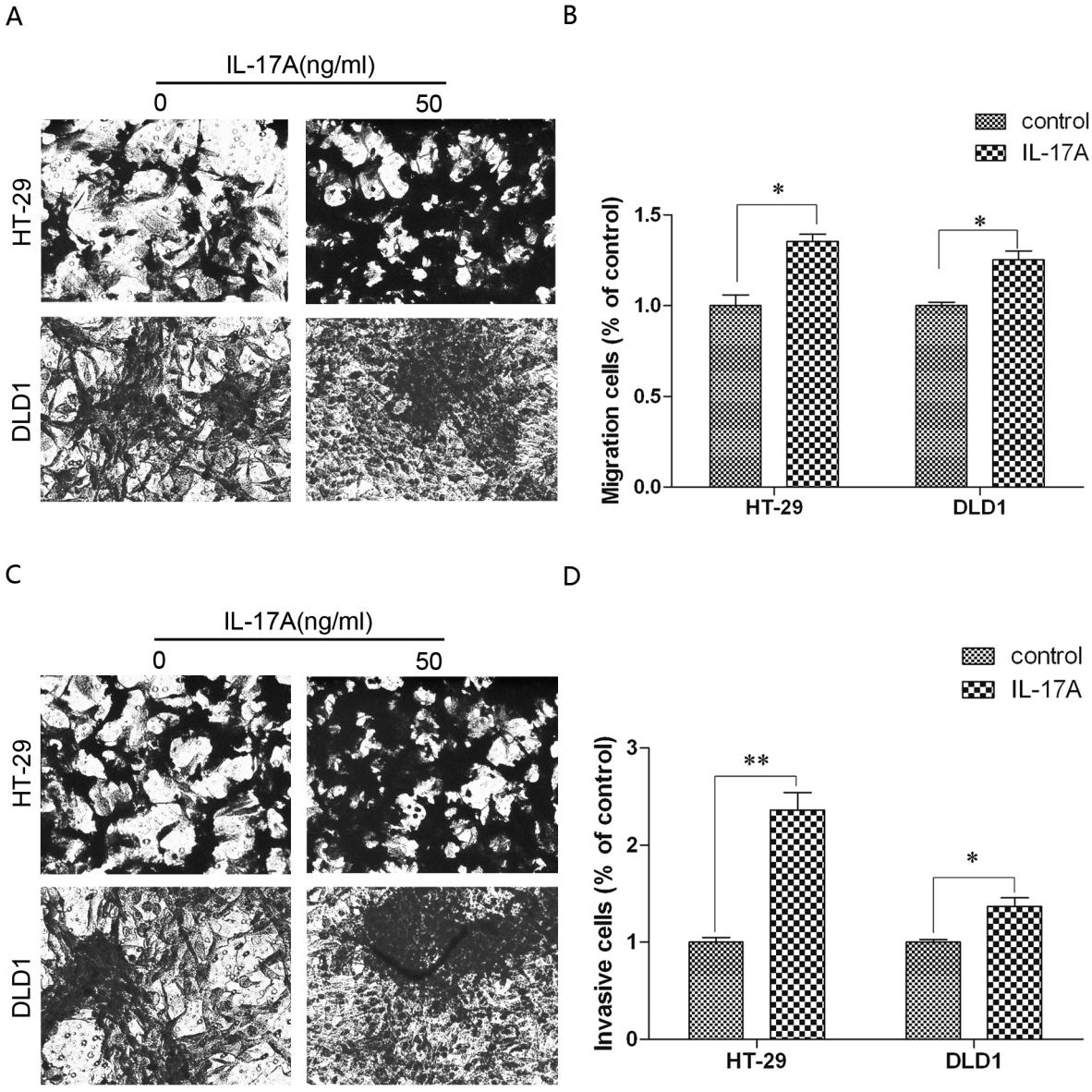


Figure 1. IL-17A promotes colorectal cancer cell migration and invasion. (A, B) Compared with the control group cells, IL-17A treatment can increase the migration of colorectal cancer cells (DLD1 and HT-29) (magnification 100×). (C, D) By cell invasive assay, the effect of IL-17A on cell invasion was detected (magnification 100×). Total invasive cell number of treated group was expressed as the percentage of control group. Data are represented as means ± SD of three independent experiments performed in triplicate. *$p<0.05$ and **$p<0.01$ compared with control group, respectively.

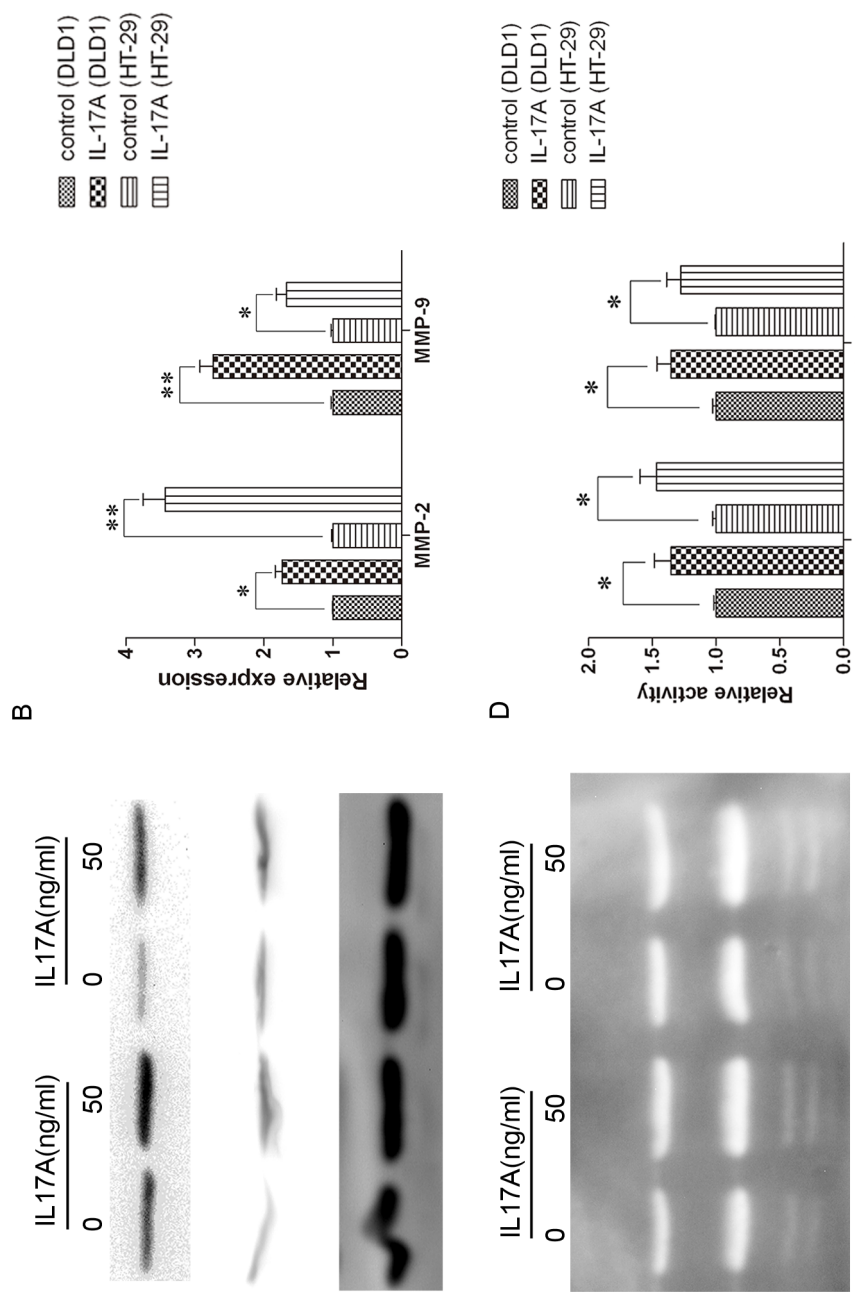
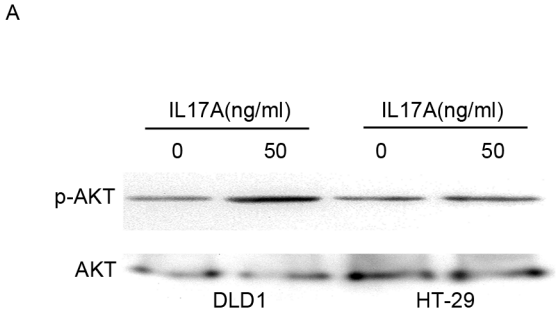
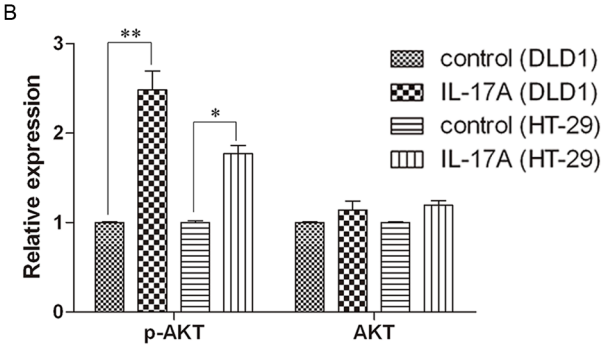
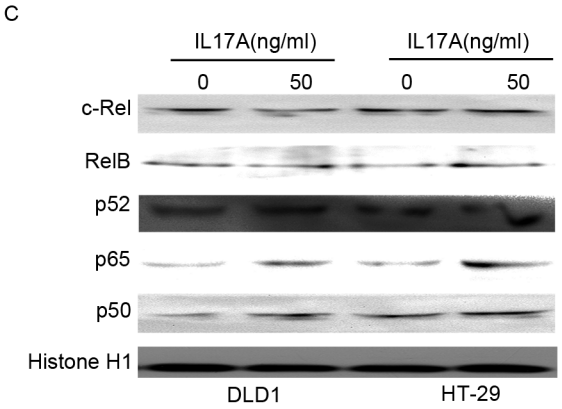
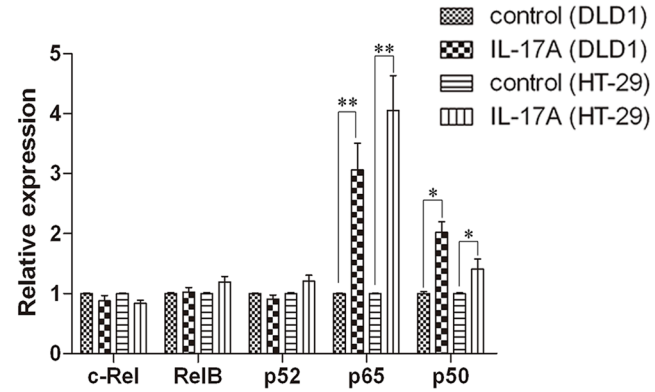


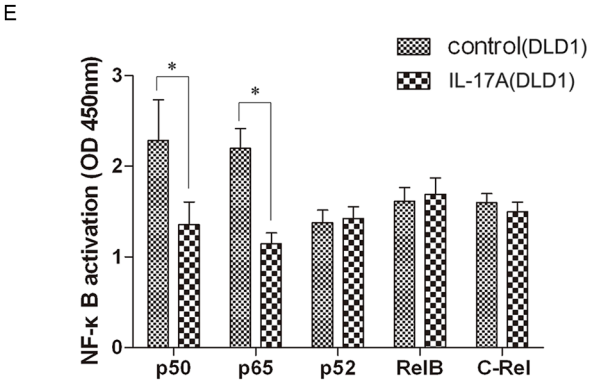
Figure 2. IL-17A upregulated the expressions and activities of MMP-2 and MMP-9. (A) After treating with IL-17A for 24 h, the expression of MMP-2/9 was detected by Western blot analysis in colorectal cancer cells. (B) Quantification of the protein levels of MMP-2/9. (C) The effects of IL-17A on the activity of MMP-2/9 were analyzed via zymography assay. (D) Quantification of the activities of MMP-2/9. Data are represented as means ± SD of three independent experiments performed in triplicate. $^{*}p<0.05$ and $^{**}p<0.01$ compared with the control group, respectively.

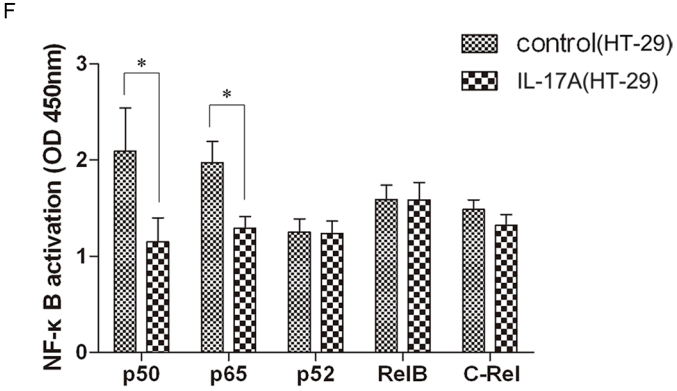

A
IL17A(ng/ml)
IL17A(ng/ml)
0
50
0
50
p-AKT
AKT
DLD1
HT-29


B
Relative expression
control (DLD1)
IL-17A (DLD1)
control (HT-29)
IL-17A (HT-29)
p-AKT
AKT


C
IL17A(ng/ml)
IL17A(ng/ml)
0
50
0
50
c-Rel
RelB
p52
p65
p50
Histone H1
DLD1
HT-29


D
Relative expression
control (DLD1)
IL-17A (DLD1)
control (HT-29)
IL-17A (HT-29)
c-Rel
RelB
p52
p65
p50


E
NF-κ B activation (OD 450nm)
control(DLD1)
IL-17A(DLD1)
p50
p65
p52
RelB
C-Rel


F
NF-κ B activation (OD 450nm)
control(HT-29)
IL-17A(HT-29)
p50
p65
p52
RelB
C-Rel

chambers were coated with Matrigel (BD Biosciences, San Jose, CA, USA).

Western Blotting

After treatment with IL-17A, PDTC, or LY294002, 2×10^6 cells were suspended in 200 µl of lysis buffer (1 mmol/L EDTA, 40 mmol/L Tris-HCl, 150 mmol/L KCl, 1% Triton X-100, 100 mmol/L $NaVO_3$, 1 mmol/L PMSF). The proteins (80 µg) were separated by 10% or 12% DS-polyacrylamide gel electrophoresis and then transferred onto PVDF membranes. After being blocked in defatted milk (5% in Tris-buffered saline with Tween-20 buffer) at 37°C, the membranes were incubated with various antibodies against MMP-2, MMP-9, AKT, p-AKT, NF-κB-p50, p65/RelA, P52, c-Rel, RelB, histone H1, or β-actin overnight at 4°C. The membranes were then incubated with appropriate secondary antibodies for 1 h at room temperature. The bands were detected and expressed as arbitrary units (a.u.).

Evaluation of NF-κB Activity

DNA-binding activity of NF-κB p50, p52, p65, RelB, and c-Rel in IL-17A-treated cells was assessed by TransAM NF-κB ELISA (Active Motif, Carlsbad, CA, USA). The assay was performed according to the manufacturer's protocol.

Zymography

The assay was carried out as previously described (17); after treating with IL-17A at 37°C for 24 h, samples of conditioned media of cells were collected and separated in 0.1% gelatin–8% SDS-PAGE electrophoresis. Then the gels were washed in 2.5% Triton X-100 for 40 min twice at room temperature and then incubated in reaction buffer (40 mM Tris-HCl, 10 mM $CaCl_2$, and 0.01% NaN_3, pH 8.0) at 37°C for 14 h. The gels were stained with Coomassie brilliant blue R-250 gel stain. The intensities of bands on the gels were calculated using an image analysis system (Bio-Rad Laboratories, Richmond, CA, USA). The last volumes of samples were adjusted according to the vital cell number.

Statistical Analysis

All data are shown as the means ± SD. Statistical significance was analyzed using the Student's *t*-test. All statistical tests and corresponding *p* values were two sided. A value of $p<0.05$ was considered as statistically significant.

RESULTS

IL-17A Increased the Motility of Colorectal Cancer Cells

The effect of IL-17A on cell motility was investigated by migration and Matrigel invasion assays. As shown in Figure 1A, the mobility was significantly increased after 24 h of incubation in the presence of IL-17A compared with the control parental cells in both two tested colorectal cell lines. Quantification analysis indicated that the difference is significant (Fig. 1B). Moreover, the invasion assay showed that IL-17A could also facilitate the invasion of colorectal cancer cells (Fig. 1C, D). These data showed that IL-17A could enhance colorectal cancer cell migration and invasiveness.

IL-17A Upregulated the Expression and Activity of MMP-2/9 in Colorectal Cancer Cells

MMPs, particularly MMP-2 and MMP-9, play an important role in cancer metastasis and invasiveness. In light of this, we evaluated the effect of IL-17A on MMP expression in colorectal cancer cells. As shown in Figure 2A and B, the expressions of MMP-2 and MMP-9 were upregulated in IL-17A-treated cells (DLD1 and HT-29) compared with controls. Furthermore, the activity of MMP-2 and MMP-9 in colorectal cancer cells treated with or without IL-17A was measured by gelatin zymography assay. The results showed that IL-17A enhanced the activity of MMP-2 and MMP-9 in colorectal cancer cells (Fig. 2C, D). These results suggested that the metastasis-promoting function of IL-17A might be through remodeling the extracellular MMP expression.

IL-17A Upregulated MMP-2/9 Expression Through Activating the NF-κB Pathway

The PI3K/AKT/NF-κB signaling pathway plays an important role in the invasion of colorectal cancer cells via regulating MMP-9, and NF-κB has been reported as a downstream target of the IL-17A signaling pathway in many cells, which is able to upregulate MMP-2 and MMP-9 expressions. In this study, we found that IL-17A could increase the phosphorylation level of AKT (Fig. 3A, B) and have no effect on the expression of total

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Figure 3. IL-17A activates PI3K/AKT/NF-κB signaling pathways in colorectal cancer cells. (A) Expressions of AKT and p-AKT were detected by Western blot in DLD1 and HT-29 cells treated with or without IL-17A for 24 h. (B) Quantification of the protein levels of AKT and p-AKT in DLD1 and HT-29 cells. (C) Western blotting analysis was used to detect nuclear p50, p65, p52, c-Rel, and RelB expression in DLD1 and HT-29 cells treated with IL-17A (50 ng/ml) at indicated time points. (D) Quantification of the protein levels of nuclear p50, p65, p52, c-Rel, and RelB. (E, F) The DNA-binding capacity of NF-κB was also detected in DLD1 and HT-29 cells. Data are represented as means ± SD of three independent experiments performed in triplicate. $*p<0.05$ and $**p<0.01$ compared with the control group, respectively.

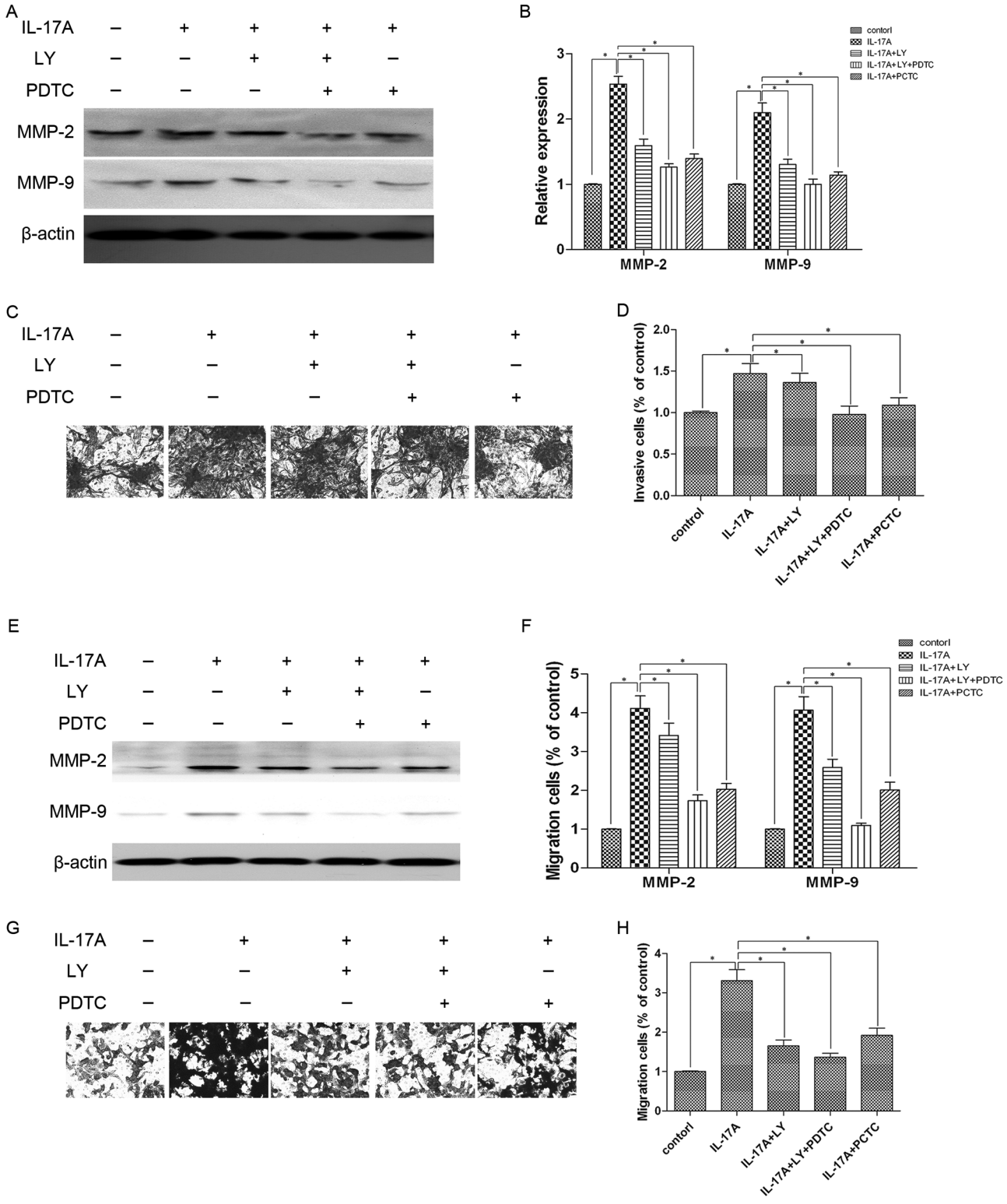

Figure 4. Effects of PI3K/AKT inhibitor (LY294002), NF-κB inhibitor (PDTC), and IL-17A on cell invasion and MMP-2/9 expression in colorectal cancer cells. DLD1 (A) and HT-29 (E) cells were pretreated with LY294002 (20 μM) and PDTC for 30 min, then incubated in the presence or absence of IL-17A (50 ng/ml) for 24 h. The cell invasive abilities were performed by Transwell assay. The percentage of invasive rate of DLD1 (B) and HT-29 (F) cells was expressed as a percentage of control. DLD1 (C, D) and HT-29 (G, H) cells were treated and then subjected to Western blot to analyze the protein levels of MMP-2/9. Data are represented as means ± SD of three independent experiments performed in triplicate. *$p < 0.05$ and **$p < 0.01$ compared with control group, respectively.

AKT. Furthermore, the result showed that the level of p50 and p65 in the nuclei was dramatically elevated in colorectal cancer cells after IL-17A treatment (Fig. 3C, D). The DNA-binding activity of NF-κB p50 and p65 was also increased in IL-17A-treated colorectal cancer cells (Fig. 3E, F).

Next, colorectal cancer cells were treated with LY294002 (a PI3K/AKT inhibitor) and PDTC (a NF-κB inhibitor) before IL-17A treatment, and the invasive ability and MMP-2/9 expression were analyzed. The result demonstrated that both LY294002 and PDTC can partly reverse the invasion increased by IL-17A (Fig. 4A, B). In addition, Western blot analysis revealed that the pretreatment of LY294002 and PDTC abolished the upregulation of MMP-2/9 induced by IL-17A (Fig. 4C, D). Similar results were observed in HT-29 cells (Fig. 4E–H). Taken together, the results demonstrate that IL-17A regulated MMP-2/9 expression and invasion of colorectal cancer cells via activating PI3K/AKT/NF-κB signaling pathway.

DISCUSSION

Increasing evidence indicates that chronic inflammation relates to an increased risk of colorectal cancer (18–20). IL-17A is one of the important inflammatory cytokines in the development of many inflammatory diseases and is also frequently detected in the tumor microenvironment (21–24).

In this study, IL-17A was found to be a promoter of the migration and invasion of colorectal cancer cells. Previous research found that IL-17A could promote the migration and invasion abilities of human gastric cancer and hepatocellular carcinoma cells (25,26). Our results suggest that IL-17A is closely correlated with the migration and invasion of colorectal cancer cells. MMPs, especially MMP-2/9, are responsible for breaking down the ECM (27,28). IL-17A was reported to promote the invasion of cancer cells via upregulating the expression of MMP-2/9 (26). Negative MMP-9 expression levels are correlated with longer survival time and lower risk of developing disease recurrence (29). Positive tissue expression of MMP-2 was a significant prognostic factor for colorectal cancer patients' survival (30,31). In order to clarify the related mechanisms, we investigated whether the promoting effect of IL-17A on cell invasion is through regulating expression of MMP-2/9. Our results showed that IL-17A can upregulate the expression and activity of MMP-2/9.

PI3K/AKT signaling pathway plays an important role in the invasion of colorectal cancer cells via regulating MMP-9 (32). The PI3K/AKT signaling pathway has also been reported as a downstream target of IL-17A in other cells (33,34). To further clarify the possible mechanism(s) of IL-17A's promoting effect on colorectal cancer cell invasion, we detected the effect of IL-17A on the phosphorylation of AKT. The results showed that IL-17A could upregulate the phosphorylation level of AKT. NF-κB was reported as a downstream target of IL-17A in many cells (25,35,36), which is able to promote MMP-2/9 expression (25,37). IL-17A has also been reported to increase the expression of MMPs via activating the NF-κB signaling pathway in many cells (25,37). So we investigated whether the upregulation of the expression of MMP-2/9 by IL-17A is through activating NF-κB. The results showed that IL-17A promoted nuclear translocation and DNA-binding activity of the p65 and p50 subunits of colorectal cancer cells. Results also showed that pretreatment of LY294002 and PDTC abrogated the upregulation of MMP-2/9 induced by IL-17A.

In conclusion, our findings suggested that IL-17A could increase cell motility by regulating MMP-2/9 via activating the PI3K/AKT-NF-κB signaling pathway. IL-17A could serve as a promising therapeutic target for colorectal cancer.

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