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ORIGINAL RESEARCH

CCL20 Expression via AKT-ERK1/2-AP1 Pathway in *Mycoplasma Pneumoniae* Infection: Implications for EMT and Cell Migration

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Purpose: *Mycoplasma pneumoniae*, a clinically significant respiratory pathogen, primarily causes community-acquired pneumonia and contributes to asthma development, with its persistent infection frequently resulting in fibrotic pulmonary changes and structural airway abnormalities. This study investigates the signaling pathways regulating CCL20 expression in THP-1 cells following *M. pneumoniae* infection and its impact on cell migration and epithelial-mesenchymal transition (EMT).

Methods: THP-1 cells were infected with *M. pneumoniae*, and the expression of CCL20 was measured over time and at various doses. In addition, co-culture experiments were performed using *M. pneumoniae*-infected THP-1 cells and bronchial epithelial cells to assess EMT and cell migration.

Results: *M. pneumoniae* infection significantly upregulated CCL20 production in THP-1 cells via the AKT-ERK1/2-AP1 pathway, a process that was both time- and dose-dependent. Furthermore, co-culturing *M. pneumoniae*-infected THP-1 cells with 16HBE cells promoted EMT and increased cell migration, a process that is believed to be associated with CCL20.

Conclusion: This study provides insights into the molecular mechanisms linking CCL20 to cell migration, highlighting potential therapeutic targets for *M. pneumoniae*-related lung diseases.

Keywords: *M. pneumoniae*, CCL20, EMT, AKT-ERK1/2-AP1, cell migration

Introduction

Mycoplasma pneumoniae is a significant pathogen that plays a major role in community-acquired pneumonia as well as both acute and chronic respiratory infections in humans.[1] This pathogen demonstrates extensive clinical variability, presenting with respiratory symptoms spanning from mild bronchial inflammation to life-threatening pulmonary infections, while frequently inducing systemic manifestations involving diverse organ systems such as neurological, cardiovascular, dermatological, and hepatorenal functions.[2] Recent studies have provided in-depth insights into the core pathogenic mechanisms of *M. pneumoniae*, including its adhesion to and invasion of host cells, immune-mediated tissue damage, and immune evasion capabilities.[3] The immune response triggered by *M. pneumoniae* infection involves the complex regulation of multiple factors, particularly through the cytokine network mediated by various components of the mycoplasma.[4] Although the precise pathogenesis remains incompletely understood, a key feature of *M. pneumoniae* infection is the induction of cytokine production under both acute and chronic conditions.[5]

Chemokines play a central regulatory role in immune system development and homeostasis maintenance, extensively participating in both protective and destructive immune-inflammatory responses in the organism.[6] Beyond mediating classical chemotaxis, these factors can regulate various cellular migration behaviors, including haptotaxis, chemotaxis, and adhesion-driven migration.[6] Research has demonstrated that *M. pneumoniae* infection can recruit and activate

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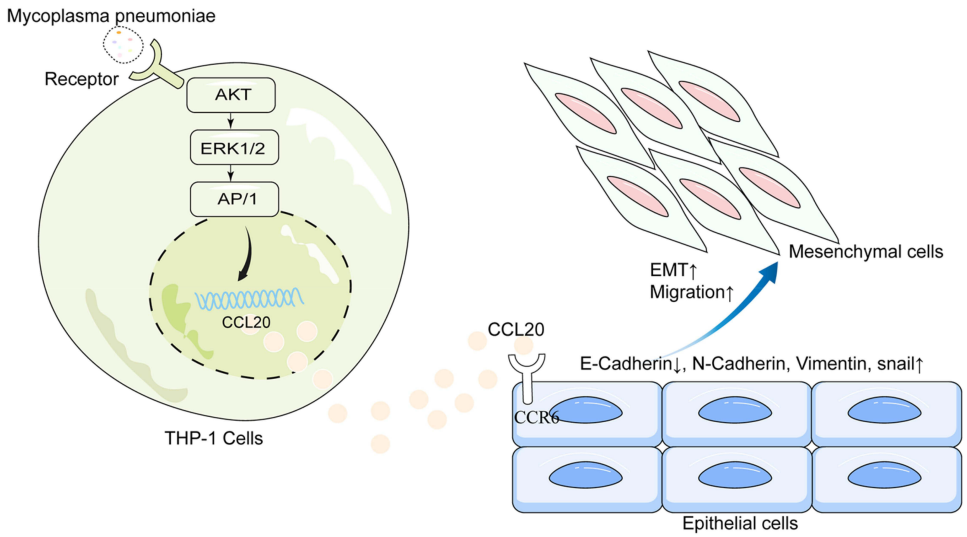
Graphical Abstract



alveolar macrophages, thereby triggering a cascade of chemokine and cytokine responses.[7] Among these, CCL20—also known as LARC, MIP-3α, or Exodus-1—is a chemokine that binds to its specific receptor, CCR6, and plays a critical role in immune regulation.[8] CCL20 production can be induced under inflammatory or hypoxic conditions, as well as by bacterial and viral infections or pro-inflammatory cytokines.[9] The regulation of CCL20 expression has been a longstanding research focus due to its association with chronic inflammation. However, the mechanisms by which different pathogens modulate CCL20 expression vary significantly. For instance, house dust mite infection upregulates CCL20 in BEAS-2B cells through the AKT-ERK1/2-C/EBPβ signaling pathway;[10] while *Brucella* induces CCL20 expression in THP-1 cells via activation of the JNK1/2 and NF-κβ pathways.[11] Similarly, serum amyloid A promotes CCL20 secretion in THP-1 cells through the MAPK pathway.[12] Despite these findings, it remains unclear whether *M. pneumoniae* infection induces CCL20 expression through these specific pathways.

Given the critical role of CCL20 in immune regulation and inflammation, its involvement in *M. pneumoniae*-related diseases warrants further investigation. Although *M. pneumoniae* infection typically presents as a self-limiting disease, it can progress to severe or even life-threatening conditions in certain individuals.[13] Research indicates that the CCL20/CCR6 pathway plays a critical role in cancer and autoimmune conditions through the regulation of cell proliferation, migration, and immune microenvironment remodeling.[14,15] Specifically, within the tumor microenvironment, CCL20 secreted by tumor-associated macrophages (TAMs) can activate CCR6 expression in tumor cells, thereby inducing pathological processes such as cell migration and EMT.[16] Clinical research data indicate that high expression of CCL20 is a significant marker of poor prognosis in lung adenocarcinoma patients, potentially due to its role in enhancing EMT, promoting inflammatory responses, and activating the TNF signaling pathway.[17] Notably, *M. pneumoniae* infection also has the potential to induce epithelial cell migration and EMT, processes that may contribute to the persistence of inflammation and the progression of tissue remodeling. From a molecular perspective, *M. pneumoniae* infection activates the host's innate immune response, promoting the secretion of various cytokines, such as TGF-β, TNF-α, and IL-6,[18] which are capable of inducing cell migration and EMT. These cytokines drive the EMT process by activating related signaling pathways such as Smad, Wnt/β-catenin, and NF-κβ, which regulate the expression of EMT-related transcription factors.[19,20] In summary, the role of the CCL20/CCR6 signaling axis in *M. pneumoniae* infection and related diseases is gradually being elucidated. This not only deepens our understanding of host-pathogen interactions but also provides new theoretical foundations and potential therapeutic targets for related diseases. Future

research should further explore the specific regulatory mechanisms of this signaling axis in *M. pneumoniae* infection to offer more precise directions for clinical interventions.

In this study, we propose that the cytokine CCL20, induced by *M. pneumoniae*, can drive the migration and EMT of host epithelial cells, thereby exacerbating inflammatory responses and tissue damage. To further investigate the impact of this CCL20-rich microenvironment on epithelial cell migration and EMT, we established a high-expression CCL20 model by infecting THP-1 cells with *M. pneumoniae*. This model not only provides a valuable tool for elucidating the role of CCL20 in *M. pneumoniae* infection but also lays an experimental foundation for further research into the molecular mechanisms of inflammation and tissue remodeling.

Materials And Methods

Cell Cultivation

The 16HBE (human bronchial epithelial cells) and THP-1 (human monocytic leukemia cells) cell lines were generously provided by the School of Public Health and the Institute of Pathogen Biology at the University of South China, respectively. The use of these cell lines has been approved by the Research Ethics Committee of Nanhua Hospital, affiliated with the University of South China. THP-1 cells were maintained in RPMI 1640 medium, while 16HBE cells were cultured in DMEM medium. Both media were supplemented with 10% fetal bovine serum (FBS) and incubated under standard conditions of 37°C and 5% CO_2. The culture media were purchased from GIBCO (USA), and FBS was obtained from ExCell Bio (China).

Cultivation and Infection of M. Pneumoniae

The *M. pneumoniae* M129 strain (ATCC) was passaged in PPLO medium at a 1:10 dilution until the color of the medium transitioned from red to orange. The bacteria were then harvested by centrifugation (12,000 rpm, 10 minutes, 4°C). The pellet was resuspended in serum-free medium, filtered to disperse the mycoplasma. For infection experiments, 1×10^6 cells were seeded in 12-or 24-well plates and exposed to *M. pneumoniae* at varying multiplicities of infection (MOIs) for specific time intervals.

RNA Extraction and RT-qPCR Reaction

Total RNA was extracted using the RNA-easy kit (Vazyme, China), and 1 μg of RNA was reverse-transcribed into complementary DNA (cDNA). Subsequently, RT-qPCR was performed using the SYBR kit (Vazyme, China), with each reaction containing 2 μL of cDNA template. GAPDH was used for normalization. The $2^{-\Delta\Delta Ct}$ method was used to calculate the final product expression levels as determined by RT-qPCR. Primers were designed and synthesized by Sangon Biotech (China), and the sequences are as follows: E-Cadherin, 5'-GCCATCGCTTACACCATCCTCAG-3' (Forward) and 5'-CTCTCTCGGTCCAGCCCAGTG-3' (Reverse) and N-Cadherin, 5'-AGCCTGGAACATATGTGATG-3' (Forward) and 5'-TTGGAGCCTGAGACACGATT-3' (Reverse) and Snail 5'-ACTCCTACGGGAGGCAGCAGTAG-3' (Forward) and 5'-AGATGAGCATTGGCAGCGAG-3' (Reverse) and Vimentin, 5'-TGCAGGCTCAGATTCAGGAA-3' (Forward) and 5'-AGATGAGCATTGGCAGCGAG-3' (Reverse) and CCL20, 5'-TGCTGTACCAAGAGTTTGCTC-3' (Forward) and 5'-CGCACACAGACAACTTTTTCTTT-3' (Reverse) and GAPDH 5'-ACCACAGTCCATGCCATCAC-3' (Forward) and 5'-TCCACCACCCTGTTGCTGTA-3' (Reverse).

Western Blot Analysis

The infected cells were lysed using lysis buffer (Beyotime Biotechnology, China) supplemented with 1% protease and phosphatase inhibitors (APExBIO Technology, USA). After adjusting the protein concentration, the samples were mixed with loading buffer (Epizyme Biotech, China) at a 1:5 ratio and denatured by boiling at 100°C for 5 minutes. Equal amounts of protein were separated by SDS-PAGE (Epizyme Biotech, China) and then transferred onto a PVDF membrane (Cytiva, USA). The membrane was blocked with 5% skim milk at room temperature for 2 hours. Subsequently, the membrane was incubated overnight at 4°C with primary antibodies, including HY-P80276 P-AKT, HY-P80009 AKT, HY-P80813 P-ERK1/2, HY-P80393 ERK1/2, HY-P80084 c-jun, HY-P80616 c-fos, and HY-P80112

E-cadherin (all from MCE, USA, diluted at 1:800), as well as HY-P80438 β-actin (from MCE, USA, diluted at 1:10,000) and D155001 p-c-jun, D151325 p-c-fos, and D121239 snail (from Sangon Biotech, China, diluted at 1:800). After washing, the membrane was incubated with secondary antibodies (1:5000 dilution; mouse antibodies from Epizyme Biotech and rabbit antibodies from Sangon Biotech, D110058) for 2 hours. Protein bands were visualized using an ECL chemiluminescent substrate (NCM Biotech, China). Additionally, the inhibitors LY294002, PD98059, and T-5224 used in this experiment were all sourced from MCE (USA). Human CCL20/MIP-3α Antibody was obtained from R&D Systems (USA).

ELISA Analysis

To investigate the expression of CCL20 following *M. pneumoniae* infection, we employed enzyme-linked immunosorbent assay (ELISA) to quantitatively measure CCL20 levels. After infecting cells with *M. pneumoniae* for the specified time periods, the supernatants were collected by centrifugation at 4°C and 3000 rpm for 10 minutes. Using the human CCL20 ELISA kit (4A Biotechnology, China), the CCL20 protein levels in the cell supernatants and standards were quantified strictly following the manufacturer's instructions. Absorbance was measured at 450 nm using a microplate reader (Molecular Devices, USA). The concentration of CCL20 in the samples was determined by interpolating the absorbance values of the samples into the standard curve.

Wound Healing Assay

After trypsin digestion (GIBCO, USA), 16HBE cells were seeded into pre-labeled 12-well plates. When the cells reached 80% confluence (10^5 cells/well), a scratch was made perpendicular to the marked line using a 10 μL pipette tip guided by a steel ruler. The wells were washed with PBS and cultured in serum-free medium. For co-culture experiments, polycarbonate Transwell (Corning, USA) inserts with a pore size of 0.4 μm were utilized. THP-1 cells were seeded in the upper chamber of the Transwell, while 16HBE cells were cultured in the lower chamber. After incubation at 37°C with 5% CO_2 for 0 h and 48 h in serum-free conditions, cell migration distances were recorded using a Nikon microscope (Nikon, Japan).

Data Analysis and Graphical Presentation

Data are presented as the mean ± standard deviation (mean ± SD). The significance of differences between two groups was analyzed using the *t*-test, while comparisons among multiple groups were performed using analysis of variance (ANOVA). All data in this study were analyzed using GraphPad Prism 8.0.2 software. Graphical Abstract was created using Adobe Illustrator v27.

Results

M. Pneumoniae Induces CCL20 Expression

To investigate the regulatory mechanisms of CCL20 expression, we examined the effects of *M. pneumoniae* on THP-1 cells. Three hours post-infection, CCL20 protein levels were significantly upregulated (Figure 1A), and its mRNA expression increased in a dose-dependent manner with higher multiplicities of infection (MOIs) (Figure 1B). ELISA results demonstrated that CCL20 expression peaked at 24 hours and significantly increased with higher MOIs (Figure 1C and D). These findings indicate that *M. pneumoniae* effectively induces the expression and secretion of CCL20 in THP-1 cells.

To further validate the induction mechanism of CCL20, cells were treated under different conditions, including live and heat-inactivated bacteria, at a fixed MOI of 100. RT-qPCR analysis revealed a significant increase in CCL20 mRNA levels 3 hours post-infection (Figure 1E). ELISA further confirmed a marked elevation in CCL20 protein expression in the culture supernatant 12 hours post-infection (Figure 1F). Notably, CCL20 induction was significant regardless of bacterial viability, suggesting that the induction of CCL20 by *M. pneumoniae* is not entirely dependent on the pathogen's activity.

Additionally, we compared CCL20 expression levels across different cell types and found that CCL20 expression was significantly higher in THP-1 cells than in 16HBE cells (Figure 1G and H).

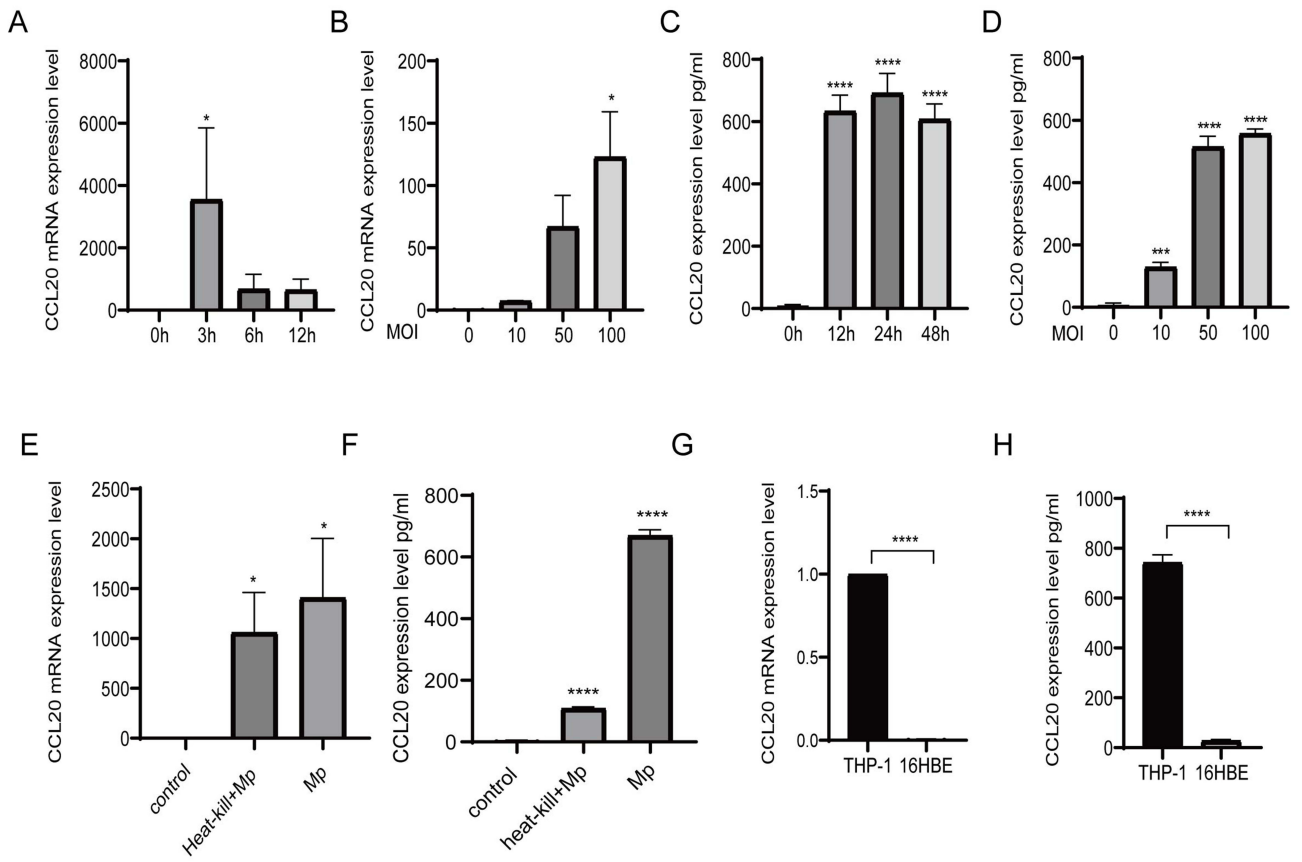


Figure 1 *M. pneumoniae* induces secretion of CCL20. In the RT-qPCR experiments, THP-1 cells were infected with *M. pneumoniae* at an MOI of 100 for 0, 3, 6, and 12 h (**A**); alternatively, cells were infected with MOIs of 0, 10, 50, and 100 for 3 h (**B**). In the ELISA experiments, THP-1 cells were infected with *M. pneumoniae* at an MOI of 100 for 0, 12, 24, and 48 h (**C**); or with MOIs of 0, 10, 50, and 100 for 12 h (**D**). RNA and supernatant were collected from THP-1 cells infected with *M. pneumoniae* in the logarithmic growth phase or with heat-inactivated *M. pneumoniae* at 3 h or 12 h post-infection. The expression levels of CCL20 were evaluated using RT-qPCR (**E**) and ELISA (**F**). Additionally, THP-1 cells and 16HBE cells were infected with *M. pneumoniae* at an MOI of 100 for 3 h or 12 h, after which mRNA expression levels were determined by RT-qPCR (**G**) and protein expression levels were determined by ELISA (**H**). Representative results from three independent experiments are shown. *$p<0.05$, ***$p<0.001$, ****$p<0.0001$. * indicates significance compared to the control group.

M. Pneumoniae Regulates CCL20 Expression Through the AKT Signaling Pathway

AKT has been identified as a potential regulator of CCL20 expression,[21] and *M. pneumoniae* can activate the AKT signaling pathway, thereby influencing disease progression.[22] To confirm AKT's involvement in *M. pneumoniae* induced CCL20 expression, we analyzed AKT activation in THP-1 cells using Western blot. The results indicated that *M. pneumoniae* infection induced AKT phosphorylation in an MOI dependent manner, while total kinase levels remained unchanged (Figure 2A and B). Pre-treatment with the AKT inhibitor LY294002 significantly reduced AKT levels (Figure 2C and D) and suppressed both CCL20 mRNA and protein levels (Figure 2E and F).

The ERK1/2 Signaling Pathway is Involved in Regulating the Expression of CCL20

ERK1/2 is a potential downstream regulator of AKT,[23] and has been implicated in the regulation of CCL20 expression under certain conditions.[10] To investigate ERK1/2's role in *M. pneumoniae* induced CCL20 expression and its link to the AKT signaling pathway, we evaluated ERK1/2 activation. The results showed that the phosphorylation level of ERK1/2 increased with MOI of *M. pneumoniae* (Figure 3A and B). The ERK1/2 inhibitor PD98059 significantly decreased *M. pneumoniae* induced ERK1/2 expression (Figure 3C and D) and markedly reduced CCL20 mRNA and protein levels (Figure 3E and F), indicating that CCL20 expression is regulated by the ERK1/2 pathway. Additionally, pre-treatment with an AKT inhibitor significantly reduced *M. pneumoniae*-induced ERK1/2

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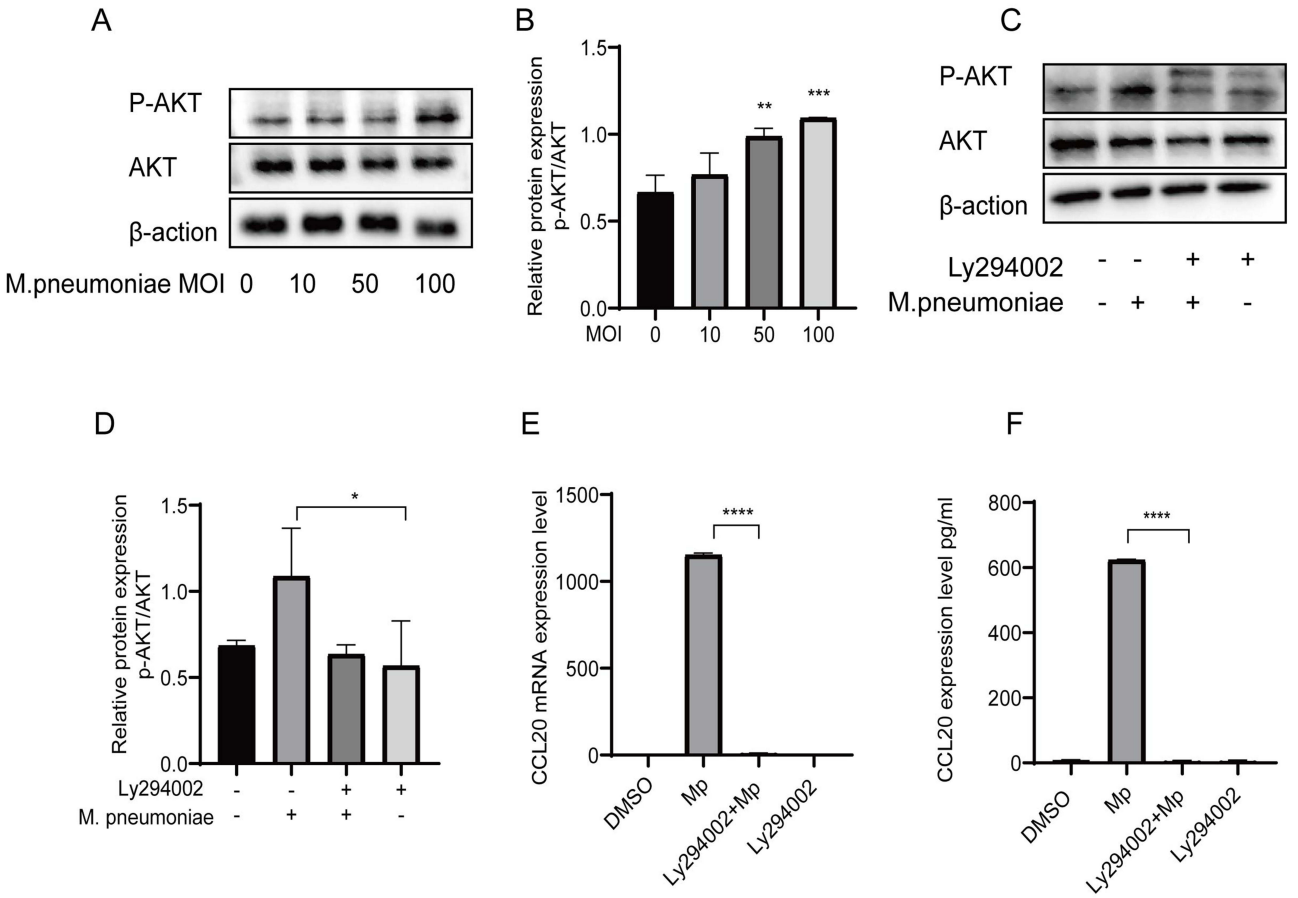

Figure 2 *M. pneumoniae* regulates CCL20 expression through the AKT signaling pathway. THP-1 cells were infected with *M. pneumoniae* at MOIs of 0, 10, 50, or 100 for 1 h, and protein expression levels were assessed by Western blot (**A** and **B**). In inhibitor experiments, cells were pretreated with 50 μM AKT inhibitor LY294002 for 1 h before infection with *M. pneumoniae* for 3 or 12 h. The inhibitory effect was detected by Western blot assay (**C** and **D**), while CCL20 mRNA and protein levels were measured by RT-qPCR (**E**) and ELISA (**F**). Representative results from three independent experiments are shown. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$. * indicates significance compared to the control group.

phosphorylation (Figure 3G and H). These findings suggest that CCL20 expression depends on the AKT-ERK1/2 signaling pathway.

M. Pneumoniae Modulates the Expression of CCL20 via the AP1 Signaling Pathway

The promoter sequence of CCL20 contains an activator protein 1 (AP1) binding site.[24] To verify AP1's role in regulating CCL20 expression, we examined the phosphorylation levels of AP1 subunits c-fos and c-jun after *M. pneumoniae* infection. The results showed that infection activated AP1 subunits in THP-1 cells, and this activation was reversed by pre-treatment with LY294002 (Figure 4A–D) and PD98059 (Figure 4E–H) inhibitors. Furthermore, using an AP1 functional inhibitor significantly suppressed CCL20 expression (Figure 4I and J). These findings indicate that *M. pneumoniae* activated AP1 operates downstream of the AKT-ERK1/2 signaling cascade to regulate CCL20 expression.

M. Pneumoniae Induces Cell Migration and EMT in a Co-Culture System

To investigate whether *M. pneumoniae* infection is associated with cell migration and EMT, 16HBE cells were co-cultured with THP-1 cells and then infected with *M. pneumoniae* at a MOI of 100. The wound healing assay results demonstrated that the co-cultured and infected group exhibited significantly enhanced cell migration compared to the control group (Figure 5A and B). Furthermore, at the protein level, the co-cultured and infected group significantly induced the down-regulation of the EMT marker E-cadherin and the upregulation of Snail expression (Figure 5C–E). At the mRNA level,

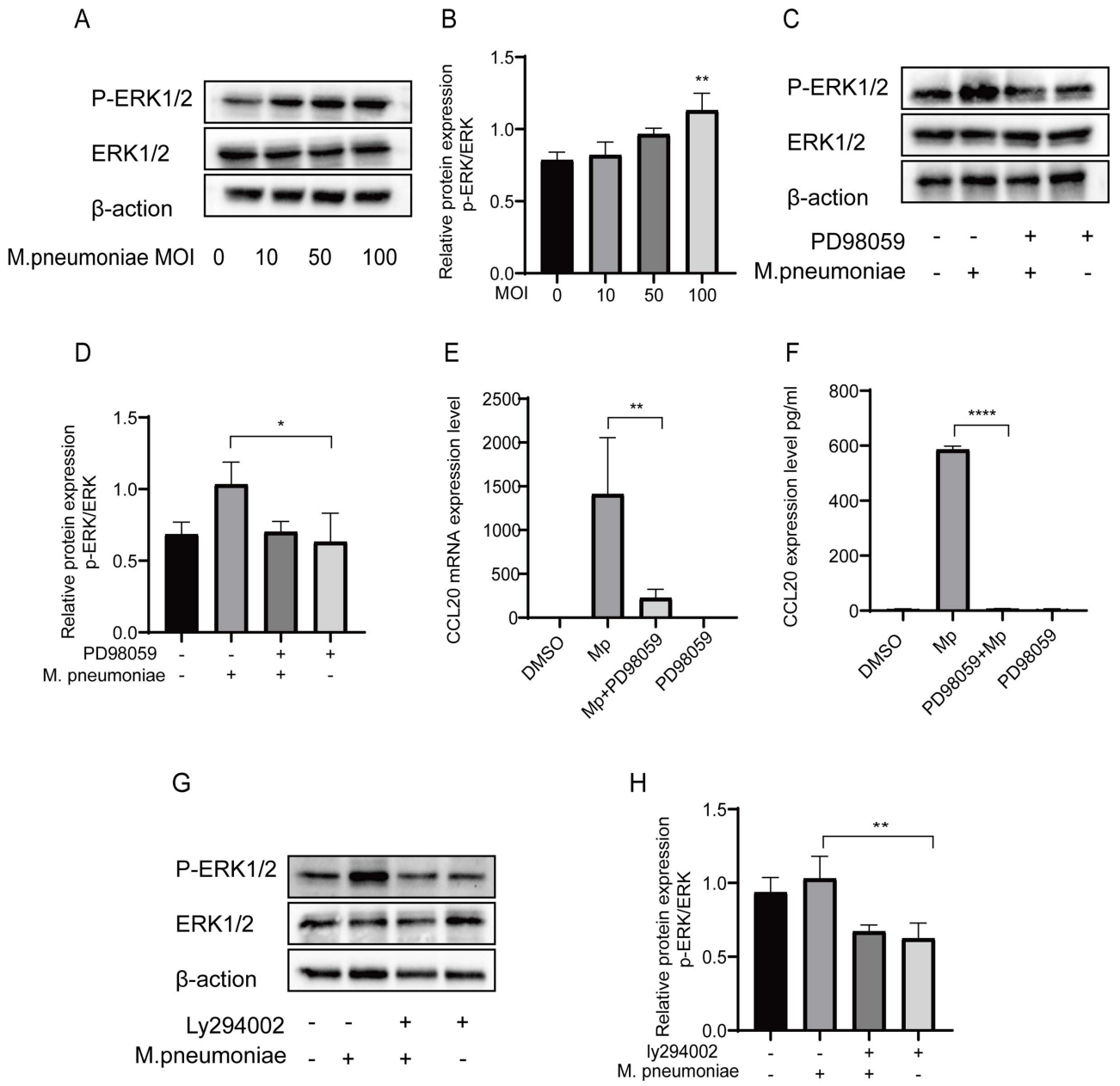

Figure 3 The ERK1/2 signaling pathway is involved in regulating the expression of CCL20. THP-1 cells were infected with *M. pneumoniae* at MOIs of 0, 10, 50, or 100 for 1 h, and protein expression levels were assessed by Western blot (**A** and **B**). In inhibitor experiments, cells were pretreated with 30 μM ERK1/2 inhibitor PD98059 for 1 h before infection with *M. pneumoniae*. The inhibitory effect was detected by Western blot (**C** and **D**). The levels of CCL20 mRNA and protein were measured by RT-qPCR (**E**) and ELISA (**F**), respectively. Additionally, cells were pretreated with 50 μM LY294002 prior to infection, and the inhibitory effect on ERK1/2 was detected by Western blot (**G** and **H**). Representative results from three independent experiments are shown. *$p<0.05$, **$p<0.01$, ****$p<0.0001$. * indicates significance compared to the control group.

E-cadherin expression was downregulated, while Snail and Vimentin expression were significantly upregulated (Figure 5F–H). These results suggest that *M. pneumoniae* infection may induce EMT and enhance cell migration.

CCL20 Induces Cell Migration and EMT

To further investigate whether *M. pneumoniae*-induced cell migration is associated with CCL20, we conducted a series of experiments to assess the role of CCL20 in this process. In the cell migration assay, the anti-CCL20 neutralizing antibody significantly inhibited cell migration (Figure 6A and B). Meanwhile, recombinant CCL20

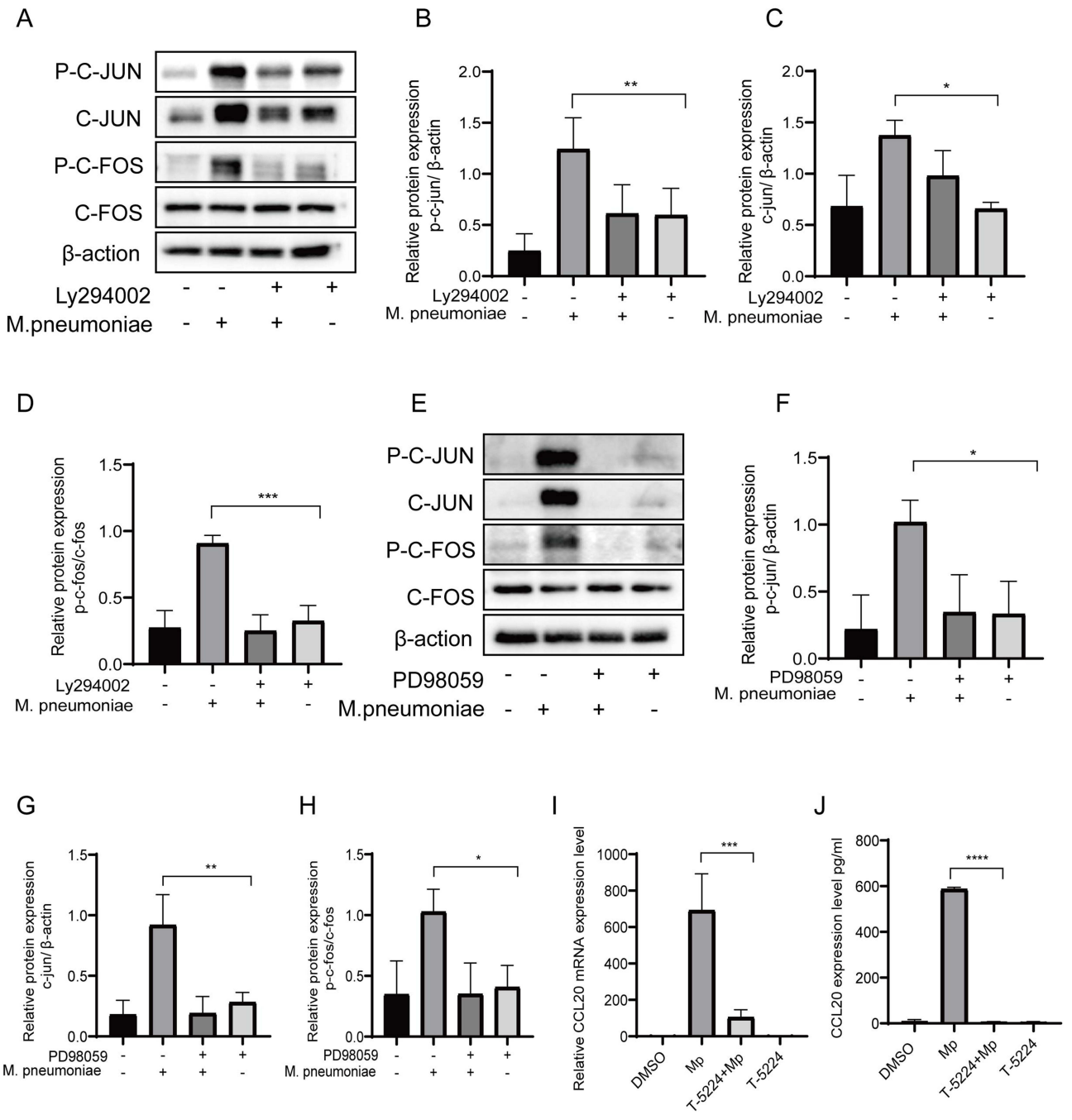

Figure 4 *M. pneumoniae* modulates the expression of CCL20 via the AP1 signaling pathway. Cells were pretreated with 50 μM LY294002 (**A-D**) or 30 μM PD98059 (**E-H**) for 1 h, followed by infection with *M. pneumoniae*. The inhibitory effects of the inhibitors were detected by Western blot. Subsequently, cells were pretreated with 30 μM AP1-specific inhibitor T-5224 for 1 h prior to infection to verify its inhibitory effect on CCL20 expression. The expression levels of CCL20 mRNA and protein were measured by RT-qPCR (**I**) and ELISA (**J**), respectively. Representative results from three independent experiments are shown. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$. * indicates significance compared to the control group.

protein enhanced cell migration (Figure 6C and D). Additionally, Western blot results (Figure 6E–G) demonstrated that recombinant CCL20 protein could induce the downregulation of the EMT marker E-cadherin and the upregulation of Snail. RT-qPCR results (Figure 6H–K) further showed that recombinant CCL20 induced the downregulation of E-cadherin and the upregulation of N-cadherin, Vimentin, and Snail. These findings suggest that *M. pneumoniae*-induced cell migration and EMT are associated with CCL20.

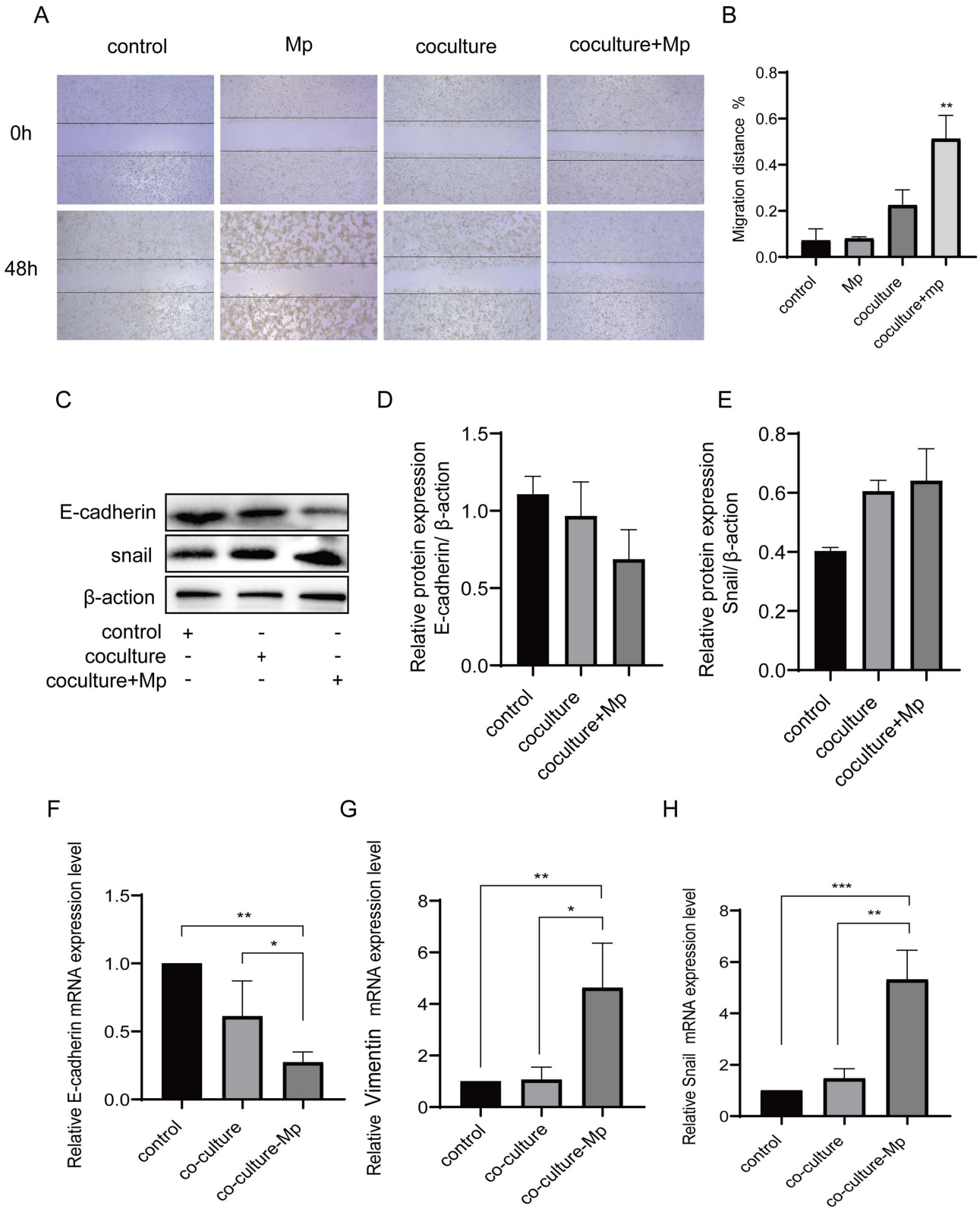

Figure 5 *M. pneumoniae* induces Cell Migration and EMT in a co-culture system. THP-1 cells and 16HBE cells were co-cultured at a 1:1 ratio in a Transwell system, with THP-1 cells seeded in the upper chamber and 16HBE cells in the lower chamber. THP-1 cells were infected with *M. pneumoniae* at an MOI of 100 for 48 h. After infection, the migration distance of 16HBE cells was assessed under a microscope (**A** and **B**). To investigate the role of EMT in this process, cells were cultured and infected under the same conditions for 48 h. Proteins from 16HBE cells were collected, and the expression levels of EMT markers were detected by Western blot for E-cadherin and Snail (**C-E**) and by RT-qPCR for E-cadherin, Vimentin, and Snail (**F-H**). Representative results from three independent experiments are shown. *$p<0.05$, **$p<0.01$, ***$p<0.001$. * indicates significance compared to the control group.

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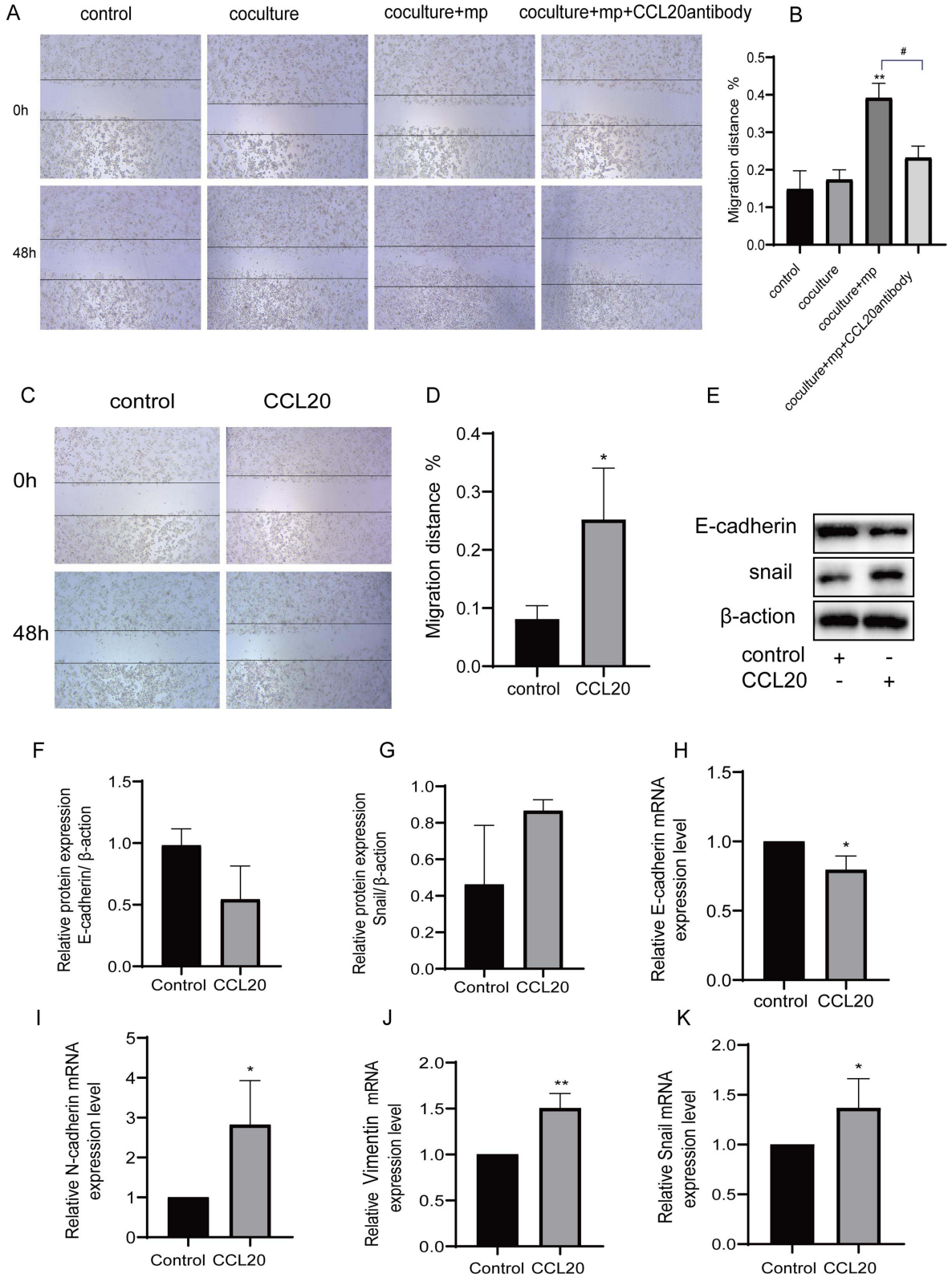

Figure 6 CCL20 Induces Cell Migration and EMT. THP-1 and 16HBE cells were co-cultured at a 1:1 ratio in a Transwell plate. To investigate the role of CCL20, 16HBE cells were pre-treated with an anti-CCL20 neutralizing antibody (30 μg/mL) in serum-free medium 3 h before *M. pneumoniae* infection, and cell migration distance was assessed under a microscope 48 h later (**A** and **B**). Additionally, 16HBE cells were treated with 40 ng/mL of recombinant CCL20, and cell migration distance was evaluated under a microscope after 48 h (**C** and **D**). To further explore the impact of CCL20 on EMT, cells were treated with 40 ng/mL recombinant CCL20 for 48 h. The expression levels of EMT markers were detected by Western blot for E-cadherin and Snail (**E-G**) and by RT-qPCR for E-cadherin, N-cadherin, Vimentin, and Snail (**H-K**). Representative results from three independent experiments are shown. *$p<0.05$, **$p<0.01$. * indicates significance compared to the control group. $^{\#}p<0.05$. $^{\#}$ Indicates significance compared to the CCL20antibody group.

Discussion

This study observed a significant increase in CCL20 expression in THP-1 cells following *M. pneumoniae* infection, with higher expression in THP-1 cells compared to 16HBE cells. Upon antigenic stimulation, immune cells consistently exhibit a more pronounced response compared to barrier cells. In alignment with previous studies, the chemokine CCL20 exhibits increased expression in immune cells under various conditions, including Brucella infection,[11] lipopolysaccharide (LPS) stimulation,[25] and during chemotherapy.[16] In addition to antigenic stimulation, many cytokines, such as IL-1β, TNF-α, and IL-17α, can dose-dependently stimulate CCL20 expression and exhibit synergistic effects.[26] Since *M. pneumoniae* infection significantly increases the levels of cytokines, particularly in immune cells, this could explain the observed differences in CCL20 expression between THP-1 and 16HBE cells following infection in this study.

CCL20 is a crucial chemokine that recruits immune cells, particularly dendritic cells (DCs) and T cells, to the site of infection, thereby promoting the immune response.[14] We demonstrated that *M. pneumoniae* infection induces the activation of AKT and ERK1/2, leading to the phosphorylation of the transcription factor AP1. Phosphorylated AP1 then binds to the promoter region of the CCL20 gene, enhancing its transcription. The activation of AKT mainly depends on PIP3 (phosphatidylinositol trisphosphate) produced by PI3K (phosphoinositide 3-kinase), and this process maintains AKT activity by inhibiting the dephosphorylation mediated by PTEN (phosphatase and tensin homolog).[27] In THP-1 cells, *M. pneumoniae* infection significantly increases AKT phosphorylation levels by activating the PI3K/AKT pathway. AKT indirectly affects ERK activity by modulating the phosphorylation state of MEK.[28] ERK (extracellular signal-regulated kinase) is an important member of the MAPK family, and its activation usually occurs through the Ras-RAF-MEK cascade, ultimately regulating the expression of downstream genes via phosphorylation.[29] ERK enhances the transcriptional activity of AP1 by directly phosphorylating the c-Jun and c-Fos subunits within AP1, a mechanism that has been validated in our study.[30] Further research indicates that the pathogenicity of *M. pneumoniae* may result from the combined effects of various bacterial components rather than relying solely on its viability, as even heat-inactivated *M. pneumoniae* is capable of inducing CCL20 expression and secretion in THP-1 cells. As a pathogen, *M. pneumoniae's* pathogenicity is not limited to its viability; its extracellular toxins, such as CARDS toxin,[31] lipoproteins,[31] and adhesive molecules,[32] can also cause similar pathogenic effects. This discovery provides new perspectives on the molecular mechanisms of *M. pneumoniae* infection and may offer clues for the design of future therapeutic strategies.

In experimental models, the addition of recombinant CCL20 protein was shown to downregulate E-cadherin expression and upregulate EMT marker proteins, highlighting the potential of CCL20 to induce EMT. In this study, we found that the anti-CCL20 neutralizing antibody did not significantly inhibit EMT, but it did effectively reduce cell migration. This suggests that CCL20 plays an important role in cell migration. However, the limited effect on EMT indicates that CCL20 may not be the main factor driving EMT. In addition to CCL20, multiple factors in the co-culture system promote the occurrence of EMT. When immune cells are co-cultured with lung cells, IL-6, HBEGF, linc00668, etc. in the microenvironment can promote the progression of EMT.[33–35] These findings suggest that multiple factors in the co-culture system can induce EMT. In the microenvironment created by *M. pneumoniae* infection, CCL20 may act as a secondary factor to induce EMT, but it plays a major role in the progression of cell migration. Future research will further explore which specific factors drive EMT in the co-culture system during *M. pneumoniae* infection.

In summary, our findings elucidate the mechanism by which *M. pneumoniae* stimulates the expression of CCL20 in THP-1 cells, subsequently inducing EMT and cell migration in 16HBE cells. Our data support the following conclusions: first, the expression of CCL20 is associated with the AKT-ERK1/2-AP-1 signaling cascade; second, co-culture of THP-1 cells with 16HBE cells can induce EMT and cell migration in bronchial epithelial cells. This suggests that the interaction between inflammatory cytokines and chemokines expressed by cells during bacterial infection is a critical area of research. Elucidating these interactions and their consequences plays a crucial role in future medical studies.

Conclusion

This study elucidates the molecular mechanism by which *M. pneumoniae* induces the expression of CCL20 in THP-1 cells through the AKT-ERK1/2-AP1 signaling pathway, further promoting EMT and cell migration in 16HBE cells. Specifically, *M. pneumoniae* infection significantly upregulates CCL20 expression in THP-1 cells, with higher expression levels compared to 16HBE cells. Although CCL20 plays a critical role in cell migration, its contribution to EMT is

relatively limited, suggesting that EMT may depend on the synergistic effects of multiple factors in the co-culture system. Additionally, the pathogenicity of *M. pneumoniae* is not solely dependent on its viability. These findings provide new insights into the mechanisms of cell migration and EMT associated with *M. pneumoniae* infection and offer potential targets for the design of future therapeutic strategies for related diseases.

Data Sharing Statement

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

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Author Contributions

All authors have substantially contributed to this work through various roles, including conceptualization, experimental design, implementation, data collection, analysis, and interpretation. Each author actively participated in manuscript preparation, critical revision, and final approval for publication. Furthermore, all contributors consented to submit the manuscript to this journal and take full responsibility for the integrity of the research.

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Disclosure

The authors declare no conflicts of interest.

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