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Hyperoside inhibits proinflammatory cytokines in human lung epithelial cells infected with *Mycoplasma pneumoniae*

Fang Liu[1] · YuHua Zhao[1] · JieMin Lu[1] · ShuangHui Chen[1] · XinGuang Zhang[2] · WenWei Mao[3]

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Abstract

Mycoplasma pneumoniae pneumonia (MPP) is the most common respiratory infection in young children and its incidence has increased worldwide. In this study, high expression of chemokine ligand 5 (CCL5) was observed in the serum of MPP patients, and its expression was positively correlated to DNA of *M. pneumoniae* (MP-DNA). In vitro, *M. pneumoniae* (MP) infection to A549 cells induced the expression of CCL5, chemokines receptor 4 (CCR4), nuclear factor-κB (NF-κB) nuclear protein, and phosphorylation of NF-κB-p65 (p-NF-κB-p65), whereas NF-κB cytoplasmic protein was decreased. On the contrary, treatment of hyperoside counteracted the induction of MP infection and promoted the proliferation of MP-infected A549 cells. Similarly, MP-induced IL-8 and TNF-α production was also markedly reduced by hyperoside. And CCR4 inhibitor AZD2098 had a better effect than hyperoside. In addition, CCL5 recombinant protein inhibited the effect of hyperoside to promote IL-8 and TNF-α production and CCR4 expression. These results indicated that CCL5 may be involved in the progression of MPP, and hyperoside was beneficial for MPP probably through CCL5–CCR4 interactions, which may provide a potential effective therapy for MPP.

Keywords Hyperoside · CCL5–CCR4 · *Mycoplasma pneumoniae* pneumonia · NF-κB · AZD2098

Introduction

Mycoplasma pneumoniae pneumonia (MPP), causing respiratory tract infection in persons of all ages [1, 2], particular in young children [3], is recognized as a worldwide cause of primary atypical pneumonia. In 1944, from tissue culture of a primary atypical pneumonia, *M. pneumoniae* (MP) was first isolated [4] and initial described as a human pathogen. Accumulated studies have showed that up to 10–40% of cases of all community-acquired pneumonia are due to the infection of *M. pneumoniae* [5–7]. Since 2010, it is reported that in Asia, Europe, and United States, the incidence of *M. pneumoniae* infection is increasing [8–10]. However, we know little about the molecular mechanisms of *M. pneumoniae* infection besides adherence to host epithelial cells [7].

Chemokine ligand 5 (CCL5, also known as RANTES), belonging to CC-chemokines family, is an agonist of four chemokines receptors of CCR1, CCR3, CCR4, and CCR5, and is an effective chemokine critical to immune and inflammatory response [11]. As an inflammatory chemokines, CCL5 is known to associate with various inflammatory diseases including inflammatory bowel disease and cancer [12, 13]. It is reported that the synthesis of CCL5 in human corneal keratocytes is induced by proinflammatory cytokines [14]. In human detrusor smooth muscle cells, IL-6, IL-8, and CCL5 are released to response to proinflammatory cytokines [15]. Nuclear factor-κB (NF-κB) is a frequent target of anti-inflammatory molecules and plays a key role in inflammatory diseases. Studies have related NF-κB activation to the production of proinflammatory cytokines such as IL-8 and TNF-α [16, 17]. In human lung cancer cells A549, *M. pneumoniae* infection induces proinflammatory cytokines expression [18], indicating the important role of inflammatory response in MPP.

✉ WenWei Mao
wwmaolf@163.com

[1] Department of Pediatrics, The Affiliated East Hospital of Tongji University, Shanghai 200123, People's Republic of China

[2] Department of Pediatrics, Shanghai Municipal Hospital of Traditional Chinese Medicine, Shanghai 200071, People's Republic of China

[3] School of Pharmacy, Shanghai Jiaotong University, NO. 800, DongChuan Road, MinHang District, Shanghai 200240, China

Flavonoids, a group of polyphenols, are found in various plants including fruit, vegetables, as well as beverages of plant origin. It is well known that flavonoids display a wide range of activities including anti-inflammatory [19, 20]. Hyperoside (also known as quercetin 3-*O*-β-D-galactoside) is a flavonoid compound with pharmacological properties, mainly found in Hypericum and Crataegus [21]. A large number of studies have shown that hyperoside exerts multiple activities including anti-inflammatory [22–24]. However, the effect and mechanism of hyperoside on MPP through CCL5 expression remain unclear.

In this study, we found that CCL5 expression was significantly elevated in MPP patients, and there was a positive correlation between CCL5 expression and MP-DNA. In A549 cells, MP infection significantly increased the expression of CCL5, CCR4, nucleoprotein NF-κB, and phosphorylation of NF-κB-p65 (p-NF-κB-p65) in a time-dependent manner, and decreased NF-κB cytoplasmic protein. Hyperoside counteracted the effect of MP infection and promoted cell proliferation in a dose-dependent manner, and the effect of CCR4 inhibitor AZD2098 was stronger. Further, MP-induced IL-8 and TNF-α production was inhibited by hyperoside and promoted by CCL5 recombinant. We speculated that hyperoside promoted the cell proliferation probably by regulating CCL5–CCR4 interactions via NF-κB pathway.

Materials and methods

Samples of peripheral blood

Thirty MPP patients who were treated at Shanghai Jiaotong University were enrolled in this study. After the informed consent was obtained, samples of peripheral blood were respectively collected from MPP patients and healthy controls. The expression of CCL5 in serum of MPP patients and healthy controls was detected by specific enzyme-linked immunosorbent assay (ELISA). All experiments of this study were approved by Ethics Committee of Shanghai Jiaotong University.

Cell culture

A549, a lung cancer cell line, was purchased from Cell Bank of Chinese Academy of Science (Shanghai, China). The cells were cultured with RPMI-1640 medium (HyClone, SH30809.01B, USA) containing 10% fetal bovine serum (FBS, GIBCO, USA) and 1% double-antibiotics (penicillin and streptomycin, Solarbio, P1400-100, China), and then incubated in a 37 °C, 5% CO_2 incubator (Thermo, Thermo Forma 3111). The medium was refreshed according to the demands of the cells.

Experimental grouping

To investigate the effect of hyperoside on MP-infected A549 cells, gradient concentrations of hyperoside (0, 25, 50, 100, 200, 400, and 1000 μg/ml, Aladdin, Q109801) were used. After 0, 12, 24, and 48 h of treatment, assays of cell proliferation were carried out. Subsequently, the level of CCL5 in the supernatant of medium was detected.

Further, four concentrations of hyperoside (0, 100, 200, and 400 μg/ml) and CCR4 inhibitor ADZ2098 were utilized to treat the MP-infected A549 cells. The assays of western blot and ELISA were performed after 24 h of treatment.

To explore the effect of CCL5 on MP-infected A549 cells, the cells were divided into five groups and randomly treated with medium, MP, MP + hyperoside, MP + hyperoside + CCL5, CCL5. After 24 h of treatment, the assays of ELISA and western blot were performed to detect the expression of IL-8, TNF-α, and CCR4.

Proliferation assay

A549 cells in logarithmic growth phase were digested with 0.25% trypsin (Solarbio, T1300-100) and counted under a microscope to make a cell suspension of 3×10^4 cells/ml. Later, the suspension seeded in 96-well culture plates at a density of 3×10^3 cells/well with 3 identical wells as duplicate wells, and incubated in a 5% CO_2 humidified incubator at 37 °C overnight. The next day, the cells were treated with gradient concentrations of hyperoside (0, 25, 50, 100, 200, 400, and 1000 μg/ml). After 0, 12, 24, and 48 h of treatment, 100 μl mixture of Cell Counting Kit-8 (CCK-8, SAB, CP002, USA) and serum-free medium in a volume ratio of 1:10 was added to each well, and then incubated at 37 °C in a 5% CO_2 incubator for 1 h. Finally, the optical density (OD) of the absorbance at 450 nm was measured by a microplate reader (Perlong, DNM-9602, Beijing).

Real-time polymerase chain reaction (RT-PCR)

The total RNA of MP-infected A549 cells was extracted by Trizol reagent (Invitrogen, 1596-026, USA) and quantified. The integrity of extracted RNA was confirmed by 1% electrophoresis and then reversed into cDNA by a reverse transcriptase kit (Fermentas, #K1622). Later, RT-PCR reactions with cDNA as templates were conducted on a Real-time detector (ABI, ABI-7300, USA) using a SYBR Green PCR kit (Thermo, #K0223, USA). The mRNA levels of CCL5 and CCR4 normalized to GAPDH were analyzed on software of ABI-7300 using $2^{-\Delta\Delta CT}$ method. Primers are as follows: CCL5, 5′ GCCAACCCAGAGAAGAAATG 3′ and 5′ GGACAAGAGCAAGCAGAAAC 3′; CCR4, 5′ CCT

TCCTGGCTTTCTGTTC 3′ and 5′ CATCTTCACCGCCTTGTTC 3′; GAPDH, 5′ AATCCCATCACCATCTTC 3′ and 5′ AGGCTGTTGTCATACTTC 3′. In addition, the following are procedure: 95 °C, 10 min (95 °C, 15 s; 60 °C, 45 s) × 40; 95 °C, 15 s; 60 °C, 1 min; 95 °C, 15 s; 60 °C, 15 s [25].

Western blot analysis

Treated-cells were collected and lysed in RIPA buffer (Solarbio) which containing protease and phosphatase inhibitors on ice for 30 min to full homogenize. After centrifuged at 12,000×*g* at 4 °C for 10 min, the total proteins of cells were collected. At meantime, Nuclear and Cytoplasmic Extraction Reagents (NE-PER^{TM}, Thermo, #78,835) was applied to extract the cytoplasmic or nuclear proteins. The collected cells were added pre-cooled Cytoplasmic Reagent I (CER I) with 15 s of severe concussion and incubated on ice for 10 min. Following the 1-min incubation of pre-iced Cytoplasmic Reagent II (CER II), the lysates were centrifuged at 16,000×*g* at 4 °C for 5 min, and then the supernatants were collected in a new tube. The remaining sediments were added pre-cooled nuclear protein extraction reagent (NER), mixed vigorously for 15 s, incubated on ice for 40 min, and mixed every 10 min for 15 s. And then the nuclear or cytoplasmic proteins were collected. Later, the proteins were quantified by a BCA protein kit (Thermo, PICPI23223). After separation of the proteins by electrophoresis on 10% SDS-PAGE, they were semi-dried by electroblotting onto polyvinylidene fluoride (PVDF) membranes (Millipore, HATF00010). Subsequently, the transferred membranes were blocked in 5% skim milk (BD Biosciences, BYL40422, USA) for 1 h at room temperature, followed by incubation with primary antibodies against CCL5 (1:1000, Abcam, Ab189841), CCR4 (1:1000, Abcam, Ab1669), NF-κB/NF-κB-p65 (1:1000, Abcam, Ab16502), p-NF-κB-p65 (1:1000, Abcam, Ab76302), GAPDH (1:2000, Cell Signaling Technology [CST], #5174), H3 (1 : 2000, CST, #4499S) at 4 °C with gentle shaking overnight. After 5–6 times of washing, the membranes were incubated with corresponding secondary antibodies (1 : 1000, Beyotime) at room temperature for 1 h. Finally, washed again and incubated with chemiluminescent reagent for 5 min, the target protein bands were visualized by an ECL imaging system (Tanon, Shanghai, China), and the protein levels were calculated by Image J software of version 1.47v (Bethesda, MD, USA).

Enzyme-linked immunosorbent assay (ELISA)

The peripheral blood from patients or treated-cells were collected and centrifuged at 2000–3000 rpm/min for approximately 20 min. Later, the serum or supernatants were collected in a new and clean tube. Following the ELISA Kit instructions, the concentrations of CCL5, IL-8, and TNF-α in serum or cell supernatants were respectively detected.

Statistical analysis

The data in this study were analyzed by the software of GraphPad prism 7.0 (GraphPad Software, USA). Statistical analyses of student's *t* tests were used to determine the difference between two comparisons. And the difference among three or more comparisons was analyzed by the methods of one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison. The correlation between two comparisons was determined by Pearson's analysis. With three independent experiments, all data were expressed as mean ± SD, and $P < 0.05$ was considered statistically significant.

Results

CCL5 was significantly increased in the serum of MPP patients and positively correlated with MP-DNA expression

CCL5, an inflammatory chemokines, is reported to involve in various diseases including lung cancer [12, 13]. Thus, CCL5 expression in serum of MPP patients was detected by ELISA. As shown in Fig. 1, compared to healthy control, the level of CCL5 in serum of MPP patients was significantly increased (Fig. 1a). Importantly, there was a positive correlation between CCL5 and MP-DNA by Pearson's analysis (Fig. 1b). It suggested that CCL5 was probably implicated in the progression of MPP.

MP infection induced CCL5, CCR4, NF-κB nuclear protein and p-NF-κB-p65 expression and decreased NF-κB cytoplasmic protein

Previous study has shown that *M. pneumoniae* infection may contribute to chronic asthma by inducing CCL5. Thus, 10^7 of *M. pneumoniae* strain were used to infect A549 cells in vitro. After 0, 12, 24, and 48 h of infection, the expression of CCL5, CCR4, and NF-κB was detected. Data shown in Fig. 2 revealed that the mRNA (Fig. 2a, b) and protein (Fig. 2d) levels of CCL5 and CCR4, as well as CCL5 level in cell supernatants (Fig. 2c) and NF-κB nuclear protein (Fig. 2e) were significantly increased in a time-dependent manner, whereas NF-κB cytoplasmic protein was decreased (Fig. 2f). In addition, MP infection increased the levels of p-NF-κB-p65 in A549 cells in a time-dependent manner, while NF-κB-p65 was unaltered (Fig. 2g). Accumulated studies had closely related NF-κB pathway to the progression of lung-related diseases [26–28]. These indicated that CCL5, CCR4, and NF-κB pathway were functioned in MP-infected A549 cells.

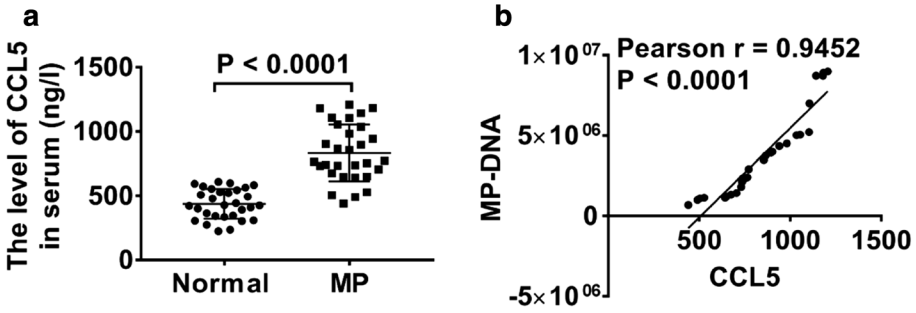

Fig. 1 CCL5 was significantly increased in serum of MPP patients and positive correlated with MP-DNA expression. Samples of peripheral blood from thirty MPP patients and healthy control was collected. **a** After the serum obtained, the level of CCL5 in serum of patients and healthy was detected by ELISA. **b** The correlation between CCL5 and MP-DNA was determined by Pearson's analysis. The data were presented as mean ± SD and $P < 0.0001$

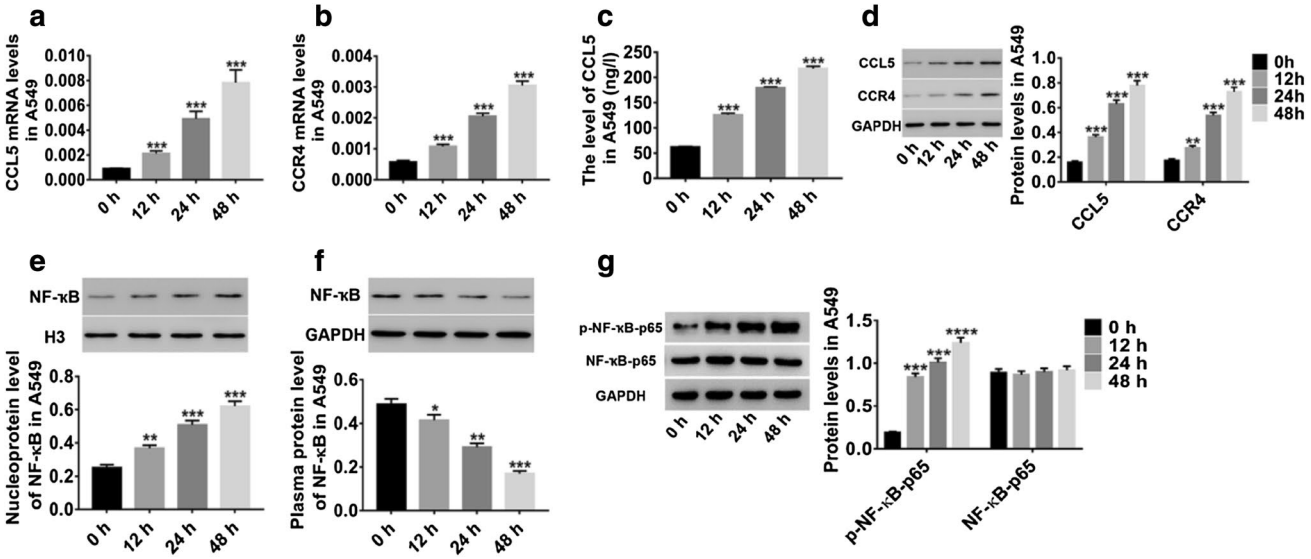

Fig. 2 MP infection induced CCL5, CCR4, NF-κB nuclear protein and p-NF-κB-p65 expression and decreased NF-κB cytoplasmic protein. The A549 cells were infected with 10^7 of *M. pneumoniae* strain for 0, 12, 24, and 48 h. The expression of CCL5 (**a**) and CCR4 (**b**) was quantified by RT-PCR while the concentration of CCL5 in cell supernatants was detected by ELISA (**c**). And the protein levels of CCL5, CCR4 (**d**), NF-κB nuclear (**e**) and cytoplasmic (**f**) proteins, and p-NF-κB-p65 (**g**) were determined by western blot analysis. All results were expressed at mean ± SD, and $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$ were compared to 0 h

Hyperoside promoted the cell proliferation and inhibited CCL5 expression in MP-infected A549 cells

Hyperoside is a natural product with various pharmacological properties, usually isolated from Hyperium or Crataegus [21]. Study has showed that hyperoside induces apoptosis and suppresses inflammatory response through NF-κB pathway to inhibit lung cancer [29]. Here, gradient concentrations of hyperoside (0, 25, 50, 100, 200, 400, and 1000 μg/ml) were used to investigate its effects on MP-infected A549 cells. As shown in Fig. 3, hyperoside potently promoted the cell proliferation (Fig. 3a) and inhibited CCL5 expression (Fig. 3b) in MP-infected A549 cells in a dose-dependent manner, suggesting that hyperoside may facilitate the proliferation of MP-infected A549 cells by suppression of CCL5 expression, which further evidenced the critical important role of CCL5 in MPP.

Hyperoside inhibited IL-8 and TNF-α production by suppressing CCR4 expression through NF-κB pathway in MP-infected A549 cells

Further, after treatment of hyperoside, the expression of CCR4 and NF-κB in MP-infected A549 cells was detected. As shown in Fig. 4, hyperoside obviously suppressed the expression of CCR4 (Fig. 4a) and NF-κB nuclear protein

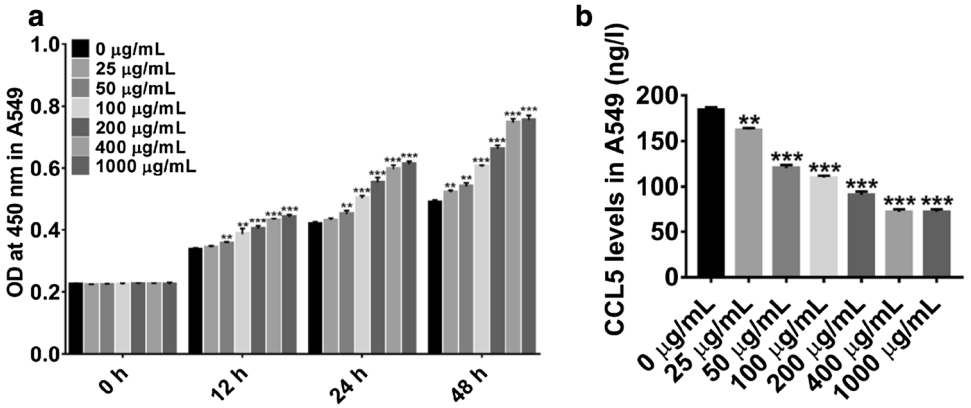

Fig. 3 Hyperoside promoted the cell proliferation and inhibited CCL5 expression in MP-infected A549 cells. MP-infected A549 cells were treated with gradient concentrations of hyperoside (0, 25, 50, 100, 200, 400, and 1000 μg/ml). **a** After 0, 12, 24, 48 h, the cell proliferation were assessed CCK-8 assays. **b** The concentration of CCL5 in cell supernatants was detected by ELISA 24 h later. The results were shown as mean ± SD, and $^{**}P < 0.01$, $^{***}P < 0.001$ were compared to 0 μg/ml treatment

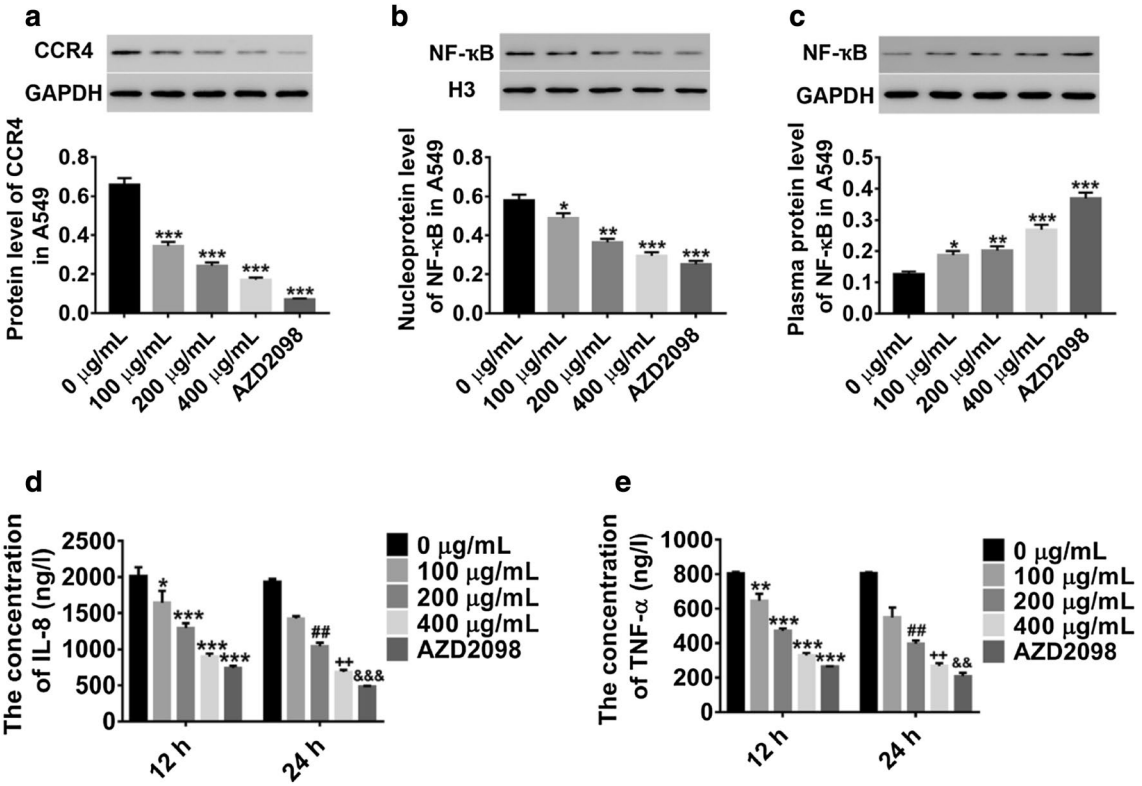

Fig. 4 Hyperoside inhibited IL-8 and TNF-α production by suppressing CCR4 expression through NF-κB pathway in MP-infected A549 cells. A549 cells MP-infected A549 cells were treated with hyperoside (0, 100, 200, and 400 μg/ml) and CCR4 inhibitor AZD2098. After 24 h, the protein levels of CCR4 (**a**), NF-κB nuclear (**b**) and cytoplasmic (**c**) proteins were quantified by western blot analysis. And the concentrations of IL-8 (**d**) and TNF-α (**e**) in cell supernatants were detected at 12 and 24 h by ELISA. All data were presented as mean ± SD, and $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ were compared to 0 μg/ml treatment

(Fig. 4b), whereas NF-κB cytoplasmic protein (Fig. 4c) was increased, and CCR4 inhibitor AZD2098 had a better effect than hyperoside. In addition, the production of IL-8 (Fig. 4d) and TNF-α (Fig. 4e) in cell supernatants was markedly reduced by hyperoside. In recent years, studies have found that *M. pneumoniae* causes diseases in part through the

induction of inflammatory reactions that produce cytokines such as IL-8 and TNF-α [18]. CCR4, one of the chemokine receptors to CCL5, is suggested to involve in lung diseases [30]. These indicated that hyperoside was beneficial for MP-infected A549 cells, which may inhibit CCR4 expression and IL-8 and TNF-α production through the NF-κB pathway.

Hyperoside inhibited IL-8 and TNF-α production via regulating CCL5–CCR4 interactions

To further explore the mechanism of hyperoside on MPP, the MP-infected A549 cells were treated with 200 μg/ml hyperoside and CCL5 recombinant protein. After 24 h of treatment, the expression of IL-8, TNF-α, and CCR4 was detected. As shown in Fig. 5, the production of MP-induced IL-8 (Fig. 5a) and TNF-α (Fig. 5b) was significantly reduced by hyperoside, and the effect of hyperoside was strongly counteracted by the treatment of CCL5 recombinant protein. Furthermore, hyperoside and CCL5 recombinant protein showed a similar effect on CCR4 expression (Fig. 5c). The results further demonstrated that hyperoside inhibited IL-8 and TNF-α production in MP-infected A549 cells probably through CCL5–CCR4 interactions, which may benefit for MPP treatment.

Discussion

MPP, the most severe respiratory infection by *M. pneumoniae*, is most commonly occurred in older children and young adults [31]. Study has shown that second only to *Streptococcus pneumoniae, M. pneumoniae* infection is responsible for about one-third of pneumonias overall [32, 33]. In this study, we discovered that CCL5 was highly expressed in serum of MPP patients; hyperoside significantly decreased MP-induced CCL5/CCR4 expression and IL-8/TNF-α production in A549 cells and promoted cell proliferation, which probably by regulating CCL5–CCR4 interactions through NF-κB pathway.

CCL5, produced by various cells including epithelial cells and lymphocytes, is suggested to modulate pneumococcal immunity and carriage [34]. Here, we found significant increased CCL5 in serum of MPP patients, and its expression was positive correlated to MP-DNA. In vitro, the levels of CCL5, CCR4, NF-κB nuclear protein, and p-NF-κB-p65 were significantly increased by MP infection in A549 cells in a time-dependent manner, whereas NF-κB cytoplasmic protein was decreased. These suggested that CCL5, associated with CCR4 and NF-κB pathway, was involved in MPP progression and high CCL5 in serum may be as a predictor of MPP prognosis. Further, MP-induced the expression of CCL5, CCR4, and NF-κB nuclear protein was markedly reduced by hyperoside whereas NF-κB cytoplasmic protein was increased, and CCR4 inhibitor AZD2098 had a better effect than hyperoside. NF-κB is reported to be a transcription factor that affects the severity of pneumonia [35]. There is study shown that the F0F1 ATPase derived from mycoplasma cell membrane activates NF-κB via Toll-like receptor (TLR)-1, -2, and -6 [36]. And increased expression of NF-κB was observed in MP-infected asthmatic mice [37]. In addition, *M. pneumoniae* directly activates and induces the production of

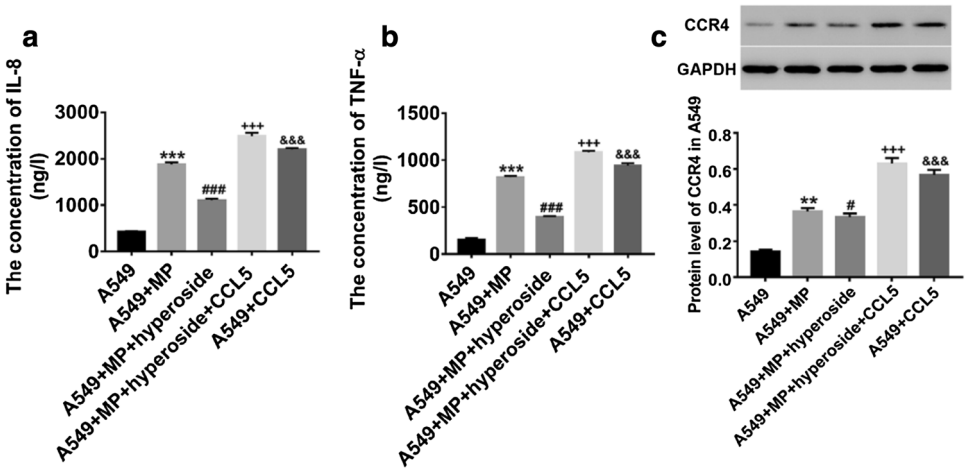

Fig. 5 Hyperoside inhibited IL-8 and TNF-α production via regulating CCL5–CCR4 interactions. A549 cells were randomly divided and treated with medium, MP infection, MP + hyperoside, MP + hyperoside + CCL5 recombinant, and CCL5 recombinant. After 24 h of treatment, the production of IL-8 (**a**) and TNF-α (**b**) in cell supernatants was detected by ELISA, while CCR4 protein level was quantified by western blot analysis (**c**). The results were shown as mean ± SD, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to A549; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ compared to A549 + MP; $^{+++}P < 0.001$ compared to A549 + MP + hyperoside; $^{\&\&\&}P < 0.001$ compared to A549 + MP + hyperoside + CCL5

cytokines, for instance, IL-8 and TNF-α are produced by *M. pneumoniae*-infected human lung cells, subsequently modulating the inflammatory infiltrates activity [7]. It appears that the activation of TNF-α production by phagocytes does not require cell contact and can be induced with the culture supernatant of mycoplasma [38]. These are consistent with our results that the IL-8 and TNF-α production in MP-infected A549 cells was significantly increased, and the induction of MP infection was counteracted by hyperoside. Furthermore, treatment of CCL5 recombinant protein showed a similar effect to MP infection. These demonstrated that hyperoside inhibited MP-induced IL-8 and TNF-α production probably by regulating CCL5–CCR4 interactions through NF-κB pathway.

In summary, we demonstrated the beneficial effect of hyperoside on MP-infected A549 cells, which possible by regulating CCL5–CCR4 interactions through the NF-κB pathway. It shows that hyperoside may be a potential active ingredient of drugs that treat or ameliorate MPP.

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Compliance with ethical standards

Conflict of interest We have no conflict of interest to declare.

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