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YKL-40 Induces IL-8 Expression from Bronchial Epithelium via MAPK (JNK and ERK) and NF-κB Pathways, Causing Bronchial Smooth Muscle Proliferation and Migration

Hao Tang,*[,1] Yu Sun,[†,1] Zhaoquan Shi,*[,1] Hai Huang,* Zheng Fang,* Jiquan Chen,* Qingyu Xiu,* and Bing Li*

Recently, the serum levels of YKL-40, a chitinase-like glycoprotein, have been shown to be significantly elevated in asthmatics and are associated with asthma severity. Although these studies raise the possibility that YKL-40 may influence asthma, the mechanisms remain unknown. This study firstly investigated the mechanisms involved in YKL-40–mediated inflammation in human bronchial epithelial cells (HBECs) and analyzed the soluble factors secreted by bronchial epithelial cells exposed to YKL-40 that were responsible for increasing proliferation and migration of primary normal human bronchial smooth muscle cells (BSMCs). YKL-40–induced inflammation was assayed in two HBECs (BEAS-2B cell line and primary HBECs). In addition, we treated BEAS-2B cells and HBECs with YKL-40 and added the conditioned culture media to BSMCs. The proliferation and migration of BSMCs were determined by premixed WST-1 cell proliferation reagent (Clontech Laboratories) and QCM chemotaxis migration assay (Millipore), respectively. Bronchial epithelial cells treated with YKL-40 resulted in a significant increase of IL-8 production, which was dependent on MAPK (JNK and ERK) and NF-κB pathways activation. YKL-40–induced IL-8 was found to further stimulate proliferation and migration of BSMCs, and the effects were inhibited after neutralizing IL-8. Through investigating the interaction of airway epithelium and smooth muscle, our findings implicate that YKL-40 may be involved in the inflammation of asthma by induction of IL-8 from epithelium, subsequently contributing to BSMC proliferation and migration. Moreover, inhibition of IL-8 signaling is a potential therapeutic target for YKL-40–induced inflammation and remodeling of asthma. *The Journal of Immunology*, 2013, 190: 438–446.

Asthma is traditionally defined as a chronic disease characterized by bronchial hyperresponsiveness and lung inflammation (1). It is now believed that chronic inflammation drives the remodeling response, leading to structural alterations responsible for pathogenesis and clinical manifestations of asthma (2). Among these alterations, increased bronchial smooth muscle cell (BSMC) mass may represent a key feature contributing to airflow obstruction (3). Features of hyperplasia (increased BSMC number) (4) and hypertrophy (increased BSMC size) (5) have been observed in affected tissue. Additionally, the migration of BSMCs toward the epithelium may be an additional factor that enhances or perpetuates the BSMC remodeling process (6, 7). However, the mechanisms underlying such BSMC remodeling remain to be elucidated.

YKL-40 is a chitinase-like glycoprotein expressed and secreted by a variety of cell types (8). Since it was identified in 1993 as a secreted product of articular chondrocytes and synovial cells (9), a growing body of evidence has shown that high serum levels of YKL-40 are associated with various pathological conditions such as allergic diseases (10–12). In terms of lung diseases, high serum levels of YKL-40 are associated with asthma (13, 14), chronic obstructive pulmonary disease (15), and lung cancer (16). In asthma, increased serum levels and lung expression of YKL-40 are correlated with disease severity, airway remodeling, and decreased pulmonary function (17). Our previous study also showed that the elevated serum YKL-40 levels in asthmatics were correlated positively with total serum IgE levels and the percentage of peripheral blood eosinophils and correlated inversely with lung functions (18). Based on these findings, YKL-40 is believed to be involved in the pathogenesis of asthma and has attracted the attention of many groups during last few years. Using BRP-39 (YKL-40 homolog in mouse) null mutant mice, YKL-40–transgenic mice, and mice that lack BRP-39 but produce YKL-40 only in their pulmonary epithelium, the study of Lee et al. (19) demonstrated that BRP-39/YKL-40 played a critical role in the pathogenesis of aeroallergen-induced Th2 inflammation and IL-13–induced inflammation and remodeling of the lung. Moreover, human macrophages stimulated with YKL-40 produced IL-8 (15), providing evidence that YKL-40 may contribute to the inflammatory response. Although the studies of the role of YKL-40 on asthma are limited, from current studies available, it could be speculated that YKL-40 is more than a simple biomarker in asthma, but rather an active player in inflammation of asthma.

Other studies showed that serum YKL-40 level was positively related to the degree of liver fibrosis, and staining of YKL-40 was

Table I. Primers used for real-time PCR

Gene Name	Forward Primer	Reverse Primer
IL-8	5′-ATGACTTCCAAGCTGGCCGTGGCT-3′	5′-TCTCAGCCCTCTTCAAAAACTTCTC-3′
Eotaxin	5′-CCCAACCACCTCCTGCTTTAAC-3′	5′-CCAGATACTTCATGGAATCCTGCAC-3′
RANTES	5′-TGCTTTGCCTACATTGCCCCG-3′	5′-CTGGGGAAGGTTTTGTAACTGC-3′
TNF-α	5′-CCGAGTGACAAGCCTGTAGC-3′	5′-GAAGGAGACGGTAAAGTTGTTGA-3′
β-actin	5′-GTGGGGCGCCCCAGGCACCA-3′	5′-CTCCTTAATGTCACGCACGATTTC-3′

higher in areas with fibrosis, particularly in areas with active fibrogenesis (20, 21). Along this line, it is possible that YKL-40 also plays a role in the tissue-remodeling process of asthma. However, up to now, only one new report from Bara et al. (22) showed that YKL-40 could promote BSMC proliferation and migration directly via a protease-activated receptor-2–dependent pathway. Our very limited understanding of the biology of YKL-40 makes it difficult to explain how YKL-40 contributes to the pathological responses of asthma, characterized by airway inflammation and remodeling. Further investigations are needed to explore the mechanisms of YKL-40–mediated inflammation and structural changes of remodeling.

The bronchial epithelium is known to play a critical regulatory role in the maintenance of airway function and integrity (23–25). Bronchial epithelium can produce various cytokines/chemokines to regulate other systems or tissues, including BSMCs (26). As a recent study (22) showed that YKL-40 was secreted by epithelial cells and macrophages, particularly in severe asthmatics, we hypothesized that YKL-40 may cause bronchial epithelial cells to produce soluble factors (cytokines/chemokines) that in turn in-

crease proliferation and migration of BSMCs, adding an indirect role of YKL-40 on BSMC remodeling in asthma to the direct role reported by Bara et al. (22). In the current study, we investigated the molecular mechanisms involved in YKL-40–mediated IL-8 production from human bronchial epithelial cells (HBECs) and analyzed the soluble factors (including IL-8) secreted by bronchial epithelial cells after YKL-40 exposure that were responsible for increasing proliferation and migration of primary normal human BSMCs.

Materials and Methods

Materials and reagents

Purified human YKL-40 protein was purchased from Quidel (San Diego, CA). Abs against phospho-p38, p38, phospho-JNK, JNK, phospho-ERK, ERK, phospho–NF-κB p65, NF-κB p65, phospho-IκBα, IκBα, and actin were ordered from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant human IL-8 (rhIL-8) and anti–IL-8 Ab were purchased from R&D Systems (Minneapolis, MN). c-JNK inhibitor (SP600125), p38 inhibitor (SB239063), and NF-κB inhibitor (Bay 11-7082) were purchased from Sigma-Aldrich (St. Louis, MO). ERK inhibitor (UO126) was ordered from Cell Signaling Technology (Beverly, MA).
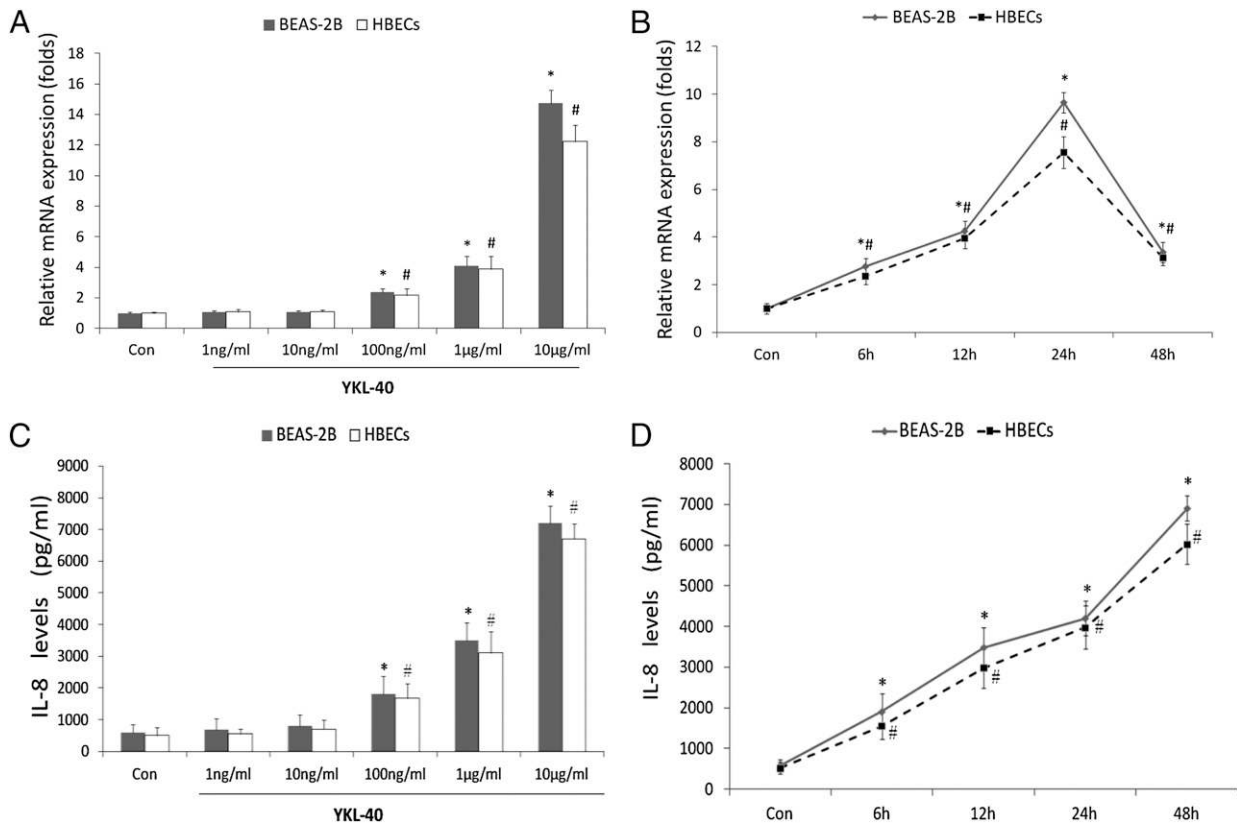
FIGURE 1. The effect of YKL-40 on IL-8 production in HBECs (BEAS-2B cells and HBECs). Beas-2B cells and HBECs were treated with various concentrations of YKL-40 for 6 h or stimulated with YKL-40 (100 ng/ml) at different time points as indicated. YKL-40 increased the expression of IL-8 mRNA in a concentration- (**A**) and time-dependent (**B**) manner in BEAS-2B cells and HBECs. YKL-40 also induced the release of IL-8 from BEAS-2B cells and HBECs in a concentration- (**C**) and time-dependent (**D**) mode. All experiments were performed independently at least three times. $*p < 0.05$ versus control (Con) of BEAS-2B; $^{\#}p < 0.05$ versus Con of HBECs.

Cell culture

We used two HBECs in present study, immortalized, nontumorigenic HBECs (cell line BEAS-2B) obtained from cell banks of the Chinese Academy of Sciences and primary normal HBECs purchased from Lonza (Walkersville, MD). Both BEAS-2B cells and HBECs were cultured in bronchial epithelial growth medium (Lonza). BEAS-2B cell cultures were maintained in 60-/100-mm dishes (CellBIND surface; Corning, Lowell, MA). Primary normal human BSMCs were also obtained from Lonza. BSMCs were cultured in SmGM-2 smooth muscle medium (Lonza).

To obtain various conditioned media (CM), BEAS-2B cells and HBECs (2×10^6) were treated with vehicle control or various concentrations of YKL-40 for 6 h. After treatment, the medium was replaced, and the supernatants were harvested after 24 h of incubation, namely CM of BEAS-2B treated with YKL-40 (YKL-40–BEAS-2B-CM) and CM of HBECs treated with YKL-40 (YKL-40–HBECs-CM). IL-8 depletion from YKL-40–BEAS-2B-CM and YKL-40–HBECs-CM was performed using anti–IL-8 (2 µg/ml) Ab and Sepharose A/G beads following regular immunoprecipitation techniques. Cytokine depletion was confirmed by IL-8 ELISA assay.

ELISAs

The levels of IL-8, RANTES, eotaxin, TNF-α, and IL-6 were determined by ELISA kits (R&D Systems). The levels of IL-8, RANTES, eotaxin, and TNF-α were assayed by treatment of BEAS-2B cells and HBECs with YKL-40. For experiments employing inhibitors, BEAS-2B cells were pretreated with SB239063 (10 µM), UO126 (20 µM), SP600125 (20 µM), or Bay 11-7082 (5 µM) for 1 h and then exposed to YKL-40 to assay levels of IL-8. The levels of IL-8, RANTES, eotaxin, and IL-6 were determined for BSMCs treated with YKL-40–BEAS-2B-CM for 12 h.

NF-κB reporter assay

NF-κB reporter activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Fitchburg, WI). BEAS-2B cells (4×10^4) were seeded into 24-well culture plates. After 48 h at 70% confluency, BEAS-2B cells were cotransfected with 1 µg NF-κB–responsive reporter plasmid containing a promoter construct consisting of three tandem repeats of an NF-κB binding site linked to the luciferase gene (pNFκB-luc; Stratagene, La Jolla, CA) along with 0.01 µg control plasmid linked to the Renilla luciferase (pRL-TK; Promega) by using jetPEI transfection Reagent (Polyplus-transfection SA). At 24 h after transfection, cells were washed and stimulated with YKL-40 for an additional 6, 12, and 24 h. The luciferase activities of the NF-κB luciferase and Renilla luciferase were measured, and results were presented as luciferase activity normalized to Renilla luciferase activity.

Cell proliferation

BSMCs (4×10^3) were plated in 96-well culture plates. After 24 h incubation, BSMCs were treated with control medium, YKL-40–BEAS-2B-CM, YKL-40–HBECs-CM, IL-8–depleted YKL-40–BEAS-2B-CM, IL-8–depleted YKL-40–HBECs-CM, or medium containing rhIL-8 (20 ng/ml) for 72 h. The proliferation of BSMCs was determined by Premixed WST-1 Cell Proliferation Reagent (Clontech Laboratories, Mountain View, CA). For inhibitor experiments, YKL-40–BEAS-2B-CM and YKL-40–HBECs-CM pretreated with UO126 (20 µM), SP600125 (20 µM), or Bay 11-7082 (5 µM) for 1 h was collected to stimulate BSMCs for an additional 72 h.

Cell migration assay

Cell migration was carried out using the QCM Chemotaxis 8-µm cell migration assay system (Millipore, Bedford, MA). BSMCs were seeded
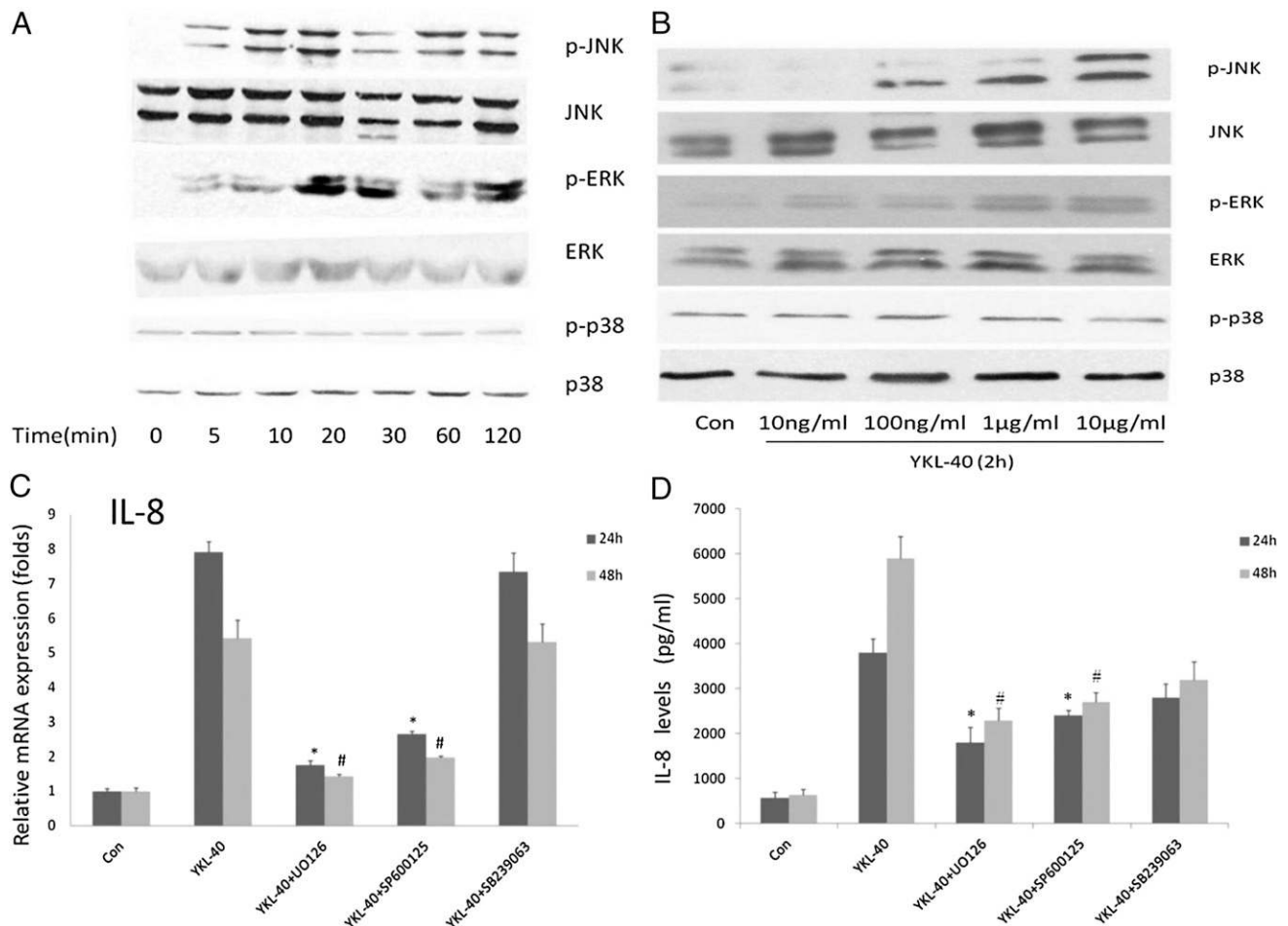
FIGURE 2. Involvement of MAPK pathway in YKL-40–mediated IL-8 production in BEAS-2B cells. YKL-40 induced JNK and ERK but not p38 phosphorylation in a time- (**A**) and concentration-dependent (**B**) manner in BEAS-2B cells. The results represent one of similar results from four independent experiments. The effects of JNK, ERK, and p38 inhibitors on YKL-40–mediated IL-8 mRNA expression (**C**) and IL-8 release (**D**) from BEAS-2B cells. Each point represents mean ± SD of triplicate values from three independent experiments. *$p < 0.05$ versus YKL-40 stimulation for 24 h without inhibitors; #$p < 0.05$ versus YKL-40 stimulation for 48 h without inhibitors. Con, Control.

into the migration chamber. Control medium, YKL-40–BEAS-2B-CM, YKL-40–HBECs-CM, IL-8–depleted YKL-40–BEAS-2B-CM, IL-8–depleted YKL-40–HBECs-CM, or medium containing rhIL-8 (20 ng/ml) was placed in the lower chamber. After allowing cell migration for 24 h, BSMCs that had migrated through the membrane were stained, lysed, and quantified on a microplate at 520 nm. For experiments employing inhibitors, YKL-40–BEAS-2B-CM and YKL-40–HBECs-CM pretreated with UO126 (20 μM), SP600125 (20 μM), or Bay 11-7082 (5 μM) for 1 h were placed in the lower chamber, and BSMCs were subjected to the migration assay.

RNA extraction and real-time PCR analysis

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA), and 2 μg total RNA was reverse-transcribed using Reverse Transcription Reagents (Fermentas). The mRNA levels of the indicated genes were analyzed using SYBR Green PCR Master Mix (Toyobo) on a Mastercycler ep realplex (Eppendorf). The primer sequences used can be found in Table I. Thermal cycling conditions consisted of an initial denaturing step (95°C, 2 min) followed by 40 cycles of denaturing (95°C, 15 s), annealing (60°C, 15 s), and extending (72°C, 45 s). The specificity of the product made was controlled via a melting curve. The mRNA levels were normalized to β-actin (internal control) using the formula Δ threshold cycle (Ct) = Ct target − Ct reference. The differential expression signal was calculated as $\Delta\Delta Ct = \Delta Ct$ (gene of YKL-40–treated group) − ΔCt (gene of untreated group) and expressed as relative fold of change using the formula $2^{\Delta\Delta Ct}$.

Western blot analysis

Whole cells were prepared in RIPA lysis buffer, and cleared lysates were equally loaded on 12% SDS-polyacrylamide gel and transferred to nitrocellulose membrane (Millipore). The blots were stained with 0.2% Ponceau S red to ensure equal protein loading. After blocking with 5% nonfat milk in TBS Tween-20, the membranes were incubated with primary Ab overnight at 4°C followed by HRP-conjugated secondary Ab (Rockland Immunochemicals). All blots were reprobed with anti-actin Ab (Sigma-Aldrich Chemicals) to confirm equal loading among samples. Detection was visualized using an ECL assay kit (Pierce).

Statistical analysis

Data are presented as mean ± SD of at least three determinations. Statistical comparisons of the results were made using ANOVA. Significant differences ($p < 0.05$) between the means of the two test groups were analyzed by Dunnett's test.

Results

Stimulation of YKL-40 increased IL-8 production in HBECs

Increased inflammatory response in epithelium plays an important role in development of asthma (27). We first assessed whether YKL-40 increased the production of inflammatory mediators in HBECs (BEAS-2B and HBECs). We found that YKL-40 significantly increased the mRNA expression of IL-8 in both BEAS-2B and HBECs in a concentration- and time-dependent manner (Fig. 1A, 1B), with a maximum increase at a concentration of 10 μg/ml and at a time point of 24 h. Further ELISA assays showed that YKL-40 stimulation led to the release of IL-8, also in a concentration- and time-dependent fashion, with a maximum induction at 10 μg/ml and at 48 h in BEAS-2B and HBECs (Fig. 1C, 1D). On the contrary, YKL-40 failed to affect RANTES, eotaxin, and TNF-α expression on both mRNA and protein levels in BEAS-2B and HBECs (data not shown).

Involvement of MAPK pathway in YKL-40–increased IL-8 production in BEAS-2B

The MAPK family is well known to play a key role in mediating inflammatory responses by secretion of chemokines such as IL-8 (28). To verify the involvement of the MAPK pathway in YKL-40–mediated IL-8 production, BEAS-2B cells stimulated with
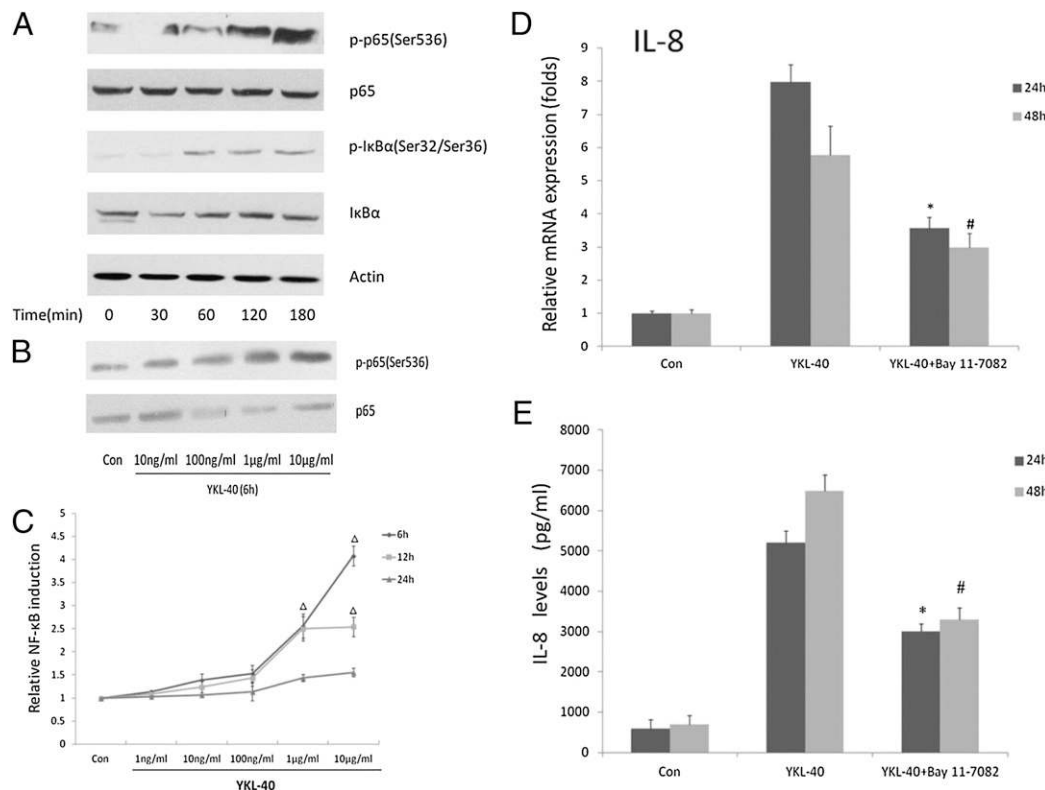
FIGURE 3. Involvement of NF-κB pathway in YKL-40–mediated IL-8 production in BEAS-2B cells. YKL-40 induced p65 and IκBα phosphorylation in a time-dependent manner in BEAS-2B cells (**A**). YKL-40 also induced p65 phosphorylation in a concentration-dependent mode in BEAS-2B cells (**B**). The results represent one of similar results from three independent experiments. YKL-40 induced transactivation of NF-κB in BEAS-2B cells (**C**). The effect of NF-κB inhibitor on YKL-40–mediated IL-8 mRNA expression (**D**) and IL-8 release (**E**) from BEAS-2B cells. Each point represents mean ± SD of triplicate values from three independent experiments. $^{\triangle}p < 0.05$ versus control (Con), $*p < 0.05$ versus YKL-40 stimulation for 24 h without inhibitor; $^{\#}p < 0.05$ versus YKL-40 stimulation for 48 h without inhibitor.

YKL-40 (100 ng/ml) were harvested for protein extraction at indicated time points. YKL-40 induced rapid and marked phosphorylation of JNK and ERK, but not p38 (Fig. 2A). Phosphorylation of JNK and ERK reached a maximum at 10–20 min after YKL-40 treatment and then declined thereafter. Another submaximal increase in JNK and ERK phosphorylation was observed at 60 and 120 min, respectively. Furthermore, we found that YKL-40 significantly induced phosphorylation of JNK and ERK in a concentration-dependent manner after 2 h stimulation (Fig. 2B).

Further experiments using SP600125, U0126, and SB239063, specific and potent inhibitors of JNK, ERK, and p38, respectively, showed that treatment of BEAS-2B cells with SP600125 and U0126, but not SB239063, before YKL-40 (100 ng/ml) stimulation caused a significant inhibition of IL-8 mRNA expression (Fig. 2C) and release (Fig. 2D) at both 24 and 48 h. These results indicated that both the JNK and ERK pathways were responsible for YKL-40–mediated IL-8 production by BEAS-2B cells and that the p38 pathway was dispensable for this production.

Involvement of NF-κB pathway in YKL-40–increased IL-8 production in BEAS-2B

The promoter of the IL-8 gene contains potential binding sites for NF-κB (29). We then assessed NF-κB pathway in BEAS-2B cells after YKL-40 (100 ng/ml) exposure. IκBα phosphorylation was induced after 30–60 min of YKL-40 (100 ng/ml) stimulation, and p65 phosphorylation was observed after 60–120 min (Fig. 3A). Treatment with YKL-40 also significantly induced p65 phosphorylation in a concentration-dependent manner in BEAS-2B cells (Fig. 3B). To further investigate mechanisms of YKL-40–mediated IL-8 transcription, BEAS-2B cells transfected with an NF-κB–responsive luciferase reporter showed a significant increase in luciferase activity in response to YKL-40 in a concentration-dependent mode at indicated time points. The peak NF-κB luciferase activity obtained when BEAS-2B cells were stimulated with YKL-40 at a concentration of 10 μg/ml for 6 h (Fig. 3C). Collectively, these data suggested that YKL-40 induced IL-8 via regulation of transcription in BEAS-2B cells.

Moreover, using an NF-κB inhibitor (Bay 11-7082), we found that pretreatment with Bay 11-7082 significantly reduced the IL-8 mRNA expression and the IL-8 release from BEAS-2B cells after YKL-40 (100 ng/ml) exposure (Fig. 3D, 3E). These results suggested that YKL-40–induced phosphorylation of IκBα led to degradation of IκBα, thereby releasing NF-κB from the complex (p50/p65), followed by translocation of NF-κB to the nucleus to activate the IL-8 gene through binding to the site specific for this transcription factor at the IL-8 promoter.

The CM of both YKL-40–treated BEAS-2B cells and YKL-40–treated HBECs increased proliferation and migration of BSMCs

An increase in BSMC mass and decreased distance between BSMC and epithelium are hallmark features of the remodeled wall in asthmatic airways (2). These changes in BSMCs may be induced by factors produced by epithelium (30). We harvested the CM of both BEAS-2B cells and HBECs cultured with various concentrations of YKL-40 for 6 h (YKL-40–BEAS-2B-CM and YKL-40–HBECs-CM) and then assessed the effects of CM on the proliferation and migration of BSMCs. Fig. 4A showed that both YKL-40–BEAS-2B-CM and YKL-40–HBECs-CM increased the proliferation of BSMCs in a concentration-dependent manner after 72 h treatment. These two CM also increased the migration of BSMCs in a concentration-dependent mode after 24 h induction (Fig. 4B). As the significant difference started from the concentration of 100 ng/ml of YKL-40, and the circulating levels of
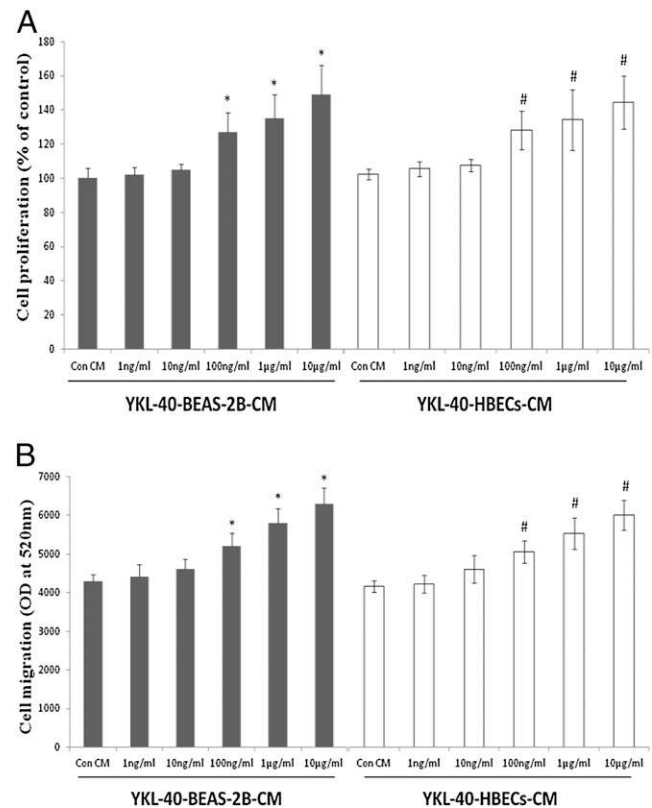

FIGURE 4. The effect of YKL-40–treated BEAS-2B cells or HBECs-CM (YKL-40–BEAS-2B-CM or YKL-40–HBECs-CM) on the proliferation and migration of BSMCs. Both YKL-40–BEAS-2B-CM and YKL-40–HBECs-CM increased the proliferation (**A**) and migration (**B**) of BSMCs in a concentration-dependent manner. Both experiments were repeated three times. *$p < 0.05$ versus Con CM of Beas-2B; #$p < 0.05$ versus Con CM of HBECs. Con CM, Control CM; OD at 520nm, OD measured at a wavelength of 520 nm.

YKL-40 range from 30–160 ng/ml in asthmatic patients (17), we used the concentration of YKL-40 (100 ng/ml) in the following experiments concerning the proliferation and migration of BSMCs.

IL-8 is a potential factor of YKL-40–BEAS-2B-CM and YKL-40–HBECs-CM in increasing BSMC proliferation and migration

Fig. 1 showed that YKL-40 only increased the IL-8 in HBECs, whereas other important chemokines or cytokines like RANTES, eotaxin, and TNF-α, which were reported to participate in the BSMC remodeling of asthma were not increased under this experimental condition. Thus, we speculated that IL-8 could be a potential factor of YKL-40–BEAS-2B-CM and YKL-40–HBECs-CM in increasing BSMC proliferation and migration. To address the role of IL-8 on BSMC proliferation and migration, we depleted IL-8 from both YKL-40–BEAS-2B-CM and YKL-40–HBECs-CM. The successful depletion of IL-8 from these two CM was confirmed by ELISA assay (data not shown). Both IL-8–depleted YKL-40–BEAS-2B-CM and IL-8–depleted YKL-40–HBECs-CM failed to stimulate proliferation of BSMCs (Fig. 5A). Similarly, increased migration of BSMCs by YKL-40–BEAS-2B-CM and YKL-40–HBECs-CM was completely abrogated upon IL-8 depletion (Fig. 5B). Moreover, we found that rhIL-8 increased both proliferation and migration of BSMCs (Fig. 5C, 5D).

Figs. 2 and 3 of this study showed that the JNK, ERK, and NF-κB pathways were involved in YKL-40–induced IL-8 production in BEAS-2B cells. Therefore, we collected the CM of YKL-40–
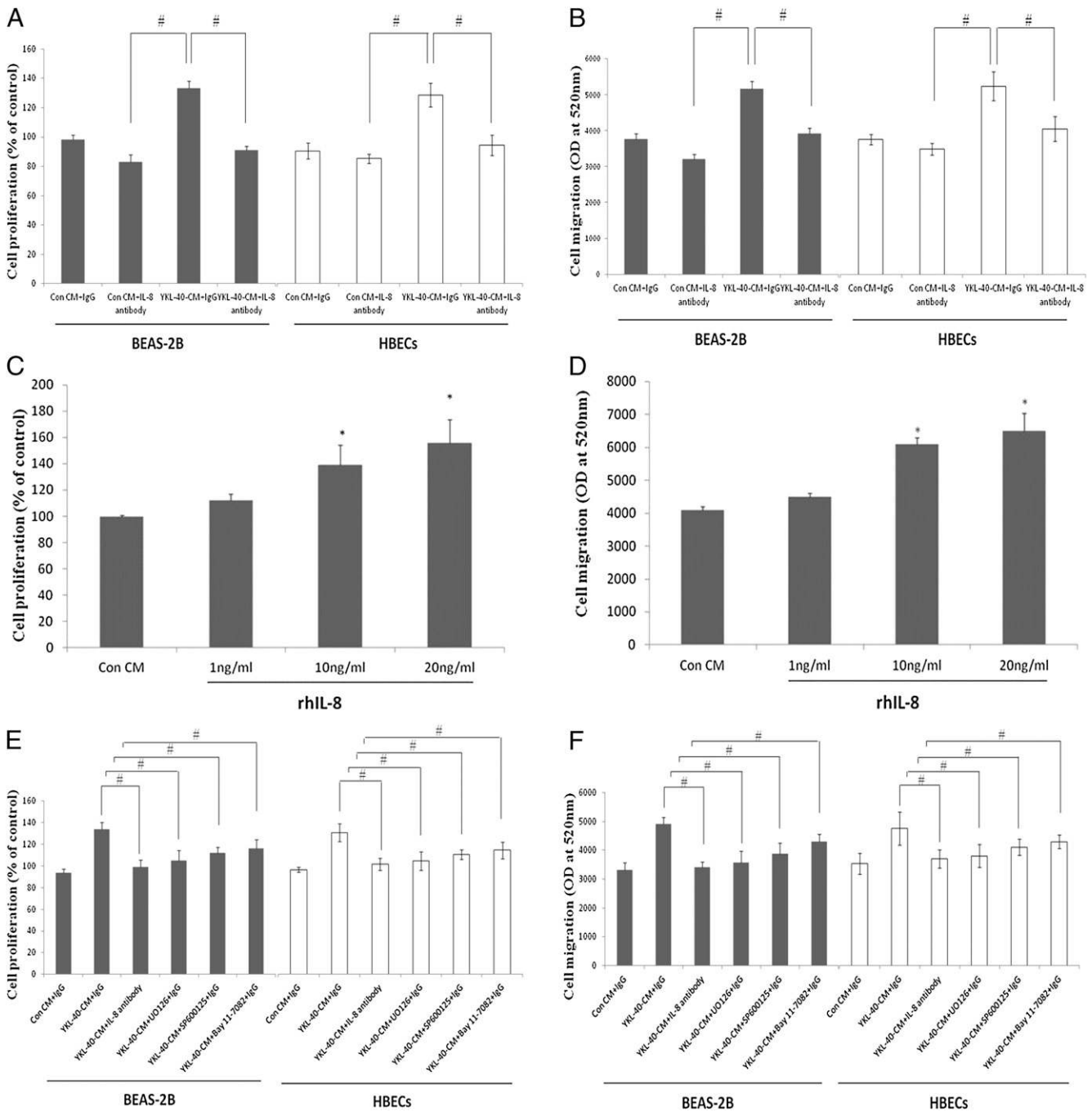
FIGURE 5. Involvement of IL-8 in proliferation and migration of YKL-40–BEAS-2B-CM or YKL-40–HBECs-CM. Depletion of IL-8 from YKL-40–BEAS-2B-CM or YKL-40–HBECs-CM failed to induce proliferation (**A**) and migration (**B**) of BSMCs induced by YKL-40–BEAS-2B-CM or YKL-40–HBECs-CM. The addition of rhIL-8 increased proliferation (**C**) and migration (**D**) of BSMCs. YKL-40–BEAS-2B-CM or YKL-40–HBECs-CM pretreated with ERK, JNK, or NF-κB inhibitor failed to increase proliferation (**E**) and migration (**F**) of BSMCs. All experiments were repeated three times. *$p < 0.05$ versus Con CM; #$p < 0.05$. Con CM, Control CM; OD at 520nm, OD measured at a wavelength of 520 nm; YKL-40–CM, YKL-40–treated CM.

BEAS-2B-CM and YKL-40–HBECs-CM pretreated with various inhibitors and investigated the effects on proliferation and migration of BSMCs. Fig. 5E showed that all of the YKL-40–BEAS-2B-CM pretreated with inhibitors of JNK, ERK, or NF-κB failed to stimulate proliferation of BSMCs when compared with treatment of YKL-40–BEAS-2B-CM. IL-8–depleted YKL-40–BEAS-2B-CM exhibited the most significant reduction in the proliferation of BSMCs compared with inhibitor-pretreated YKL-40–BEAS-2B-CM. Among the three inhibitors, the efficacy of the ERK inhibitor to prevent proliferation of BSMCs was the greatest, and the NF-κB inhibitor had the weakest effect. The CM of YKL-40–HBECs-CM pretreated with inhibitors showed similar reversing effects on increased proliferation of BSMCs caused by YKL-40–HBECs-CM (Fig. 5E). Furthermore, all of the inhibitor-pretreated YKL-40–BEAS-2B-CM significantly decreased the ability of YKL-40–BEAS-2B-CM to stimulate migration of BSMCs. The tendency of inhibitor-pretreated and IL-8–depleted YKL-40–BEAS-2B-CM to fail to stimulate migration of BSMCs was similar to that of those on preventing proliferation of BSMCs (Fig. 5F). The study of inhibitor-pretreated YKL-40–HBECs-CM on migration of BSMCs obtained similar results as treatment of inhibitor-pretreated YKL-40–BEAS-2B-CM on BSMCs (Fig. 5F).

YKL-40–BEAS-2B-CM increased cytokine and chemokine production in BSMCs

Increased inflammatory response of BSMCs is a key pathological feature in the development of airway remodeling (30). We found that YKL-40–BEAS-2B-CM strongly increased the secretion of IL-8, RANTES, eotaxin, and IL-6 in BSMCs (Fig. 6).

Discussion

To our knowledge, the current study is the first to investigate the interaction of airway epithelium and bronchial smooth muscle after YKL-40 exposure. We found that stimulation of HBECs (BEAS-2B cells and HBECs) with YKL-40 resulted in a significant increase of IL-8 mRNA expression and release in a concentration- and time-dependent manner. Moreover, YKL-40 induced phosphorylation of JNK, ERK, p65, and IκBα, but not p38, in BEAS-2B cells. Transfection using an NF-κB luciferase reporter also showed that YKL-40 induced IL-8 at the transcriptional level. Furthermore, BEAS-2B cells pretreated with inhibitors of JNK, ERK, or NF-κB showed a significant decrease in IL-8 release upon YKL-40 treatment. In addition, we treated BEAS-2B cells and HBECs with YKL-40 and added the conditioned culture media (YKL-40–BEAS-2B-CM and YKL-40–HBECs-CM) to BSMCs, which led to increased proliferation and migration of BSMCs. However, IL-8–depleted or inhibitor-pretreated YKL-40–BEAS-2B-CM and YKL-40–HBECs-CM failed to induce the proliferation and migration of BSMCs. Taken together, our data provide evidence of YKL-40–induced IL-8 expression in HBECs via the JNK, ERK, and NF-κB pathways, and IL-8 released from BEAS-2B cells or HBECs after YKL-40 exposure was found to further stimulate the proliferation and migration of BSMCs. Our results raise the possibility that YKL-40 may play a role in remodeling of asthma by inducing IL-8 production.

YKL-40/BRP-39 has been regarded as a prototype of chitinase-like proteins in mammals (31). Exaggerated levels of YKL-40 have been noted in a number of diseases characterized by inflammation, tissue remodeling, and aberrant cell growth (32, 33). Recently, the novel regulatory role of YKL-40/BRP-39 in the pathogenesis of asthma has been demonstrated both in human studies and allergic animal models (19, 22, 34). However, up to now, the role of YKL-40 in inflammation or remodeling of asthma remains largely unknown. Previous studies about YKL-40 mostly concentrated on inflammatory cells (T cells, macrophages, and eosinophils) in

asthma (35). So far, there is only one report demonstrating the role of YKL-40 on BSMCs (22), one of the structural cells in the lung that plays an important role in tissue remodeling of asthma. However, the effects of YKL-40 on other key structural cells (epithelial cells and fibroblasts) involved in pathogenesis of asthma have not yet been elucidated. Although a report by Bara et al. (22) indicated that YKL-40 could actively participate in the tissue-remodeling process by directly promoting BSMC proliferation and migration, other possibilities like indirectly modulating the process by YKL-40 through regulation of some inflammatory mediators have not been explored. In the present study, we study the role of YKL-40 on HBECs (BEAS-2B cells and HBECs) and investigate the effect of soluble factors (cytokines and chemokines) secreted from BEAS-2B cells and HBECs upon YKL-40 stimulation on BSMCs. Our preliminary results suggest that the epithelial cells may be one of the cellular targets of YKL-40 in asthma, contributing to IL-8 expression in the airways. The induced IL-8 not only participates in the inflammation of asthma, probably by recruitment of neutrophils, but also has a role in tissue remodeling by increasing the proliferation and migration of BSMCs.

IL-8, a chemokine belonging to the CXC family, also called CXCL8, is produced by various cell types including epithelial cells. As a chemoattractant, its primary function is to attract neutrophils to the site of inflammation. Increased production of IL-8 and enhanced neutrophil infiltration in the airways have been associated with allergic diseases such as asthma (36, 37). Our current study shows that YKL-40 induces IL-8 in HBECs (BEAS-2B cells and HBECs), but not other chemokines (RANTES, eotaxin) or cytokine (TNF-α) analyzed, thus revealing a new pathway for induction of IL-8 within the airways. These results are partly in agreement with a previous study in which YKL-40 induced IL-8 as well as RANTES, eotaxin, and TNF-α production in colonic epithelial cells (38). As we used the bronchial epithelial cell line BEAS-2B and primary normal HBECs, the effect of YKL-40 on cytokine/chemokine production may be epithelial cell specific. Further experiments are needed to explore whether the effect of YKL-40 on IL-8 induction is special to the lung in vivo.

The MAPK family is fundamental in mediating numerous changes in cell function such as cytokine expression, proliferation, and apoptosis (39, 40). p38, JNK, and ERK play a central role in these cell responses (41–43). Using pharmacological inhibitors of MAPK and investigating the kinetics of phosphorylation of p38, JNK, and ERK, we demonstrate that YKL-40–induced IL-8 release in BEAS-2B cells involves JNK and ERK but not p38 pathways. IL-8 is one of the genes activated by NF-κB, as the promoter of the IL-8 gene contains a potential binding site for NF-κB (44). We also found that YKL-40 induced IL-8 production through activation of NF-κB apart from JNK and ERK phosphorylation. Other limited numbers of studies reported that YKL-40 activated the ERK and PI3K/protein kinase B pathways (31, 35, 45, 46). It is plausible that YKL-40 may act very differently depending on cell type, and these activities induced by YKL-40 may be mediated through differential signaling pathways. Although the activation of these pathways is initiated by ligand binding to a cell-surface receptor, no cell-surface protein has been identified that binds YKL-40 (31, 35).

BSMCs play a key role in the modulation of airway tone. They not only increase secretory and proliferative ability, but also migrate to the subepithelial area in the asthmatic airway (30). In addition, BSMCs release inflammatory mediators, which are responsible for the progression of asthma pathogenesis (23). IL-8 has been reported to trigger calcium release, contraction, and migration in BSMCs (47–49) through its functional receptors (50, 51). Halwani et al. (52) reported that chemokines (including IL-8)
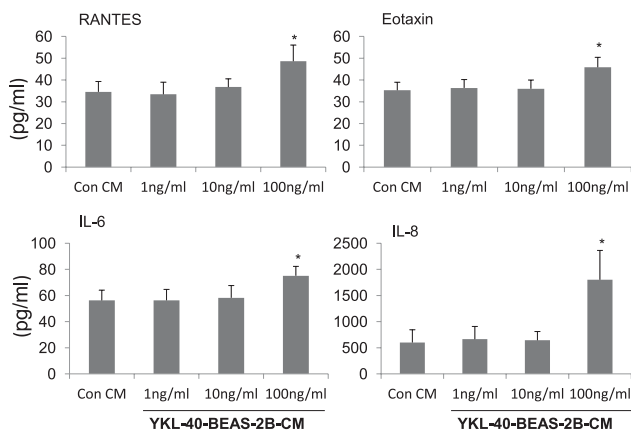
FIGURE 6. YKL-40–BEAS-2B-CM increased inflammatory and chemotactic response in BSMCs. BSMCs were treated with YKL-40–BEAS-2B-CM (1, 10, and 100 ng/ml), and the amounts of various proteins were detected by ELISA. The experiments were repeated three times. *$p < 0.05$ versus Con CM. Con CM, Control CM.

may contribute to airway remodeling of asthma by enhancing the number and survival of BSMCs. However, the role of IL-8 on YKL-40–related remodeling of asthma remains unknown. We show in this study that YKL-40 increased IL-8 secretion from BEAS-2B cells and HBECs, which in turn increased the proliferation and migration of BSMCs, and these effects were further confirmed by direct stimulation of BSMCs with rhIL-8. Furthermore, IL-8–depleted or inhibitor-pretreated (JNK, ERK, or NF-κB) YKL-40–BEAS-2B-CM and YKL-40–HBECs-CM failed to induce the proliferation and migration of BSMCs. Although it seems that the JNK, ERK, or NF-κB inhibitor reversing the proliferation and migration of BSMCs caused by YKL-40–treated CM in BEAS-2B cells and HBECs through reduction of IL-8 production, other possibilities like inhibitor-related direct effects on remodeling of BSMCs need to be explored further. As IL-8 has been widely accepted to be a strong chemokine, our results will add an indirect role of YKL-40 on BSMCs, which is associated with IL-8 production from HBECs, to the direct role of YKL-40–induced BSMC remodeling in asthma reported by Bara et al. (22).

In conclusion, our current study shows that YKL-40 increased IL-8 production in HBECs via MAPK (JNK and ERK) and NF-κB pathways, and induced IL-8 resulted in enhanced proliferation and migration of BSMCs. These findings implicate that YKL-40 may be involved in inflammation by induction of IL-8 in epithelium, subsequently contributing to BSMC remodeling in asthma. Moreover, inhibition of IL-8 signaling is an attractive potential therapeutic target for YKL-40–induced inflammation and remodeling of asthma.

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Disclosures

The authors have no financial conflicts of interest.

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