Withanolides are C$_{28}$ steroidal lactones isolated from plants that exhibit potent anti-cancer activity. The chemokine receptor CCR7 is important for lymphatic invasion of cancer cells and is overexpressed in metastatic breast cancer cells. A bioactive withanolide tubocapsanolide A (Tubo A) suppressed NF-κB-mediated CCR7 expression in breast cancer cells and attenuated their migration toward lymphatic endothelial cells. Chromatin immunoprecipitation assay confirmed that binding of NF-κB to the consensus site localized at the −398/−389 of human CCR7 promoter was repressed by Tubo A. Tubo A inhibited IκB kinase (IKK) and p38 kinase and downstream mitogen and stress-activated protein kinase 1 (MSK1) activity to reduce IκB degradation and to suppress NF-κB activation. Co-expression of IKK and MSK1 fully rescued Tubo A-induced inhibition. In addition, ectopic expression of transforming growth factor-β-activating kinase (TAK1), the common upstream kinase of IKK and MSK1, also completely reversed the inhibition by Tubo A. Most importantly, Tubo A reduced NF-κB activation, CCR7 expression, and lymph node metastasis of breast cancer in vivo. We conclude that Tubo A inhibits TAK1 to repress NF-κB-induced CCR7 expression in breast cancer cells and suggest that Tubo A may be useful for the prevention of lymphatic invasion of breast cancer cells.

Withanolides are steroidal lactones that were originally isolated from Withania somnifera, one of the most important herbs used as a traditional remedy for several illnesses in Asian countries (1). These compounds are biologically active and have been shown to inhibit the enzymatic activity of cyclooxygenase-2 and suppress inflammation (2). In addition, recent studies demonstrate that withanolides exhibit anti-cancer effect on human lung, colon, and breast cancer cells in vitro and exert immunopotentiating activity in vivo (3, 4). Several potential mechanisms have been implicated in the inhibition of tumorigenesis by withanolides. First, withanolides induce growth arrest and apoptosis in cancer cells (4, 5). Second, withanolides can inhibit angiogenesis by suppressing endothelial cell proliferation (6). Third, withanolides can reduce cancer cell invasion and metastasis (7). These data suggest that withanolides may be developed as a novel class of anti-cancer drugs.

Lymph node invasion by cancer cells is an important step for tumor metastasis and is frequently correlated with early recurrence and poor prognosis. However, the underlying mechanism by which cancer cells metastasize into peripheral lymphatic capillaries is poorly defined. Recent studies indicate that the interaction between chemokine CCL21 and its cognate receptor CCR7 may play an important role in this process (8, 9). The hypothesis suggests that lymphatic endothelial cells (LEC) express and release chemotactic factors such as CCL19 and CCL21 to direct cancer cells with high expression of chemokine receptor CCR7 to grow and migrate toward lymphatic capillaries (10–12). Indeed, many highly metastatic cancer cells express large amount of CCR7 receptor (13, 14), and CCR7 expression is associated with lymph node metastasis in breast, lung, gastric, esophageal cancer, and melanoma (15–19). It is possible that inhibition of CCR7 expression or block of CCL21-CCR7 interaction may cause reduction of lymph node invasion and tumor metastasis. However, the control of CCR7 gene transcription in cancer cells is largely unclear, and no natural products have been shown to regulate CCR7 expression.

We have isolated a new bioactive withanolide tubocapsanolide A (Tubo A) from Tubocapsicum anomalum (20). Tubo A exhibited cytotoxic activity on various types of human cancer cells (20). In addition, our recent results demonstrated that Tubo A suppressed the transcription of Skp2 oncogene and up-regulated cyclin-dependent kinase inhibitory proteins p27

The abbreviations used are: LEC, lymphatic endothelial cell; Tubo A, tubocapsanolide A; FCS, fetal calf serum; MSK1, mitogen and stress-activated protein kinase 1; TAK1, transforming growth factor-β-activating kinase; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IκB, IκB kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH$_2$-terminal kinase; MEKK1, mitogen-activated protein kinase kinase 1.

This study was supported by the National Sun Yat-Sen University-Kaohsiung Medical University Joint Research Center and Center for Gene Regulation and Signal Transduction Research, National Cheng Kung University (to W.-C. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S6.
In this study, we address the following objectives: (a) the effect of Tubo A on the expression of CCR7 in breast cancer cells, (b) the effect of Tubo A on lymphatic migration of breast cancer cells, and (c) the underlying mechanism by which Tubo A regulates CCR7.

**EXPERIMENTAL PROCEDURES**

**Plant Material**—The initial collection of *T. anomalum* (Solanaceae) was made on July 2003 near NanTao County and identified by Dr. Hsin-Fu Yen (National Museum of Natural Science, Taichung, Taiwan). A larger amount of the same plant was collected at the Da-Han Mountain, Kaohsiung, in October 2004, and identified by Dr. Ming-Ho Yen (Graduate Institute of Natural Products, Kaohsiung Medical University, Taiwan). The samples were authenticated and deposited in the Graduate Institute of Natural Products, Kaohsiung Medical University, Taiwan. Extraction and isolation of Tubo A were performed as described previously (20).

**Cell Culture and Experimental Agents**—MCF-7 and MDA-MB-231 breast cancer cells were obtained from the cell bank of the National Health Research Institute (Miaoli, Taiwan). Cells were cultured in Dulbecco’s modified Eagle’s medium/F-12 medium containing 10% charcoal-stripped fetal calf serum (FCS) and antibiotics. Normal human lymphatic endothelial cells (C-12218) were purchased from PromoCell (Heidelberg, Germany). Anti-CCR7 (MAB197) antibody and recombinant human tumor necrosis factor-α (210-TA) were purchased from R&D (Minneapolis, MN). Anti-NF-κB (SC-8008), anti-IκB (SC-371), anti-MSK1 (SC-25417), anti-TAK1 (SC-7967), and anti-IKKα/β (SC-7607) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phospho-MSK1 (Thr-581, no. 9595), phospho-IKKα/β (no. 2697), p38 (no. 9212), and phospho-p38 (Thr-180/Tyr-182, no. 9216) were purchased from Cell Signaling (Danvers, MA). An antibody (ab7504) against EpCAM, an epithelial-specific antigen and a cell surface glycoprotein, was purchased from Abcam (Cambridge, UK). Withaferin A (CT-104) was obtained from BIOMOL (Plymouth Meeting, PA). Anti-LYVE-1 antibody was purchased from Upstate (Temecula, CA). IKK-α and -β expression vector was provided by Dr. Michael Karin (University of California San Diego), and MSK1 expression vector was obtained from Dr. Dario R. Alessi (University of Dundee, UK). The TAK1 expression vector was kindly provided by Dr. Xin Lin (M. D. Anderson Cancer Center). The NF-κB luciferase reporter plasmid (no. 219078, Stratagene) and antibody specific for NF-κB (p65) phosphorylated at Ser-276 (no. 3037, Cellular Signaling) were provided by Dr. Chih-Hsin Tang (China Medical University, Taiwan).

**In Vitro Invasion Assay**—In vitro invasion assay was performed as described previously (22). 3000 cells in 100 μl of medium with vehicle or Tubo A (0.5 μM) were placed in the upper part of the Transwell unit and allowed to invade for 24 h. The lower part of the Transwell unit was filled with LECs. After incubation, invaded cells on the bottom surface of the membrane were fixed in formaldehyde, stained with Giemsa solution, and counted under a microscope. In some experiments, cells transfected with various expression vectors were collected by trypsinization and placed into the upper well in the absence or presence of Tubo A for invasion assay.

**RT-PCR**—Total RNA was subjected to RT-PCR analysis as described previously (22). The primer sequences: CCR7-forward, 5′-GGCACTTGGGAAACCAAT-3′; CCR7-reverse, 5′-GCCAGGTGAGCAGGTAGTT-3′; GAPDH-forward, 5′-GAGCTCAGCGATTGTGCTGTG-3′; and GAPDH-reverse, 5′-TGTGTTCTATGAGCTCTTCCA-3′. Amplified cDNA products were run on 2% agarose gels, stained with ethidium bromide, and visualized under UV light.

**Immunoblotting**—Extraction of cellular proteins and immunoblotting were performed as previously described (23).

**Preparation of Cytoplasmic and Nuclear Fractions**—Cells were plated in 75-cm² flasks and grown to 70–80% confluence. Treated cells were washed with cold phosphate-buffered saline and lysed in hypotonic buffer (10 mM HEPES-KOH, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride). Nuclei were spun down, and the supernatant was collected as the cytoplasmic fraction. Nuclei were further extracted in cold buffer (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride) on ice for 20 min. Cellular debris was clarified by centrifugation, and the supernatant was collected as nuclear fraction.

**Flow Cytometry**—For detection of CCR7 expression, cells were washed with ice-cold phosphate-buffered saline and incubated with biotinylated anti-CCR7 antibody at 4 °C for 30 min. After washing, cells were incubated with fluorescein isothiocyanate-conjugated avidin and subjected to flow cytometric analysis as described previously (24).

**Promoter Activity Assay**—In brief, cells were plated onto 6-well plates at the density of 100,000 cells/well and grown overnight. Cells were transfected with 1 μg of NF-κB or AP-1 luciferase reporter plasmid. After transfection, cells were treated with vehicle (0.1% DMSO) or various concentrations of Tubo A in 10% FCS medium for 24 h. Promoter activity was determined and normalized for the concentration of cellular proteins.

**Analysis of Protein Stability**—Protein stability of IκB was measured by blocking protein synthesis with cycloheximide and harvesting the cells at various times. Cells were incubated with 10% FCS medium containing vehicle or various concentrations of Tubo A (0 or 0.5 μM) for 24 h. Cells were treated with 10 μg/ml cycloheximide, and cellular proteins were harvested at various times. Protein level of IκB was determined by immunoblotting.

**Chromatin Immunoprecipitation Assay**—Vehicle- or Tubo A-treated cells were fixed with 1% formaldehyde at 37 °C for 10 min. Cells were washed twice with ice-cold phosphate-buffered saline containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml peptatin A), scraped, and pelleted by centrifugation at 4 °C. Cells were resuspended in a lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris-HCl, pH 8.1), incubated for 10 min on ice, and sonicated to shear DNA. After sonication, lysate was centrifuged for 10 min at 13,000 rpm at 4 °C. The supernatant was diluted in ChIP dilution buffer (0.01% SDS, 1% Triton X-100, 2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl, and protease inhibitors).
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Anti-NF-κB (p65) or anti-Myc (negative control) antibodies were added to the supernatant and incubated overnight at 4 °C with rotation. ChIP assays were performed as described previously (23). DNA fragments were recovered and were subjected to PCR amplification by using the primers specific for the human CCR7 gene promoter. The sequences for the primers: forward, 5'-AGAAGGCGATGTTGAAGGTCA-3'; reverse, 5'-ACTCCCTCTCAAGAACCCTGT-3'.

In Vivo Xenograft Study—We tested the anti-metastatic ability of Tubo A in nude mice and animal studies were approved by the Animal Care and Ethics Committee of the National Sun Yat-Sen University. Female BALB/cAnN-Foxn1 null mice (5 weeks old) were obtained from National Laboratory Animal Center (Taipei, Taiwan). MDA-MB-231 cells (1 × 10^6 cells/mouse) were injected into left second thoracic mammary fat pad of nude mice. Tumor volumes were measured every 3 days from the second week after injection and were calculated using the formula, \( V = (\text{length}) \times (\text{width})^2 \times 0.5 \). After 4 weeks, tumors grew to \( \approx 0.18 - 0.2 \text{ cm}^3 \). Mice were randomly divided into two groups (\( n = 5 \) for each group) and subjected to treatment. Animals of the treatment group received intraperitoneal injection of Tubo A (4 mg/kg) every 3 days. The chemical structure of Tubo A is shown in supplemental Fig. S1. We found that CCR7 was highly expressed in metastatic MDA-MB-231 cells (Fig. 1A). Conversely, CCR7 expression was low in non-metastatic MCF-7 cells. Flow cytometric analysis demonstrated a positive correlation between CCR7 mRNA and protein located at the same side of tumor injection site) were excised and fixed in 4% paraformaldehyde. In addition, right axillary, brachial, and inguinal lymph nodes (defined as distal lymph nodes because they located at the counter side of tumor injection site) were also excised. All lymph nodes were embedded in paraffin using routine procedures and subjected for immunohistochemical analysis.

**Immunohistochemical Analysis**—Lymph nodes were cut into 3-μm sections, and paraffin was removed by xylene. Tissues were placed in citrate buffer and incubated at 85 °C for 5 min for antigen retrieval. Tissues were blocked for 15 min with a 3% hydrogen peroxide solution to inhibit endogenous peroxidase activity and washed with phosphate-buffered saline. Tissues were probed with antibodies directed against lymphatic marker LYVE-1 for the confirmation of lymphatic tissues or epithelial marker EpCAM for the identification of lymphatic invasion of breast cancer cells. EpCAM (also referred as CD326) is a glycoprotein of \( \approx 40 \text{ kDa} \) that was originally identified as a marker for carcinoma, attributable to its high expression on rapidly proliferating tumors of epithelial origin (25). MDA-MB-231 cells have been shown to express a high level of EpCAM (26), whereas lymph node tissues do not express this epithelial marker (27). Bound primary antibodies were detected with horseradish peroxidase-conjugated secondary antibody and then developed by dianobenzidine substrate. Finally, sections were co-stained with hematoxylin. In addition, tissues were also subjected to hematoxylin & eosin stain for identify tumor metastasis. We also addressed the effect of Tubo A on NF-κB activation in vivo. Tumor tissues were stained with anti-NF-κB antibody, and the cell number with positive staining was counted for each tumor section. Percentages of positive staining cells with nuclear signal, which indicated nuclear translocation and activation of NF-κB, were determined and compared between control and the Tubo A-treated group.

**Statistical Analysis**—Student’s \( t \) test was used to evaluate the difference between various experimental groups. Differences were considered to be significant at \( p < 0.05 \).

**RESULTS**

**Tubo A Inhibited CCR7 Expression in Breast Cancer Cells and Attenuated Their Migration toward LECs**—The chemical structure of Tubo A is shown in Fig. S1. We found that CCR7 was highly expressed in metastatic MDA-MB-231 cells (Fig. 1A). Conversely, CCR7 expression was low in non-metastatic MCF-7 cells. Flow cytometric analysis demonstrated a positive correlation between CCR7 mRNA and protein...
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expression in these two breast cancer cell lines (Fig. 1A). Because MDA-MB-231 cells expressed high level of CCR7, we used this cell line to study the regulation of CCR7 and lymphatic invasion by Tubo A. Our previous studies have demonstrated that Tubo A at the concentration higher than 0.5 μM showed significant cytotoxic activity on various human cancer cell lines (20, 21). Because we addressed the anti-metastatic effect of Tubo A in this study, we consistently used low doses (0–0.5 μM) of Tubo A to treat cells to prevent the nonspecific effect caused by cell death. Tubo A at the concentrations of 0.125, 0.25, and 0.5 μM inhibited CCR7 expression in a dose- and time-dependent manner (Fig. 1B). In addition, CCR7 mRNA level was also down-regulated by Tubo A in a dose-dependent manner (Fig. 1C). In addition to inhibit basal CCR7 expression in MDA-MB-231 cells, Tubo A also repressed tumor necrosis factor-α-induced CCR7 expression (supplemental Fig. S2). Therefore, Tubo A inhibited both basal and extracellular signal-stimulated CCR7 expression. We also compared the effect of another bioactive withanolide Withaferin A on CCR7 expression and found that these two withanolides exhibited similar potency on the inhibition of CCR7 (supplemental Fig. S3). We next tested whether CCR7 was required for the migration of breast cancer cells toward LECs. Primary cultured LECs used in these assays expressed high levels of CCL21 (supplemental Fig. S4). LECs were placed on the lower part of a Transwell unit, and MDA-MB-231 cells were added on the upper part. As shown in Fig. 1D, Tubo A suppressed the invasion of MDA-MB-231 cells toward LECs in a dose-dependent manner. Our previous results also showed that knockdown of CCR7 expression in MDA-MB-231 cells reduced their migration toward LECs (28). Our data suggested that Tubo A inhibited CCR7 expression in breast cancer cells and reduced their migration toward LECs.

Tubo A Attenuated IkB Degradation and NF-κB (p65) Nuclear Translocation—Because CCR7 has been found to be a target gene for NF-κB (29) and a bioactive withanolide withaferin A has been shown to inhibit NF-κB-mediated gene transcription (30), we tested the effect of Tubo A on the IkB degradation and NF-κB activation. As shown in Fig. 2A, Tubo A up-regulated IkB protein level in MDA-MB-231 cells. Pulse-chase analysis indicated that protein stability of IkB was increased by Tubo A in a dose-dependent manner and Tubo A at the concentration of 0.5 μM effectively reduced IkB degradation (Fig. 2A). On the contrary, the protein level of NF-κB was

FIGURE 2. Inhibition of IkB degradation and NF-κB nuclear translocation by Tubo A. A, MDA-MB-231 cells were incubated with 10% FCS medium containing vehicle (0.1% DMSO) or various concentrations of Tubo A for 24 h. Cells were treated with 10 μg/ml cycloheximide, and cellular proteins were harvested at 0, 2, and 4 h. Protein levels of IkB and NF-κB were determined by Western blotting. B, cells were treated with vehicle (0.1% DMSO) or Tubo A (0.5 μM) for 24 h. Nuclear and cytoplasmic fractions were prepared. The subcellular distribution of NF-κB and phosphor-276 NF-κB was studied by immunoblotting. Retinoblastoma (Rb) and Raf-1 kinase were used as internal control to verify purification of nuclear and cytoplasmic fractions and equal loading of proteins. C, cells were transfected with 1 μg of NF-κB (N) or AP-1 (A) luciferase reporter plasmids. After transfection, cells were untreated (−) or treated with vehicle (D, 0.1% DMSO) or various concentrations of Tubo A (T1, 0.125 μM; T2, 0.25 μM; and T3, 0.5 μM) in 10% FCS medium for 24 h. Promoter activity was determined and normalized for the concentration of cellular proteins. Results of three independent assays were expressed as mean ± S.D., p < 0.05 when the Tubo A-treated groups compared with the control group without Tubo A treatment. D, vehicle- or Tubo A-treated cells were fixed with 1% formaldehyde at 37 °C for 10 min. Cells were harvested and resuspended in a lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris-HCl, pH 8.1), incubated for 10 min on ice, and sonicated to shear DNA. Anti-NF-κB (p65) or anti-Myc (negative control) antibodies were added to the supernatant and incubated overnight at 4 °C with rotation. DNA fragments were recovered and were subjected to PCR amplification by using the primers specific for the detection of the NF-κB (−398/−389) site of human CCR7 gene promoter. Cell transfected with short hairpin RNA targeting luciferase gene (C) or NF-κB gene (sh-NF) were harvested at 48 h after transfection and subjected to ChIP assay as described above. Western blot analysis was performed to verify the down-regulation of NF-κB in transfected cells.

FIGURE 3. Partial reversion of Tubo A-induced down-regulation of NF-κB promoter binding and CCR7 expression by ectopic expression of IKK-β. A, MDA-MB-231 cells were incubated with 10% FCS medium containing vehicle (DMSO, 0.1%) or Tubo A (0.5 μM) for 24 h, and the phosphorylation status of IKK-α and -β was investigated by phospho-specific antibodies. B, cells were transfected with pcDNA or IKK-β expression vector for 48 h. The phosphorylation status of IKK and the protein level of IkB were examined by Western blotting. C, pcDNA or IKK-β-transfected cells were treated with DMSO (D, 0.1%) or Tubo A (T, 0.5 μM) for 24 h. ChIP assay was performed as described in Fig. 2D. D, CCR7 protein level of pcDNA or IKK-β-transfected cells treated with Tubo A (T, 0.5 μM) was investigated by flow cytometry and compared with the DMSO-treated and pcDNA-transfected control cells (peak with gray color).
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FIGURE 4. Inhibition of p38 and MSK1 signaling pathway by Tubo A. A, MDA-MB-231 cells were incubated with 10% FCS medium containing vehicle or Tubo A (0.5 μM) for 24 h, and the phosphorylation status of ERK and p38 was investigated by phospho-specific antibodies. B, cells were also harvested for the detection of the phosphorylation of Ser-581 of MSK1 and Ser-276 of p38 using phospho-specific antibodies. C, cells were transfected with pcDNA or MSK1 expression vector for 48 h. The increase of MSK1 protein level and phosphorylation was assessed by Western blotting. Cells were also treated with DMSO (D, 0.1%) or Tubo A (T, 0.5 μM) for 24 h, and ChIP assay was performed to study the binding of NF-κB to CCR7 promoter. D, CCR7 protein level of pcDNA or MSK1-transfected cells treated with Tubo A (T, 0.5 μM) was investigated by flow cytometry and compared with the DMSO-treated and pcDNA-transfected control cells (peak with gray color).

FIGURE 5. Complete reversion of Tubo A-induced down-regulation of NF-κB promoter binding and CCR7 expression by ectopic expression of IKK-β and MSK1. A, cells were transfected with pcDNA or co-transfected with IKK-β and MSK1 expression vector for 48 h. Cells were treated with DMSO (D, 0.1%) or Tubo A (T, 0.5 μM) for 24 h and ChIP assay was performed to study the binding of NF-κB to CCR7 promoter. B, CCR7 protein level of IKK-β+MSK1-transfected cells were treated with Tubo A (T, 0.5 μM) for 24 h, and CCR7 protein level was investigated by flow cytometry and compared with the DMSO-treated and pcDNA-transfected control cells (peak with gray color). C, cells were transfected with pcDNA, pcDNA+IKK-β, pcDNA+MSK1, or pcDNA+IKK-β+MSK1 for 48 h. Cells were collected by trypsinization and subjected to in vitro invasion assay in the presence of vehicle (0.1% DMSO, D) or Tubo A (T, 0.5 μM). After 24 h, the invaded cell number was counted, and results of three independent assays are expressed as mean ± S.D.

Infection with IKK-β-transfected cells (selected by puromycin) were used to study the binding of NF-κB to CCR7. A, infected with IKK-β-transfected cells (selected by puromycin) were used to study the binding of NF-κB to CCR7. B, infected with IKK-β-transfected cells (selected by puromycin) were used to study the binding of NF-κB to CCR7. C, infected with IKK-β-transfected cells (selected by puromycin) were used to study the binding of NF-κB to CCR7.

not significantly changed. Increase of IκB might sequester NF-κB in cytoplasm to attenuate gene transcription. Therefore, we investigated the effect of Tubo A on nuclear translocation of NF-κB. Fig. 2B showed that NF-κB was mainly located in the nucleus of MDA-MB-231 cells possibly due to constitutive activation of some upstream signaling pathways, and this nuclear accumulation was attenuated by Tubo A. On the contrary, Tubo A treatment induced cytoplasmic NF-κB. To verify that NF-κB in the nucleus was active, we used an antibody that specifically recognized the phosphorylated (at Ser-276) NF-κB to perform immunoblotting and confirmed that phosphorylated NF-κB was located in the nucleus of DMSO-treated cells while Tubo A treatment reduced nuclear accumulation (Fig. 2B). In agreement with these data, the NF-κB-dependent reporter activity in MDA-MB-231 cells was also inhibited by Tubo A in a dose-dependent manner (Fig. 2C). This effect is NF-κB-specific, because Tubo A did not significantly inhibit AP-1-mediated reporter activity under the same experimental condition. Analysis of the human CCR7 promoter sequence revealed two potential NF-κB binding sites at −797/−787 and −398/−389 regions. Prediction of potential NF-κB consensus sites in human CCR7 promoter had also been reported by Hopken et al. (29). We studied the in vivo binding of NF-κB to CCR7 promoter by ChIP assays and found that binding of NF-κB to the −797/−787 consensus sequence was very weak (data not shown). Conversely, NF-κB constitutively bound to the −398/−389 region of CCR7 promoter, and its binding was attenuated by Tubo A (Fig. 2D). Binding of NF-κB to this consensus site was specific, because knockdown of NF-κB by short hairpin RNA reduced the binding of NF-κB to this site while a control short hairpin RNA targeting luciferase had no effect. Because no Myc binding site was found in the CCR7 promoter region amplified by PCR primers used in our study, addition of anti-Myc antibody did not get any nonspecific signal, which further verified the specificity of our ChIP assays (Fig. 2D). These data indicated that Tubo A repressed CCR7 by suppressing NF-κB activation.

Ectopic Expression of IKK-β Only Partly Counteracted Tubo A-induced Down-regulation of CCR7—Our aforementioned data suggested that Tubo A reduced IκB degradation to attenuate NF-κB-mediated transcription of CCR7. The main upstream kinase that phosphorylates IκB and controls its protein stability is IKK, so we tested the effect of Tubo A on IKK activity. Phosphorylation on Ser-177/181 of IKK-β or Ser-176/180 on IKK-α is an indication for kinase activation. We used anti-phospho-IKK antibody to
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FIGURE 6. Complete reversion of Tubo A-induced down-regulation of NF-κB promoter binding and CCR7 expression by ectopic expression of TAK1. A, cells were transfected with pcDNA or TAK1 expression vector for 24 h. CCR7 protein level was investigated by flow cytometry and compared with the DMSO-treated and pcDNA-transfected control cells (peak with gray color). C, cells were co-transfected with 1 μg of NF-κB (N) luciferase reporter plasmid and pcDNA or TAK1 expression vector. After transfection, cells were untreated (-) or treated with vehicle (0.1% DMSO) or 0.5 μM of Tubo A (T) in 10% FCS medium for 24 h. Promoter activity was determined and normalized for the concentration of cellular proteins. Results of three independent assays were expressed as mean ± S.D. *, p < 0.05 when the Tubo A-treated groups compared with the DMSO-treated control group. D, cells were transfected with pcDNA or TAK1 for 48 h. Cells were collected by trypsinization and subjected to in vitro invasion assay in the presence of vehicle (0.1% DMSO, D) or Tubo A (0.5 μM). After 24 h, invaded cell number was counted, and results of three independent assays were expressed as mean ± S.D. *, p < 0.05 when the Tubo A-treated groups compared with the DMSO-treated control group.

investigate the activation of IKK, and our data indicated that IKK-α and -β activity were attenuated by Tubo A (Fig. 3A). Ectopic expression of IKK-β in MDA-MB-231 cells increased IKK-β activity and induced degradation of IkB in these cells (Fig. 3B). In addition, overexpression of IKK-β increased the binding of NF-κB to CCR7 promoter (Fig. 3C). However, reduction of NF-κB promoter binding by Tubo A was only partly reversed. Moreover, Tubo A-induced down-regulation of CCR7 protein on the cell surface was only partly reversed by IKK-β (Fig. 3D). Similar results were observed when IKK-α was ectopically expressed in MDA-MB-231 cells (data not shown). Our data suggested that IKK was not the only target for Tubo A to inhibit CCR7.

Tubo A Inhibited p38 and MSK1 Activation and Reduced NF-κB Phosphorylation—Because enhancement of IkB degradation by overexpression of IKK could not fully rescue Tubo A-induced inhibition of NF-κB, we thought that Tubo A affected other signaling pathways that might directly regulate NF-κB phosphorylation and activation. By using phospho-specific antibodies, we found that Tubo A suppressed the activation of p38, but not extracellular signal-regulated kinase (ERK) was also fully rescued after co-expression of IKK-β and MSK1 (Fig. 5C). These results suggested that Tubo A simultaneously inhibited IKK and p38/MSK1 signaling pathways to suppress CCR7 expression.

Ectopic Expression of TAK1 Also Fully Rescued the Inhibitory Effect of Tubo A—A common upstream kinase of IKK and p38 is JNK. We next tested whether overexpression of JNK-1 and -2 might counteract the inhibitory effect of Tubo A. Our data showed that overexpression of JNK-1 and -2 totally reversed Tubo A-induced inhibition of NF-κB binding to CCR7 promoter and CCR7 expression (Fig. 4, A and B). In addition, the migration ability of MDA-MB-231 cells toward LECs was also fully rescued after co-expression of IKK-β and MSK1 (Fig. 5C). These results suggested that Tubo A simultaneously inhibited IKK and p38/MSK1 signaling pathways to suppress CCR7 expression.

Tubo A Effectively Reduced Lymph Node Metastasis of Breast Cancer in Vivo—We next used a xenograft animal model to address the anti-metastatic activity of Tubo A in vivo. MDA-MB-231 cells were injected into left second thoracic mammary fat pad of nude mice to induce tumor growth and lymph node in MDA-MB-231 cells (Fig. 4A). Phosphorylation status of c-Jun N-terminal kinase (JNK) was not changed by Tubo A (data not shown). A main downstream effector kinase of p38, which can directly phosphorylate NF-κB, is MSK1. We found that phosphorylation of MSK1 on Ser-581, a major phosphorylation site for p38, was also reduced by Tubo A (Fig. 4B). NF-κB was phosphorylated by MSK1 on Ser-276 (31), and we found that Tubo A reduced Ser-276 phosphorylation of NF-κB (Fig. 4B). So, p38 and MSK1 were involved in the inhibition of CCR7 by Tubo A. Ectopic expression of MSK1 partly reversed Tubo A-induced inhibition of NF-κB binding to CCR7 promoter and CCR7 expression (Fig. 4, C and D).

Co-activation of IKK-β and MSK1 Fully Rescued the Inhibition of NF-κB—Promoter Binding, CCR7 Expression, and Cell Migration by Tubo A—We next tested whether combinational expression of IKK-β and MSK1 might fully counteract the inhibition of Tubo A. Our data showed that co-expression of IKK-β and MSK1 totally reversed Tubo A-induced inhibition of NF-κB binding to CCR7 promoter and CCR7 expression (Fig. 5, A and B). In addition, the migration ability of MDA-MB-231 cells toward LECs was also fully rescued after co-expression of IKK-β and MSK1 (Fig. 5C). These results suggested that Tubo A simultaneously inhibited IKK and p38/MSK1 signaling pathways to suppress CCR7 expression.
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FIGURE 7. Inhibition of lymph node metastasis of breast cancer by Tubo A. A, MDA-MB-231 cells (1 × 10⁶ cells/mouse) were injected into left second thoracic mammary fat pad of nude mice. Tumor volumes were measured every 3 days from the second week after injection. After 4 weeks (time zero indicated in the figure), tumors grew to ~0.18–0.2 cm³, and drug treatment was initiated. Animals of the control group (A–A) received intraperitoneal injection of 0.1% of DMSO, and animals of the treatment group (■–■) received intraperitoneal injection of Tubo A (4 mg/kg) every 3 days. Tumor volumes were also monitored continuously. B, tumors were excised and subjected to RT-PCR analysis to study the expression of CCR7 in two tumors of DMSO-treated (D1 and D2) and Tubo A-treated (T1 and T2) groups was shown. The averaged CCR7 level of five tumors of DMSO-treated and Tubo A-treated groups were expressed as mean ± S.D. *, p < 0.05 when the Tubo A-treated groups compared with the DMSO-treated control group. C, effect of Tubo A on NF-κB activation was studied by staining the subcellular localization of NF-κB. Tumor tissues were stained with anti-NF-κB antibody, and percentages of positive-staining cells with nuclear signal, which indicated nuclear translocation, and activation of NF-κB were determined and compared between control and Tubo A-treated groups. *, p < 0.05 when the Tubo A-treated groups compared with the DMSO-treated control group. D, left (proximal) and right (distal) axillary lymph nodes of DMSO-treated (D) or Tubo A-treated (T) group were subjected to immunohistochemical analysis for the expression of EpCAM. The percentages of proximal (n = 15) and distal (n = 15) lymph nodes with tumor metastasis of DMSO-treated (D) and Tubo A-treated (T) groups were shown.

metastasis. To mimic real drug treatment, intraperitoneal injection (but not direct intratumor injection) was used as the drug administration route. After a 21-day treatment, mice were sacrificed and tumors were removed. Our data indicated that intraperitoneal injection of Tubo A (4 mg/kg) did not inhibit tumor growth at the injection site (Fig. 7A). In addition, no significant alterations of the weights of body, liver, lung, heart, kidney, and pancreas were found in Tubo A-treated group (data not shown). However, an 80% of reduction of CCR7 expression was detected in the tumors of Tubo A-treated group (Fig. 7B). We investigated whether reduction of CCR7 in the tumors was due to attenuation of NF-κB activation. Our data indeed demonstrated that nuclear translocation of NF-κB was inhibited by 57% by Tubo A in vivo (Fig. 7C). Left and right axillary, brachial, and inguinal lymph nodes of each mouse were excised and were confirmed to be lymphatic tissues by using the specific marker LYVE-1 (data not shown). Lymphatic metastasis of breast cancer was studied by using EpCAM as a marker. Because MDA-MB-231 cells expressed high level of EpCAM (26) and lymph node tissues did not express this epithelial marker (27), we thought it is a suitable marker for detection of lymph node metastasis of MDA-MB-231 cells. Similar application had also been used by others for the detection of lymph node metastasis of cancer cells (26, 27, 32, 33). Lymphatic metastasis of MDA-MB-231 was detected in 100% of left lymph nodes (n = 15, three lymph nodes of left side for each mouse) of the DMSO-treated group (Fig. 7D). Hematoxylin & eosin stain also confirmed the tumor nest in the lymph node (supplemental Fig. S5). Conversely, only 53% of lymph nodes exhibited metastasis in Tubo A-treated group. Moreover, breast cancer cells (EpCAM-positive staining cells) detected in lymph nodes were much less in Tubo A-treated group (compared the staining signals of the two experimental groups, Fig. 7D). We defined left axillary, brachial, and inguinal lymph nodes as proximal lymph nodes, because they were located at the same side of tumor injection site. Right axillary, brachial, and inguinal lymph nodes (referred as distal lymph nodes because they were located on the counter side of the tumor injection site) were also studied. Our data showed that 83% of right lymph nodes of the DMSO-treated group had tumor metastasis. On the contrary, only 40% of right lymph nodes of the Tubo A-treated group exhibited tumor metastasis. Collectively, we conclude that Tubo A effectively inhibited lymph node metastasis of breast cancer in vivo.

DISCUSSION

Recent studies indicate that withanolides may repress NF-κB-mediated gene transcription via repression of IKK activation (30, 34). Consistent with these studies, we found that withanolides (Tubo A in this study) inhibit IKK activation by suppressing the phosphorylation of Ser-177/181 on IKK-β or Ser-176/180 on IKK-α, which are required for their activation. Although reduction of IKK activity and IkB degradation are commonly observed in withanolide-treated cells, in vitro kinase assay indicated that IKKs are not direct targets for withanolides (30, 34). For the first time, we demonstrate that withanolides may inhibit the p38/MSK1 signaling pathway. A previous study reported that treatment of withaferin A reduced the phosphorylation of NF-κB (p65) (30). The authors showed that tumor necrosis factor-induced phosphorylation of NF-κB on Ser-536 was attenuated by withanolides. In this study, we demonstrated that the Tubo A inhibited p38 activation in MDA-MB-231 cells. We propose a link between p38 activation and NF-κB phosphorylation and find a possible candidate, MSK1, because MSK1 can be activated by p38 and may directly phosphorylate the Ser-276 of NF-κB (31, 35). Our data indeed demonstrated that...
inhibition of p38 led to a reduction of phosphorylation of MSK1 on Ser-581, a specific phosphorylation site for p38. In addition, the phosphorylation of Ser-276 of NF-κB was also attenuated. The functional importance of the p38/MSK1 signaling in the inhibition of CCR7 by Tubo A was further strengthened by the results that ectopic expression of MSK1 might reverse the inhibitory effect of Tubo A (Fig. 4D).

Because both IKK and p38 kinase activities are attenuated by Tubo A, we hypothesize that Tubo A may target a common upstream kinase of these two kinases. Several kinases, including NF-κB-inducing kinase, NF-κB-activating kinase, mitogen-activated protein kinase kinase 1 (MEKK1), MEKK3, and TAK1 have been shown to phosphorylate the IKK complex and induce NF-κB activation when overexpressed in cells (36–39). Interestingly, TAK1 can also activate the main upstream activating kinases for p38, MKK3, and MKK6 (40). TAK1, originally identified as a transforming growth factor-β-activating kinase, plays critical roles in the regulation of diverse cellular processes (41). Activation of TAK1 required its binding proteins (TAB1–3) and is ubiquitin-dependent (42). So, we address the role of TAK1 and show that activation of TAK1 fully reverses the inhibitory effect of Tubo A on CCR7 expression. We conclude that TAK1 is a molecular target for Tubo A (supplemental Fig. S6).

The interaction between chemokines and their cognate receptors is important for tumor metastasis. For example, the chemokine receptor CXCR4 and its ligand stroma-derived factor-1 have been shown to be critical for the dissemination of cancer cells (8, 9). CCR7 is important for the adhesion and chemotaxis of leukocyte and dendritic cell to lymph nodes. Because LECs express high level of CCR7 ligands CCL19 and CCL21, up-regulation of CCR7 in cancer cells may promote the migration of cancer cells toward LECs and enhance lymph node metastasis. Indeed, many metastatic cancer cells expressed large amount of CCR7 (13, 14), and CCR7 expression is associated with lymph node metastasis in many cancers (15–19). Thus, inhibition of CCR7 expression or block of CCL21-CCR7 interaction may cause reduction of lymph node invasion and tumor metastasis. Indeed, we have shown that knockdown of CCR7 by small interference RNA in metastatic MDA-MB-231 cells reduced their migration toward lymphatic cells (28). However, treatment of human diseases by small interference RNA gene targeting is still at a very premature stage, and local application of small interference RNA for therapy of age-related macular degeneration has just undergone phase I clinical trial (43). Therefore, development of drugs for the inhibition of CCR7 or CCR7-CCL21 interaction is urgently needed. However, very little chemical compounds or natural products have been shown to suppress CCR7 expression. One potential compound is triptolide. This compound is a major active component isolated from the Chinese herb *Tripterygium wilfordii* Hook F, which has been used in traditional Chinese medicine for the treatment of autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus (44, 45). A very recent study demonstrated that triptolide might repress CCR7 expression and lymphatic migration of dendritic cells (46).

In this study, we provide the first evidence that Tubo A, a bioactive withanolide, inhibits CCR7 expression and lymphatic invasion of breast cancer *in vitro* and *in vivo*. Lack of inhibition of primary tumor growth by Tubo A is not unexpected, because the dose used for treatment in this study was very low (4 mg/kg). Previous studies showed that another bioactive withanolide Withaferin A needed almost a 10-fold dose (~30–50 mg/kg) to inhibit the growth and angiogenesis of melanoma *in vivo* (7, 47). However, Tubo A at this low concentration still exhibited potent inhibitory effect on lymph node metastasis of breast cancer *in vivo*. These data are encouraging, because lymph node metastasis is a very poor prognostic factor for breast cancer and no effective drugs have been reported to reduce the lymph node metastasis at present. Taken together, we conclude that Tubo A is an effective compound for the inhibition of CCR7 and may be useful for the prevention lymphatic invasion of breast cancer.

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