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C/EBPβ Mediates TNF-α-Induced Cancer Cell Migration by Inducing MMP Expression Dependent on p38 MAPK

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ABSTRACT

Tumor necrosis factor (TNF)-α is a pleiotropic cytokine that triggers cell proliferation, cell death, or inflammation. Besides its cytotoxic effect on cancer cells, TNF-α exerts tumor promoting activity. Aberrant TNF-α signaling promotes cancer cell motility, invasiveness, and enhances cancer metastasis. Exaggerated tumor cell migration, invasion, and metastasis by TNF-α has been attributed to the activation of NF-κB signaling. It is yet to be elucidated if other signaling pathways and effector molecules are involved in TNF-α-induced cancer cell migration and metastasis. Expression of C/EBPβ, a transcription factor involved in metabolism, inflammation, and cancer, is increased upon TNF-α treatment. TNF-α induces C/EBPβ expression by enhancing its transcription and protein stability. Activation of p38 MAPK, but not NF-κB or JNK, is responsible for TNF-α-induced stabilization of C/EBPβ protein. C/EBPβ is involved in TNF-α-induced cancer cell migration. Knockdown of C/EBPβ inhibits TNF-α-induced cell migration, while overexpression of C/EBPβ increases migration of cancer cells. C/EBPβ is translated into transcriptional activator LAP1 and LAP2 and transcriptional repressor LIP utilizing alternative in-frame translation start sites. Despite TNF-α induces expression of all three isoforms, LAP1/2, but not LIP, promote cancer cell migration. TNF-α induced MMP1/3 expression, which was abrogated by C/EBPβ knockdown or p38 MAPK inhibition. MMP inhibitor or knockdown of MMP1/3 diminished TNF-α- and C/EBPβ-induced cell migration. Thus, C/EBPβ mediates TNF-α-induced cancer cell migration by inducing MMP1/3 expression, and may participate in the regulation of inflammation-associated cancer metastasis. J. Cell. Biochem. 116: 2766–2777, 2015.

KEY WORDS: TNF-α; CELL MIGRATION; C/EBPβ; MMP; p38 MAPK; INFLAMMATION

Tumor necrosis factor (TNF)-α is a pleiotropic cytokine that triggers cell proliferation, cell death, or inflammation through cell surface TNF receptors and downstream effector signaling pathways [De Smaele et al., 2001; Kamata et al., 2005; Jurisic et al., 2006; Balkwill, 2009; Jurisic et al., 2011; Cabal-Hierro and Lazo, 2012]. The biological consequences of TNF-α are dependent on the cellular context [De Smaele et al., 2001; Kamata et al., 2005; Jurisic et al., 2006; Balkwill, 2009; Jurisic et al., 2011; Cabal-Hierro and Lazo, 2012]. Binding of TNF-α to TNFR1 activates MAPK-AP1 and IKK-NF-κB pathways that in turn induces expression of genes involved in inflammation and cell survival. Unlike rapid apoptosis induced by FAS ligand and TRAIL, cell apoptosis is a late response to TNF-α. Prolonged activation of JNK1 is essential for TNF-α-induced cell death when NF-κB activation is impaired [De Smaele et al., 2001; Kamata et al., 2005]. TNF-α has been considered as candidate therapeutic agent for cancer [Balkwill, 2009]. TNF-α induced necrosis of both syngeneic and xenograft tumors [Pennica et al., 1984; Brouckaert et al., 1986]. Although systemic TNF-α administration was associated with severe toxicity [Balkwill, 2009], regional delivery of TNF-α, for example, isolated hepatic perfusion in unresectable liver tumors, enabled organ-targeted therapy with minimal systemic toxicity [Bellavance and Alexander, 2009].

Intriguingly, TNF-α exerts tumor promoting activity as a major mediator of cancer-related inflammation [Karin and Greten, 2005; Balkwill, 2006; Karin, 2006]. TNF-α is frequently detected in human cancer biopsies [Balkwill, 2006]. TNF-α produced by tumor cells or stromal cells is involved in tumor development, and is associated with poor prognosis [Balkwill, 2002; Szlosarek and Balkwill, 2003].

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Lipopolysaccharide enhanced metastasis of colon cancer and breast cancer in experimental metastasis mouse models, which was dependent on TNF-α production by hematopoietic cells in tumor microenvironment [Luo et al., 2004; Li et al., 2012]. TNF-α treatment enhanced lung metastasis of fibrosarcoma [Orosz et al., 1993; Orosz et al., 1995]. Overexpression of TNF-α conferred invasive properties on xenograft tumors [Malik et al., 1990]. Conversely, lung metastasis was reduced by neutralizing endogenous TNF-α [Malik et al., 1990; Orosz et al., 1993]. Mice deficient of TNF-α receptor were resistant to the development of liver metastasis of experimental colon cancer [Kitakata et al., 2002]. TNF-α promotes cancer cell motility, invasiveness, and metastatic potential [Karin and Greten, 2005; Balkwill, 2006; Karin, 2006]. The exaggerated tumor cell migration, invasion, and metastasis by TNF-α have been attributed to the activation of NF-κB signaling. Activation of NF-κB stabilizes Snail or transcriptionally upregulates Twist1 expression, which subsequently promotes cancer cell epithelial–mesenchymal transition (EMT), cell migration, invasion, and tumor metastasis [Cho et al., 2009; Wu et al., 2009; Li et al., 2012; Zhu et al., 2014]. It is yet to be elucidated if signaling pathways and effector molecules other than NF-κB are involved in TNF-α-induced tumor progression and metastasis.

CCAAT/enhancer binding protein β (C/EBPβ) is a member of C/EBP family of leucine-zipper transcription factors [Zahnow, 2009]. C/EBPβ is translated into three isoforms utilizing alternative in-frame translation start sites [Grimm and Rosen, 2003; Zahnow, 2009]. The liver-enriched activating protein 1 and 2 (LAP1 and LAP2) isoforms are transcriptional activators, whereas the liver enriched inhibitory protein (LIP) isoform lacks the N-terminal transactivation domain, and therefore generally functions as a dominant-negative inhibitor of transcription [Grimm and Rosen, 2003; Zahnow, 2009]. C/EBPβ plays important roles in cellular proliferation, differentiation, inflammation, metabolism, and tumorigenesis [Grimm and Rosen, 2003; Zahnow, 2009]. Although expression of C/EBPβ is not altered in breast cancers compared with normal breast tissues, high C/EBPβ expression is associated with metastatic breast cancer and overall poorer prognosis [van de Vijver et al., 2002; Zahnow, 2009]. Overexpression of LAP2 in normal mammary epithelial cells led to EMT and transformation [Bundy and Sealy, 2003]. Increased LIP expression was linked to a defective transforming growth factor β-dependent cytostatic response in metastatic breast cancer cells [Gomis et al., 2006].

In this study, we demonstrate TNF-α treatment enhances C/EBPβ expression by stabilizing C/EBPβ protein. TNF-α-induced expression and stabilization of C/EBPβ is dependent on p38 MAPK activation, but not NF-κB activity. C/EBPβ and its downstream matrix metalloproteinases 1/3 (MMP1/3) are required for TNF-α-induced cancer cell migration. Thus, TNF-α activates multiple signaling pathways, including NF-κB and C/EBPβ to promote cancer cell migration.

MATERIALS AND METHODS

REAGENTS AND CELL CULTURE

TNF-α was purchased from R&D Systems (USA). p38 MAPK inhibitor SB203580 was purchased from Selleckchem (USA). TPCA-1, SP600125, and GM6001 were provided by SIBCB Chemical Biology Core Facility. Cycloheximide (CHX), MG132, and NH_4Cl were purchased from Sigma–Aldrich (USA). MDA-MB-231 and MDA-MB-435 were originally from ATCC (USA) and cultured in DMEM (Invitrogen, USA) supplemented with 10% FBS (Hyclone, USA) and 1% penicillin/streptomycin (Invitrogen, USA) at 37°C, 5% CO_2.

PLASMIDS

The human *CEBPB* sequences were amplified from MDA-MB-231 cDNA using primers 5′-AGCGAATTCATGCAACGCCTGGTGGCCTGGGACC-3′ (forward) and 5′-CGCGGATCCCTACTTGTCGTCATCGTCTTTG-TAGTCGCAGTGGCCGGAGGAGGCGAGCAG-3′ (reverse) with a FLAG-tag at the C-terminus. Amplicon was inserted into pCDH-CMV-MCS-EF1-puro. Such construct expresses all three C/EBPβ isofoms (LAP1, LAP2, and LIP). Methionine at positions 24 and 199 was mutated to Alanine to disrupt the second and third alternative start codons. Such M24A/M199A mutant only expresses the LAP1 isoform. The primers used to generate the LAP1 (M24A/M199A) expression vector are 5′- AGCGAATTCATGCAACGCCTGGTGGCCTGGGACC-3′, 5′-GTAGAAGTTGGCCACTTCCGCGGATTTAAA-3′, 5′-CCGCCTGCCT-TTAAATCCGCGGAAGTGGCC-3′, 5′-GTACGGGAAGCCCGCCGCCGC-GCCTGCGCC-3′, 5′-CCGGGCGGCGGCGCAGGCGCGGCGGCGGGC-3′, and 5′-CGCGGATCCCTACTTGTCGTCATCGTCTTTGTAGTCGCAGTGG-CCGGAGGAGGCGAGCAG-3′. The LAP2 and LIP expression vectors were constructed by amplifying fragment corresponding to residue 24–345 and 199–345 from the LAP1 construct, respectively. The primers used are 5′-AGCGAATTCATGGAAGTGGCCAACTTCTACTACG-3′ (LAP2, forward); 5′-AGCGAATTCATGGCGGCGGGCTTCCCGTACG-CGC-3′ (LIP, forward) and 5′-CGCGGATCCCTACTTGTCGTCATC-GTCTTTGTAGTCGCAGTGGCCGGAGGAGGCGAGCAG-3′ (reverse). LAP1, LAP2, and LIP were also tagged by a FLAG-tag at the C-terminus.

The shRNAs against human *CEBPB*, *MMP1*, and *MMP3* were cloned into pLKO.1 (Addgene). The target sequences are as follows:

sh-h*CEBPB*-1: 5′-GTTGATGCAATCGGTTTAA-3′;
sh-h*CEBPB*-4: 5′-GTGTACAGATGAATGATAA-3′;
sh-h*MMP1*-1: 5′-GGCAAGGGATAACTCTTCT-3′;
sh-h*MMP1*-4: 5′-GGATAGGCAAGGGATAACT-3′;
sh-h*MMP3*-2: 5′-GGCTGATATAATGATCTCT-3′;
sh-h*MMP3*-4: 5′-GCTTGCACCTTGTCACATA-3′.

RNA EXTRACTION AND QUANTITATIVE REAL-TIME PCR

Total RNA was extracted using Trizol reagent (Invitrogen, USA) and was reverse-transcribed to obtain cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). The cDNA was amplified using Bestar qPCR Mastermix SYBR green (DBI, Germany). Quantitative RT-PCR was performed as previously described [Gao et al., 2010]. Primers used are *CEBPB*, 5′- GCGACGAGTACAA-GATCCGG-3′ (forward) and 5′-AGCTGCTTGAACAAGTTCCG-3′ (reverse), and *GAPDH*, 5′-ATGCCTCCTGCACCACCAAC-3′ (forward) and 5′-GGCAGTGATGGCATGGACTG-3′ (reverse). All q-PCR data were normalized to *GAPDH*. All experiments were performed in triplicates at least three times.

MICROARRAY ANALYSIS

Expression microarray analysis was carried out with commercially available Agilent Human 4 × 44 K Gene Expression Arrays (Agilent technologies, Santa Clara, CA). Total RNA was extracted using

TRIZOL Reagent (Life technologies, USA) following the manufacturer's instructions and checked for RNA integration by an Agilent Bioanalyzer 2100 (Agilent technologies, USA). Total RNA was amplified and labeled by Low Input Quick Amp Labeling Kit, One-Color (Agilent technologies, USA). Hybridization was performed using Gene Expression Hybridization Kit (Agilent technologies, USA). Data were extracted with Feature Extraction software 10.7 (Agilent technologies, USA). Raw data were normalized by Quantile algorithm, Gene Spring Software 11.0 (Agilent technologies, USA). Detailed gene expression data are available at the GEO website under accession number GSE67425.

WESTERN BLOT

For total cell lysates, cells were harvested in hot SDS sample buffer. To collect conditioned medium, cells were cultured to confluence, washed, and placed in serum-free media containing indicated stimuli. Proteins in conditioned medium were precipitated with 10% trichloroacetic acid and solubilized in SDS sample buffer after washing the pellet with acetone. Total proteins (30 μg) were separated by SDS–PAGE. After electrophoresis, the proteins were transferred to nitrocellulose membrane. The membrane was blocked, incubated overnight at 4°C with primary antibodies, washed with TBS-T (TBS with 0.1% Tween-20), and incubated with HRP-conjugated secondary antibodies at room temperature for 1 h. Immuno-reactive protein was detected using SuperSignal® West Pico Chem KIT (Thermo Scientific, USA). Primary antibodies used in this study were against C/EBPβ (Santa Cruz, USA), p38, p-p38 (Cell Signaling, USA), MMP1 (R&D Systems, USA), MMP3 (ABclonal Technology, USA), Flag and Tubulin (Sigma, USA). Horseradish peroxidase-conjugated secondary antibodies were purchased from Cell Signaling (USA). Western blots were analyzed with Image J.

CELL MIGRATION ASSAY

The cell migration assay was performed using Transwell migration chambers (Corning, USA). The lower section was filled with culture medium containing 10% FBS with or without TNF-α (10 ng/mL). Serum-starved cancer cells were placed in the upper section of the transwell chamber in serum-free medium with chemical inhibitors (10 μM SB203580, 10 μM TPCA-1, 1 μM SP600125, or 10 μM GM6001), and were incubated for 20 h at 37°C. The cells were fixed with 4% PFA for 15 min, and stained with 0.1% crystal violet for 15 min. Cells on the upper surface of the transwell chamber were removed with a cotton swab. Migrated cells on the lower surface of the transwell chamber were observed under a light microscope, and cells in five randomly selected fields were counted. All experiments were performed in triplicates and repeated at least three times.

For scratching wound healing assay, cells were pretreated with 10 ng/mL TNF-α and 10 μM SB203580 for 20 h before scratching with a 20 μL tip. Cells were washed with PBS, and cultured with 10 ng/mL TNF-α and 10 μM SB203580 for additional 8 h. Images were obtained under a light microscope, and migration velocity was analyzed with Image J. Experiments were performed six times.

MTT ASSAY

Cell proliferation was assessed using the MTT assay. Briefly, cells were seeded in 24-well plates (3,000 cells per well), treated with or without 10 ng/mL TNF-α for 0, 24, 48, 72 or 96 h. MTT solution was added into each well and continuously incubated for 4 h. After aspiration of the supernatant, the resulting crystals were dissolved in DMSO, and the absorbance was recorded at 570 nm with a microplate reader. Experiments were performed four times.

STATISTICAL ANALYSIS

Transwell and RT-PCR experiments were performed in triplicates and repeated three times. All other experiments were performed at least three times. Data were presented as the mean ± SD. Data were analyzed using the Anova, and considered statistically significant when the *P*-value was less than 0.05.

RESULTS

TNF-α INDUCES CANCER CELL MIGRATION

TNF-α is a potent stimulator of tumor metastasis. To test if TNF-α induces cancer cell migration, MDA-MB-231 and MDA-MB-435 cells were treated with TNF-α and subjected to modified Boyden chamber transwell assay. TNF-α treatment significantly increased the number of migrated MDA-MB-231 and MDA-MB-435 cells in a dose-dependent manner (Fig. 1). TNF-α significantly increased the migration velocity of MDA-MB-231 cells in the wound healing assay as well (Fig. S1A).

TNF-α INDUCES C/EBPβ EXPRESSION

C/EBPβ is closely associated with breast cancer metastasis [van de Vijver et al., 2002; Zahnow, 2009]. To examine whether TNF-α could induce C/EBPβ expression in cancer cells, C/EBPβ expression was analyzed in TNF-α-treated MDA-MB-231 and MDA-MB-435 cells. TNF-α treatment induced C/EBPβ expression (Fig. 2). LAP1, LAP2, and LIP isoforms are translated from a single C/EBPβ mRNA. TNF-α treatment enhanced the protein levels of all three isoforms in MDA-MB-231 and MDA-MB-435 cells (Fig. 2B and D). Upon TNF-α treatment, C/EBPβ mRNA level was slightly induced (1.2–3 fold; Fig. 2A and C). However, C/EBPβ protein level was more significantly increased (3–7 fold; Fig. 2B and D). Induction of C/EBPβ protein levels required low concentration (1 ng/mL) TNF-α (Fig. 2B), and were prior to the increase of mRNA levels (Fig. 2D) in MDA-MB-231 cells.

P38 MAPK ACTIVATION IS REQUIRED FOR TNF-α-INDUCED C/EBPβ EXPRESSION

TNF-α signaling activates multiple intracellular signaling cascades including NF-κB and JNK [Balkwill, 2006]. Inhibition of NF-κB, but not JNK, activation impaired TNF-α-induced cell migration (Fig. S2A). NF-κB was reported to stabilize C/EBPβ protein in hepatocytes [Wang et al., 2010]. To examine if NF-κB and JNK signaling are involved in regulation of TNF-α-induced C/EBPβ expression in cancer cells, MDA-MB-231 cells were treated with IKK inhibitor TPCA-1 or JNK inhibitor SP600125 (Fig. S2B and S2C). Unlike hepatocytes, inhibition of IKK or JNK had no significant effect on TNF-α-induced C/EBPβ expression in cancer cells (Fig. S2B and S2C). Besides NF-κB and JNK, TNF-α activates p38 MAPK in a context dependent manner [Balkwill, 2006]. TNF-α treatment increased p38 MAPK phosphorylation levels in MDA-MB-231 and

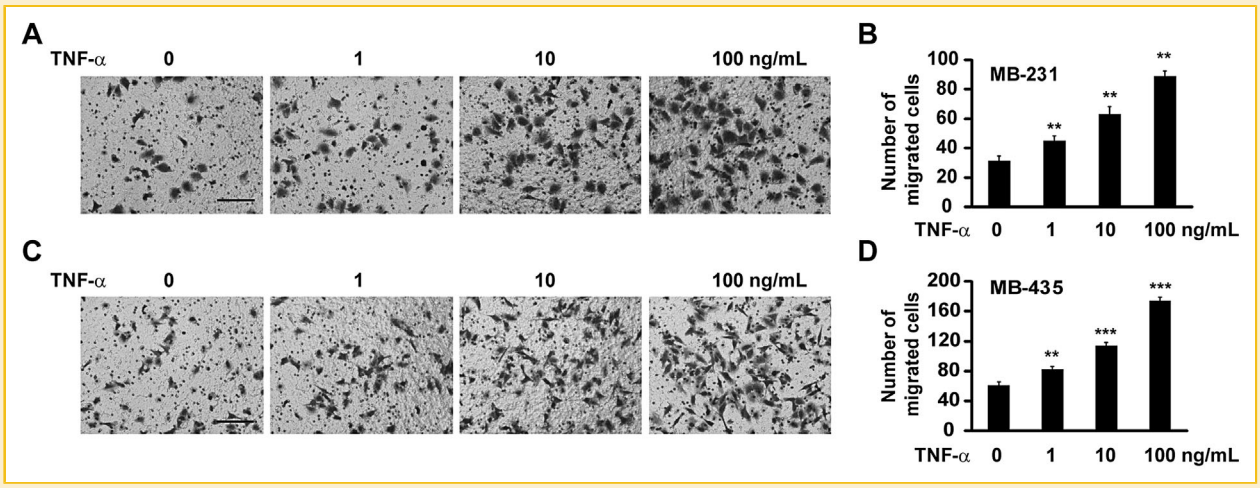

Fig. 1. TNF-α induces cancer cell migration. (A) MDA-MB-231 cells (3×10^4 cells/well in a 24-well Transwell chamber) were treated with different concentrations of TNF-α (0, 1, 10, and 100 ng/mL) in Transwell assay for 20 h. Scale bar: 200 μm. (B) Cell migration was quantified by counting the number of cells that migrated to the lower side of the filter within five randomly selected microscope fields. (C) MDA-MB-435 cells (5×10^4 cells/well in a 24-well Transwell chamber) were treated with different concentrations of TNF-α (0, 1, 10, and 100 ng/mL) in Transwell assay for 20 h. Scale bar: 200 μm. (D) Cell migration was quantified by counting the number of cells that migrated to the lower side of the filter within five randomly selected microscope fields. The experiments were performed in triplicates, and repeated three times. Data are presented as mean ± SD of three independent experiments.

MDA-MB-435 cells (Fig. 3A and B). p38 MAPK phosphorylation level was increased upon low concentration (1 ng/mL) TNF-α treatment (Fig. 3A), and as early as 0.5 h after TNF-α treatment (Fig. 3B). The parallel of enhanced p38 MAPK phosphorylation level with C/EBPβ protein levels suggested that p38 MAPK activation may be involved in regulating TNF-α-induced C/EBPβ expression. To investigate if activation of p38 is required for TNF-α-induced C/EBPβ expression, cancer cells were treated with TNF-α along with p38 MAPK inhibitor SB203580. Western blot showed that SB203580 treatment efficiently diminished C/EBPβ expression induced by TNF-α in both cells (Fig. 3C).

Ectopic expression of C/EBPβ in MDA-MB-231 and MDA-MB-435 cells resulted in overexpression of LAP1, LAP2, and LIP isoforms (Fig. 3D). Interestingly, inhibition of p38 MAPK decreased protein levels of exogenous C/EBPβ (LAP1, LAP2, and LIP) in MDA-MB-231 and MDA-MB-435 cells (Fig. 3D). In addition, TNF-α treatment further increased protein levels of exogenous LAP in MDA-MB-231 cells (Fig. 3E). These data collectively suggested that TNF-α may regulate C/EBPβ expression via p38 MAPK-dependent post-transcriptional mechanism. C/EBPβ proteins contain multiple phosphorylation sites [Zahnow, 2009; Tsukada et al., 2011]. To test if phosphorylation of C/EBPβ upon TNF-α treatment and p38 MAPK activation is required for increased C/EBPβ protein levels, Thr235 and Ser261, two phosphorylation sites essential for C/EBPβ function [Nakajima et al., 1993; Chinery et al., 1997], were mutated to Alanine (Fig. 3E and F). TNF-α less potently increased the protein levels of C/EBPβ T235A and S261A mutants, compared to wild-type C/EBPβ (Fig. 3E). Inhibition of p38 MAPK activity did not affect the protein levels of these mutants (Fig. 3F), suggesting that phosphorylation of C/EBPβ on Thr235 and Ser261 downstream of p38 MAPK may be essential to the increase of C/EBPβ expression upon TNF-α treatment.

C/EBPβ expression regulation is achieved primarily via post-transcriptional mechanisms [Zahnow, 2009]. The much more significant increase of C/EBPβ protein levels than the mRNA levels upon TNF-α treatment as well indicates that TNF-α may also induce C/EBPβ expression via mechanisms other than transcriptional regulation. We, therefore, examined the stability of C/EBPβ by incubating the cells with cycloheximide (CHX) (Fig. 4A). Slower decline of C/EBPβ protein levels was observed upon TNF-α treatment (Fig. 4A and B), suggesting that TNF-α induced C/EBPβ expression at least partially by stabilizing C/EBPβ proteins. To study if p38 MAPK is involved in such regulatory process, CHX treatment experiment was performed in the presence of p38 MAPK inhibitor SB203580. The half-life of C/EBPβ was significantly shorter in the presence of SB203580 upon TNF-α treatment (Fig. 4C and D), suggesting that p38 MAPK activation is necessary for TNF-α-induced stabilization of C/EBPβ protein. To study the mechanism of C/EBPβ degradation, MDA-MB-231 cells were incubated with lysosome or proteasome inhibitor. Lysosome inhibitor NH_4Cl had minimal effect on C/EBPβ protein levels (Fig. 4E and F). Proteasome inhibitor MG132 treatment increased both basal and TNF-α-induced C/EBPβ protein levels (Fig. 4E and F). MG132 treatment could also rescue SB203580 inhibition on TNF-α-induced C/EBPβ expression (Fig. 4E and F). Therefore, activation of p38 MAPK downstream of TNF-α may stabilize C/EBPβ by inhibiting its proteasome-dependent degradation.

C/EBPβ IS REQUIRED FOR TNF-α-INDUCED CELL MIGRATION

To investigate the function of C/EBPβ in TNF-α-induced cell migration, C/EBPβ was knocked down in MDA-MB-231 cells (Fig. 5A). The migration of MDA-MB-231 cells expressing scramble shRNA was enhanced upon TNF-α treatment in both transwell and wound healing assays (Fig. 5B and S1A). However, TNF-α was

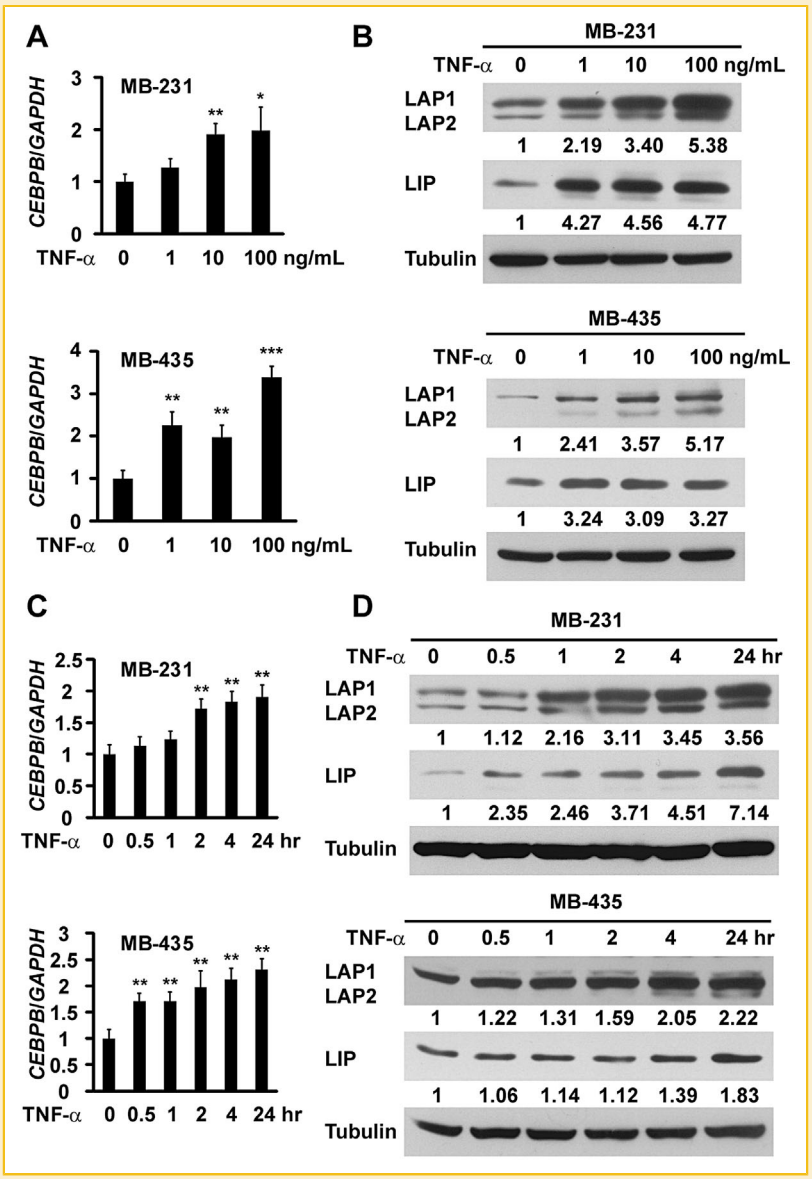

Fig. 2. TNF-α induces C/EBPβ expression. (A and B) Quantitative RT-PCR (A) and western blot (B) analyses of C/EBPβ expression in MDA-MB-231 and MDA-MB-435 cells treated with different concentrations of TNF-α for 24 h. (C and D) Quantitative RT-PCR (C) and western blot (D) analyses of C/EBPβ expression in MDA-MB-231 and MDA-MB-435 cells treated with 10 ng/mL TNF-α for 0, 0.5, 1, 2, 4, or 24 h. *GAPDH* was used to normalize *CEBPB* mRNA levels. Data are presented as mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Western blots are representative of three independent experiments.

unable to induce the migration of MDA-MB-231 cells expressing C/EBPβ shRNAs (Fig. 5B and S1A). To further determine if C/EBPβ could induce cancer cell migration, C/EBPβ was overexpressed in MDA-MB-231 cells. Expression of C/EBPβ increased migration of MDA-MB-231 cells (Fig. 5C, D and S1C). C/EBPβ isoforms (LAP1, LAP2, and LIP) are translated from a single mRNA using alternative start codons. All three isoforms were overexpressed in MDA-MB-231-C/EBPβ cells (Fig. 5C). To assess the roles of individual C/EBPβ isoforms in cell migration, mutations were introduced to the alternative start codons to specifically express LAP1, LAP2, or LIP in MDA-MB-231 cells (Fig. 5C). Expression of LAP1 and LAP2 induced MDA-MB-231 cell migration (Fig. 5D). LIP lacks the transactivation domain, and generally functions as a dominant-negative inhibitor of transcription [Grimm and Rosen, 2003; Zahnow, 2009]. Consistently, overexpression of LIP inhibited MDA-MB-231 cell migration (Fig. 5D). TNF-α is a multifunctional cytokine that may regulate cell survival and apoptosis as well. TNF-α treatment or manipulation of C/EBPβ expression did not result in cell death. Altered C/EBPβ expression did not affect MDA-MB-231 cell proliferation, as assessed by the MTT assay (Fig. S3). Therefore, changes in the migration ability observed in the C/EBPβ-knockdown or -overexpressing cancer cells are likely not the indirect consequence of altered cell proliferation and/or cell apoptosis.

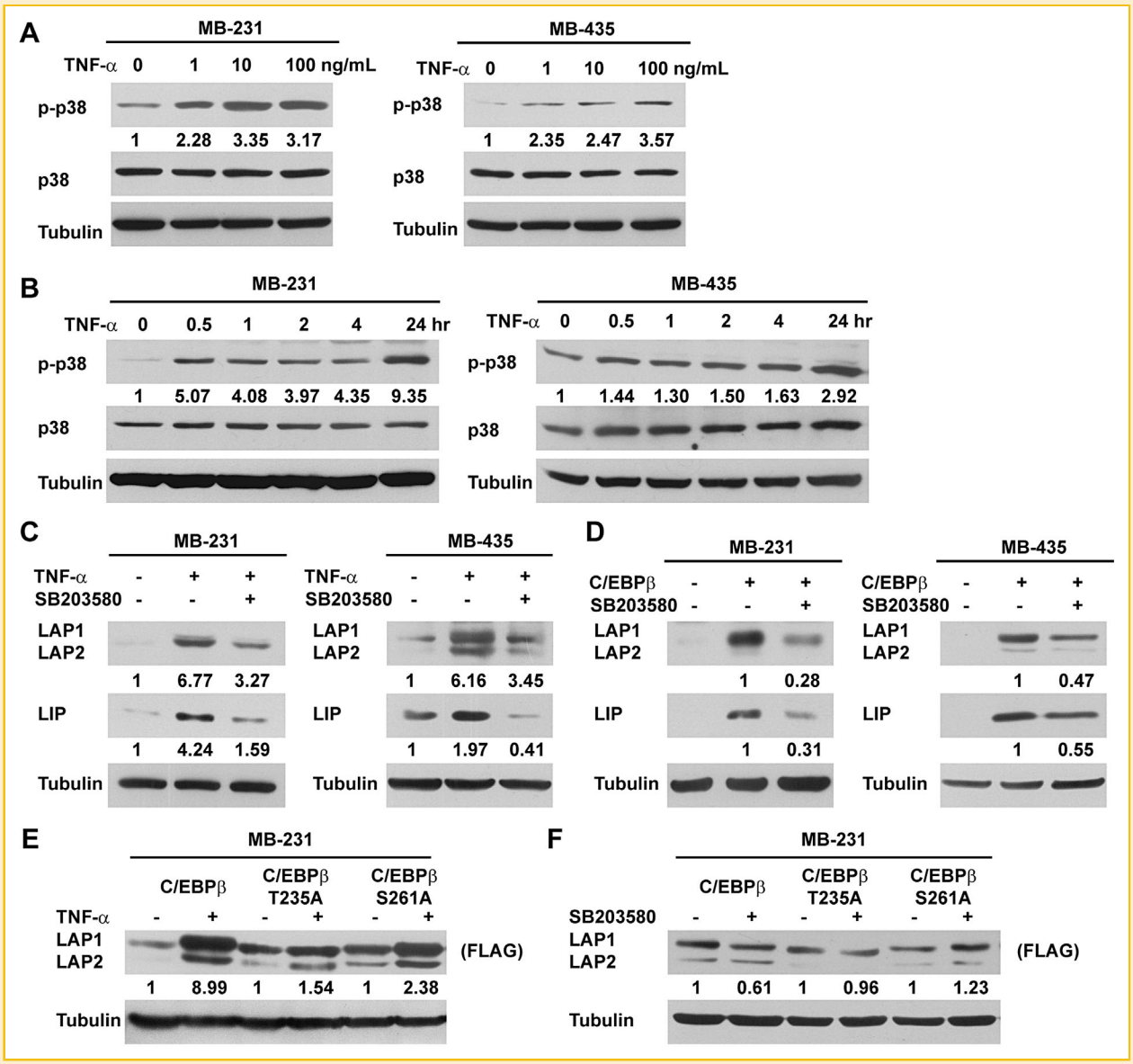

Fig. 3. Activation of p38 MAPK is required for TNF-α-induced C/EBPβ expression. (A) Western blot of p-p38 in MDA-MB-231 and MDA-MB-435 cells treated with different concentrations of TNF-α for 24 h. (B) Western blot of p-p38 in MDA-MB-231 and MDA-MB-435 cells treated with 10 ng/mL TNF-α for 0, 0.5, 1, 2, 4, or 24 h. (C) Western blot of C/EBPβ in MDA-MB-231 and MDA-MB-435 cells treated with 10 ng/mL TNF-α alone or together with 10 μM p38 MAPK inhibitor SB203580 for 24 h. (D) Western blot with FLAG antibody to detect exogenous FLAG-tagged C/EBPβ in C/EBPβ-expressing MDA-MB-231 and MDA-MB-435 cells treated with 10 μM SB203580 for 24 h. (E) Western blot with FLAG antibody to detect exogenous FLAG-tagged wild-type, T235A, or S261A C/EBPβ in C/EBPβ-expressing MDA-MB-231 cells treated with 10 ng/mL TNF-α for 24 h. (F) Western blot with FLAG antibody to detect exogenous FLAG-tagged wild-type, T235A, or S261A C/EBPβ in C/EBPβ-expressing MDA-MB-231 cells treated with 10 μM SB203580 for 24 h. Western blots are representative of three independent experiments.

Since inhibition of p38 MAPK diminished the induction of C/EBPβ expression by TNF-α, we next examined if p38 MAPK inhibition affects TNF-α-induced cell migration. Indeed, SB203580 treatment inhibited TNF-α-induced cell migration (Fig. 5E and S1B). In addition, SB203580 treatment inhibited cell migration induced by C/EBPβ overexpression in MDA-MB-231 and MDA-MB-435 cells as well (Fig. 5F and S1C).

C/EBPβ MEDIATES TNF-α-INDUCED MMP1/3 EXPRESSION

To study which effectors downstream of TNF-α-C/EBPβ signaling are responsible for TNF-α-induced cell migration, global gene expression was analyzed by microarray (Fig. S4 and Table S1). Gene Ontology (GO) analysis on the gene expression profile indicated that GO terms enriched in C/EBPβ expressing MDA-MB-231 cells were mainly related to pathways regulating cell migration (Fig. S4).

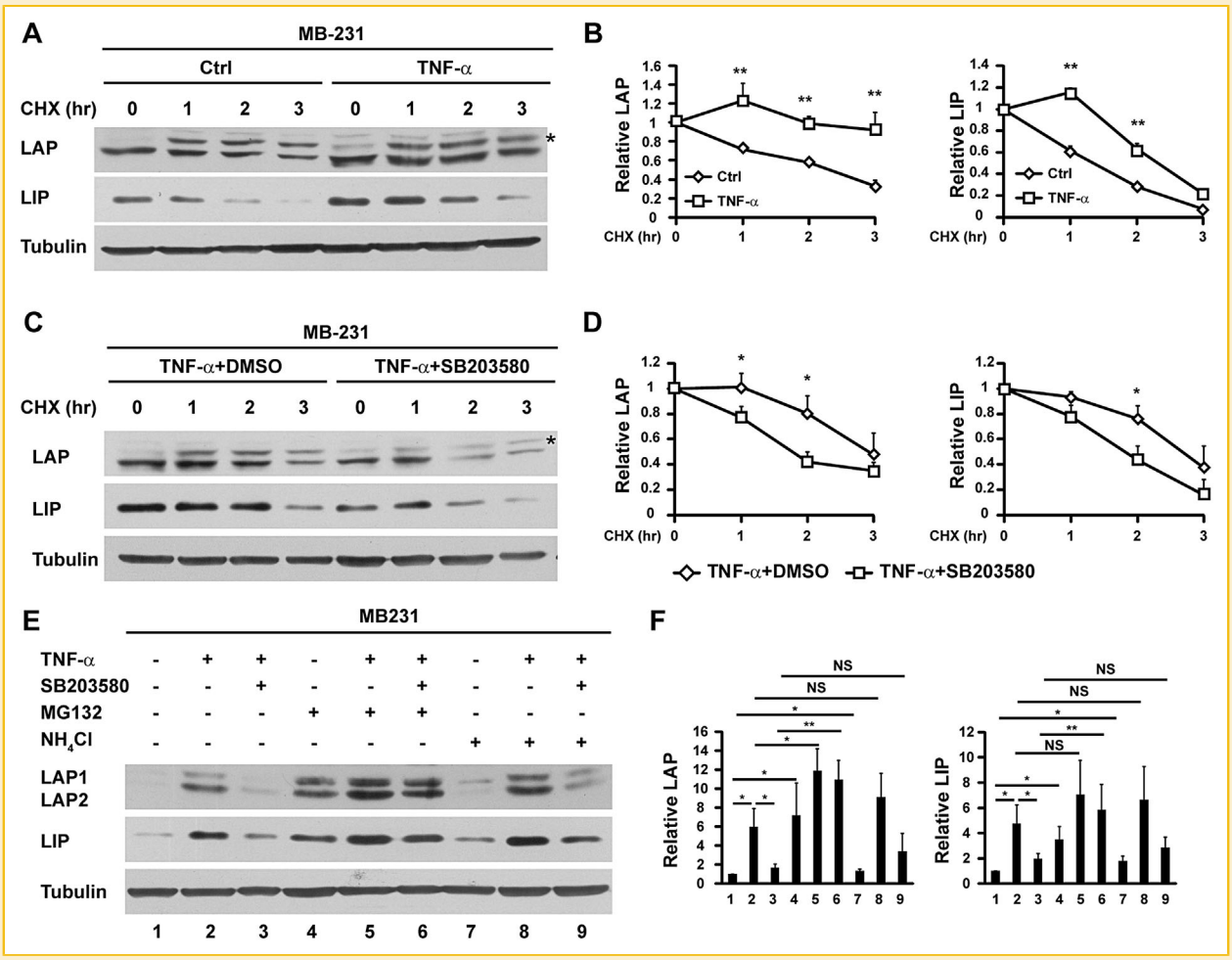

Fig. 4. TNF-α-induced activation of p38 MAPK stabilizes C/EBPβ. (A) MDA-MB-231 cells were pretreated with or without 10 ng/mL TNF-α for 24 h before 0.1 mg/mL CHX treatment. C/EBPβ protein levels were analyzed by western blot. (B) Relative protein levels of LAP and LIP after CHX treatment in (A). (C) MDA-MB-231 cells were pretreated with 10 ng/mL TNF-α with or without 10 μM SB203580 for 24 h before 0.1 mg/mL CHX treatment. C/EBPβ protein levels were analyzed by western blot. (D) Relative protein levels of LAP and LIP after CHX treatment in (C). (E) Western blot of C/EBPβ in MDA-MB-231 cells treated with 10 ng/mL TNF-α with or without 10 μM SB203580 for 24 h in the presence of 10 μM proteasome inhibitor MG132 or 20 mM lysosome inhibitor NH_4Cl. (F) Relative protein levels of LAP and LIP in (E). Data are presented as mean ± SD of three independent experiments. *$P < 0.05$, **$P < 0.01$. Western blots are representative of three independent experiments.

Matrix metalloproteinases (MMPs)-1 and -3 were among the most upregulated genes in MDA-MB-231 cells expressing C/EBPβ (Table S1). MMPs are a family of zinc-dependent proteases that degrade extracellular matrix and play important roles in cancer cell migration, invasion, and metastasis. MMP1 and MMP3, members of MMPs, have been reported to promote cell migration [Ganea et al., 2007; Gialeli et al., 2011; Iguchi, 2012]. MMP1 and MMP3 were expressed at low levels in MDA-MB-231 and MDA-MB-435 cells. TNF-α treatment dramatically increased MMP1 and MMP3 protein levels in conditioned medium from MDA-MB-231 and MDA-MB-435 cells (Fig. 6A). Knockdown of C/EBPβ inhibited TNF-α-induced MMP1 and MMP3 expressions (Fig. 6A), suggesting that C/EBPβ mediates MMP1 and MMP3 expression upon TNF-α treatment. Consistently, expression of exogenous C/EBPβ enhanced MMP1 and MMP3 expressions in MDA-MB-231 and MDA-MB-435 cells (Fig. 6B). p38 MAPK regulates C/EBPβ protein stability and TNF-α-induced cell migration. SB203580 treatment significantly decreased MMP1 and MMP3 protein levels in cells treated with TNF-α (Fig. 6C) or cells expressing C/EBPβ (Fig. 6B).

MMP1 AND MMP3 ARE REQUIRED FOR TNF-α-INDUCED CELL MIGRATION

To determine whether MMP1 and MMP3 are required for TNF-α-induced cell migration, MDA-MB-231 and MDA-MB-435 cells were treated with pan-MMP inhibitor GM6001 in the Transwell assay. GM6001 treatment inhibited cell migration induced by TNF-α in both cancer cells (Fig. 7A). In addition, GM6001 treatment also inhibited cell migration induced by ectopic C/EBPβ expression (Fig. 7B). GM6001 inhibits the activity of a group of MMPs. To investigate the specific functions of MMP1 and MMP3 in TNF-α-induced cell

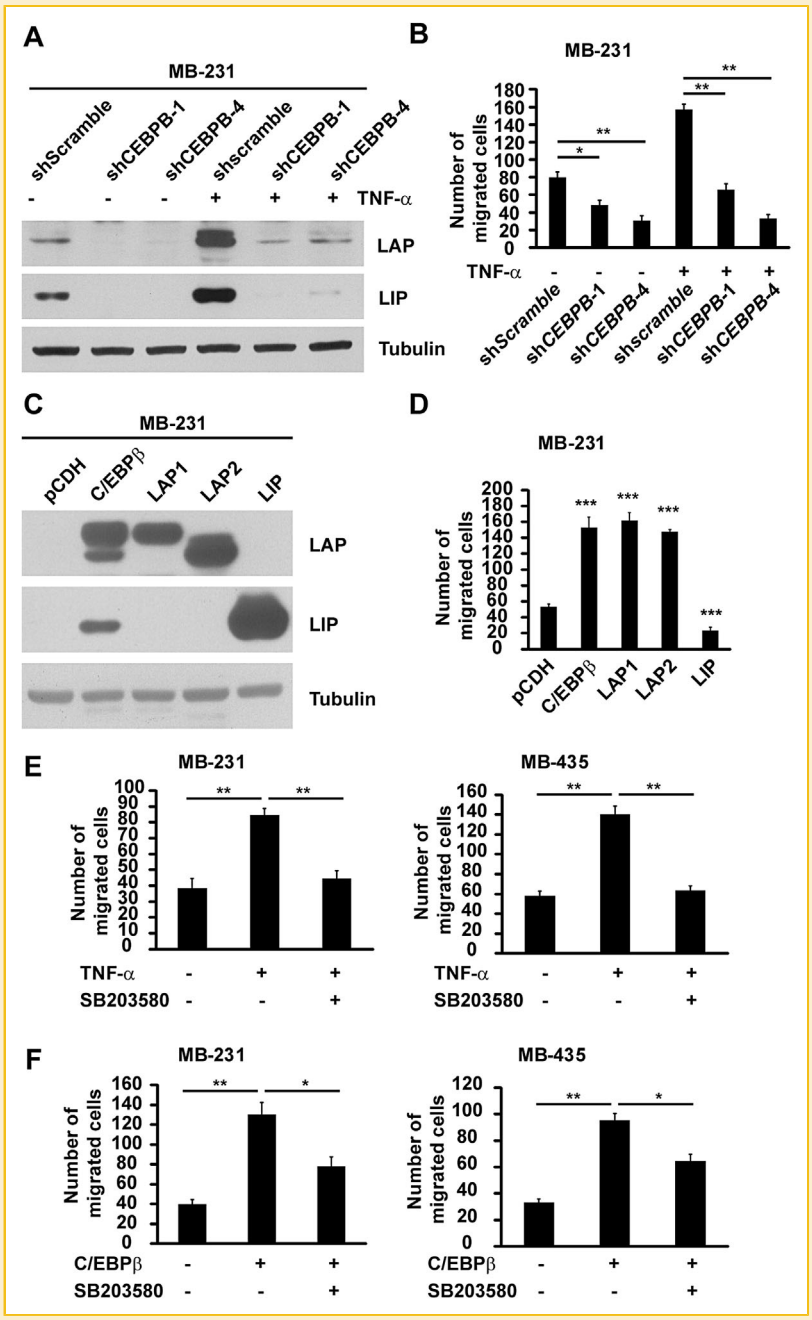

Fig. 5. C/EBPβ is required for TNF-α-induced cell migration. (A) Western blot of C/EBPβ in MDA-MB-231 cells expressing Scramble or C/EBPβ shRNAs treated with 10 ng/mL TNF-α for 24 h. (B) Migration of MDA-MB-231 cells expressing Scramble or C/EBPβ shRNAs treated with 10 ng/mL TNF-α for 20 h. (C) Western blot of C/EBPβ in MDA-MB-231 cells expressing C/EBPβ, LAP1, LAP2, or LIP. (D) Migration of MDA-MB-231 cells expressing C/EBPβ, LAP1, LAP2, or LIP for 20 h. (E) Migration of MDA-MB-231 and MDA-MB-435 cells treated with 10 ng/mL TNF-α and 10 μM SB203580 for 20 h. (F) Migration of C/EBPβ-expressing MDA-MB-231 and MDA-MB-435 cells treated with 10 μM SB203580 for 20 h. Data are presented as mean ± SD of three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Western blots are representative of three independent experiments.

migration, MMP1 or MMP3 were knocked down in MDA-MB-231 cells with two individual shRNAs. MMP1 and MMP3 shRNAs efficiently decreased the expression of MMP1/MMP3 in MDA-MB-231 cells (Fig. 7C). Knockdown of MMP1 or MMP3 inhibited cell migration induced by TNF-α in MDA-MB-231 cells (Fig. 7D), indicating that MMP1 and MMP3 are required for TNF-α-induced cell migration. Knockdown of MMP1 or MMP3 significantly inhibited cell migration induced by C/EBPβ overexpression as well (Fig. 7E and F). Taken together, MMP1 and MMP3 mediate TNF-α-induced cell migration downstream of C/EBPβ.

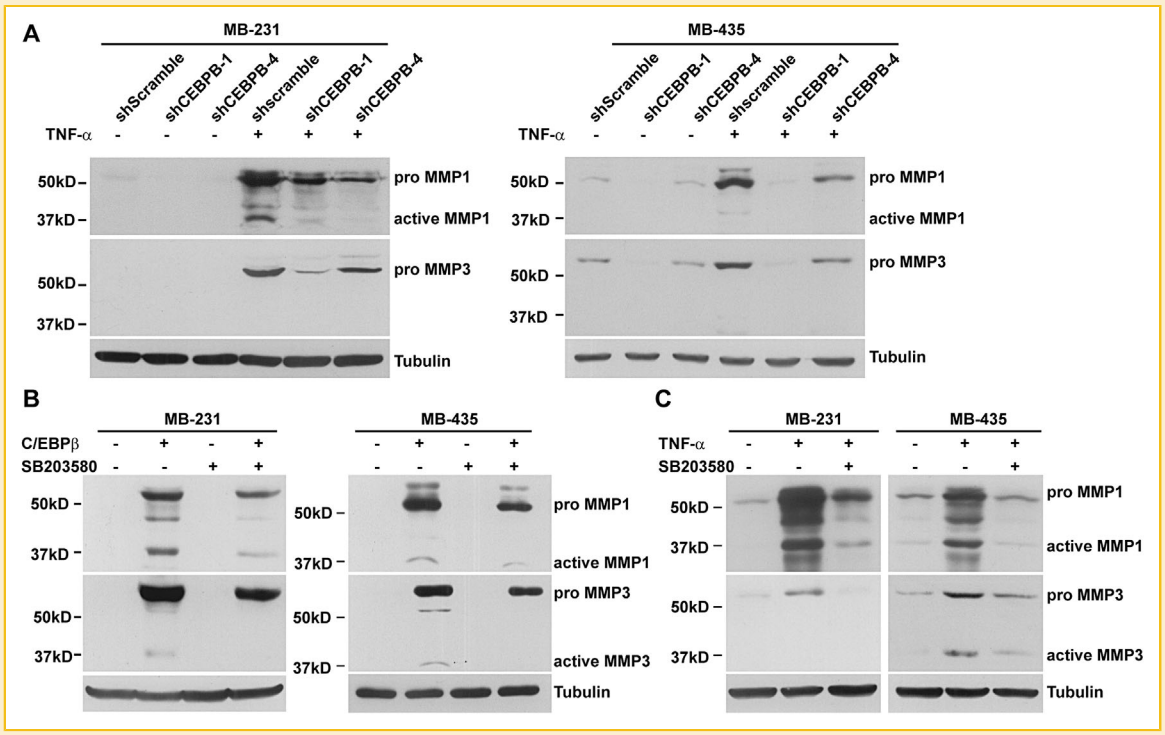

Fig. 6. C/EBPβ mediates TNF-α-induced expression of MMP1 and MMP3. (A) Western blot of MMP1 and MMP3 in conditioned medium from MDA-MB-231 and MDA-MB-435 cells expressing Scramble or C/EBPβ shRNAs that were treated with or without 10 ng/mL TNF-α for 24 h. (B) Western blot of MMP1 and MMP3 in C/EBPβ-expressing MDA-MB-231 and MDA-MB-435 cells treated with 10 μM SB203580 for 24 h. (C) Western blot of MMP1 and MMP3 in MDA-MB-231 and MDA-MB-435 cells treated with 10 ng/mL TNF-α with or without 10 μM SB203580 for 24 h. Tubulin in cell lysates were probed to normalize loading. Western blots are representative of three independent experiments.

DISCUSSION

TNF-α is one of the key cytokines mediating inflammation-associated cancer. TNF-α is not only involved in cancer initiation and development, but also contributes substantially to cancer metastasis at advanced stages [Balkwill, 2006]. TNF-α enhanced the motility of breast cancer cells [Wu et al., 2009; Li et al., 2012]. Aberrant TNF-α signaling enhanced cancer metastasis [Malik et al., 1990; Orosz et al., 1993; Orosz et al., 1995], while blocking TNF-α signaling abolished distal metastasis in mouse models [Malik et al., 1990; Orosz et al., 1993; Kitakata et al., 2002].

Multiple effectors are involved in TNF-α-induced cell migration. In this report, we identified that TNF-α treatment increased C/EBPβ expression in cancer cells (Fig. 2). TNF-α regulated C/EBPβ expression at both transcriptional and post-transcriptional levels (Figs. 2 and 4). Activation of p38 MAPK was required for stabilization of C/EBPβ protein upon TNF-α stimulation (Fig. 3), in which process C/EBPβ phosphorylation may be necessary (Fig. 3). C/EBPβ is a transcription factor involved in metabolism, inflammation, and cancer [Grimm and Rosen, 2003; Zahnow, 2009]. C/EBPβ expression is modestly increased in estrogen receptor-negative breast cancers, compared to estrogen receptor-positive breast cancers [Zahnow, 2009] and high C/EBPβ expression is associated with breast cancer metastasis [van de Vijver et al., 2002; Zahnow, 2009], suggesting that C/EBPβ may act as a candidate regulator of inflammation-associated cancer metastasis. C/EBPβ is not a single protein. Three isoforms, including transcriptional activator LAP1 and LAP2 and transcriptional repressor LIP, are encoded by a single mRNA by alternative translation [Grimm and Rosen, 2003; Zahnow, 2009]. It is yet to be elucidated the specific functions of each isoform. Overexpression of LAP2 in normal mammary epithelial cells led to EMT and transformation [Bundy and Sealy, 2003]. However, LAP proteins were also reported to suppress EMT and cell migration [Johansson et al., 2013; Miura et al., 2014]. Epigenetic silencing of C/EBPβ by MiR-155 promoted TGF-β-induced breast cancer cell EMT, invasion, and metastasis [Johansson et al., 2013]. Overexpression of LAP2 partially inhibited TGF-β-induced breast cancer cell EMT [Johansson et al., 2013]. The balance between LAP and LIP was important in TGF-β-induced mouse mammary epithelial cell EMT that LIP promoted EMT and LAP promoted mesenchymal–epithelial transition (MET) [Miura et al., 2014]. In the experimental system used in this study, LAP, but not LIP, was the effector molecule in mediating TNF-α-induced cancer cell migration, although TNF-α induced expression of both LAP and LIP.

TNF-α activates multiple intracellular signaling pathways. Activation of the NF-κB transcription factor has been emphasized as the key event in inflammation-associated cancer progression and metastasis [Karin and Greten, 2005; Balkwill, 2006; Karin, 2006]. TNF-α activated NF-κB in the cancer cells to promote their motility,

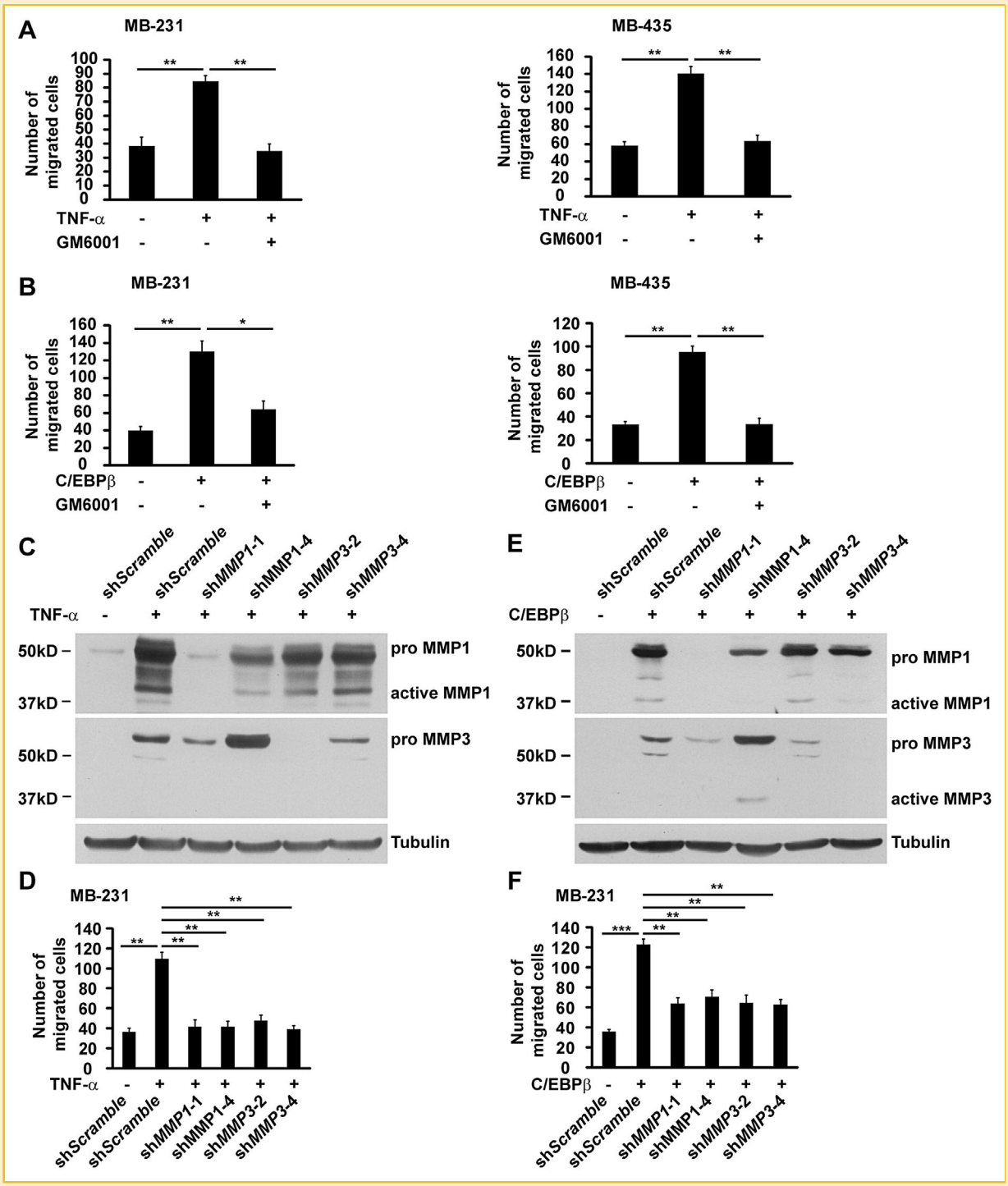

Fig. 7. MMP1 and MMP3 are required for TNF-α-induced cell migration. (A) Migration of MDA-MB-231 and MDA-MB-435 cells treated with 10 ng/mL TNF-α with or without 10 μM pan-MMP inhibitor GM6001 for 20 h. (B) Migration of C/EBPβ-expressing MDA-MB-231 and MDA-MB-435 cells treated with 10 μM GM6001 for 20 h. (C) Western blot of MMP1 and MMP3 in conditioned medium from MDA-MB-231 cells expressing Scramble, MMP1 or MMP3 shRNAs. Tubulin in cell lysates were probed to normalize loading. (D) Migration of MDA-MB-231 cells expressing scramble, MMP1, or MMP3 shRNAs treated with 10 ng/mL TNF-α for 20 h. (E) Western blot of MMP1 and MMP3 in MDA-MB-231-C/EBPβ cells expressing Scramble, MMP1, or MMP3 shRNAs. Tubulin in cell lysates were probed to normalize loading. (F) Migration of MDA-MB-231-C/EBPβ cells expressing Scramble, MMP1, or MMP3 shRNAs for 20 h. Data are presented as mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Western blots are representative of three independent experiments.

invasion, and metastasis by inducing EMT [Kulbe et al., 2005; Wu et al., 2009; Li et al., 2012]. NF-κB activation prevented ubiquitination and degradation of Snail transcription factor [Wu et al., 2009]. NF-κB activation also upregulated the transcription of another EMT-related transcription factor Twist1 [Li et al., 2012]. Although NF-κB was suggested to function upstream of C/EBPβ by stabilizing C/EBPβ protein in TNF-α-induced hepatocyte death [Wang et al., 2010], inhibition of NF-κB pathway did not affect TNF-α-induced C/EBPβ expression in cancer cells (Fig. S2). C/EBPβ and NF-κB were both required for TNF-α-induced cancer cell migration (Fig. 5 and S2). C/EBPβ and NF-κB may cooperate to regulate cancer cell migration. Both C/EBPβ and NF-κB were required for IL-17-induced MMP1 expression [Cortez et al., 2007]. CXCR4 and its ligand CXCL12 are implicated in metastasis in a wide range of cancers. C/EBPβ upregulated CXCR4 expression and modulated breast cancer migration [Park et al., 2013]. It was also reported that TNF-α induced CXCR4 expression and ovarian cancer cell migration dependent on NF-κB [Kulbe et al., 2005]. All these collectively suggest that C/EBPβ and NF-κB may function in concert to mediate inflammation-induced cancer cell migration and metastasis. C/EBPβ forms heterodimers with multiple transcription factors, including NF-κB [LeClair et al., 1992; Zahnow, 2009; Tsukada et al., 2011]. It requires further investigation whether C/EBPβ and NF-κB function in a complex in regulating TNF-α-induced cancer cell migration and inflammation-associated cancer metastasis.

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