TIPE2 inhibits TNF-α-induced hepatocellular carcinoma cell metastasis via Erk1/2 downregulation and NF-κB activation

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Abstract. Tumor necrosis factor-α-induced protein 8-like 2 (TNFAIP8L2, TIPE2), which belongs to the TNF-α-induced protein 8 family, is a negative regulator of immune homeostasis. Although pro-inflammatory cytokines such as TNF-α have been reported to be involved in liver carcinoma metastasis, the effect of TIPE2 on hepatocellular carcinoma metastasis remains unknown. We demonstrate that TNF-α clearly augments MMP-13/MMP-3 expression and promotes cell migration in HepG2 cells through activation of the Erk1/2-NF-κB pathways. Interestingly, in addition to human PBLs, macrophages and fibroblasts, liver cancer cells specifically express TNF-α following LPS treatment. Most importantly, TIPE2 overexpression efficiently abrogates the effects of LPS on TNF-α secretion and abolishes the effects of TNF-α on MMP-13/MMP-3 upregulation, cell migration and Erk1/2-NF-κB activation. Taken together, these findings demonstrate that TIPE2 was able to suppress TNF-α-induced hepatocellular carcinoma metastasis by inhibiting Erk1/2 and NF-κB activation, indicating that both TNF-α and TIPE2 might be potential targets for the treatment of HCC metastasis.

Introduction

Hepatocellular carcinoma (HCC), which shows a close relationship with HBV- and HCV-induced inflammation (1), is associated with a high potential for vascular invasion, metastasis and recurrence (2). Although inflammation is closely associated with hepatic cell carcinogenesis (3), the involvement of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), in liver carcinoma metastasis and nutritional status has also been documented (4,5), indicating that TNF-α plays a particularly important role in the tumor microenvironment and promotes tumor cell migration and invasion (6). As tumor tissues are composed of cancer cells, fibroblasts, vasculature and immune cells, the finding that activated macrophages secrete TNF-α and promote tumor metastasis (3) does not exclude the possibility that other components of the liver cancer microenvironment might secrete TNF-α and promote HCC metastasis in an autocrine or paracrine manner. Hence, the role of microenvironment components in TNF-α-facilitated HCC metastasis remains elusive.

Extracellular matrix (ECM) degradation and basement membrane destruction are prerequisites for tumor invasion and metastasis, which are known to involve matrix metalloproteinases (MMPs) (7,8). Stromelysin-1 (MMP-3) is an MMP with broad substrate specificity that has been shown to degrade fiber mucin (FN), laminin (LN), casein, and proteoglycans and to activate gelatinase B (MMP-9)/collagenase. MMP-3 has been found to be associated with early oral squamous cell carcinoma and breast cancer (9,10). Furthermore, involvement of MMP-3 in hepatocyte growth factor-induced invasion has also been documented (11,12). Additionally, human collagenase 3 (MMP-13), which represents the third member of the collagenase subfamily, has been detected in gastric cancer and breast cancer (10,13,14). MMP-13 can be activated by MMP-2/MMP-3, and activated MMP-13, in turn, has the ability to activate MMP-2 and MMP-9 (8), indicating that MMP-13 plays a central role in the MMP activation cascade (15). However, few studies conducted thus far have explored the roles of MMP-13 and MMP-3 in inflammation-associated HCC metastasis.

Tumor necrosis factor-α-induced protein 8-like 2 (TNFAIP8L2, TIPE2), which belongs to the TNF-α-induced protein 8 family, could inhibit the release of pro-inflammatory cytokines, such as TNF-α, IL-4, IL-12 and IFN-γ (16). TIPE2 inhibition of MMP-9 expression (17) and reduction of F-actin polymerization (18) have also been documented, indicating that TIPE2 might play an important role in tumor metastasis. However, little is currently known concerning the effect of TIPE2 on inflammation-associated metastasis, which is clearly an important issue for treating HCC metastasis.

The aim of the present study was to identify the functions of TNF-α and TIPE2 in HCC metastasis by studying changes in MMP expression and to examine their relationships with Erk1/2 and NF-κB pathway activation. For this purpose,

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we investigated the effects of both TNF-α and TIPE2 on MMP-13/MMP-3 expression and cell migration as well as Erk1/2-NF-κB activation under exogenous TNF-α conditions. We found that TNF-α was able to increase both MMP-3/MMP-13 expression and enhance the migratory ability of HepG2 cells by activating the Erk1/2-NF-κB pathways. However, when TIPE2 was overexpressed, these TNF-α-induced effects were inhibited. These results indicated that TIPE2 might play a negative role in TNF-α-accelerated HCC metastasis, suggesting that TIPE2 could be a potential new target for the clinical treatment of liver cancer.

Materials and methods

Reagents. Lipopolysaccharides (LPS) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Recombinant human TNF-α, brefeldin A solution, and fluorescein-conjugated antibodies against mouse or human TNF-α were purchased from eBioscience (San Diego, CA, USA). Western blot antibodies and the Erk1/2 inhibitor U0126 were acquired from Cell Signaling Technology (Beverly, MA, USA). The NF-κB inhibitor Bay-7082 was purchased from Cayman Chemical (Ann Arbor, MI, USA). SYBR® Premix Ex Taq™, TRIzol and PrimeScript reverse transcriptase were purchased from Takara (Dalian, China). Goat anti-rabbit IgG-FITC and IgG-PE were obtained from Abcam (San Francisco, CA, USA). A transwell migration system was purchased from Corning (Corning, NY, USA). Lipofectamine 2000 was purchased from Invitrogen (Grand Island, NY, USA). DMEM and fetal bovine sera were obtained from Hyclone (Logan, UT, USA). The NE-PER nuclear and cytoplasmic extraction reagents were purchased from Pierce (Rockford, IL, USA).

Cell lines. The human hepatoblastoma cell line HepG2 was obtained from the Shanghai Cell Bank (Shanghai, China). Cells were cultured in DMEM supplemented with 10% fetal bovine serum at 37˚C under 5% CO_2. Cells were synchronized by serum starvation for at least 12 h before TNF-α or LPS treatment.

Establishment of a TIPE2-overexpressing HepG2 cell line. TIPE2-overexpressing HepG2 cells were established as described previously (18). Briefly, the pEGFP-C1-TIPE2 plasmid was generated by inserting full-length TIPE2 into the pEGFP-C1 vector. HepG2 cells plated at a density of $6x10^5$ cells/well were transfected with the plasmid using Lipofectamine 2000, and resistant cells were selected for using 500 μg/ml G418 antibiotic. Separated cell clones were confirmed via RT-PCR and stored for further experiments.

Flow cytometric measurements. The effects of LPS or TIPE2 on TNF-α expression were assayed as described previously (19). Briefly, cells plated at a density of $2x10^4$ cells/well were stimulated with LPS for 4 h. For PBLs, the final concentration of LPS was 100 ng/ml. For other cells, the final concentration of LPS was 1 μg/ml. BFA was added to inhibit TNF-α secretion. After LPS treatment, the cells were permeabilized and fixed for 30 min using BD Cytofix/Cytoperm™ fixation/permeabilization solution (BD Biosciences, San Jose, CA, USA). Then, the cells were washed with Perm/Wash buffer and stained with specific antibodies for 30 min on ice. Flow cytometry was performed using a FACSCalibur flow cytometer, and the data were analyzed using CellQuest software.

Table I. Primer sequence table.

Genes	F/R	Sequence
β-actin	F	5'-ACCGTGGAGAAGAGCTACGA-3'
β-actin	R	5'-GTACTTGCGCTCAGAAGGAG-3'
TIPE2	F	5'-CACCGCAATGGCTCCTTT-3'
TIPE2	R	5'-CACCAACTCTAGCAGCACATC-3'
TNF-α	F	5'-GAAAGCATGATCCGGGACGTG-3'
TNF-α	R	5'-GATGGCAGAGAGGAGGTTGAC-3'
MMP-13	F	5'-TTGTTGCTGCGCATGAGTTCG-3'
MMP-13	R	5'-GGGTGCTCATATGCAGCATCA-3'
MMP-3	F	5'-CCTGCTTTGTCCTTTGATGC-3'
MMP-3	R	5'-TGAGTCAATCCCTGGAAAGTC-3'

Confocal immunofluorescence assays. The effects of TNF-α or TIPE2 on p65, Erk1/2 phosphorylation and c-Fos expression were investigated using immunofluorescence assays (19). Briefly, cells were treated with TNF-α (2 ng/ml), fixed and permeabilized in 100% methanol. Then, the cells were blocked with 10% non-fat milk and incubated with primary antibodies (anti-phospho-p65, anti-phospho-Erk, or anti-c-Fos; 1:100 dilution) overnight at 4˚C, followed by staining with FITC-conjugated IgG (1:100 dilution) or PE-conjugated IgG (1:1,000 dilution). Finally, the cells were mounted in mounting medium containing DAPI. Images were captured using a confocal fluorescence microscope at 488 or 546 nm.

RT-PCR and qPCR. The effects of TIPE2 on MMP-3/MMP-13 and TNF-α expression were investigated via RT-PCR and real-time PCR analyses as described previously (20). Briefly, whole cellular RNA was extracted, and reverse-transcription was performed using PrimeScript reverse transcriptase. PCR amplification was conducted using the following conditions: 95˚C for 3 min, followed by 30 cycles of 95˚C for 5 sec, 60˚C for 30 sec and 72˚C for 60 sec and a final extension at 72˚C for 10 min. β-actin was used as an internal control. The PCR products were run on 1.5% agarose gels and analyzed under ultraviolet (UV) light after ethidium bromide staining. To quantify gene amplification, real-time PCR analysis was performed using an ABI 7000 Sequence Detection System in the presence of SYBR Green. The cycling parameters were 95˚C for 3 min, followed by 40 cycles of 95˚C for 5 sec, 60˚C for 30 sec, and 72˚C for 60 sec, with a final extension at 72˚C for 10 min; a melting curve analysis was subsequently conducted. The relative expression levels (defined as fold-changes) of the target genes were normalized to the fold-value of the corresponding control cells. The following primer sequences were used in these assays (Table I).

Isolation of cytoplasmic and nuclear extracts. Cytoplasmic and nuclear extracts were prepared from whole-cell extracts as described previously (19). Briefly, following TNF-α (2 ng/ml)

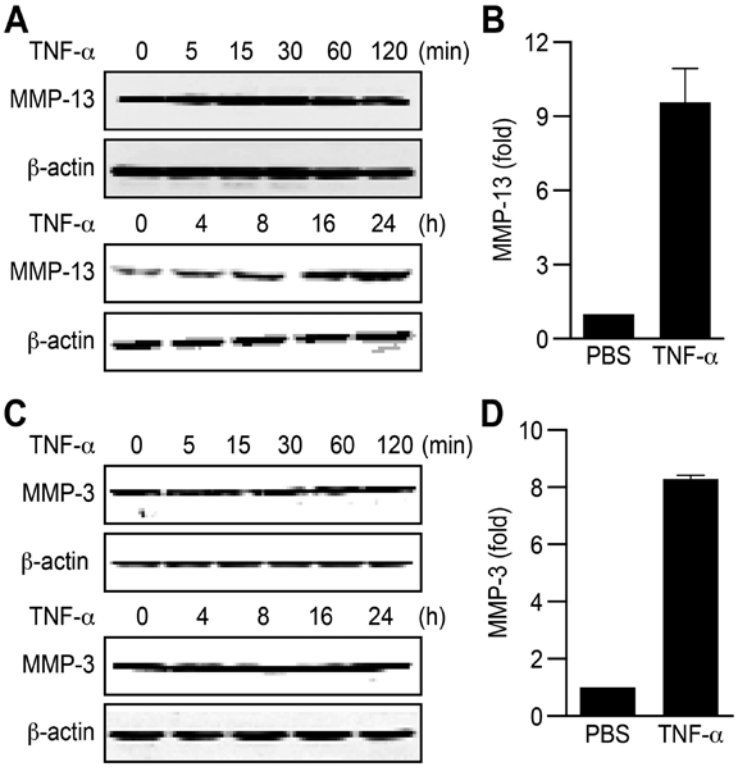

Figure 1. TNF-α upregulates MMP-3 and MMP-13 expression in HepG2 cells. HepG2 cells were exposed to TNF-α (2 ng/ml), and whole cellular protein or RNA was extracted. The effects of TNF-α on MMP-13 and MMP-3 expression were determined via western blot analysis (A and C) and real-time PCR analyses (B and D). Representative results from 1 of 3 independent experiments are shown. β-actin was used as an internal control. The data are presented as the mean ± SEM (B and D).

treatment, the cells were suspended and vortexed in CER (cytoplasmic extraction reagent) buffer. The cytosolic fraction was separated via centrifugation (16,000 x g, 5 min, 4˚C), and nuclear proteins were separated by incubating the insoluble fraction with NER (nuclear extraction reagent) for 40 min, followed by centrifugation at 16,000 x g for 10 min. The obtained protein concentration was estimated, and the nuclear extract was further analyzed by western blot analysis.

Western blot analysis. The phosphorylation of Erk1/2-c-Fos and NF-κB and the expression of related proteins were determined via western blot analysis (21). Briefly, cells were pretreated with kinase inhibitors 2 h before TNF-α (2 ng/ml) stimulation, and whole cellular protein was extracted. Then, the protein was loaded onto 10-12% SDS-PAGE gels and transferred onto a PVDF membrane. After blocking with 5% non-fat milk, the membrane was incubated with primary antibodies, followed by HRP-conjugated secondary antibodies. The bands were developed using an ECL chemiluminescence substrate. β-actin, tubulin or histone 3 was used as a loading control.

Transwell migration assay. The effects of TNF-α and TIPE2 on cell migration were investigated in a transwell migration assay as previously described (17). Briefly, $4x10^3$ cells were seeded into the top chamber in serum-free medium (12-well insert, 12 μm; Corning) and pretreated with related kinase inhibitors 2 h before TNF-α (2 ng/ml) stimulation. After 48 h of incubation, cell migration was determined by calculating the number of migrated cells in three visual fields per well via microscopy at x100 magnification.

ELISA. To determine the effect of TNF-α on MMP-3 and MMP-13 activities, cells ($3x10^6$) were treated with TNF-α (2 ng/ml) for 24 h, and the supernatant was collected. MMP-3/MMP-13 activities were determined via ELISA (22). Briefly, the plates were pre-coated with a coating antibody at 4˚C overnight, washed with PBST, and blocked with assay buffer. Then, the samples, MMP-3/MMP-13 standard proteins, assay buffer, and biotin-conjugate detector antibodies were added to the wells, followed by incubation for 2 h. After incubation, each plate was washed with PBST, and streptavidin-HRP was added, followed by incubation for 1 h. The color reaction was developed with TMB solution, and absorbance was measured at 450 nm.

Statistical analysis. All experiments were repeated at least three times to confirm the results. The data are presented as the mean ± SEM. Student's t-test, one-way ANOVA with the Newman-Keuls post-test or two-way ANOVA was applied. Differences were considered significant at $p<0.05$.

Results

TNF-α enhances the migration of hepatocellular carcinoma cells. MMPs are involved in the processes of angiogenesis, tumor progression and metastasis (10). In this study, the effect of TNF-α on MMP-13 and MMP-3 expression was determined. Compared with controls, TNF-α efficiently upregulated MMP-13/MMP-3 expression at both the protein (Fig. 1A and C) and RNA levels (Fig. 1B and D). Taken together, these findings indicate that pro-inflammatory cytokines, such as TNF-α, play an important role in HCC metastasis.

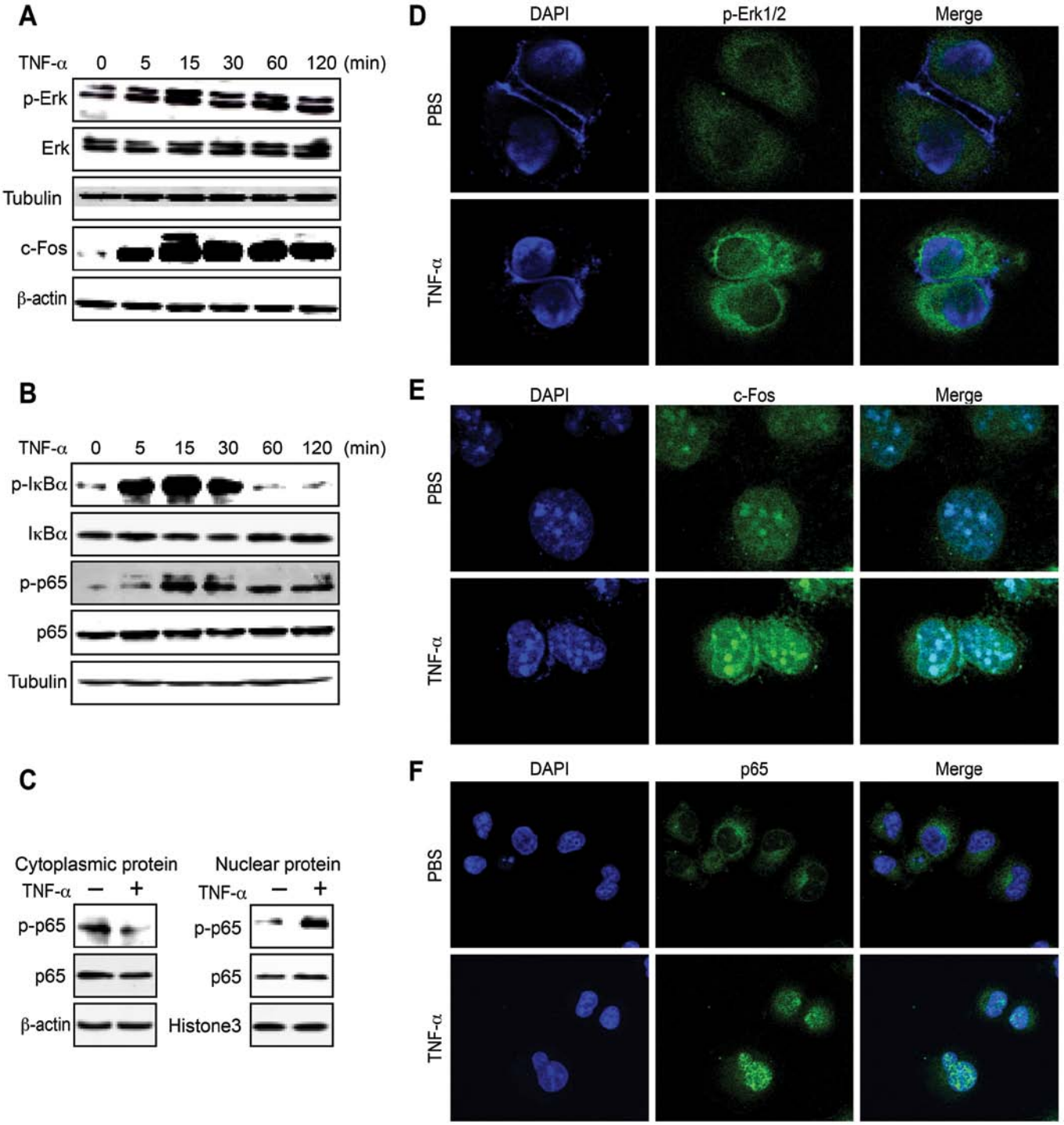

Figure 2. TNF-α activates the Erk1/2 and NF-κB pathways in HepG2 cells. HepG2 cells were exposed to TNF-α (2 ng/ml). Whole cellular protein (A and B) or the cytosolic and nuclear fractions (C) were extracted, and Erk1/2, p65, and IκBα phosphorylation and p65 translocation were determined by western blot analysis (A-C) and confocal microscopy analyses (D-F), respectively. β-actin, tubulin or histone 3 was used as an internal control. Representative results from 1 of 3 independent experiments are shown.

TNF-α activates the Erk1/2 and NF-κB pathways. Although the MAPK and NF-κB pathways are involved in TNF-α-augmented cell migration in VSMCs and macrophages (23,24), the exact mechanism of TNF-α-augmented cell migration remains uncertain. Our results revealed that Erk1/2 phosphorylation and c-Fos expression were clearly increased following TNF-α treatment (Fig. 2A), as were the levels of IκBα and p65 phosphorylation (Fig. 2B) and p65 translocation (Fig. 2C). The confocal microscopy results led to a similar conclusion (Fig. 2D-F), and taken together, these findings indicate that TNF-α activates the Erk1/2-c-Fos and NF-κB pathways in HepG2 cells.

Inhibition of Erk1/2 and NF-κB activation abrogates the effects of TNF-α on cell migration. To understand the role of Erk1/2 and NF-κB activation in cell migration, inhibitors

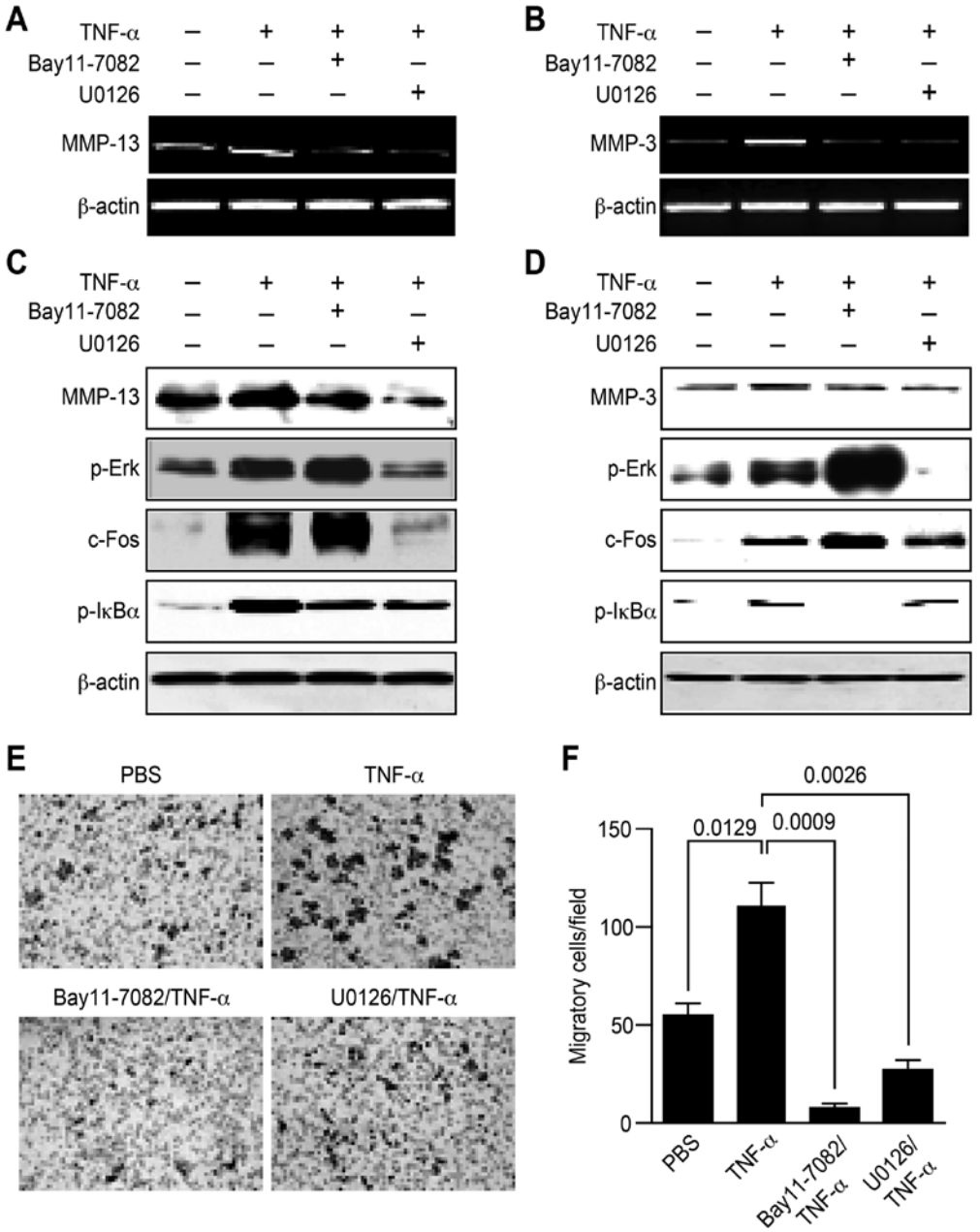

Figure 3. Inhibition of Erk1/2 and NF-κB activation abrogates TNF-α increased cell migration in HepG2 cells. HepG2 cells were pretreated with 20 μM Bay11-7082 or U0126 prior to TNF-α (2 ng/ml) treatment. The roles of Erk1/2 and NF-κB activation in MMP-3/MMP-13 expression (A-D) and cell migration (E and F) were determined via RT-PCR (A and B), western blot analysis (C and D) and transwell migration assays (E and F). The data are presented as the mean ± SEM. $p<0.05$, $p<0.01$, $p<0.001$, one-way ANOVA with the Newman-Keuls post-test. Representative results from 1 of 3 independent experiments are shown. β-actin was used as the loading control.

were used to inhibit related kinase activities. These inhibitors were invariably able to abolish Erk1/2 or NF-κB kinase activity. The inhibition of Erk1/2 or NF-κB activity clearly abrogated the effect of TNF-α on MMP-13/MMP-3 expression at both the transcriptional (Fig. 3A and B) and translational (Fig. 3C and D) levels, indicating that TNF-α augments MMP-13/MMP-3 expression by activating the Erk1/2 and NF-κB pathways. Additionally, the inhibition of Erk1/2 or NF-κB activity led to a 77.8 or 93.1% rate of inhibition of cell migration, respectively (Fig. 3E, $p<0.05$, $p<0.01$, $p<0.001$, one-way ANOVA with the Newman-Keuls post-test). The observation that inhibition of Erk1/2 or NF-κB activity abolished the effect of TNF-α on MMP-13/MMP-3 expression and cell migration indicates that the Erk1/2 and NF-κB pathways are involved in MMP-13/MMP-3-mediated cell migration in HepG2 cells.

TIPE2 abolishes the effect of TNF-α on cell migration by downregulating MMP-13/MMP-3 expression. HCC exhibits lower TIPE2 expression than ambient tissues (18), and the finding that TIPE2 inhibits F-actin depolymerization (18) and downregulates MMP-9 expression (17) therefore raises the question of whether TIPE2 plays an important role in HCC metastasis. Although TNF-α clearly augmented cell migration, TIPE2 overexpression led to approximately 50% inhibition of cell migration (Fig. 4A). Moreover, TIPE2 efficiently abrogated

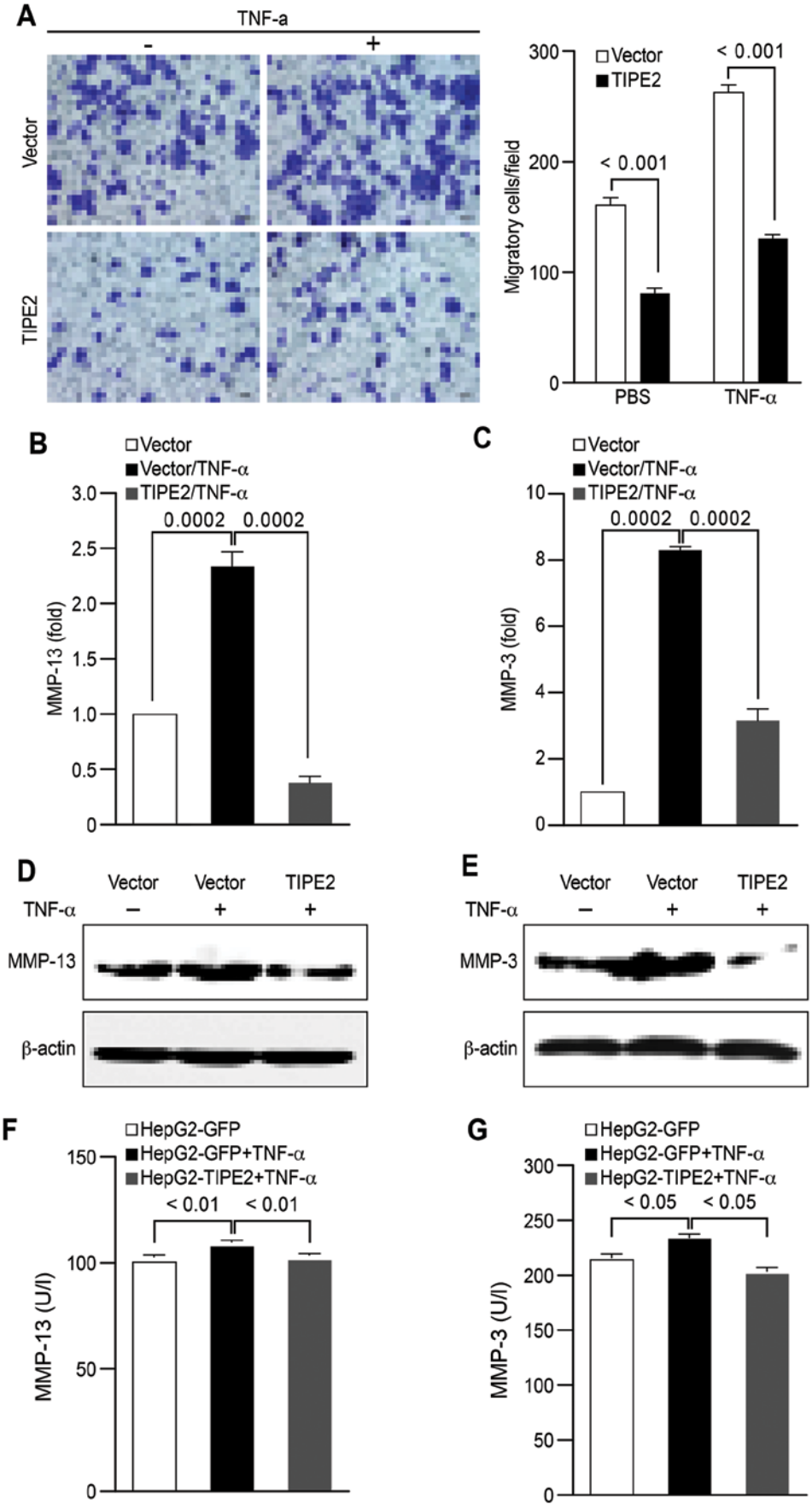

Figure 4. TIPE2 inhibits TNF-α increased cell migration in HepG2 cells. TIPE2 overexpressing HepG2 and control cells were treated with TNF-α (2 ng/ml), and the effects of TIPE2 on cell migration, MMP-13 and MMP-3 expression and MMP-3 and MMP-13 activities were determined via transwell migration assays (A), real-time PCR (B and C), Western blot (D and E) and ELISA analyses (F and G), respectively. For the cell migration assays, $6x10^3$ cells were seeded and exposed to TNF-α for 48 h. $p<0.01$, $p<0.001$, one-way ANOVA with the Newman-Keuls post-test or two-way ANOVA. Representative results from 1 of 3 independent experiments are shown. β-actin was used as a loading control.

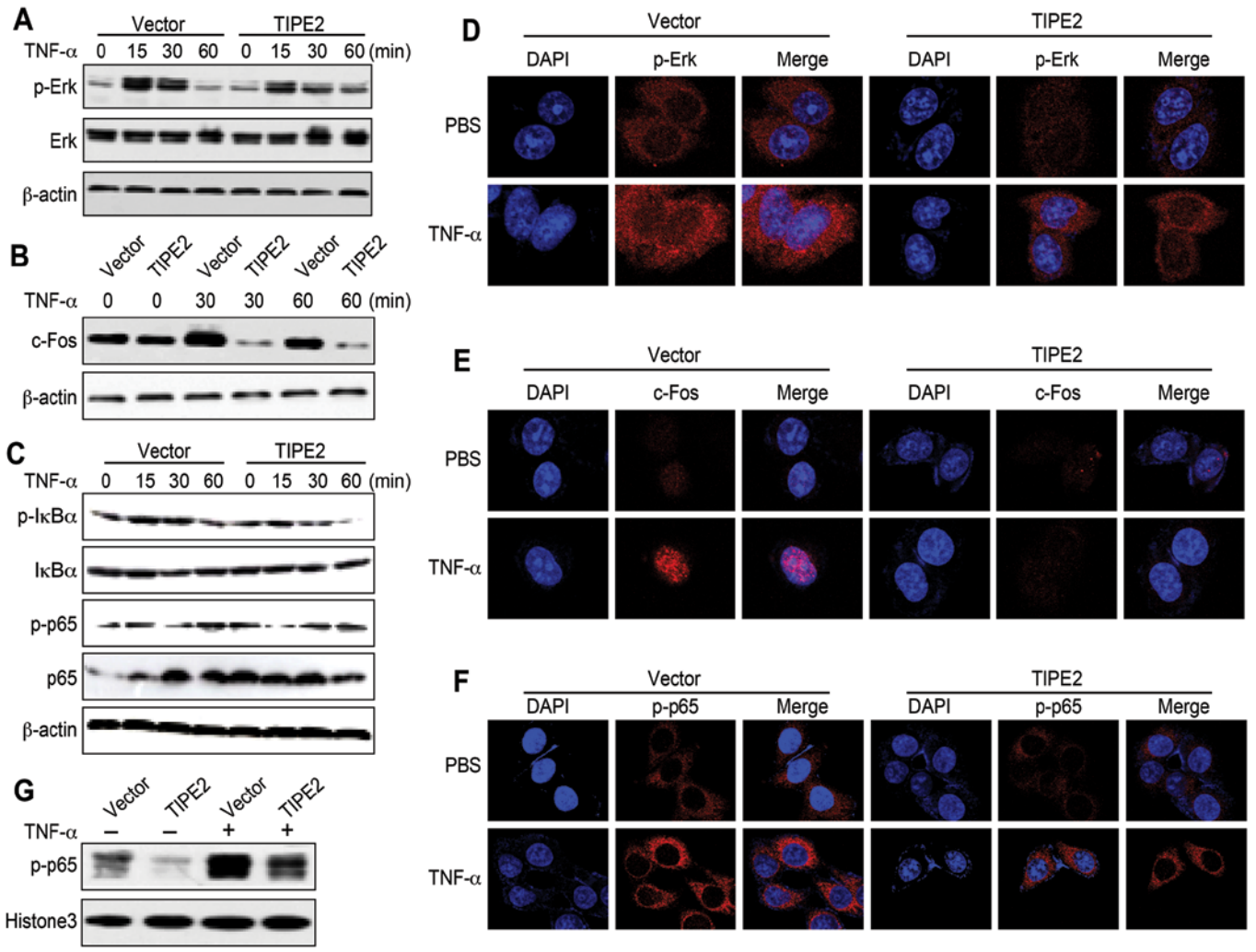

Figure 5. TIPE2 abolishes the effect of TNF-α on Erk1/2 and NF-κB pathway activation. (A-F) TIPE2 overexpressing and control cells were treated with TNF-α (2 ng/ml), and the activation of Erk1/2 (A and D), c-Fos (B and E) and NF-κB (C and F) and the translocation of phosphorylated p65 (G) were determined by western blot analysis (A, B, C and G) and confocal microscopy analyses (D-F), respectively. Representative results from 1 of 3 independent experiments are shown. β-actin was used as a loading control.

the effect of TNF-α on the expression of MMP-13 and MMP-3 at both the RNA (Fig. 4B and C) and protein (Fig. 4D and E) levels. Additionally, TIPE2 significantly inhibited MMP-13 and MMP-3 activities (Fig. 4F and G) ($p<0.01$, $p<0.001$, one-way ANOVA with the Newman-Keulspost test). The data presented in this study indicate that TIPE2 inhibits the effect of TNF-α on HCC metastasis by inhibiting MMP expression.

TIPE2 inhibits the effect of TNF-α on Erk1/2 and NF-κB activation. The Erk/AP-1 and NF-κB pathways play important roles in TNF-α-induced MMP-13/MMP-3 expression and cell migration (23,24). Hence, we examined the effect of TIPE2 on TNF-α-augmented Erk1/2-NF-κB activation. As shown in Fig. 5, TIPE2 efficiently inhibited the effect of TNF-α on Erk1/2 (Fig. 5A), c-Fos expression (Fig. 5B) and IκBα/p65 phosphorylation (Fig. 5C). Confocal microcopy demonstrated similar results (Fig. 5D-F). As phosphorylated p65 translocation was impeded by TIPE2 (Fig. 5G), the data presented in this study indicate that TIPE2 abolishes the effect of TNF-α on Erk1/2-NF-κB activation in HepG2 cells.

The primary components of the HCC microenvironment express TNF-α following LPS treatment. Kupffer cells and pit cells (hepatic natural killer cells), which are primary immune cells of the HCC microenvironment, contribute to elevated serum TNF-α levels (25). To elucidate the source of TNF-α, the primary components of the tumor microenvironment were treated with LPS, and the expression of TNF-α was analyzed. Consistent with another report (25), LPS treatment increased TNF-α expression in macrophages (Fig. 6A and B), fibroblasts (Fig. 6C and D) and human peripheral blood lymphocyte (PBL) (Fig. 6E and F) at both the transcriptional (Fig. 6A, C and E) and translational (Fig. 6B, D and F) levels. To our surprise, the expression of TNF-α in HepG2 (Fig. 6G and H) was notably upregulated following LPS treatment, indicating that liver cancer itself secretes TNF-α in an autocrine manner and promotes metastasis under LPS treatment conditions.

TIPE2 suppresses the effect of LPS on TNF-α expression in HCC cells. TIPE2, which is a negative regulator of immune homeostasis, can inhibit the release of the pro-inflammatory

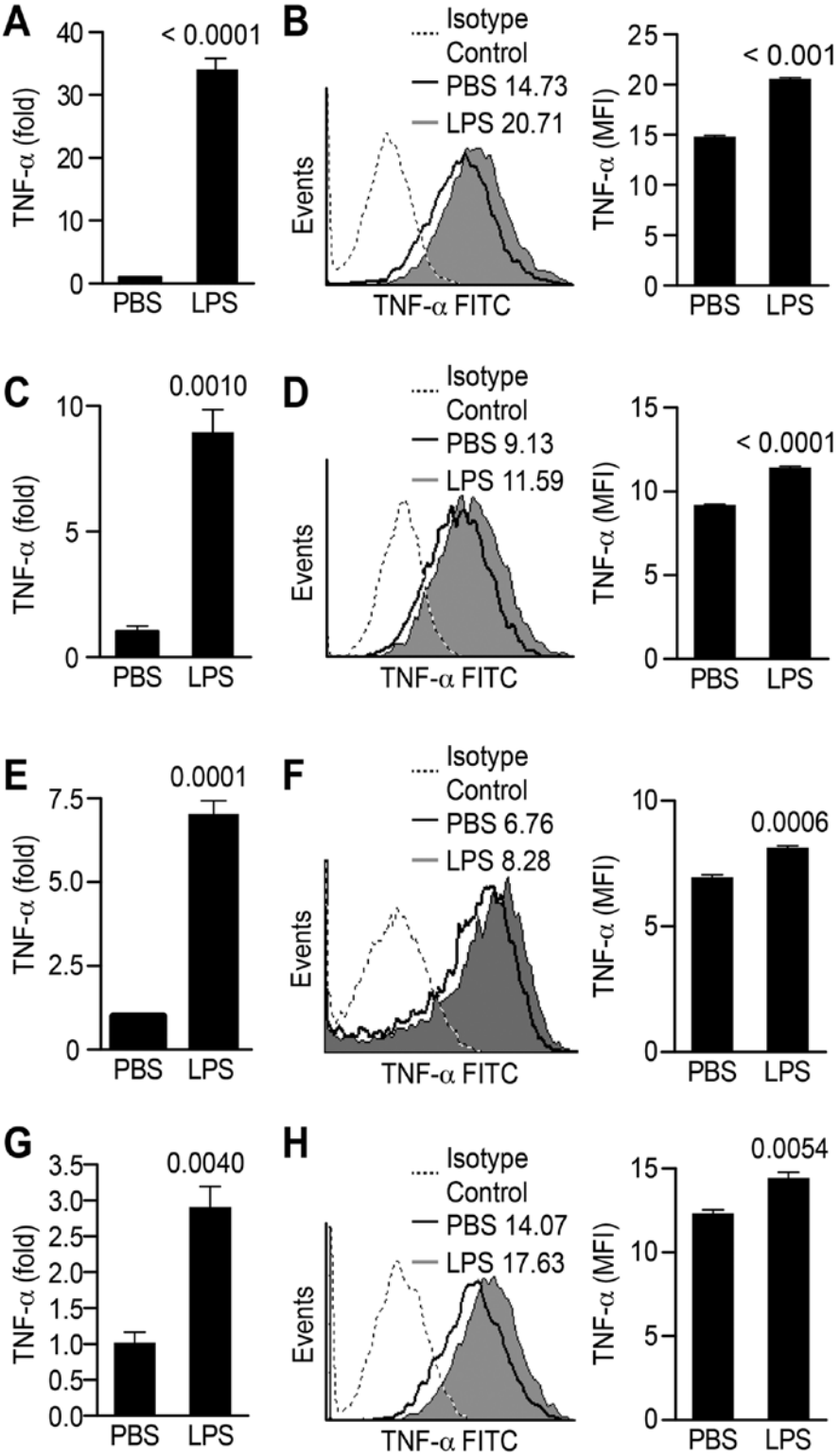

Figure 6. The components of the tumor microenvironment secrete TNF-α following LPS treatment. The primary components of the HCC microenvironment, such as Raw 264.7 (A and B), NIH-3T3 (C and D) and HepG2 (G and H) cells and peripheral blood lymphocytes (PBLs) (E and F) were exposed to LPS, and TNF-α expression was determined via real-time PCR (A, C, E and G), and flow cytometry analyses (B, D, F and H). For the flow cytometry analysis, the numbers in the histogram indicate the geometric mean fluorescence (MFI) of the tested samples. Positive percentages of TNF-α expression and the MFI are shown. The data are presented as the mean ± SEM, $p<0.05$, $p<0.01$, Student's t-test. Representative results from 1 of 3 independent experiments are shown. β-actin was used as a loading control. LPS, lipopolysaccharide.

cytokine TNF-α (16). The finding that TIPE2 is downregulated during HCC carcinogenesis (18) raises the question of whether TIPE2 could be a potential candidate for managing TNF-α-promoted HCC metastasis. Although LPS treatment significantly augmented TNF-α expression, TIPE2 successfully abrogated the effect of LPS on TNF-α expression in

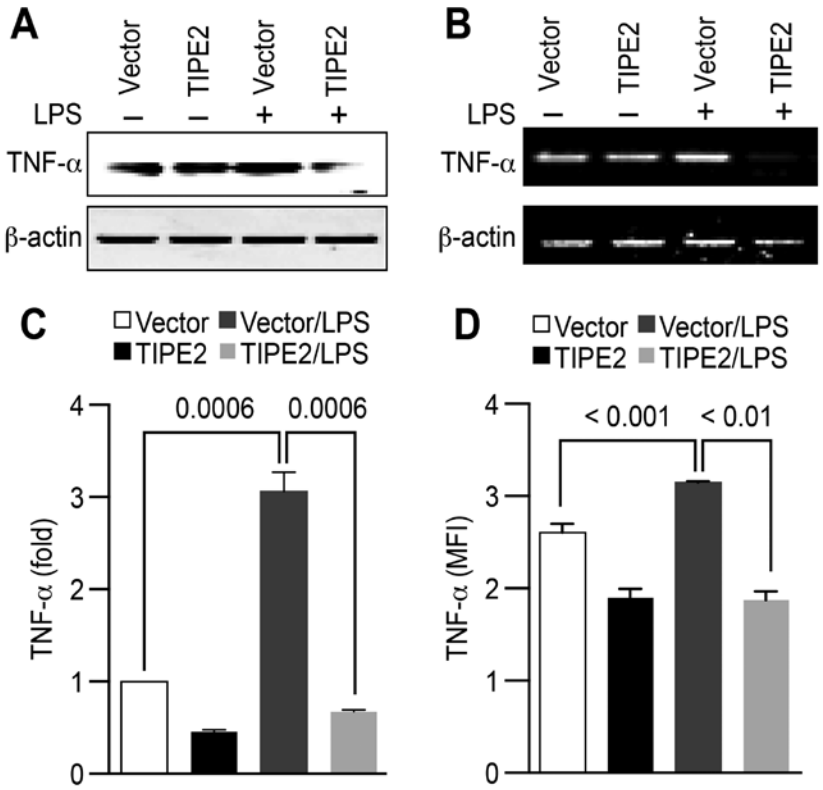

Figure 7. TIPE2 inhibits LPS-augmented TNF-α expression (A-D). HepG2 cells were treated with TNF-α (1 μg/ml), and whole cellular protein and RNA were extracted. The effect of TIPE2 on TNF-α expression was determined by western blot analysis (A), RT-PCR (B), real-time PCR (C) and flow cytometry analyses (D). The data are presented as the mean ± SEM. $p<0.01$, $p<0.001$, one-way ANOVA with Newman-Keuls post-test. Representative results from 1 of 3 independent experiments are shown. β-actin was used as a loading control.

HepG2 (Fig. 7). These results indicate that TIPE2 might be a potential therapeutic target for pro-inflammatory cytokine-associated HCC metastasis.

Discussion

HCC patients and metastatic liver carcinomas display elevated serum TNF-α levels (5,26), indicating that metastasis is influenced by the tumor microenvironment (27). TNF-α, which is a key regulator of the tumor microenvironment, has been reported to promote malignant invasion and metastasis in bladder cancer and breast cancer (14,28). The present study demonstrated that TNF-α upregulates MMP-13/MMP-3 expression and augments cell migration in HepG2 cells (Figs. 1 and 2). Moreover, inhibition of Erk1/2-NF-κB activities abrogates the effect of TNF-α on cell migration and MMP expression, indicating that TNF-α might be a candidate therapeutic target in inflammation-associated metastasis. As tumor tissues are composed of tumor cells, fibroblasts, and immune cells, the finding that Kupffer cells synthesize and secrete TNF-α cannot exclude the possibility that other components of liver cancer tissues might contribute to the elevation of serum TNF-α. In this study, all of the components of cancer tissues, such as cancer cells, fibroblasts, PBLs and macrophages, were shown to secrete TNF-α under LPS treatment conditions, indicating that TNF-α promotes metastasis in both a paracrine and autocrine manner in HCC.

Although TNF-α was found to contribute to the resistance of pathogen infection and inhibit carcinogenesis (29), a role of TNF-α in metastasis in bladder cancer and breast cancer has also been documented (14,28). These contrasting findings might be due to the concentration of TNF-α used in these studies. While a higher concentration of TNF-α (25 ng/ml) induces mouse blastocyst death (30), a lower concentration of TNF-α (1-10 ng/ml) clearly promotes prostate cancer metastasis and affects the survival of chronic lymphocytic leukemia patients (31,32).

MMPs, which are ECM degradation enzymes, play important roles in tumor migration and metastasis (10). MMP-3 has been found to directly degrade the extracellular matrix, to activate other MMPs, such as MMP-1, MMP-7 and MMP-9 (12,33), and to be related to the prognosis of HCV-associated liver cancer (12), indicating that MMP-3 is a primary MMP involved in HCC metastasis. Moreover, the ability of MMP-13, which can be activated by MMP-3, to directly degrade the extracellular matrix and to indirectly activate other MMP members, such as MMP-2/MMP-9, has also been documented, indicating that MMP-13 is another MMP involved in extracellular matrix remodeling (34). Hence, in this study, the effect of TNF-α on MMP-3 and MMP-13 expression was explored.

TIPE2 has been found to reduce F-actin polymerization and to inhibit cell migration (18). As actin polymerization is essential for the nuclear translocation of RhoA and protein trafficking (35,36), the effects of TIPE2 on MMP expression and cell migration might be due to the modulation of F-actin polymerization and epithelial-mesenchymal transition by TIPE2. In this study, we demonstrate that TIPE2 inhibits the effect of TNF-α on Erk1/2-NF-κB activation. TNF-α-

induced protein 3 (TNFAIP3, A20), which also belongs to the TNF-α-induced protein family, shows an effect similar to that of TIPE2 on NF-κB activation (37). The N-terminus of A20 encodes a deubiquitinating (DUB) domain that mediates the deubiquitination of K63-polyubiquitinated NF-κB signaling proteins, such as TRAF6 and RIP1, whereas the C-terminus of A20 encodes seven zinc-finger (ZF) motifs and confers E3 ubiquitin ligase activity (38). More recently, the binding of A20 to unanchored K63-linked polyubiquitin chains and to NEMO was shown to block the upstream kinase of IKK, TAK1 (39), indicating that the effect of A20 on NF-κB activation is a non-catalytic mechanism-dependent effect. Because TIPE2 and A20, which belong to the TNFAIP family, negatively regulate immune function and NF-κB activation, determining whether TIPE2 encodes deubiquitinase enzyme domains or antagonizes the ubiquitination of the NEMO-IκB kinase complex (IKK) regulatory subunit by interacting with NEMO requires further exploration.

Taken together, our experiments reveal that TNF-α increases MMP-13/MMP-3 expression and augments cell migration by activating the Erk1/2 and NF-κB pathways. The observed inhibition of the effects of TNF-α on MMP expression, cell migration and Erk1/2-NF-κB activation by TIPE2 indicates that TIPE2 and TNF-α might be potential targets for managing HCC metastasis.

Acknowledgements

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