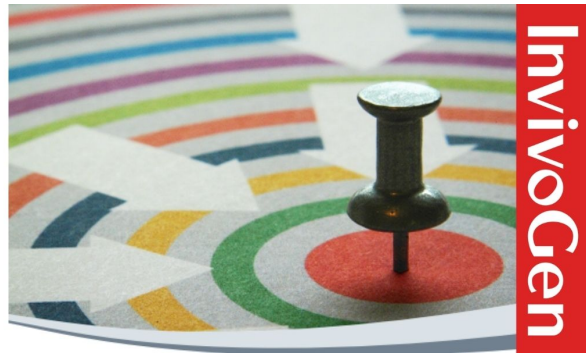


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Macrophages Induce Invasiveness of Epithelial Cancer Cells Via NF- κ B and JNK¹

Thorsten Hagemann,^{2*} Julia Wilson,* Hagen Kulbe,* Ningfeng Fiona Li,* David A. Leinster,* Kellie Charles,* Florian Klemm,[†] Tobias Pukrop,[†] Claudia Binder,[†] and Frances R. Balkwill*

Tumor-associated macrophages may influence tumor progression, angiogenesis and invasion. To investigate mechanisms by which macrophages interact with tumor cells, we developed an in vitro coculture model. Previously we reported that coculture enhanced invasiveness of the tumor cells in a TNF- α - and matrix metalloprotease-dependent manner. In this report, we studied intracellular signaling pathways and induction of inflammatory genes in malignant cells under the influence of macrophage coculture. We report that coculture of macrophages with ovarian or breast cancer cell lines led to TNF- α -dependent activation of JNK and NF- κ B pathways in tumor cells, but not in benign immortalized epithelial cells. Tumor cells with increased JNK and NF- κ B activity exhibited enhanced invasiveness. Inhibition of the NF- κ B pathway by TNF- α neutralizing Abs, an NF- κ B inhibitor, RNAi to RelA, or overexpression of I κ B inhibited tumor cell invasiveness. Blockade of JNK also significantly reduced invasiveness, but blockade of p38 MAPK or p42 MAPK had no effect. Cocultured tumor cells were screened for the expression of 22 genes associated with inflammation and invasion that also contained an AP-1 and NF- κ B binding site. EMMPRIN and MIF were up-regulated in cocultured tumor cells in a JNK- and NF- κ B-dependent manner. Knocking down either MIF or EMMPRIN by RNAi in the tumor cells significantly reduced tumor cell invasiveness and matrix metalloprotease activity in the coculture supernatant. We conclude that TNF- α , via NF- κ B, and JNK induces MIF and EMMPRIN in macrophage to tumor cell cocultures and this leads to increased invasive capacity of the tumor cells. *The Journal of Immunology*, 2005, 175: 1197–1205.

Tumors contain a dynamic combination of immune cells such as macrophages, lymphocytes and dendritic cells, endothelial cells, fibroblasts, and perivascular cells as well as malignant cells. The presence of immune cells in human tumors was first described in 1863, and researchers thought the cells reflected the onset of cancer at sites of previous chronic inflammation. It is now widely recognized that tumor-associated macrophages (TAM)³ represent a prominent component of this leukocytic infiltrate in most malignant tumors (1, 2). TAMs are derived from peripheral blood monocytes recruited into the tumor from the local circulation, rather than resident macrophages present in the healthy tissue before tumor development (3). TAMs support tumor growth by secreting factors such as cytokines and matrix metalloproteases (MMPs) that promote the proliferation, invasion, and metastasis of tumor cells, as well as tumor vascularization. TAMs also inhibit lymphocyte activity at the tumor site

by producing immunosuppressive cytokines, such as IL-10 and prostanooids (3–5). The protumor functions of TAM could explain why the extent of tumor infiltration by macrophages correlates positively with increased tumor angiogenesis and negatively with prognosis (2, 5, 6).

Recent publications (7, 8) demonstrated that stromal cell-derived inflammatory cytokines, including TNF- α , activate NF- κ B in initiated epithelial cells in vivo and that this event is crucial for malignant progression. Pikarsky et al. (8) demonstrated that inflammation triggers activation of hepatocyte NF- κ B in the early stages of tumor promotion through TNF- α up-regulation in adjacent endothelial and inflammatory cells. Suppression of NF- κ B activation, through anti-TNF- α treatment or induction of hepatocyte I κ B, inhibited progression to hepatocellular carcinoma.

To define further interactions between macrophages, inflammatory cytokines, and tumor cells we developed an in vitro coculture model. In a previous publication, we reported that coculture of tumor cells and macrophages increases invasiveness of transformed cells in a TNF- α - and MMP-dependent manner (9). To investigate the signaling pathways involved, cocultured tumor cells were screened for transcription factor activation. In this paper we report that coculture of tumor cells with macrophages increases tumor cell invasiveness TNF- α -dependent via Rel A (NF- κ B-p65) and JNKII-induced extracellular matrix metalloprotease inducer (EMMPRIN) and macrophage migration inhibitory factor (MIF) expression in malignant cells. These in turn stimulate macrophage MMP secretion and activity in the coculture supernatant.

As described, there is considerable evidence that macrophages not only fail to kill tumor cells but also may even contribute to tumor progression. Our results suggest that TNF- α is essential for this tumor-promoting interaction and crucial for extracellular matrix reorganization. Inhibition of this pathway may provide a suitable option for cancer treatment.

*Cancer Research U.K., Translational Oncology Laboratory, Barts and The London, Queen Mary's School of Medicine and Dentistry, London, United Kingdom; and [†]Department of Haematology and Oncology, Georg-August-University, Göttingen, Germany

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² Address correspondence and reprint requests to Dr. Thorsten Hagemann, Cancer Research U.K. Translational Oncology Laboratory, Barts and The London, Queen Mary's School of Medicine and Dentistry, John Vane Science Centre, Charterhouse Square, London EC1M 6BQ, U.K. E-mail address: t.hagemann@qmul.ac.uk

³ Abbreviations used in this paper: TAM, tumor-associated macrophage; MMP, matrix metalloprotease; EMMPRIN, extracellular matrix metalloprotease inducer; ECM, extracellular matrix; MIF, macrophage migration inhibitory factor; EGFP, enhanced GFP.

Materials and Methods

Cells lines and reagents

If not otherwise indicated, all substances were purchased from Sigma-Aldrich. The MCF-7 human breast cancer cell line was obtained from the American Type Culture Collection, the IGROV1 human ovarian cancer cell line was from the Benard et al. study (10), the telomerase-immortalized benign human breast epithelial cell line hTERT-HME1 from BD Clontech, and the telomerase-immortalized benign human ovarian epithelial cell line was a gift from N. F. Li (Queen Mary's School of Medicine and Dentistry, London, U.K.). All cell lines were grown in RPMI 1640 media supplemented with 10% FCS, which was treated with gelatin immobilized on cross-linked 4% beaded agarose as described before (9). All experiments were performed under endotoxin-free conditions. Cell viability was assessed using the Beckman Coulter ViCell XR Counter.

Transfection of tumor cells

Tumor cells were seeded in six-well plates to be 60–90% confluent on the day of transfection (typically $1-4 \times 10^5$ cells/35-mm plate incubated for 24 h at 37°C and 5% CO₂). Cells were transfected with the p κ B-EGFP vector (BD Clontech). The SUPER RNA interference (RNAi) plasmids RelA (L19067), JNKII (MAPK9, GenBank accession no. NM_139070), p42/ERK2 (NM_002745), p38 MAPK (NM_139012), EMMPRIN (NM_001728), and MIF (NM_002415) were isolated according to the protocols previously described (11) and were transfected directly into the target cells. For each well, 4 μ g of plasmid DNA in 250 μ l of serum-free Opti-MEM (Invitrogen Life Technologies) medium were mixed with 10 μ l of Lipofectamine 2000 transfection reagent in 250 μ l of Opti-MEM (Invitrogen Life Technologies) and incubated for 20 min at room temperature. Serum-free medium (1.5 ml) was mixed with the Lipofectamine 2000/DNA solution, added to the target cells, and incubated at 37°C. For stable transfections, medium was removed after 4 h and replaced with 2 ml of fresh medium supplemented with 10% FCS. Antibiotic selection for stable cell lines started at 48–72 h after transfection. p κ B-EGFP clones were selected under 500 μ g/ml G418 (Invitrogen Life Technologies) for 30 days. For stable transfections with SUPER RNAi plasmids the optimal concentration of puromycin was dependent on the cell type used and varied from 1.5–3 μ g/ml. SUPER RNAi plasmid stably expressing cells were selected in puromycin for 30 days. Positive cell clones with significant enhanced GFP (EGFP) expression were sorted under the fluorescence microscope for additional experiments. SUPER RNAi plasmid-expressing clones were selected after Western blot confirmation of silencing the gene of interest. Mock-transfected cells were used as controls in experiments.

Luciferase transcription factor reporter assay and β -galactosidase assay

We used the pNF- κ B-Luc or pAP-1-Luc vector (BD Clontech) containing multiple copies of the NF- κ B consensus sequence to monitor NF- κ B activation. Transient transfections were performed by the calcium phosphate method (12). Cells (5×10^4 per well of six-well plates) were transfected with 1 μ g of pNF- κ B-Luc, 1 μ g of pAP-1-Luc, or 1 μ g of pTAL-Luc as a negative control plus 1 μ g of reference plasmid β -galactosidase control (p β gal). Two independent wells were lysed 16–20 h posttransfection and luciferase reporter gene activity was determined by the Luciferase Reporter assay (BD Clontech) or β -galactosidase activity (BD Clontech) was measured to monitor transfection efficiency according to the manufacturer's instructions.

Ab agents

Anti-phospho-Ser15-p53 (cat. no. 9284), anti-NF- κ B-p65 (cat. no. 3034), anti-JNKII (cat. no. 4672), anti-I κ B- α Ab (cat. no. 9242), anti-p38 MAPK (cat. no. 9212), and p42 MAPK (cat. no. 9108) Abs were purchased from New England Biolabs and anti-MIF (clone 12302) and anti-EMMPRIN (clone 109403) Abs were purchased from R&D Systems.

Preparation of cytosolic and nuclear tumor cell extracts

Cytosolic and nuclear fractions were prepared as described before (13–15). All steps were performed at 4°C unless otherwise specified. Briefly, cells were homogenized in ice-cold cytosolic lysis buffer (10 \times Pre-Lysis buffer: 100 mM HEPES (pH 7.9), 15 mM MgCl₂, 100 mM KCl, 0.1 M DTT, protease inhibitor mixture) using an Ultra-Turrax T8 homogenizer, followed by a 15-min incubation on ice. Homogenates were centrifuged (10,000–11,000 \times g; 20 min, 4°C) and the supernatant (cytosolic fraction) stored at –70°C.

The pellet was resuspended in ice-cold nuclear lysis buffer extraction buffer (Pre-Extraction buffer: 20 mM HEPES (pH 7.9), 1.5 mM MgCl₂,

0.42 M NaCl, 0.2 mM EDTA, 25% (v/v) glycerol, 0.1 M DTT, 1.5 μ l protease inhibitor mixture; final volume 150 μ l extraction buffer) and incubated for 30 min at 4°C. Cellular debris was removed by centrifugation (20,000–21,000 \times g for 5 min, 4°C) and the supernatant (nuclear fraction) was stored at –70°C. Protein concentrations were estimated using the Bio-Rad protein assay, based on the method of Bradford (16).

Western blotting

Normalized cell homogenates were prepared by boiling with one-quarter volume of concentrated Laemmli sample buffer (100°C, 5 min). Cell extracts (10 μ g) were resolved on a 10% denaturing gel using running buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS). See-Blue markers (Invitrogen Life Technologies) were used to determine protein size. Proteins were transferred to nitrocellulose membranes (Hybond ECL; Amersham). Blots were blocked, and Ab hybridizations were performed according to the manufacturer's protocol. Proteins were visualized using ECL reagent (Amersham). Protein concentration equivalence was confirmed after probing by amido black staining and β -actin Western blot.

Isolation of human monocytes from peripheral blood

Human macrophages were derived from PBMC by CD14-positive selection (Miltenyi Biotec) and incubated in Teflon bags (Süd-Laborbedarf) with AIM-V medium (Invitrogen Life Technologies) plus 2% human AB serum until differentiation into macrophages as assessed by morphological, functional, and FACS criteria (described in Ref. 9).

Microinvasion assay

Invasion was measured using an artificial basement membrane in a modified Boyden chamber, where the various cellular components were grown without direct cell-to-cell contact as described (9). Briefly, the membrane consisted of a polycarbonate (10- μ m pore diameter; Nucleopore) and was coated on ice with Matrigel (ECM gel) diluted 1/3 in serum-free RPMI 1640 media. MCF-7, IGROV1, hTERT-HME1, IOSE, or p κ B-EGFP or RNAi transfected cells (1×10^5 cells/ml RPMI 1640) were seeded into the upper well of the chamber, whereas the lower well was filled with RPMI 1640 plus 2% FCS. The JNK inhibitor I, II, or III (or respective negative control peptide), NF- κ B inhibitor SN50 (or control peptide SN50M), p38MAPK inhibitor SB203580, MEK1/2 inhibitor PD98059 (Calbiochem-Novabiochem), JNK inhibitor D-JNKI1 (or respective control peptide) or Z-Leu-Leu-Leu-B(OH)₂ proteasome inhibitor III (Alexis Biochemicals) were added at various concentrations as indicated and renewed every 24 h. The neutralizing TNF- α Ab 28401.111 (R&D Systems) was added and renewed every 24 h. All experiments were conducted with RPMI 1640 plus 0.5% BSA.

For coculture experiments 2×10^5 macrophages/ml RPMI 1640 were seeded in transwell inserts (Nunc). The transwells were inserted into the upper well of the Boyden chamber. At the indicated time, the floating and adherent cells in the lower well were removed and pelleted by centrifugation. The cell pellet was resolved in 200 μ l of PBS and spun down on 12-mm cover slips. After air drying, the cytospins were stained with 4',6'-diamino-2'-phenylindole (DAPI, 200 ng/ml). Cover slips were mounted in 20% mowiol 4–88 (Hoechst/Aventis, Frankfurt, Germany) on glass slides. Intact nuclei were counted by UV microscope (Nikon Labophot II microscope) and documented with a digital image editing system (Adobe Photoshop 3.0). To assess the morphology of the migrated cells, control stainings with H&E were performed. All experiments were performed at least in triplicate.

RNA extraction, transcription, and real-time RT-PCR

RNA was extracted using the guanidinium thiocyanate method (17). RNA was DNase treated with 10 U of DNase (Pharmacia Biotech) following manufacturer's instructions. DNase-treated RNA (2 μ g) was reverse transcribed with random hexamers and Moloney murine leukemia virus reverse transcriptase (Promega) according to manufacturer's instructions. Multiplex real-time analysis was performed using IL-1 α , IL-1 β , IL-6, IL-8, CXCL3, CCL2, MIF, TGF- β , M-CSF, FGF, PAI-1, MMP-1–3, MMP-7, MMP-9, MMP-12, tissue inhibitor of metalloproteinases 1–4, TNF- α , IFN- α , IFN- γ (FAM), and 18 S rRNA (VIC) specific primers and probes with the ABI PRISM 7700 Sequence Detection System instrument and software (Applied Biosystems). PCR was conducted with the TaqMan Universal PCR Master Mix (Applied Biosystems) using 2 μ l of cDNA in a 25- μ l final reaction volume. The cycling conditions were an incubation at 50°C for 2 min, followed by 10 min at 95°C and 60 cycles of 15 s at 95°C, and 1 min at 60°C. Experiments were performed in triplicate for each sample. The relative gene expression levels were normalized to the 18S

RNA, and the fold differences were calculated as described before (18). Primers for EMMPRIN were designed using the "Primer3" program (19) and synthesized by MWG Biotech. The specific primers for human EMMPRIN: forward 5'-ACTCCTCACCTGCTCCTTGA-3' and reverse 5'-GCGAGGAACCTACGAAGAAC-3', amplifying a 380-bp fragment (60°C annealing temperature).

Real-time RT-PCR for EMMPRIN was performed on the Light Cycler PCR Analysis System (Roche Diagnostics). The detailed procedure for quantification has been previously described (20). All experiments were performed at least in triplicate.

ELISA

The cell culture supernatants were harvested under sterile conditions, frozen and stored at -20°C . TNF- α concentration was determined using a commercial ELISA kit (R&D Systems) according to the manufacturer's instructions.

MMP activity assay

Biologically active MMP-2 and MMP-9 protein levels were measured in cell culture supernatants using a MMP-2 and MMP-9 ELISA kit (Amersham Biosciences) according to the manufacturer's instructions. The assay allows quantification of both the active and total protein. The assay sensitivity was <0.5 ng/ml. After the addition of chromogen, plates were read in a Dynatech MR 5000 plate reader at 405 nm.

DNA-binding activity of multiple transcription factors

A signaling profiling assay was used to identify transcription factors induced by coculture. This ELISA has 10-fold higher sensitivity than traditional gel-shift assays with less false negative results (sensitivity for NF- κB >0.3 nM) (21). TransFactor kits (BD Mercury TransFactor Profiling Assay and BD Mercury TransFactor Individual Assay) identify DNA-protein interactions (22) and were used for rapid, high-throughput detection of NF- κB -p65 and -p50, c-Fos, CREB-1, ATF2, c-Rel, FosB, JunD, SP-1, STAT1 and c-Jun transcription factor activities in MCF-7 and IGROV1 nuclear extracts (25 μg) after 24 h coculture. After NF- κB -p65 and c-Jun were identified as crucial we assessed these individual transcription factor activities in depth with single factor kits in a time course. After the addition of chromogen, plates were read in a Dynatech MR 5000 plate reader at 650 nm.

Statistical analysis

Results are expressed as means with SD. Graphs were created with the Sigma Plot for Windows graphic system (version 1.1; Jandel). Data were analyzed with the Student *t* test. A value for $p < 0.05$ was considered significant.

Results

Macrophages increase tumor cell invasiveness but have no effect on immortalized epithelial cells

To assess whether coculture of tumor cells with macrophages increased invasiveness of tumor cells, a breast cancer cell line MCF-7, an immortalized mammary epithelial cell line hTERT-HME1, an ovarian cancer cell line IGROV1, and an immortalized ovarian epithelium cell line IOSE were grown on Matrigel. Human monocyte-derived macrophages were placed in transwell inserts in a modified Boyden chamber without direct cell-to-cell contact with the tumor cells.

After 72 h coincubation with macrophages, the invasive capacity of the tumor cell lines MCF-7 and IGROV1 was significantly increased (Fig. 1, A and B). Addition of neutralizing TNF- α Ab to the coculture reduced the invasiveness to almost the control levels ($p < 0.0001$, Fig. 1, A and B). The immortalized cell lines hTERT-HME1 and IOSE did not exhibit any invasive behavior either with or without macrophages (Fig. 1, A and B).

Coculture increased c-Jun and NF- κB activity in malignant cells

To address which signaling pathways were activated in tumor cells upon coculture with macrophages, the BD Mercury TransFactor Profiling Assay was used. The activity of the transcription factors NF- κB -p65 and -p50, c-Fos, CREB-1, ATF2, c-Rel, FosB, JunD,

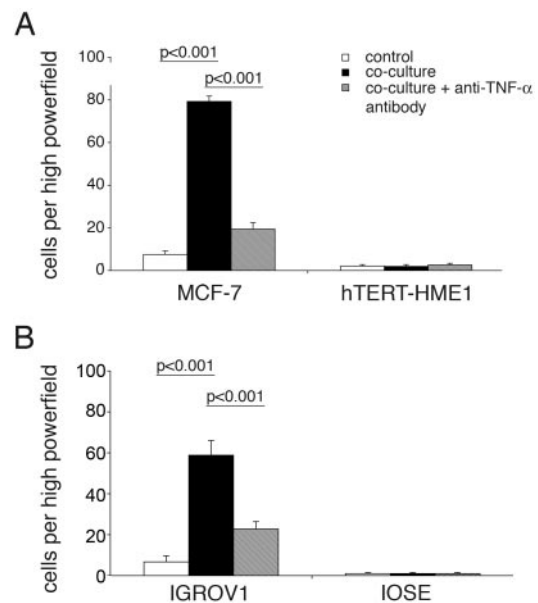


FIGURE 1. Invasion assay. *A*, Invasiveness of MCF-7 and hTERT-HME1 cells following coculture with macrophages. Cell invasion through Matrigel and the influence of TNF- α neutralization (Ab used at 1 $\mu\text{g}/\text{ml}$) on in vitro invasiveness was measured. Results are the mean \pm SD ($n = 6$). Statistical significance ($p < 0.001$). *B*, Influence of coculture with macrophages on invasiveness of IGROV1 and IOSE cells. Results are mean \pm SD ($n = 6$; $p < 0.001$).

SP-1, STAT1 and c-Jun were assessed in the MCF-7 and IGROV1 cell lines after 24 h coculture with macrophages. NF- κB -p65 and -p50, JunD, ATF2, and c-Jun activities significantly increased in

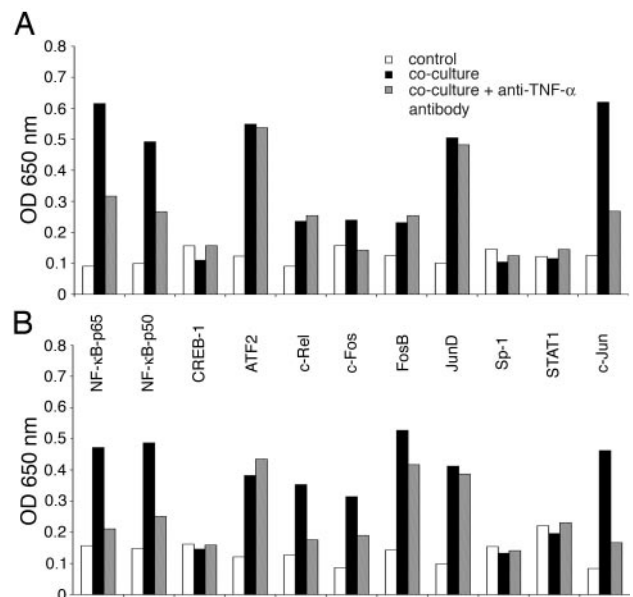


FIGURE 2. Transcription factor profiling assay. After 24 h coculture of tumor cells with macrophages \pm TNF- α -neutralizing Ab (1 $\mu\text{g}/\text{ml}$), nuclear extracts (25 μg) were analyzed with the Transcription Factor Profiling Assay in MCF-7 (*A*) and IGROV1 (*B*) cells. Activity was measured by an ELISA-based assay at 650 nm. Activity in control cells (\square), after coculture (\blacksquare), and upon coculture and addition of the neutralizing anti-TNF- α Ab (\square) are indicated. Coculture induced NF- κB -p65, NF- κB -p50, and c-Jun activity in a TNF- α -dependent manner. Differences were statistically significant ($p < 0.001$).

MCF-7 cells after coculture with macrophages (Fig. 2A). However, only NF- κ B-p65 and -p50 and c-Jun induction could be inhibited by 1 μ g/ml of the neutralizing TNF- α Ab (Fig. 2A). Similar results were obtained from cocultured IGROV1 cells (Fig. 2B). To assess when transcription factor activity increased, we studied the activity of c-Jun and NF- κ B-p65 over a 24 h time course. The role of JNK was assessed indirectly and levels of phosphorylated c-Jun activity were used as a readout of the JNK activity. These data demonstrated that NF- κ B-p65 and c-Jun activity in MCF-7 and IGROV1 (Fig. 3), increased rapidly upon coculture with macrophages (Fig. 3). Activation of both transcription factors could be prevented by the addition of neutralizing TNF- α Ab to the coculture (Fig. 3).

To monitor NF- κ B activation, tumor cells were transiently transfected with a pI κ B-EGFP vector. The pI κ B-EGFP vector encodes an I κ B-EGFP signaling probe, which is a fusion of EGFP and the inhibitor of κ B, I κ B. When the NF- κ B pathway is inactive, I κ B and NF- κ B exist as an inactive complex in the cytosol. Upon stimulation, I κ B is degraded and NF- κ B is translocated to the nucleus. In cocultured MCF-7 cells transfected with pI κ B-EGFP, degradation of the I κ B-EGFP fusion protein was observed as a decrease in EGFP fluorescence (Fig. 4A). Similar results were obtained for cocultured IGROV1 cells (data not shown).

MCF-7 cells transiently transfected with a luciferase reporter plasmid for NF- κ B or AP-1 provided measurement of the binding of the transcription factors to their consensus region in vitro. Coculture of transfected MCF-7 tumor cells induced binding of transcription factors NF- κ B or AP-1 to their DNA consensus sequence, as displayed by an increase in luciferase activity (Fig. 4B). The pTAL-Luc vector was used as a negative control and showed no activation upon coculture. Similar results were obtained for cocultured IGROV1 cells (data not shown). We confirmed these observations by Western blotting of protein extracts of untreated cells. I κ B α protein was degraded after coculture in the tumor cells. Addition of the neutralizing TNF- α Ab protected I κ B α protein degradation (Fig. 4C).

Inhibition of coculture induced NF- κ B abrogates tumor cell invasiveness

To analyze the role of NF- κ B on in vitro invasiveness of tumor cells, we used neutralizing TNF- α Ab, a specific NF- κ B inhibitor (SN50) or the proteasome inhibitor III to prevent I κ B α degradation and subsequent NF- κ B translocation in the coculture model. The neutralizing TNF- α Ab (1 μ g/ml) significantly reduced

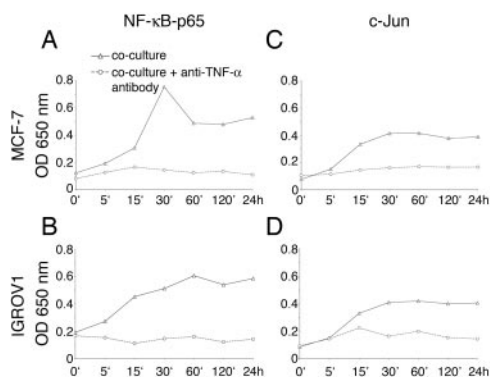


FIGURE 3. Single transcription factor assay. Cells were cocultured \pm neutralizing TNF- α Ab (1 μ g/ml) for the indicated time. A and B, Coculture with macrophages increased NF- κ B-p65 activity in a TNF- α -dependent manner in MCF-7 and IGROV1 cells after 15 min. C and D, c-Jun activity was also increased. Differences are statistically significant at $p < 0.001$.

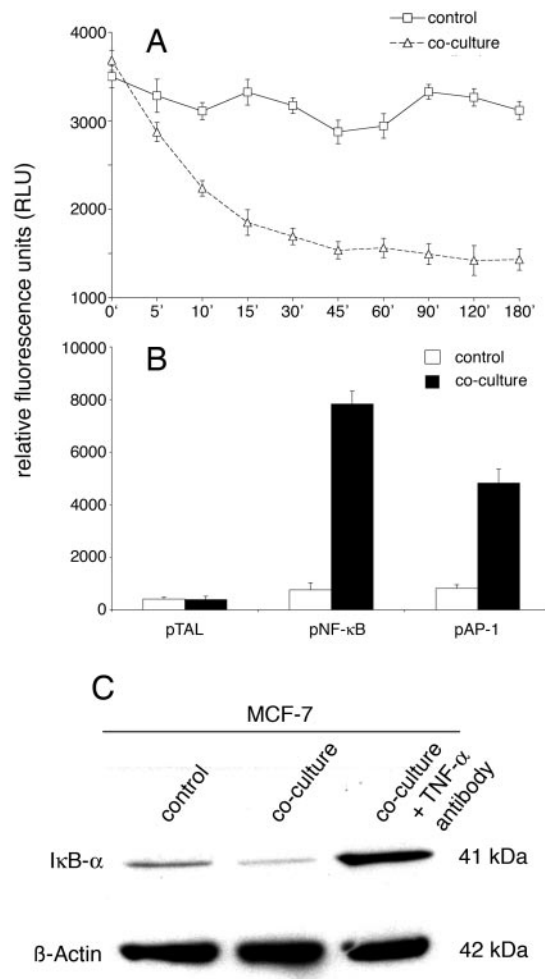


FIGURE 4. I κ B overexpression. MCF-7 cells were transiently transfected with the pI κ B-EGFP vector to monitor I κ B degradation upon coculture with macrophages. A, Control MCF-7 cells (\square , thin line) showed no I κ B degradation, whereas cocultured MCF-7 cells (Δ , dashed line) showed degradation of I κ B upon coculture with macrophages. B, Luciferase Reporter Assay for NF- κ B and AP-1 activation. The control MCF-7 cells showed no activation of NF- κ B or AP-1 pathway (\square). The 6-h cocultured MCF-7 cells showed a significant increase of NF- κ B and AP-1 activation (\blacksquare). The pTAL vector was used as a negative control. Transfection efficiency was controlled in all experiments by cotransfection with the plasmid β -galactosidase (p β gal) control vector and luciferase activity was normalized to β -galactosidase activity. C, Western blot of I κ B α protein in control, coculture, and coculture plus neutralizing TNF- α Ab (1 μ g/ml). Coculture induced I κ B α degradation could be abolished by adding the neutralizing TNF- α Ab.

MCF-7 in vitro invasiveness almost to control levels ($p < 0.001$, Fig. 5A). In experiments with the NF- κ B-inhibitor SN50 (20 mM) a significant ($p < 0.05$) effect was observed (Fig. 5, B, C, and E). The respective inactive NF- κ B SN50M control had no effect. Inhibition of I κ B α degradation and subsequent NF- κ B translocation by the proteasome inhibitor III (100 μ M) also significantly reduced invasiveness ($p < 0.001$, Fig. 5F). Neutralizing TNF- α Ab had no effect on cell viability, however, high concentrations of the NF- κ B inhibitor SN50 (>30 mM) reduced cell viability (data not shown). The proteasome inhibitor reduced cell viability above concentrations of 500 μ M.

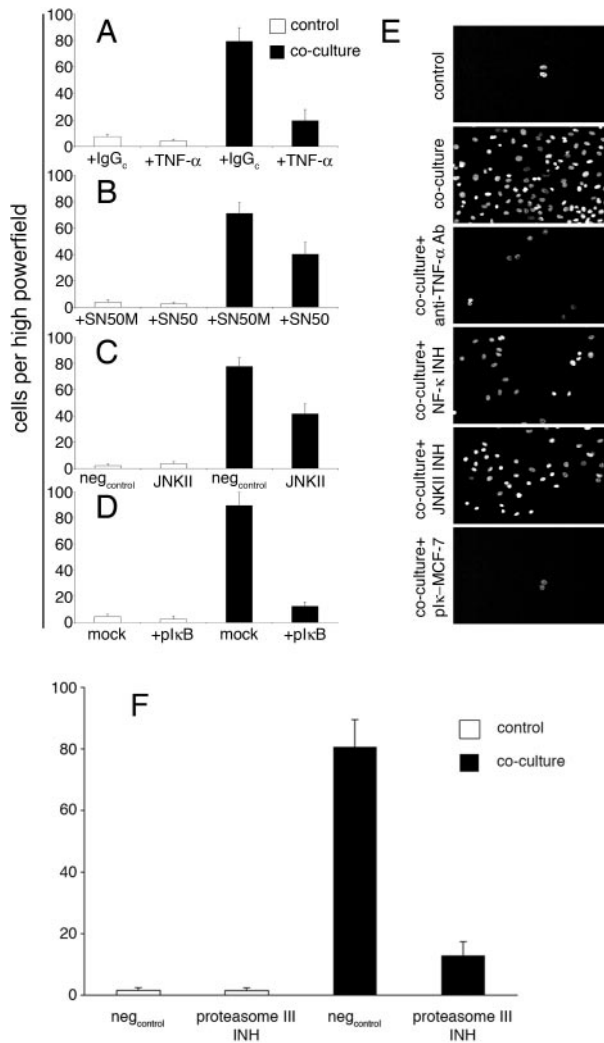


FIGURE 5. Matrigel invasion assay of cocultured MCF-7 cells. Results are mean \pm SD of 10 high-power fields in at least three independent experiments. Single cultured tumor cells (□) and invasion of cocultured tumor cells (■) are indicated. *A*, Control plus IgG (+IgG) control Ab, control plus neutralizing TNF- α mAb (+TNF- α , 1 μ g/ml), and coculture and coculture plus neutralizing TNF- α mAb (1 μ g/ml). *B*, Control plus NF- κ B inhibitor inactive peptide (+SN50M), control plus NF- κ B inhibitor (+SN50, 20 mM), coculture plus NF- κ B inhibitor inactive peptide (+SN50M), and coculture plus NF- κ B inhibitor (+SN50, 20 mM). *C*, Control plus JNKII control peptide, control plus JNKII inhibitor (60 nM), coculture plus JNKII control peptide, and coculture plus JNKII inhibitor (60 nM). *D*, Control plus mock vector, control plus pI κ B-EGFP vector (+pI κ B), coculture plus mock vector, and coculture of stable transfected MCF-7 cells with the pI κ B-EGFP vector (+pI κ B). *E*, DAPI staining of migrated MCF-7 tumor cells. Representative ($n = 10$) sections are shown. Although MCF-7 cells have no invasive behavior, coculture with macrophages lead to increased invasiveness. Inhibition of TNF- α pathway leads to a significant decrease of invasiveness. The strongest effect could be observed after stable transfection of MCF-7 cells with the pI κ B-EGFP vector, which may protect coculture MCF-7 from NF- κ B pathway activation. *F*, Control plus negative control (neg_{control}), control plus proteasome III inhibitor (100 μ M), coculture plus negative control (neg_{control}), and coculture plus proteasome III inhibitor (100 μ M).

An alternative method to investigate this involved overexpression of the physiological inhibitor I κ B. pI κ B-EGFP stably transfected MCF-7 and IGROV1 tumor cell lines were cocultured with macrophages. The presence of pI κ B-EGFP plasmid resulted in

increased I κ B expression in the tumor cells and prevented coculture induced invasiveness (Fig. 5, *D* and *E*). These results indicate that the TNF- α NF- κ B pathway is crucial for the cocultured induced invasiveness of malignant cells.

Inhibition of coculture induced JNK activation abrogates tumor cell invasiveness

In similar experiments we analyzed the role of JNK activation on the in vitro invasiveness of tumor cells, using a specific JNKII inhibitor or control peptide. c-Jun activity was used as a readout for JNK activity. In these experiments, the JNKII inhibitor (60 nM) had a significant ($p < 0.05$) effect on tumor cell invasion (Fig. 5, *B*, *C*, and *E*). The control peptide had no effect. Similar to the NF- κ B inhibitor, high concentrations of the JNKII inhibitor (>80 nM) reduced cell viability (data not shown). The JNK inhibitor D JNKII was better tolerated. These peptides tended to be toxic to cells at concentrations over 5 μ M and we used concentrations of 1 μ M. At concentrations of 1 μ M results were similar to the previous JNKII inhibitor from Calbiochem and invasiveness of tumor cells was reduced to 50% of control coculture.

To overcome the possibility of toxic effects of the signaling inhibitors, small interfering RNA was used in additional experiments.

RNAi to RelA or JNK prevents tumor cell invasion

MCF-7 and IGROV1 cells were transfected with RNAi to RelA or JNKII (Fig. 6). RNAi to RelA significantly inhibited coculture induced invasiveness of MCF-7 cells nearly to the baseline level ($p < 0.001$; Fig. 6, *A* and *B*). Western blot demonstrated that RNAi transfection resulted in reduced protein expression (Fig.

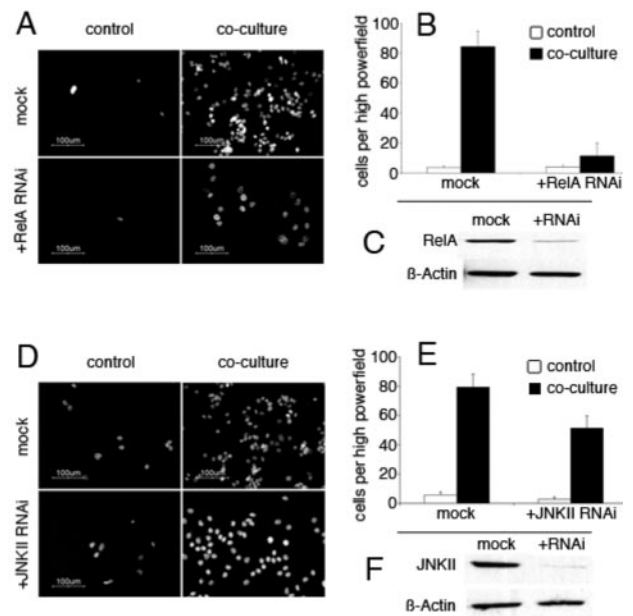


FIGURE 6. Pathway inhibition by RNAi. *A* and *B*, Matrigel invasion assay. DAPI staining of migrated MCF-7 tumor cells. Representative ($n = 10$) sections are shown. To avoid toxicity of the inhibitors, MCF-7 cells were stably transfected with RelA/NF- κ B-p65 RNAi or JNKII RNAi. Coculture of tumor cells transfected with RelA/NF- κ B-p65 RNAi reduced invasiveness nearly to the baseline ($p < 0.001$). *C*, Knockdown of RelA/NF- κ B-p65 protein expression after RNAi transfection in MCF-7 cells. *D* and *E*, Matrigel invasion assay. DAPI staining of migrated MCF-7 tumor cells. Representative ($n = 10$) sections are shown. Coculture of tumor cells transfected with JNKII RNAi also reduced invasiveness ($p < 0.05$). *F*, Knockdown of JNKII protein expression after RNAi transfection.

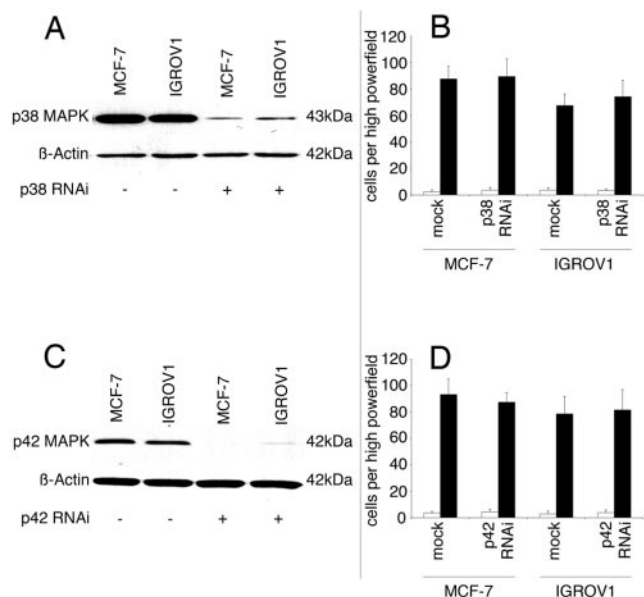


FIGURE 7. p38 and p42 MAPK pathways. RNAi to p38 (A) in the tumor cells MCF-7 and IGROV1 had no effect on in vitro invasiveness of the respective tumor cell line after coculture with macrophages (B). Similar results could be obtained for RNAi to p42 MAPK (C and D).

6C). In MCF-7 cells, JNKII down-regulation significantly inhibited in vitro invasiveness ($p < 0.05$). Compared with the parental cell line, invasion was reduced by 35% (Fig. 6, D and E). Protein knockdown was confirmed by Western blot (Fig. 6F). Similar reductions were observed, in RNAi experiments, compared with the JNKII inhibitor (Fig. 5C). Tumor cell viability was not affected by RNAi transfection.

Invasiveness of MCF-7 cells was abolished in stable RelA transfectants (Fig. 6, A and B). Similar results were obtained for the ovarian cancer cell line IGROV1 (data not shown).

Recent studies (23, 24) demonstrate that inducing RNAi by the transfection of small interfering RNA or other plasmid-based RNAi constructs also induces STAT1 expression and the potentially cytotoxic IFN responses. All stable transfected cell lines were checked by real-time RT-PCR analysis for IFN- α and IFN- γ expression. There was no increase in expression of these mRNAs upon RNAi transfection in all experiments conducted (data not shown).

The p38 MAPK inhibitor, SB203580, and the MEK1/2 inhibitor, PD98059 had no effect on coculture induced invasiveness (data not shown). Transfection of MCF-7 or IGROV1 cells with p38 MAPK (Fig. 7, A and B) or p42 MAPK (Fig. 7, C and D) RNAi had no effect on in vitro invasiveness of cocultured tumor cells, suggesting that the MAPK and MEK1/2 pathways have either no or a limited role in this system.

Coculture of malignant cells with macrophages up-regulates NF- κ B and c-Jun binding sites containing genes in the malignant cells

To determine which downstream genes might be responsible for macrophage-induced tumor cell invasiveness expression of genes involved in inflammation and tumor cell invasion containing binding sites for NF- κ B and AP-1 transcription factors was quantified using real-time RT-PCR. We screened postcoculture tumor cells for 22 AP-1- and NF- κ B-dependent genes (IL-1 α , IL-1 β , IL-6, IL-8, CXCL3, CCL2, MIF, TGF- β , M-CSF, FGF, PAI-1, MMP

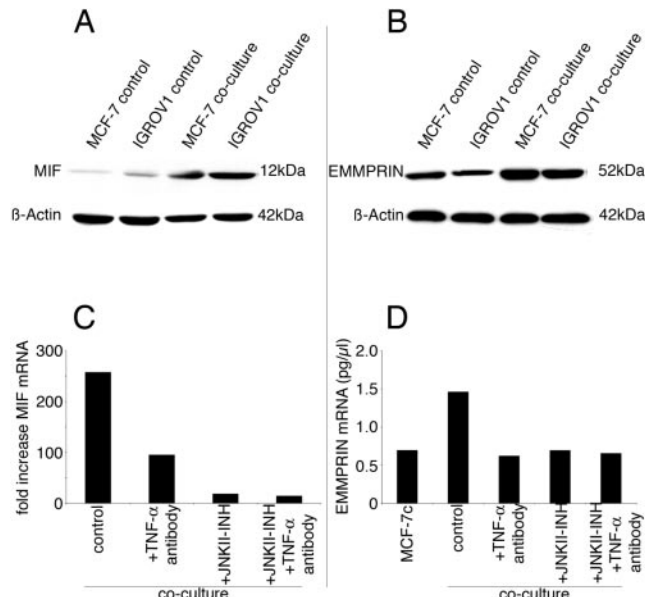


FIGURE 8. Gene induction in a NF- κ B- and JNK II-dependent manner. A, Western blot of MIF protein. Coculture induced MIF protein expression in the MCF-7 and IGROV1 tumor cell extracts. Real-time analysis confirmed these results and demonstrated that MIF mRNA increase (compared with control tumor cells) is TNF- α -dependent (shown for MCF-7 mRNA; $p < 0.05$) (A and C). B, Western Blot of EMMPRIN protein. Coculture resulted in up-regulated expression of EMMPRIN protein in the tumor cell extracts. Real-time analysis confirmed this result and demonstrated that EMMPRIN mRNA increase in the tumor cells is TNF- α - and JNKII-dependent (shown for MCF-7 mRNA, $p < 0.05$) (B and D). Real-time analysis shows one representative of three independent experiments.

1–3, MMP-7, MMP-9, MMP-12, tissue inhibitor of metalloproteinases 1–4, EMMPRIN) and found five genes induced after coculture in the tumor cells. In particular two genes, EMMPRIN and MIF, were induced in a NF- κ B- and JNK II-dependent manner (Fig. 8). MIF and EMMPRIN mRNA expressions and proteins were both increased in total cell lysates upon coculture (Fig. 8). When the specific NF- κ B or JNKII inhibitor was added to the coculture, MIF and EMMPRIN mRNA expression was significantly down-regulated (Fig. 8, C and D). Inhibition of NF- κ B by RNAi transfection led to reduced mRNA expression of MIF and EMMPRIN in cocultured tumor cells (data not shown).

Coculture induces increased MMP secretion by macrophages dependent on MIF and EMMPRIN expression in the tumor cells

We previously demonstrated that coculture of macrophages with tumor cells induced MMP expression in macrophages and increased MMP activity in the coculture supernatant (9). Tang et al. (25) demonstrated that EMMPRIN acts via its soluble form to stimulate MMP production and secretion from the surrounding stroma. Therefore we investigated whether knockdown of EMMPRIN by RNAi in the tumor cells influenced macrophage MMP secretion and subsequent tumor cell invasion.

We assessed MMP activity by ELISA in MCF-7, hTERT-HME1, IGROV1, IOSE, macrophage control supernatants and cocultured supernatants. The benign cell lines hTERT-HME1 and IOSE did not express any MMPs and coculture did not alter MMP activity (data not shown). MMP-2 and MMP-9 activity could not be detected in MCF-7 and IGROV1 control supernatants. Macrophage supernatant contained significant activity of both MMPs

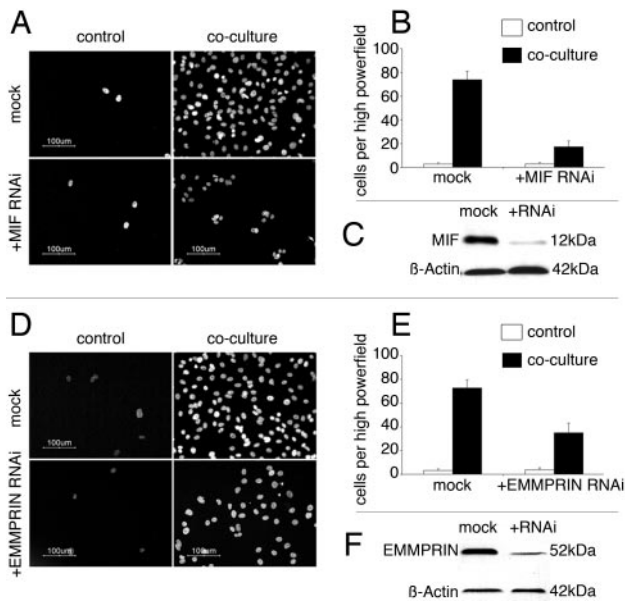


FIGURE 9. Reduced MMP activity in the coculture supernatant. *A* and *B*, Matrigel invasion assay. DAPI staining of migrated MCF-7 tumor cells. Representative ($n = 10$) sections are shown. Coculture of MCF-7 tumor cells stably transfected with MIF RNAi significantly reduced tumor cell invasiveness ($p < 0.001$). *C*, Knockdown of MIF protein expression after RNAi transfection. *D* and *E*, Matrigel invasion assay. DAPI staining of migrated MCF-7 tumor cells. Representative ($n = 10$) sections are shown. Coculture of MCF-7 tumor cells stably transfected with EMMPRIN RNAi also reduced invasiveness when cocultured with macrophages ($p < 0.05$). *F*, Knockdown of EMMPRIN protein expression after RNAi transfection.

when cultured alone (MMP-2, 6.35 ± 0.72 ng/ml and MMP-9, 6.84 ± 0.58 ng/ml). Upon coculture of MCF-7 cells and macrophages, MMP-2 and MMP-9 activities in the supernatant were significantly increased (MMP-2, 15.84 ± 1.13 ng/ml and MMP-9, 21.63 ± 1.47 ng/ml; $p < 0.01$).

MMP-2 and MMP-9 activity in the coculture supernatants decreased to control level when EMMPRIN small interfering RNA transfected MCF-7 cells were cocultured with macrophages (MMP-2, 7.28 ± 2.44 ng/ml and MMP-9, 10.32 ± 1.85 ng/ml). Coculture of MCF-7 MIF RNAi transfectants also resulted in significantly lower MMP-9 activity in the cocultured supernatant (13.62 ± 0.33 ng/ml; $p < 0.01$). Inhibition of the NF- κ B or JNK pathway upstream by RNAi in the tumor cells led to a comparable reduction of MMP-2 and MMP-9 activity (MMP-2, 4.21 ± 1.16 ng/ml and MMP-9, 5.02 ± 0.74 ng/ml). Similar results were obtained with coculture of IGROV1 and macrophages.

Knockdown of EMMPRIN and MIF resulted in reduced MMP activity in the coculture supernatant. Furthermore, reduced MMP activity significantly reduced in vitro tumor cell invasiveness ($p < 0.05$; Fig. 9, *A* and *B*). Western blot confirmed protein knockdown by RNAi transfection (Fig. 9*C*). In MCF-7 cells, EMMPRIN knockdown by RNAi also significantly inhibited in vitro invasiveness ($p < 0.05$; Fig. 9, *D* and *E*). Protein knockdown was confirmed by Western blot (Fig. 9*F*).

Discussion

The data presented in this study support the hypothesis that regulation of tumor cell invasion involves macrophages, a part of the stromal compartment. Coculture of macrophages with either a breast or ovarian cancer cell line led to a significant increase in the

in vitro invasiveness of the tumor cells. The enhanced invasion correlated with NF- κ B and c-Jun induction. Invasion was diminished by addition of a neutralizing TNF- α Ab, JNKII, or NF- κ B inhibitors and was almost completely abrogated in tumor cells transfected with RNAi for RelA or I κ B. EMMPRIN and MIF were identified as important downstream targets in this context. Blockade of MIF and EMMPRIN inhibited coculture induced invasiveness of tumor cells. Knockdown of EMMPRIN by RNAi in the tumor cells led to a decrease of MMP-2 and MMP-9 released by the macrophages (9). MIF knockdown also led to reduced MMP-9 activity. Both molecules are NF- κ B- and JNK-dependent and are involved in invasion-associated pericellular proteolysis and tumor cell migration.

TNF- α -dependent pathways are linked with inflammatory events causing tumor development and metastasis (7, 8, 26, 27). The tumor-promoting effect of TNF- α was demonstrated in mice deficient in TNF- α that were resistant to skin carcinogenesis (28). TNF- α is a critical mediator of the stress response that influences immune cell function, proliferation, differentiation, and apoptosis (29). However, TNF- α can also promote inflammation by suppressing apoptosis via a mechanism that involves both the NF- κ B and AP-1 groups of transcription factors (30, 31). Substantial evidence implicates the AP-1 group of transcription factors in many of the nonapoptotic cellular responses of TNF- α (30). AP-1 activation involves increased expression (32) and phosphorylation (33, 34) but how TNF- α regulates these functions of AP-1 proteins is unclear. However, TNF- α mediated JNK-dependent regulation of AP-1 activation has been proposed (35). One mechanism by which chronic inflammation predisposes to cancer might be that the proinflammatory cytokine TNF- α is a critical mediator of tumor promotion, acting via a PKC- α - and AP-1-dependent pathway (30).

Recent studies have demonstrated the role of NF- κ B in malignant progression (7, 8) where activation of NF- κ B in premalignant cells by TNF- α and other inflammatory cytokines from the surrounding tumor stroma can result in transformation. Indeed, constitutive activation of NF- κ B in a variety of cancers has been reported (36). Ventura et al. (37) demonstrated that JNK is critical for TNF- α regulation of the AP-1 group of transcription factors. Loss of AP-1 activation in JNK-deficient cells caused marked defects in the response of cells following treatment with TNF- α . This effect is consistent with results by Pikarsky et al. (8) who suggested that acquisition of oncogenic mutations during the tumor promotion phase renders premalignant hepatocytes particularly sensitive to apoptotic factors, which must be neutralized through NF- κ B activity. Putative apoptosis-regulating factors at this vulnerable tumor development phase are p53 and c-Jun (38), TNF- α receptor family members (39), and JNK (40).

The NF- κ B and AP-1 groups of transcription factors and MAPK pathways have implicated roles in immunity and inflammation (41, 42). Comparison of parental and RNAi knockdown cells demonstrated that NF- κ B and JNK were essential for coculture expression of the inflammatory cytokine MIF and expression of EMMPRIN. In contrast to other studies (43), we could not demonstrate a role for p38 or p42 MAPK for MIF and EMMPRIN expression in our system.

In agreement with our results, coculture of the ovarian cancer cell line PEO-1 and the monocytic cell line THP-1 led to an up-regulation of monocyte MMP-9 production (44). Furthermore, direct cocultivation of ovarian cancer cells with fibroblasts induced MMP-2 release in fibroblasts and led to an enhanced invasion of the ovarian cancer cells (45). MMP-2 is regarded as a crucial enzyme for tumor progression, invasion and metastasis due to its capability to degrade basement membrane components, and its activation process is critical for tumor development. Recently,

EMMPRIN has been reported to be highly expressed in tumor cells and induces production of MMPs from fibroblasts adjacent to the tumor cells (25). It has been identified as a tumor-derived factor that can stimulate MMP expression in fibroblasts and hence facilitate tumor invasion and metastasis. Our activity assay results indicate that tumor cells such as MCF-7 or IGROV1 express higher levels of EMMPRIN but minimal levels of MMP-2 and MMP-9, whereas macrophages express higher levels of MMP-2 and MMP-9. Upon coculture, tumor cells up-regulated EMMPRIN expression, which may stimulate active MMP-2 and MMP-9 production from macrophages. Suzuki et al. (46) assessed similar results in a fibroblast, tumor coculture model but demonstrated importance of direct cell-cell interaction. Our results outline a novel form of tumor-stromal interaction in which extracellular matrix degradation by macrophages is controlled through the release of EMMPRIN from tumor cells and is consistent with other reports (47). Blockage of EMMPRIN by RNAi inhibited tumor cell invasiveness. Kanekura et al. (48) showed Abs to EMMPRIN inhibited both the production of MMPs by fibroblasts and the invasiveness of melanoma cells.

MIF may act in a similar way to promote tumor cell invasiveness through macrophages secretion of MMP. MIF is a cytokine normally found in macrophages and lymphocytes. Several recent studies have reported the increased expression of MIF in precancerous, cancerous, and metastatic tumors (49). We demonstrated that knockdown of tumor cell-derived MIF protein decreased MMP-9 secretion by cocultured macrophages. A crucial step for invasion and metastasis is the destruction of biological barriers such as the basement membrane, which requires activation of proteolytic enzymes (9).

In conclusion, the *in vitro* interaction of tumor cells with macrophages via EMMPRIN and MIF, in a JNKII- and NF- κ B-dependent manner induces macrophages to release MMPs and to support tumor cell invasion. Additional experiments are needed to identify those soluble compounds from macrophages that initiate cross-talk and better define how these interactions are regulated and demonstrate whether similar effects play a role *in vivo*.

Disclosures

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