Regulation of Inflammation- and Angiogenesis-related Gene Expression in Breast Cancer Cells and Co-cultured Macrophages

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Abstract. *Background: Tumor-associated macrophages (TAMs) secrete key modifiers of tumor progression and their modification has been proposed as a therapeutic strategy. Phenotypic changes that may render TAMs selectively vulnerable to anti-cancer agents were examined. Materials and Methods: Gene arrays, reverse transcription-polymerase chain reaction and Western blotting were used to study inflammation- and angiogenesis-related gene expression in co-cultured breast cancer cells and macrophages and to determine how their interactions were affected by tamoxifen and aspirin. Results: MCF-7 (mammary adenocarcinoma) cells down-regulated macrophage migration inhibitory factor (MIF), but tamoxifen-pretreated MCF-7 cells up-regulated MIF in co-cultured macrophages. Two molecular variants of MIF were observed in the co-cultured MCF-7 cells. Aspirin induced IL-10 expression in the macrophages, MCF-7 and tamoxifen-pretreated MCF-7 cells. Aspirin-pretreated macrophages potently induced IL-10 expression in the MCF-7 cells. Conclusion: Because MIF is a determinant of the M1 macrophage activation state, the MCF-7-induced ablation of MIF in TAMs is suggestive of partial M2 polarization. Tamoxifen modulates MCF-7 regulation of TAM gene expression and aspirin alters macrophage regulation of MCF-7 gene expression.*

The microenvironment of epithelial tumors plays a decisive role in tumor progression (1, 2). Tumor-associated macrophages (TAMs) can initiate both angiogenesis and invasion (3-5). Two alternate activation states of macrophages are generally recognized, M1, characterized by the production of pro-inflammatory cytokines, reactive oxygen species and nitric oxide, and M2, characterized by the production of anti-inflammatory cytokines, synthesis of prostaglandin E_2 and the ability to stimulate angiogenesis (6). The specific nature of the macrophage contribution to disease may depend on its polarization status. Inflammatory macrophages can alter prostate cancer cell responsiveness to selective androgen receptor modulators (SARMs) (7), whereas M2 macrophages may play a role in reversing insulin resistance (8). Furthermore, stromal cells and epithelial cells may respond differentially to the same pharmacological agents, as has been demonstrated for hedgehog signaling in breast cancer (9).

Macrophage migration inhibitory factor (MIF), a pro-inflammatory cytokine and key modulator of immune responses, is constitutively expressed by macrophages, potentiates their inflammatory activity and sustains survival by inhibiting activation-induced, p53-mediated apoptosis (10-14). MIF is also secreted by cancer cells and has been implicated in several stages of tumor progression (14-16). MIF knockdown in murine ovarian cancer cell implants was associated with decreased expression of *TNF-α, IL-6* and *IL-10* and reduced macrophage recruitment (17). MIF prevents apoptosis of neutrophils by inhibiting the intrinsic pathway (18) and promotes the survival of primary and immortalized fibroblasts and various breast cancer cell lines, by activating the phosophoinositide-3-kinase (PI3K)-Akt pathway (19, 20). MIF may also promote monocyte recruitment and metastasis because it is a non-cognate ligand for the chemokine receptors CXCR2 and CXCR4 (21, 22). MIF signals through the CD (cluster of differentiation) 74-CD44 membrane receptor complex to activate extracellular signal-regulated kinase (ERK) 1/2 and the PI3K-Akt pathway to promote proliferation and survival (23, 24). Upon binding of MIF, the CD74 intracellular domain (ICD) translocates to the nucleus and induces activation of nuclear factor-kappa B (NF-κB) (25). MIF potentiates lipopolysaccharide (LPS) signaling by up-regulating Toll-like receptor (TLR) 4. MIF-deficient

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Key Words: Angiogenesis, inflammation, MIF, IL-10, tamoxifen, aspirin.

macrophages are hyporesponsive to LPS, as shown by a reduction in NF-κB activity and TNF-α production (26). MIF has been proposed to function both as a systemically acting, 'endocrine' cytokine and as part of a locally acting feedback loop limiting the anti-inflammatory actions of glucocorticoids (27, 28).

Because anti-cancer therapies that include targeting of stromal cells are likely to be more effective, inflammation- and angiogenesis-related gene expression in co-cultured breast cancer cells and macrophages, which may render macrophages selectively vulnerable to pharmacological agents, was examined. MIF interferes with p53 activity (13, 14), and p53 and phosphatase and tensin homologue deleted on chromosome 10 (PTEN) function collaboratively in a tumor-suppressive network (29). Therefore, the expression of *p53* and *PTEN* in recombinant human MIF (rhMIF)-treated breast cancer cells and macrophages was also examined. Additionally, the effects of tamoxifen and nonsteroidal anti-inflammatory drugs (NSAIDs) on the regulation of gene expression in macrophages and breast cancer cells were also investigated. Tamoxifen is widely used for the prevention and treatment of breast cancer and NSAIDs are thought to be effective in cancer prevention (30).

Materials and Methods

Materials. Phorbol-12 myristate 13-acetate (PMA) was obtained from Alexis® Biochemicals (San Diego, CA, USA). LPS (*Escherichia coli* 055: B5), tamoxifen citrate and aspirin were obtained from Sigma Chemical Company (St. Louis, MO, USA). Recombinant human MIF (rhMIF, 289-MF) was obtained from R&D Systems (Minneapolis, MN, USA). RPMI-1640, minimum essential medium (MEM), fetal bovine serum (FBS), penicillin, streptomycin, L-glutamine and all the primers for PCR were obtained from Invitrogen Corporation (Carlsbad, CA, USA). The rabbit anti-human MIF polyclonal antibody (sc-20121) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture. THP-1 (human monocytic leukemia) cells, MCF-10A (psuedonormal mammary epithelial) cells, MCF-7 (mammary adenocarcinoma) cells and MDA-MB-231 (mammary adeno-carcinoma) cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The THP-1 cells were differentiated to macrophages with PMA (100 nM) for 3 days, then activated with LPS (20 ng/ml) for 5 h.

The THP-1 cells were cultured in RPMI-1640 supplemented with 7.5% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin and 50 μM β-mercaptoethanol (β-EtSH). The MCF-10A, MCF-7 and MDA-MB-231 cells were propagated in MEM supplemented with 8% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin and 292 μg/ml L-glutamine. All the cells were grown at 37˚C in a humidified atmosphere of 95% air:5% CO_2.

For the co-culture experiments, 1×10^6 THP-1 cells per well were seeded in the inserts (3-μm pore size) of 6-well Transwell™ chambers (Corning Incorporated, Corning NY, USA), differentiated one day later with PMA and activated with LPS. The MCF-7 cells at 5×10^5 per well were seeded in a separate chamber, two days after the THP-1 cells were seeded. The medium of both the breast cancer cells and the activated macrophages was changed, the inserts with the activated macrophages were placed above the breast cancer cells and the chambers were incubated for 3 days. Where indicated, the cells were treated with tamoxifen (10 μM) for 24 h, aspirin (1 mM) for 24 h, and rhMIF (10 ng/ml) for 6 h prior to RNA isolation. Where pre-treatment is indicated, cells were exposed to the first agent for a specified time period and then to the second.

Cell lysis and Western blotting. The cells were washed with phosphate-buffered saline (PBS) and lysed with buffer (10 mM hydroxyethyl piperazine-ethanesulfonic acid (HEPES) pH 7.5, 1% sodium dodecyl sulfate (SDS), and 1 mM sodium orthovanadate) at 100˚C. The lysate was heated for 4 min at 95˚C, then passed through a 26-gauge needle. The protein concentration was determined by the BIO-RAD detergent-compatible protein microassay (Sigma Chemical Company, St. Louis, MO, USA). Fifty μg of lysate protein was heated at 95˚C for 5 min in Laemmli sample buffer containing 4% SDS, 20% glycerol, 10% 2-mercapto-ethanol, 0.004% bromphenol blue and 0.125 M Tris HCl pH 6.8 (Sigma Chemical Company, St. Louis, MO, USA). The samples were subjected to SDS-polyacrylamide gel electrophoresis on 4-20% Precise™ protein gels (Pierce, Rockford, IL, USA), and the separated proteins were transferred onto a nitrocellulose membrane. The membrane was blocked with Tris-buffered saline containing 0.1% Tween-20 (TBST) and 5% non-fat dry milk for 1 h at room temperature (RT) and then incubated with primary antibody diluted in TBST containing 0.5% non-fat dry milk for 1 h at RT. The membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit antibody (sc-2004, Santa Cruz Biotechnology) at RT for 1 h. The proteins were visualized using the enhanced chemiluminescence (ECL) Plus Western Blotting Detection System (Amersham Biosciences UK Limited, Buckinghamshire, England), as recommended in the manufacturer's instructions.

RNA extraction and cDNA synthesis. The cells were washed with PBS and RNA was extracted using Trizol reagent (Invitrogen Corporation), or the Versagene™ RNA Purification kit (Gentra Systems, Minneapolis, MN, USA), in accordance with the manufacturer's protocol. The cDNA was synthesized using the Advantage™ RT-for-PCR kit (Clontech Laboratories, Mountain View, CA, USA), according to the manufacturer's instructions.

Pathway-focused gene arrays. For the pathway-focused gene arrays (SuperArray Bioscience Corp., Frederick, MD, USA), 5 μg total RNA from monocytes, unactivated macrophages, co-cultured unactivated macrophages, MCF-7 cells and co-cultured MCF-7 cells was reverse transcribed into biotin-labeled cDNA. The labeled cDNA was hybridized to pathway-focused microarrays for human pro-inflammatory cytokine and receptor genes (HS-015.2), or for angiogenesis-related genes (HS-009), each containing 96 test genes and four positive control (housekeeping) genes. The membranes were incubated with alkaline phosphatase (AP)-streptavidin, visualized by chemiluminescence and exposed to x-ray film.

Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR). A 3-μl aliquot of the cDNA was used for PCR amplification together with the primer pairs (10 μM each), 10× PCR buffer, deoxynucleoside triphosphates (dNTPs) (100 μM each), $MgCl_2$ and AmpliTaq Gold DNA polymerase (1.25

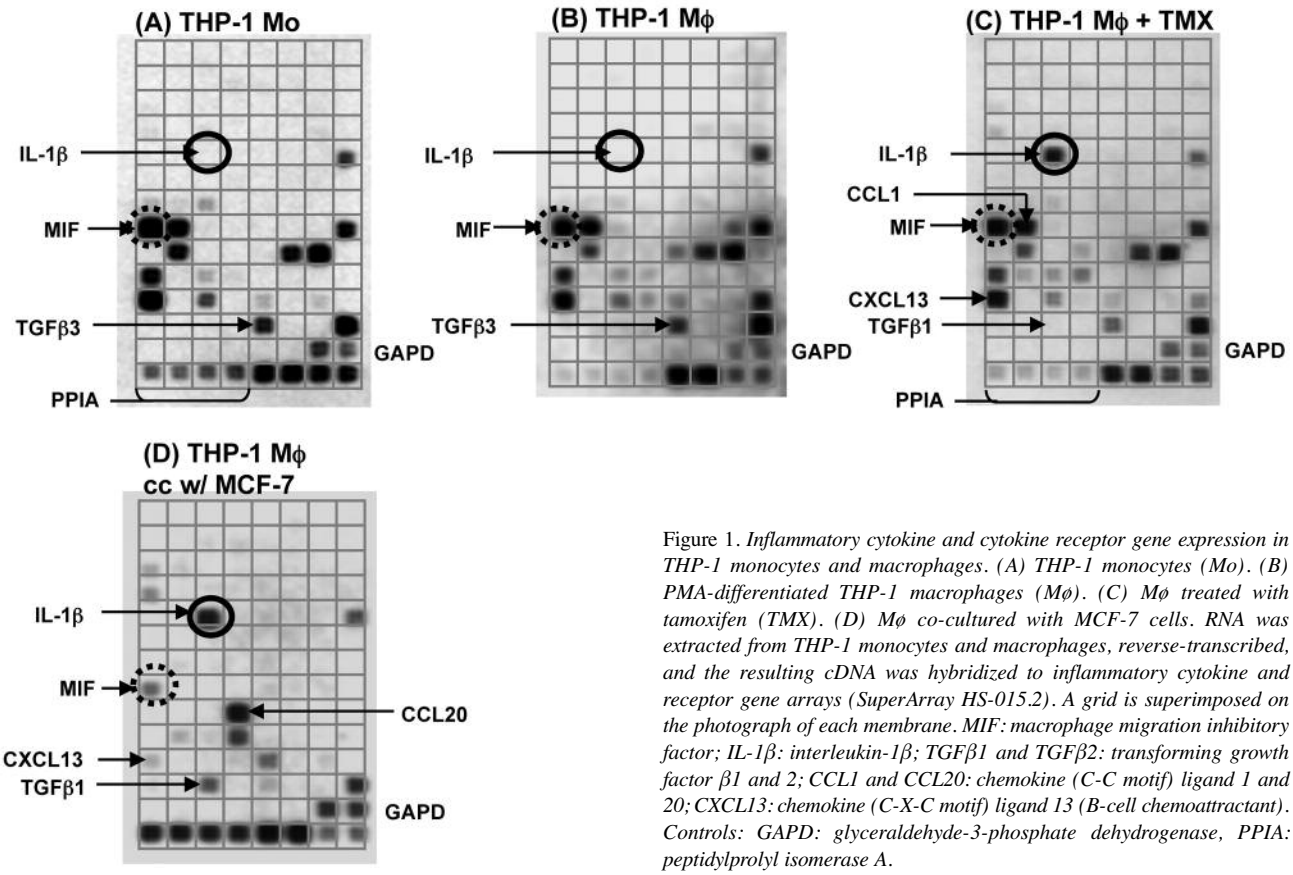

Figure 1. *Inflammatory cytokine and cytokine receptor gene expression in THP-1 monocytes and macrophages. (A) THP-1 monocytes (Mo). (B) PMA-differentiated THP-1 macrophages (Mϕ). (C) Mϕ treated with tamoxifen (TMX). (D) Mϕ co-cultured with MCF-7 cells. RNA was extracted from THP-1 monocytes and macrophages, reverse-transcribed, and the resulting cDNA was hybridized to inflammatory cytokine and receptor gene arrays (SuperArray HS-015.2). A grid is superimposed on the photograph of each membrane. MIF: macrophage migration inhibitory factor; IL-1β: interleukin-1β; TGFβ1 and TGFβ2: transforming growth factor β1 and 2; CCL1 and CCL20: chemokine (C-C motif) ligand 1 and 20; CXCL13: chemokine (C-X-C motif) ligand 13 (B-cell chemoattractant). Controls: GAPD: glyceraldehyde-3-phosphate dehydrogenase, PPIA: peptidylprolyl isomerase A.*

Units/reaction volume) (Applied Biosystems, Foster City, CA, USA), in a final volume of 25 μl. The following primers were used for PCR: *MIF*, 5'-CTCTCCGAGCTC-ACCCAGCAG-3' (forward) and 5'-CGCGT TCATGTCGTAATA-GTT-3' (reverse); p53, 5'-CAGCCAAGTCTG TGACTTGCA-CGTAC-3' (forward) and 5'-CTATGTCGAAAAGT GTTT-CTGTCATC-3' (reverse); *PTEN*, 5'-CTTCTCTTTTTTTTCT GTCC-3' (forward) and 5'-AAG-GATGAGAATTTCAAGCA-3' (reverse). The PCR conditions included: for *MIF*, 35 cycles of 94˚C, 1 min, 60˚C, 1 min, 72˚C, 1 min and 72˚C, 10 min; for *p53*, 95˚C, 15 min and 50 cycles of 94˚C, 15 s, 55˚C, 15 s, 72˚C, 15 s; for *PTEN*, 95˚C, 2 min and 35 cycles of 94˚C, 30 s, 57˚C, 30 s, 72˚C, 30 s, and 72˚C, 10 min. The expected amplicon sizes were 255 bp, 293 bp and 191 bp for *MIF*, *p53*, and *PTEN*, respectively. The PCR products were separated on 1.2% agarose gels.

Results

Inflammatory cytokine and cytokine receptor gene expression. MIF RNA was slightly down-regulated in the macrophages (Figure 1A, 1B), but dramatically down-regulated in the macrophages co-cultured with the MCF-7 cells (Figure 1D). While *IL-1β* was not expressed in the THP-1 monocytes or macrophages, it was highly induced in the macrophages co-cultured with the MCF-7 cells (Figure 1A, 1B and 1D). Surprisingly, tamoxifen potently induced *IL-1β* expression in the macrophages (Figure 1B and 1C).

Angiogenesis-related gene expression. The macrophages (Figure 2A), tamoxifen-treated macrophages (Figure 2B) and co-cultured macrophages (Figure 2D) did not express *IL-10*. Aspirin potently induced *IL-10* expression in the macrophages (Figure 2C), but not in the co-cultured macrophages (Figure 2F). However, aspirin pre-treated macrophages induced *IL-10* in both untreated and tamoxifen-pretreated MCF-7 cells (data not shown).

RT-PCR for MIF. RT-PCR was used to confirm the changes in *MIF* expression observed in the gene arrays. All the cells studied expressed *MIF* RNA. *MIF* RNA was up-regulated in the MCF-7 cells co-cultured with activated macrophages. The RT-PCR confirmed the observation that the *MIF* levels were down-regulated in the macrophages co-cultured with the MCF-7 cells (Figure 3A).

Variants of MIF protein in co-cultured MCF-7 cells. Western blot analysis showed two forms of MIF, a larger,

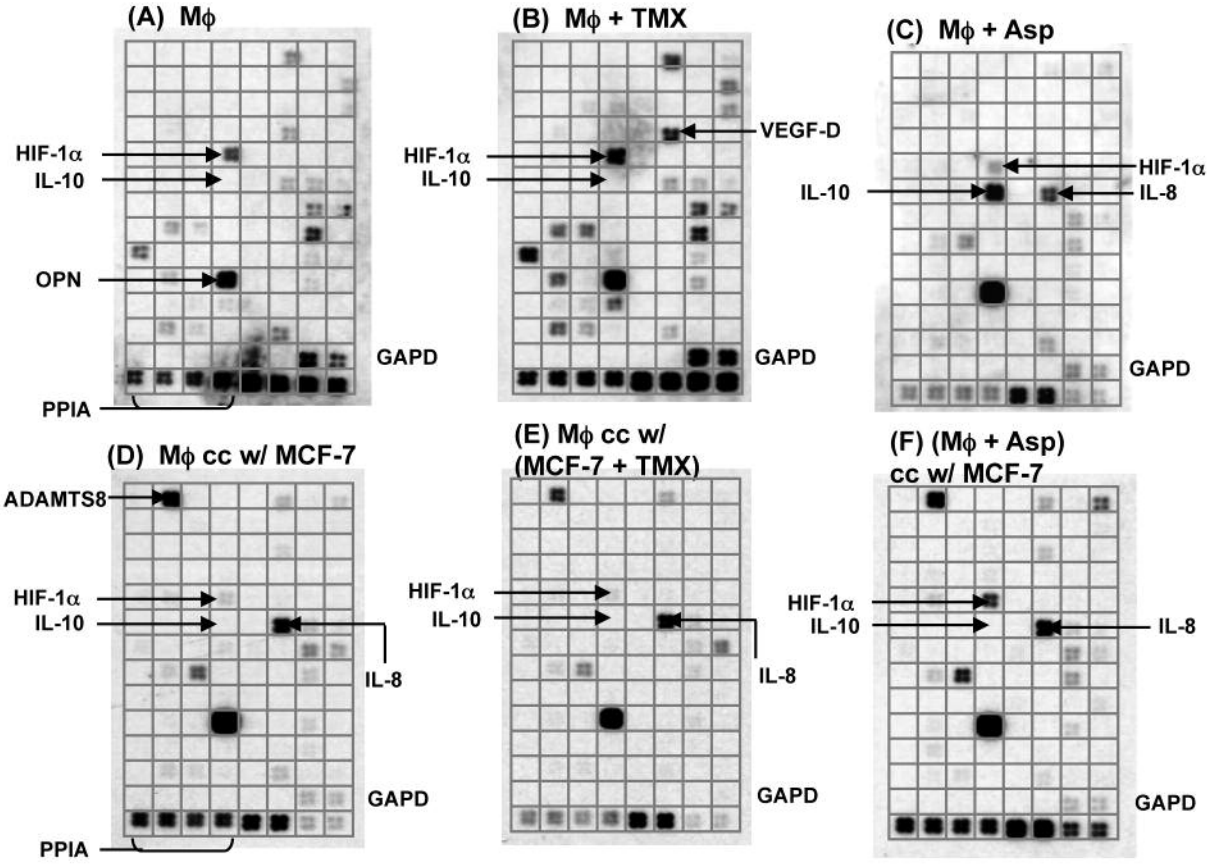

Figure 2. *Angiogenesis-related gene expression in THP-1 macrophages. (A) PMA-differentiated THP-1 macrophages (Mϕ). (B) Mϕ treated with tamoxifen (TMX). (C) Mϕ treated with aspirin (Asp). (D) Mϕ co-cultured with MCF-7 cells. (E) Mϕ co-cultured with tamoxifen-pretreated MCF-7 cells. (F) Aspirin-pretreated Mϕ co-cultured with MCF-7 cells. RNA was extracted from THP-1 macrophages and used for gene arrays (SuperArray HS-009). HIF-1α: hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor); IL-8 and IL-10: interleukin-8 and -10; VEGF-D: vascular endothelial growth factor D; OPN: osteopontin; ADAMTS8: a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 8. Controls GAPD: glyceraldehyde-3-phosphate dehydrogenase, PPIA: peptidylprolyl isomerase A.*

macrophage-type and a smaller, MCF-7-type. Although the *MIF* RNA levels were increased in the activated macrophages (Figure 3A), the MIF protein was decreased (Figure 3B). The MIF protein levels were higher in the co-cultured, activated macrophages than in the activated macrophages, although the *MIF* RNA levels were lower. Both the MIF RNA and protein levels were increased in the co-cultured MCF-7 cells, which contained both MIF variants.

Effect of recombinant human MIF on p53 and PTEN. The rhMIF down-regulated *p53* in all of the cells (Figure 4A). *p53* was detectable in THP-1 cells, and in unactivated and activated macrophages. The activated macrophages increased *PTEN* RNA in the MCF-7 cells (Figure 4B). Of the three breast cell lines studied, MCF-10A had the highest level of *PTEN* RNA, which was consistent with its non-transformed status (Figure 4B). rhMIF down-regulated *PTEN* in all of the cells (data not shown).

Discussion

The *MIF* gene was dramatically down-regulated in co-cultured macrophages, but, consistent with previous reports (31), up-regulated in co-cultured MCF-7 cells. Given the role of MIF in macrophage activation, the present results suggest that MCF-7 down-regulation of macrophage *MIF* is protective for cancer cells, while *MIF* up-regulation in cancer cells is adaptive for the tumor. The silencing of *MIF* expression observed in this study may represent a phenotypic difference that can be exploited for inhibiting TAM function.

Two variants of the MIF in co-cultured MCF-7 cells were identified, which may be the result of covalent modifications of MIF, or uptake of the macrophage form by the MCF-7 cells. It has been suggested that the genetic stability of stromal cells makes them less likely to develop drug resistance and, therefore, good targets for inhibitors of

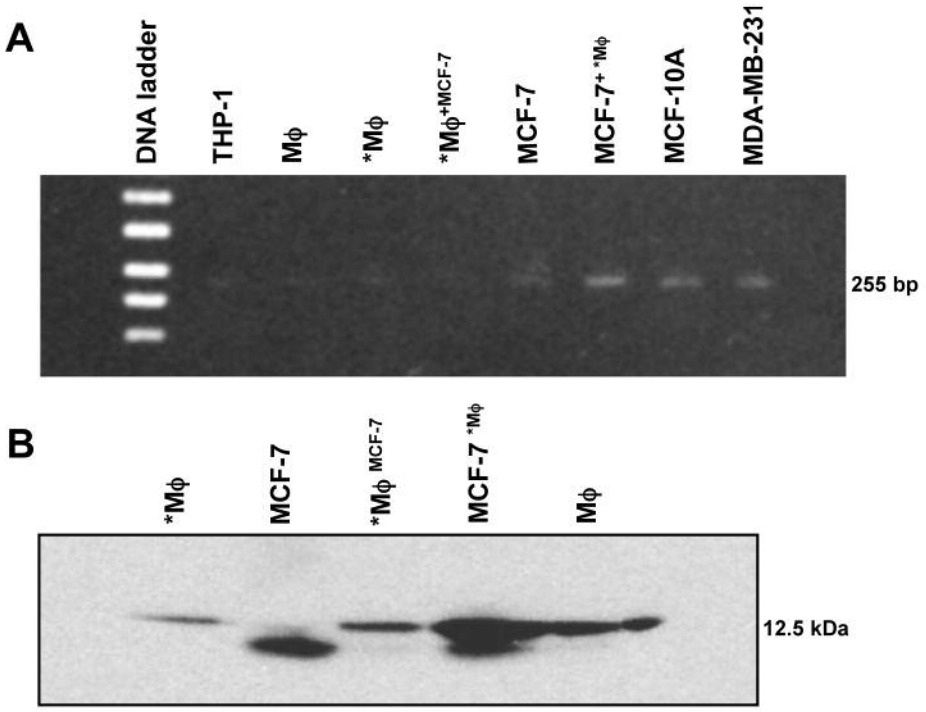

Figure 3. *Analysis of MIF RNA and protein in breast cancer cells and macrophages. (A) RT-PCR of MIF mRNA (255 bp) in breast cancer cells and macrophages. In activated macrophages (*Mϕ) co-cultured with MCF-7 cells (*Mϕ^{+MCF-7}), MIF RNA expression was down-regulated when compared with activated macrophages (*Mϕ). (B) Western blot of MIF in THP-1 macrophages and MCF-7 cells. *Mϕ, LPS-activated macrophages; *Mϕ^{MCF-7}, activated macrophages (source of protein) co-cultured with MCF-7 cells; MCF-7*Mϕ, MCF-7 cells (source of protein) co-cultured with activated macrophages and unactivated macrophages (Mϕ) were probed for MIF protein. A 50-μg aliquot of lysate protein was resolved in 4-20% SDS-PAGE gradient gels, electroblotted onto nitrocellulose membrane and probed with rabbit polyclonal anti-human MIF IgG.*

macrophage functions that contribute to tumor progression (4). The detection of *p53* in the THP-1 cells and the macrophages, in the present study, was unexpected because previous reports suggested that THP-1 cells were *p53*-negative (32). Nonetheless, if cancer cells secrete MIF, which is known to inhibit p53 function, mutations may accumulate in TAMs without *p53* loss. A frequent association of *MIF* expression with the presence of *p53* in tumors (33) suggests that there is no selective pressure for *p53* loss in stromal cells when cancer-cell MIF is present. Extracellular and intracellular forms of MIF may function in distinct molecular pathways. The rhMIF down-regulation of *p53* and *PTEN* in the breast cancer cells and macrophages suggested a mechanism by which MIF may promote survival of both transformed and nontransformed cells.

IL-10 has been shown to inhibit MIF synthesis in T-cells (34). The definitive signal that down-regulated *MIF* in co-cultured macrophages in the present study was not likely to be IL-10, because *IL-10* was not expressed in the MCF-7 cells or the activated macrophages in co-culture. Only the aspirin-treated MCF-7 cells and macrophages cultured separately expressed *IL-10*, and aspirin increased the level of *IL-10* mRNA in the MCF-7 cells and macrophages. Thus *IL-10* up-regulation may be a part of the mechanism by which aspirin exerts its anti-inflammatory effects. Additionally, the present results show that aspirin could imprint the macrophages to induce *IL-10* expression in the MCF-7 cells and suggest that NSAIDs may modulate the ability of macrophages to regulate gene expression in cancer cells.

In contrast to *MIF*, co-culture up-regulated *IL-1β* and *IL-8* expression (data not shown) in the macrophages. *IL-1β* was also up-regulated by tamoxifen in the macrophages. The reciprocal relationship between *IL-8* and *MIF* may be of physiological importance to tumor cells by allowing them to sustain a partial inflammatory microenvironment while maintaining the benefits of angiogenesis. The interactions that occurred between breast cancer cells and macrophages did not result in a complete differentiation to the M2 phenotype, but engaged selective components of each activated state (M1 and M2).

The mechanisms that underlie aggressive behavior in breast tumors are not intrinsic to cancer cells and include their ability to control the phenotypes of TAMs. Discrete stages of tumor progression are the product of evolving interactions between components of the tumor microenvironment. Consequently,

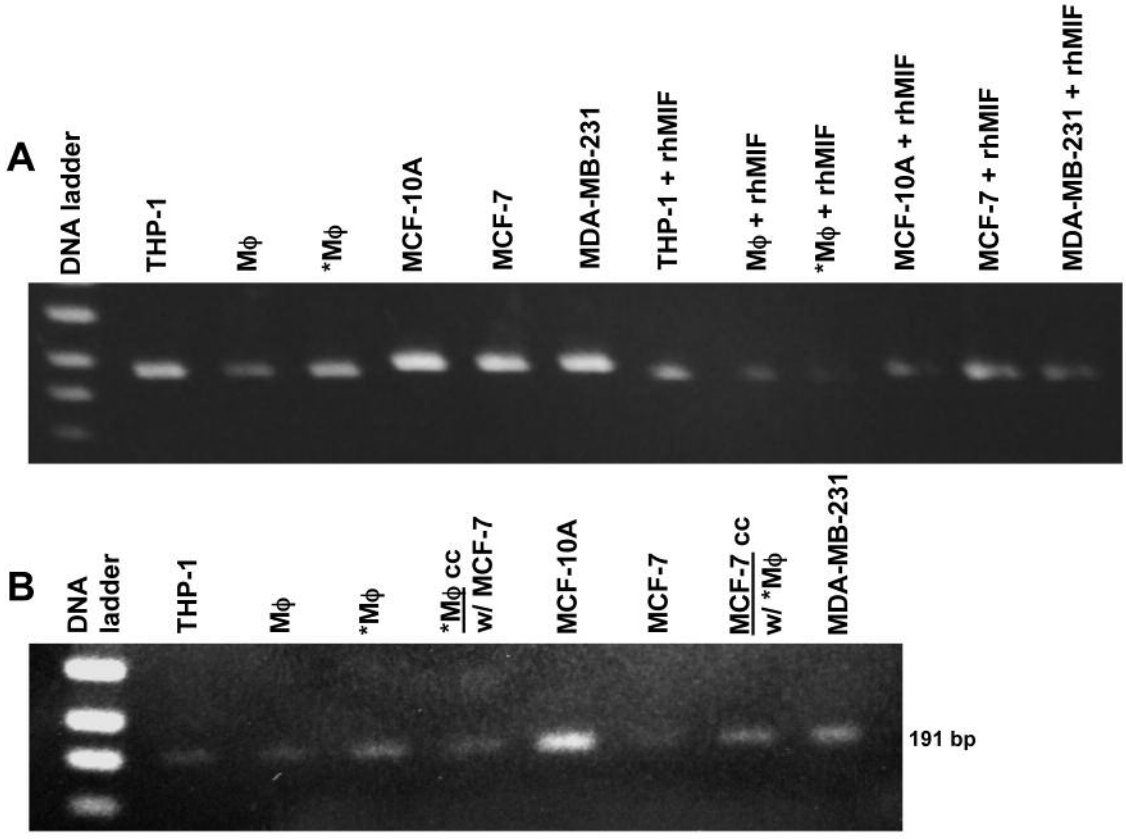

Figure 4. *p53 and PTEN expression in breast cancer cells and macrophages. (A) p53 expression in untreated and rhMIF-treated cells. Total RNA was extracted and RT-PCR for p53 was performed, as described. Mϕ, macrophage; *Mϕ, LPS-activated macrophages; MCF-10A, MCF-7, and MDA-MB-231 breast cell lines. Amplimer size 293 bp. (B) PTEN expression in THP-1 (monocytes); THP-1 macrophages (Mϕ); activated macrophages (*Mϕ); MCF-10A, MCF-7, and MDA-MB-231 breast cell lines. Cells were cultured alone, or in co-culture (cc), as indicated. A 1-μg aliquot of total RNA from each culture was reverse transcribed in a total reaction volume of 20 μl, and 3 μl of each RT mixture was used for PCR. In co-cultures, the cell line underlined was the source of RNA.*

the design of chemotherapeutic agents to target both epithelial and stromal cells should take into consideration these evolving complexities. If altered gene expression in the tumor microenvironment can enhance the tumor-promoting activities of macrophages, it is conceivable that it may also confer contextual vulnerabilities that can be exploited for pharmacological targeting. Given the importance of macrophages in the immune response, a systemic inhibition of macrophage functions would be counterproductive. It is important that anti-macrophage therapies designed to inhibit tumor progression are specific for TAMs. In this regard, a search for inhibitors of MIF-related survival signaling would be desirable.

Acknowledgements

The study was sponsored by the U.S. Army Medical Research and Acquisition Activity, 820 Chandler Street, Fort Detrick MD 21702-5014, Award NO: DAMD17-02-1-0408 to T. Bremner (PI). The content of this report does not necessarily reflect the position or the policy of the Government and no official endorsement should be inferred.

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Received January 28, 2008
Revised May 5, 2008
Accepted May 9, 2008