Research Communication

Inflammatory Cytokines Prime Adipose Tissue Mesenchymal Stem Cells to Enhance Malignancy of MCF-7 Breast Cancer Cells via Transforming Growth Factor-β1

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Abstract

Mesenchymal stem cells from human adipose tissue (hASCs) are proposed as suitable tools for soft tissue engineering and reconstruction. Although it is known that hASCs have the ability to home to sites of inflammation and tumor niche, the role of inflammatory cytokines in the hASCs-affected tumor development is not understood. We found that interferon-γ (IFN-γ) and/or tumor necrosis factor-α (TNF-α) prime hASCs to produce soluble factors which enhance MCF-7 cell line malignancy *in vitro*. IFN-γ and/or TNF-α-primed hASCs produced conditioned media (CM) which induced epithelial to mesenchymal transition (EMT) of MCF-7 cells by reducing E-Cadherin and increasing Vimentin expression. Induced EMT was accompanied by increased invasion, migration, and urokinase type-plasminogen activator (uPA) expression in MCF-7 cells. These effects were mediated by increased expression of transforming growth factor-β1(TGF-β1) in cytokines-primed hASCs, since inhibition of type I TGF-β1 receptor on MCF-7 cells and neutralization of TGF-β1 disabled the CM from primed hASCs to increase EMT, cell migration, and uPA expression in MCF-7 cells. Obtained data suggested that IFN-γ and/or TNF-α primed hASCs might enhance the malignancy of MCF-7 cell line by inducing EMT, cell motility and uPA expression in these cells via TGF-β1-Smad3 signalization, with potentially important implications in breast cancer progression. © 2016 IUBMB Life, 68(3):190–200, 2016

Keywords: *adipose tissue mesenchymal stem cells; MCF-7; inflammatory cytokines; EMT; urokinase type-plasminogen activator*

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Introduction

Beside their inherent ability to home to injured tissues, mesenchymal stem cells (MSCs) migrate toward tumors and participate in tumor stroma formation (1). MSCs interact with cells within tumor microenvironment, such as tumor-associated fibroblasts (TAFs), endothelial cells, adipocytes and macrophages, and therefore the proangiogenic, antiapoptotic and/or immunomodulatory properties of MSCs may modulate tumor growth and development, as well as interactions of tumor cells with factors of immune systems (2–5).

Breast cancer is one of the most commonly diagnosed cancers, and the second leading cause of cancer-related death in

women (6). The most abundant tissue in breast region is mammary adipose tissue constituted by resident mature adipocytes and progenitor cells, mainly preadipocytes and adipose tissue mesenchymal stem cells (hASCs) (7–9). Today it is clear that hASCs, found in the circulation of breast cancer patients and in stroma of primary breast carcinomas (8–11) do not have a passive role in the breast tumor surrounding niche (12), but they are involved in the pathogenesis, as potential suppressors (13) or promoters (14) of tumor growth.

Crosstalk between tumor cells and adjacent MSCs, including hASCs, is regulated through network of numerous soluble factors like cytokines, chemokines and growth factors, which affect the extracellular matrix remodeling, angiogenesis and epithelial to mesenchymal transition (EMT) of tumor cells, enabling them to form new tumors at distant sites (10,15–17). The process of EMT can be induced by various stimuli, including tumor-stromal cell interactions, hypoxia, cytokines and growth factors; such as transforming growth factor-β (TGF-β1) (18,19). The development of EMT may favor the capacity of cancer cells to acquire motility, through loss of cell-cell adhesion, downregulation of E-cadherin and positive expression of mesenchymal markers (20,21), thus providing the initial cancer cell with invasive and metastatic properties. Metastasis is also influenced by activity of extracellular matrix proteinases, including urokinase-type plasminogen activator (uPA) enzyme, that regulate cancer cell migration and invasion, essential steps in the process of metastasis. Therefore, high protein level of uPA is considered as prognostic marker associated with high metastasis risk (10,17,22).

It is believed that inflammatory cells infiltrated within tumor stroma may influence tumor progression, interplaying with tumor cells and other cells within the microenvironment, having anti-tumorigenic or pro-tumorigenic role (23,24). Similarly, the proinflammatory cytokines secreted by immune cells, such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α, have been shown to express different effects on tumor cells, acting either as tumor suppressors (25) or as tumor promoters (26–28). Although the influence of inflammatory cytokines on cancer cells is well recognized, it is less known how these proteins modulate MSCs paracrine regulation of breast cancer cells properties. In this study, we aimed to test our hypothesis that inflammatory cytokines activate hASCs to produce soluble factors, such as TGF-β1, which modulate EMT, motility and uPA expression in the low-invasive human breast cancer cell line MCF-7. Our findings showed that inflammatory cytokines can educate ASCs to adopt malignancy-promoting features.

Experimental Procedures

Non-Enzymatic Isolation of hASCs and Cells Cultivation

hASCs were isolated from adipose tissue samples obtained during surgical procedures from the Institute for Oncology and Radiology of Serbia, Belgrade, in accordance with ethical standards of the local ethical committee and the Declaration of Helsinki with informed consent obtained from the study subjects. hASCs were isolated by adjusted non-enzymatic method previously reported (29). In short, to remove blood and impurities, the tissues were extensively washed with Phosphate Buffered Saline (PBS, Sigma Aldrich) supplemented with 1% Penicilin/Streptomycin (P/S) and 0.25 mg/mL Amphotericin B (both from PAA, Linz, Austria). The tissues were minced to small pieces (3×3 mm) with surgical scissors and put into 25 cm^2 culture flask (Greiner Bio-One International, GmbH) for 20 min to attach in humidified atmosphere at 37°C with 5% CO_2 (standard conditions). Then, 3 mL of DMEM (Sigma-Aldrich, St.Louis, MO) supplemented with 20% Fetal Calf Serum (FCS) (Gibco, Life Technologies), 1% Hepes (PAA, Linz, Austria) and 1% P/S was added to flasks, disabling adipose tissue pieces to float. These pieces were incubated for 10 days, until first cells migrated, attached to plastic and formed colonies, when pieces were thrown out, with further medium change every 2 to 3 days. After achieving 80 to 90% confluence, hASCs were detached by 0.25% trypsin-EDTA solution (Gibco), replated at concentration 10^4 cells/cm^2 and cultivated in standard conditions in growth medium (GM) containing DMEM supplemented with 10% FCS, 1% Hepes and 1% P/S. All experiments were performed using hASCs between passage 2 and 6.

Human breast carcinoma cell line (MCF-7), was purchased from The American Type Culture Collection (ATCC, HTB-22), MD, and cultured in GM in humidified atmosphere in standard conditions.

Immunophenotypization and Multilineage Capacity of hASCs

According to the minimal criteria for defining MSCs, recommended by International Society for Cellular Therapy (ISCT), the expression of CD90, CD44, CD73 (obtained from R&D Systems, Minneapolis, MN), CD105 (Invitrogen, Carlsbad, CA), and additional hematopoietic cells markers CD34 (Dako, UK) and HLA-DR (Invitrogen) was analyzed. For cell-surface molecule analysis, 2×10^5 hASCs were washed in cold PBS supplemented with 0.5% BSA (Sigma-Aldrich), and labeled with phycoerythrin (PE) or fluorescein isothiocyannate (FITC) conjugated antibodies. To determine the level of nonspecific binding, fluorochrome-conjugated isotype control antibodies (R&D Systems) were used. Flow cytometry was performed using CyFlow CL (Partec, Münster, Germany).

For the analysis of multilineage differentiation potential, hASCs were seeded in 24-well plates at concentration of 2×10^4 cells/well in GM and incubated under standard conditions. The GM was replaced every 2 to 3 days. After reaching 80% of confluency, cells were incubated in specific differentiation medium. Osteogenic differentiation medium contained GM supplemented with 10 nM dexamethasone (AppliChem), 200 mM ascorbic acid-2-phosphate (Galenika, Belgrade, Serbia) and 10 mM β-glycerophosphate (Sigma–Aldrich). Calcified deposits and extracellular matrix mineralization were visualized by Alizarin red (2%) staining assay performed after 21 days.

To determine the hASCs capacity for adipogenic differentiation, the presence of intracellular lipid droplets in cell cultures was assessed by staining with Oil Red O (Merck, Darmstadt, Germany) after 21 days of cultivation in GM supplemented with 100 mg/mL isobutylmethylxanthine (IBMX; Sigma–Aldrich), 1 mM dexamethasone and 10 mg/mL insulin (Sigma-Aldrich). hASCs were also cultivated in chondrogenic differentiation medium consisting of GM supplemented with 5 ng/mL transforming growth factor (TGF-β) (R&D Systems), 200 mM ascorbic acid-2-phosphate and 10 nM dexamethasone for 21 days. Chondrogenic differentiation was assessed via proteoglycans stained with Safranin O (Merck). Cells were analyzed and photographed using a light microscope (Olympus, Japan).

Conditioned Media Preparation

Conditioned media (CM) from hASCs were prepared and adjusted as previously described (30,31). Briefly, hASCs were primed with 50 ng/mL of IFN-γ and/or 20 ng/mL of TNF-α (both from Invitrogen) for 24 h, and after extensive washing with PBS were cultivated in GM without cytokines for additional 24 h in standard conditions. CM were then collected and subjected to centrifugation at 1,400 rpm for 5 min and filtered by using 0.20 μm filter. CM were named according to cells treatment: CM 0, CM IFN/TNF, CM IFN and CM TNF.

Invasion and Wound Healing Assay

Transwell systems with 8.0 μm pore polycarbonate filters (Greiner Bio-One) were used for evaluation of the invasion capacity of tumor cells. MCF-7 cells were seeded at concentration 10^4 cells per transwell in 200 μL of growth medium in the upper chamber with membranes precoated with fibronectin (10 μg/mL). Growth medium (0.5 mL), or CM as chemoattractant were added in lower chamber. After 6 days, cells from upper compartment were cleaned with a cotton swab to remove the non-invading cells. Cells attached to the lower side of the membrane were fixed by immersing the transwells into ice-cold methanol. After washing with PBS, transwells were turned upside down and membranes were stained with 0.1% Crystal-violet. Stained cells were observed by inverted microscope. Quantification was performed by resolving crystal-violet in isopropanol and optical density was measured at 540 nm using an automatic reader for microtiter plates (Labsystems Multiskan PLUS, Finland).

MCF-7 at concentration 2×10^4 cells/well were seeded in fibronectin (10 μg/mL, Sigma Aldrich) precoated 24-well plates (Greiner Bio-One International) and cultured in GM in standard conditions. Wound healing assays were performed as previously described (32). Migration of the cells was documented after 6 days of cultivation (details specified in Results) by inverted light microscope and quantified using TScratch software (Computational Science and Engineering Laboratory, Swiss Federal Institute of Technology, ETH Zürich, Zürich, Switzerland).

Immunofluorescence Labeling

Cells (5×10^4 cells/coverslip) were allowed to adhere for 24 h in standard conditions, and then treated as described in Results. Immunofluorescent labeling was performed as previously described (32). Briefly, cells were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton-X100 (Sigma-Aldrich), and then immunolabeled with primary antibodies: mouse anti-TGF-β1 (dilution 1:100, Abcam, UK, kindly provided by dr. Djurdjica Jovović, Laboratory for Cardiovascular Physiology, Institute for Medical Research, Serbia), rat anti-E-cadherin (dilution 1:1,000, from Sigma-Aldrich), mouse anti-Vimentin (dilution 1:100, from Santa Cruz Biotechnology, CA) and rabbit pSmad3 (1:100, Santa Cruz Biotechnology). Samples incubated in 1% BSA in PBS served as negative control. After appropriate incubation (indicated in Results), cells were washed three times with PBS and incubated with the corresponding FITC/TRITC-coupled secondary antibodies (dilution 1:100, both from Sigma-Aldrich) and 1 μg/mL of DAPI (Sigma-Aldrich) for nucleus labeling for 1 h at room temperature. Mounted cells were analyzed using an epi-fluorescent microscope (Olympus, Japan).

Western Blot Assay

Cells (1×10^5 cells/well) were seeded in six-well plates (Greiner Bio-One International) and, after reaching 80% confluence in standard conditions, treated as indicated in Results. Cell monolayers were lysed in RIPA buffer (1% NP-40, 0.1% SDS, 1mM EDTA, 50 mM NaF, 1% sodium deoxycholate) with 10 mM of sodium orthovanadate and proteinase inhibitors. Samples were centrifuged and protein concentrations in supernatants were determined by BCA assay (Serva, GmbH). Next, 10 μg of samples were subjected to SDS-PAGE (10%) in reducing conditions, and then proteins were electrotransferred to 0.45μm pore size nitrocellulose membrane (AppliChem). Specifically, when secreted levels of TGF-β were analyzed, 100 μL of GM/CM were applied. Membranes were blocked in 5% skim nonfat milk (Sigma Aldrich) in solution of Tris Buffered Saline with 0.05% Tween 20 for 1 h and then incubated with primary antibodies: mouse anti-TGFβ1 (dilution 1:1,000, Abcam), mouse anti-α-tubulin and rat anti-E-cadherin (dilution 1:1,000, both from Sigma Aldrich), mouse anti-Vimentin (dilution 1:1,000), rabbit anti-uPA (dilution 1:500), rabbit anti-pSmad3 (dilution 1:500) and rabbit anti-tSmad3 (dilution 1:500) (all both from Santa Cruz Biotechnology). Immune complexes were detected using corresponding anti-rat (Sigma Aldrich), anti-mouse (Sigma Aldrich) and anti-rabbit (Santa Cruz Biotechnology) secondary antibodies (all dilutions 1:1,000) coupled to horseradish peroxidase (HRP) and lightened with chemiluminescent substrate (Serva Electrophoresis). Protein bands were detected and quantified.

Zymography Assays

To analyze cell-associated uPA activity, MCF-7 (10^5 cells/well) were plated in six-well plates and after reaching confluence treated with GM or CM for 6 days in standard conditions. After

TABLE 1 *Primer sets used in experiments*

Primer	*Forward sequence 5′-3′*	*Reverse sequence 5′-3′*	*Product size (bp)*	*Annealing temperature (°C)*
TGF-β1	GGGACTATCCACCTGCAAGA	CCTCCTTGGCGTAGTAGTCG	239	51
E-cadherin	GGAAGTCAGTTCAGACTCCAGCC	AGGCCTTTTGACTGTAATCACACC	290	49
Vimentin	AGATGGCCCTTGACATTGAG	TCTTGCGCTCCTGAAAAACT	345	55
uPA	GCAGGAACCCAGACAACCG	GACCCAGGTAGACGATGTAG	357	52
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA	452	52

washing twice with PBS, cells were lysed with 300 μL of lysis buffer (0.5% Triton X-100 in 0.1 mol/L Tris-HCl, pH 8.1). Cell lysates were centrifuged at 10,000 rpm for 15 min and protein concentrations were determined by BCA assay. uPA determination was assayed as described (32). Briefly, protein samples (10 μg) were subjected to SDS-PAGE (10%) under non-reducing conditions, and electrophoresis running gels were washed twice (30 min) in 2.5% Triton X-100. Then gels were laid over substrate gel composed of 1% agarose, 0.5% casein, 1.5 M Tris-HCl, pH 8.8, 1.0 M Tris HCl, pH 6.8, 10 mM $CaCl_2$ and 2 μg/mL plasminogen (Sigma-Aldrich). The gels were placed in a humidified atmosphere and incubated overnight at 37°C. Clear bands corresponding to uPA activity were detected and quantified.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

After specific cell treatments, indicated in Results, total RNA was obtained using TRIzol (Invitrogen) and complementary DNA was generated from 2 μg of RNA by RevertAid™ H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA), using oligo (dT) as a primer. The primer sets (Invitrogen) are described in Table 1. PCR products were obtained after 33 cycles of amplification with adjusted annealing temperature ranging from 49 to 55°C. Amplicons were resolved in 1.5% agarose gel and stained with ethidium bromide. GAPDH was amplified as a control for the amount of cDNA present in each sample.

Densitometry Analysis and Statistical Analysis

The intensity of the bands on gels obtained by Western blot, zymography and RT-PCR was quantified using ImageMaster TotalLab v1.11 software. Values are expressed as relative to control (given the value 1). Statistical significance was evaluated by Student's *t*-test, using the GraphPad Prism 5, with value $P < 0.05$ considered as significant.

Results

Inflammatory Cytokines Prime hASCs to Express Increased Levels of TGF-β1 and Activate TGF-β1 Signaling in MCF-7 Cells

Mesenchymal origin of isolated cells was confirmed by analysis of their immunophenotype and trilineage differentiation capacity. Positive expression of CD90, CD44, CD73, CD105 and negative expression of CD34 and HLA-DR in hASCs was observed (Fig. 1A). Also, hASCs showed ability for osteogenic, adipogenic and chondrogenic differentiation (Fig. 1B). Given that TGF-β is recognized as an immunosuppressive factor with dual role in cancer development, acting as tumor suppressor in early stages and as tumor promoter in late stages of cancer (33), we investigated whether inflammatory cytokines affect the expression of TGF-β1 in hASCs. We found that IFN-γ and/or TNF-α increased the expression of TGF-β1 both at mRNA and protein level in hASCs, as well as in their conditioned media (Figs. 1C and 1D) in comparison to non-primed hASCs. Additionally, we investigated the capacity of CM derived from hASCs to stimulate Smad3 signaling in MCF-7 cells, a canonical signaling pathway activated by TGF-β1 in tumor cells (34). Our results showed that CM derived from cytokine-primed hASCs (CM IFN/TNF, CM IFN, CM TNF) enhanced Smad3 phosphorylation (Fig. 2A), as well as pSmad3 nuclear localization in MCF-7 cells (Fig. 2B) when compared to MCF-7 cells cultivated in GM or CM 0. These results indicated the involvement of TGF-β1 in hASCs and MCF-7 cells crosstalk.

Conditioned Media from Inflammatory Cytokines-Primed hASCs Enhance EMT of MCF-7 Cells via TGF-β1

One of the hallmarks of tumor progression is the induction of EMT in epithelial cancer cells (18). Since MSCs were shown to have the capability to increase tumor malignancy (1), our next goal was to investigate whether CM from IFN-γ- and/or TNF-α-primed hASCs induce EMT in MCF-7 cells. To address this, we analyzed the expression of adherent junctions E-Cadherin, a marker for epithelial phenotype, and intermediate filament

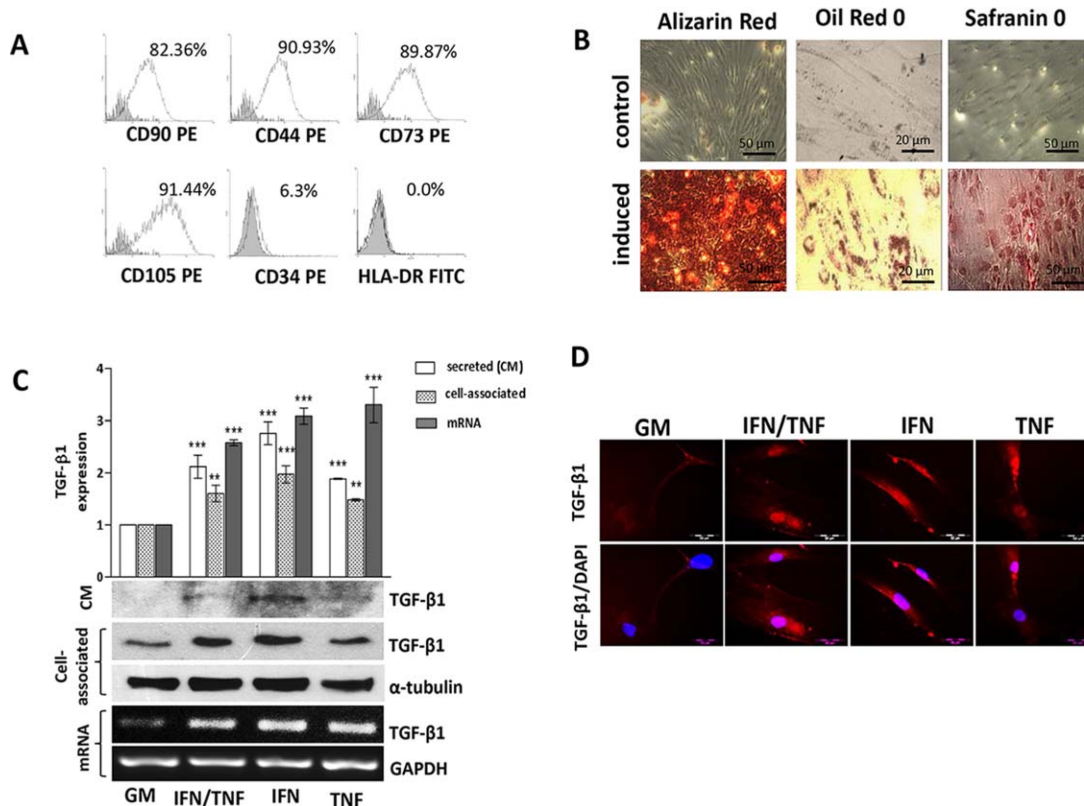

FIG 1 *Mesenchymal stem cells characteristics and expression of TGF-β1 in isolated hASCs. (A) Immunophenotype of hASCs, (B) osteogenic, adipogenic and chondrogenic differentiation potential of hASCs. (C) Confluent hASCs were treated with IFN-γ (50 ng/mL) or/and TNF-α (20 ng/mL) for 24 h and total proteins and mRNA were isolated or hASCs were subjected to immunofluorescence analyses. Conditioned media were collected as it is described in Materials and Methods. Relative TGF-β expression in hASCs was determined by Western blot and RT-PCR. α-tubulin and GAPDH were used as a gel loading controls for WB and RT-PCR, respectively. (D) Immunofluorescent labeling of TGF-β1 (red) in hASCs. Cell nuclei were labeled with DAPI (blue). Representative histograms, blots, gels and photos are shown. Graph represents TGF-β1 expression level (means±SEM from three experiments). Statistically significant changes in expression relative to control (GM): $**P < 0.01$, $***P < 0.001$.*

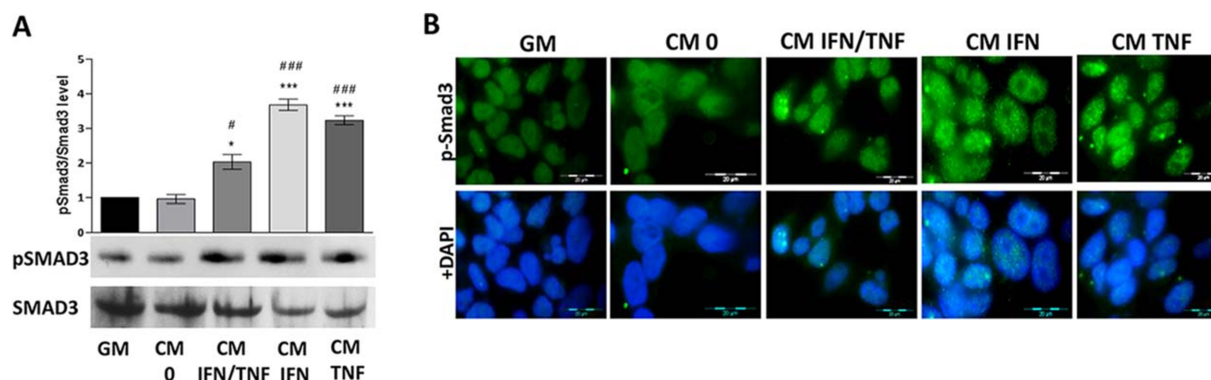

FIG 2 *CM derived from cytokines-primed hASCs enhanced Smad3 signalization in MCF-7 cells. MCF-7 cells were cultivated in GM or CM for 24h and total proteins were isolated or cells were subjected to immunofluorescence analyses. (A) Relative level of phosphorylated Smad3 was determined by Western blot. Smad3 was used as equal protein amount control. (B) Immunofluorescent labeling of phosphorylated Smad3 (green) in MCF-7 cells. Cell nuclei were labeled with DAPI (blue). Representative blots and photos are shown. Graph represents the pSmad3/Smad3 level (means±SEM for three independent experiments). Statistically significant changes in expression relative to control (GM): $*P < 0.05$, $***P < 0.001$, while relative to CM 0: $\#P < 0.05$, $\#\#\#P < 0.001$.*

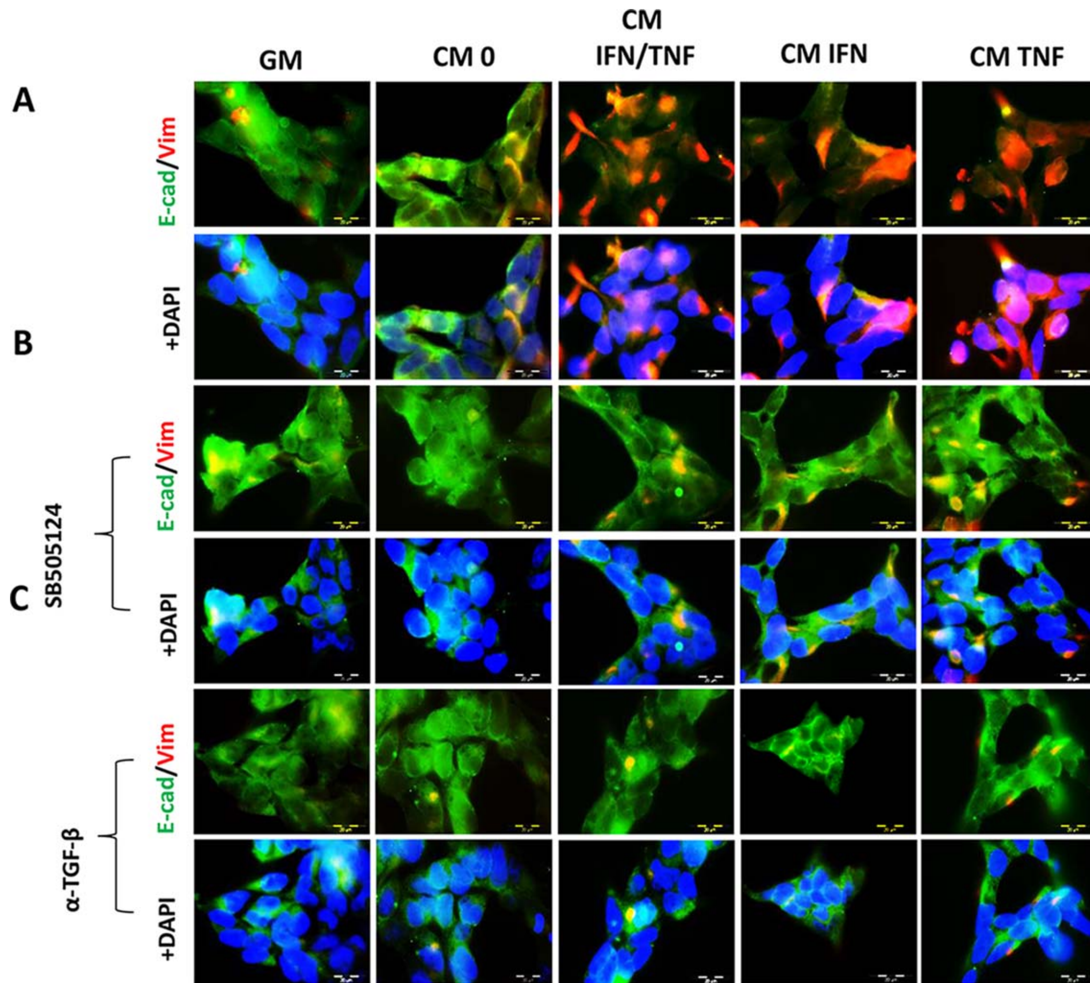

FIG 3 *CM derived from cytokines-primed hASCs induced epithelial to mesenchymal transition in MCF-7 cells. After cultivation in GM or CM for 6 days, MCF-7 cells were subjected to double-immunofluorescence labeling. Cells were incubated with primary antibodies mixture overnight at 4 °C. Expression of E-cadherin (green) and Vimentin (red) in MCF-7 cells cultivated in (A) GM or CM; (B) GM or CM in presence of SB505124 inhibitor (2 μM, Sigma-Aldrich); (C) GM/CM supplemented with neutralizing anti-TGFβ1 antibody (1 μg/mL, R&D Systems) 40 min prior to treatment of MCF-7 cells. Cell nuclei were labeled with DAPI (blue). Representative photos for at least three independent experiments performed in triplicate are shown.*

Vimentin, as a mesenchymal marker. Immunofluorescent labeling showed that CM from IFN-γ- and/or TNF-α-primed hASCs reduced E-Cadherin expression at sites of cell-cell contacts in comparison to cells grown in GM and CM 0, where E-Cadherin was localized mainly within intercellular contacts (Fig. 3A). At the same time, CM, derived from primed hASCs, enhanced Vimentin protein expression (Fig. 3A), thus giving MCF-7 cells a mesenchymal phenotype. Additionally, we demonstrated that indirect co-culture of MCF-7 and cytokines-primed hASCs stimulated EMT in MCF-7 cells, in comparison to MCF-7 cells cultivated alone or with unprimed hASCs (Supporting Information Fig. S1A).

These results were confirmed by Western blot analysis of total cell E-cadherin expression (Fig. 4A), while RT-PCR showed that CM from both primed and unprimed hASCs decreased E-cadherin mRNA expression in comparison to GM (Fig. 4B). Similarly, CM derived from primed hASCs, enhanced the Vimentin protein expression (Fig. 4C), while CM from both unprimed and primed hASCs increased Vimentin mRNA expression when compared to GM (Fig. 4D).

To investigate whether TGF-β1 was involved in the observed effects of CM of cytokines-treated ASCs on EMT in MCF-7 cells, we included a chemical inhibitor for type I TGF- β receptor (ALK5/TGF-βRI), SB505124, as well as neutralizing anti-TGF-β1 antibody in our next experiments. Results showed that 6 days cultivation of MCF-7 in CM derived from hASCs, in the presence of SB505124 (Fig. 3B) or anti-TGF-β1 antibody (Fig. 3C) failed to induce EMT in MCF-7 cells. Similar, presence of SB505124 abolished stimulatory effects of cytokines-primed hASCs on EMT of MCF-7 cells in indirect co-culture system (Supporting Information Fig. S1B). Also, SB505124 abolished the inhibitory effects of CM on E-cadherin protein and mRNA expression (Figs. 4A and 4B), as well as its stimulatory effects on Vimentin protein and mRNA expression

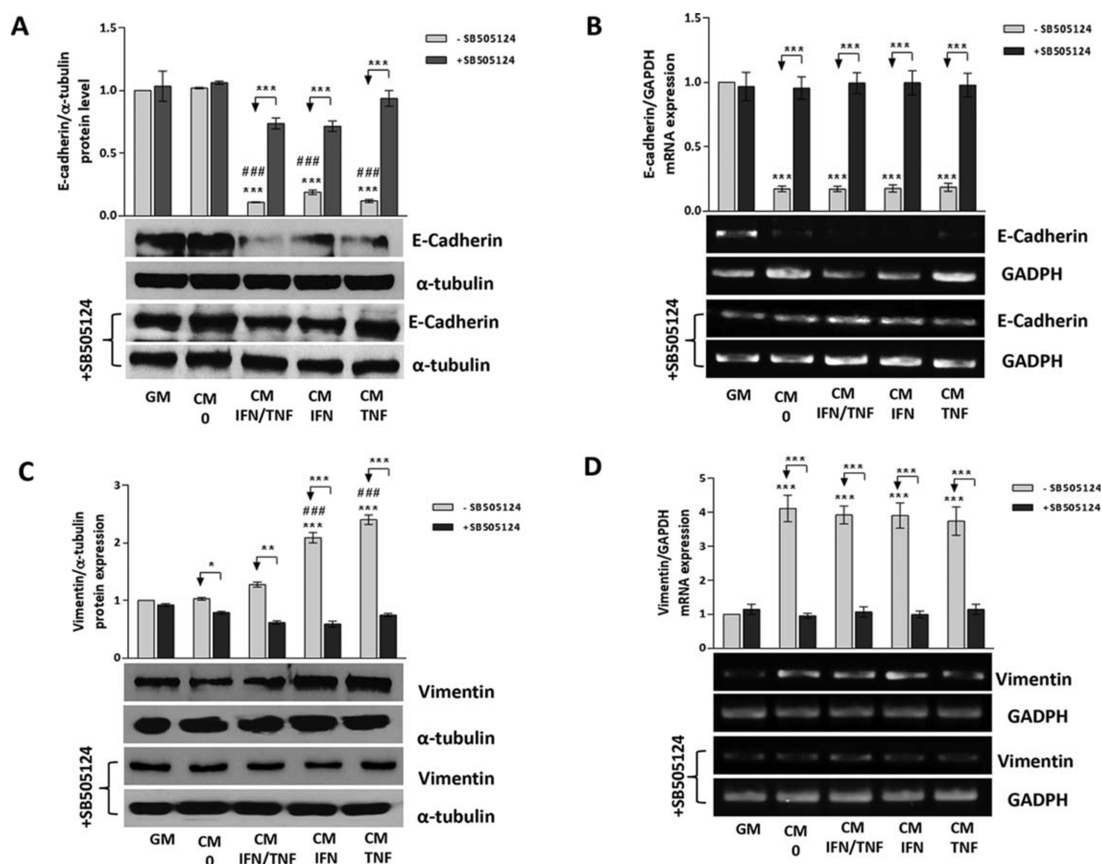

FIG 4 *Inhibition of TGF-β1 receptor reverted the effects of CM derived from hASCs on E-cadherin and Vimentin expression in MCF-7 cells. After cultivation of MCF-7 for 6 days in GM or CM, in the absence or presence of SB505124 (2 µM), (A, B) E-cadherin and (C, D) Vimentin protein and mRNA expression were detected by Western blot and RT-PCR. α-tubulin and GAPDH were used as a gel loading control for WB and RT-PCR, respectively. Representative immunoblots and gels are presented. Graphs represent E-cadherin or Vimentin expression level (means ± SEM for at least three experiments). Statistically significant changes in expression relative to control (GM w/o SB505124): *P < 0.05, **P < 0.01, ***P < 0.001, while relative to CM 0: ###P < 0.001.*

(Figs. 4C and 3D). These results indicate that inflammatory cytokines, via inducing TGF-β1 expression in hASCs, induce EMT of MCF-7.

Conditioned Media from Inflammatory Cytokines-Primed hASCs Enhance Invasion, Migration and uPA Expression in MCF-7 Cells via TGF-β1

With the increment of cell malignancy, cancer cells acquire mesenchymal phenotype that allows them to migrate and invade neighboring areas (35,36). We further analyzed whether CM from inflammatory cytokines-primed hASCs stimulate MCF-7 invasion and migration. In the invasion assay, CM derived from cytokine-primed ASCs increased the invasion capacity of MCF-7 cells, in comparison to GM and CM 0 (Figs. 5A and 5B). Addition of SB505124 inhibitor abolished stimulatory effects of CM on MCF-7 cells invasion capacity. Also, we demonstrated that in indirect co-culture, cytokines-primed hASCs stimulated invasion of MCF-7 cells in compare to MCF-7 cultivated alone or with unprimed hASCs, while presence of SB505124 abolished stimulatory effects of cytokines-primed hASCs (Supporting Information Figs. S2A and S2B). We further performed a wound healing assay, and, as shown in Figs. 5C and 5G, the CM from hASCs increased the capacity of cells to close wounded areas. Moreover, CM derived from cytokine-primed ASCs had higher stimulatory effect on migration of MCF-7 in comparison with CM 0. To determine whether the effects of CM are mediated by TGF-β1 present within, we investigated the impact of TGF-β1 receptor inhibition or neutralizing anti-TGF-β1 antibody, on the stimulation of MCF-7 cells migration. Results showed that presence of SB505124 inhibitor and anti-TGF-β1 antibody abolished the migration inducing effects of CM on tumor cells (Figs. 5D, 5E, and 5G). To investigate whether effects of ASCs-derived CM were mediated by uPA activity in MCF-7 cells, we further evaluated the migration capacity of these cells in the presence of BC-11 (inhibitor of uPA). Results showed that presence of uPA inhibitor BC-11 abolished the stimulatory effects of CM on MCF-7 cells migration (Figs. 5F and 5G). Similar, we demonstrated in indirect co-culture that hASC stimulated migration of MCF-7 cell in compare to control (MCF-7 alone), while the presence of SB505124 inhibitor abolished these effects (Supporting Information Figs. S2C and S2D).

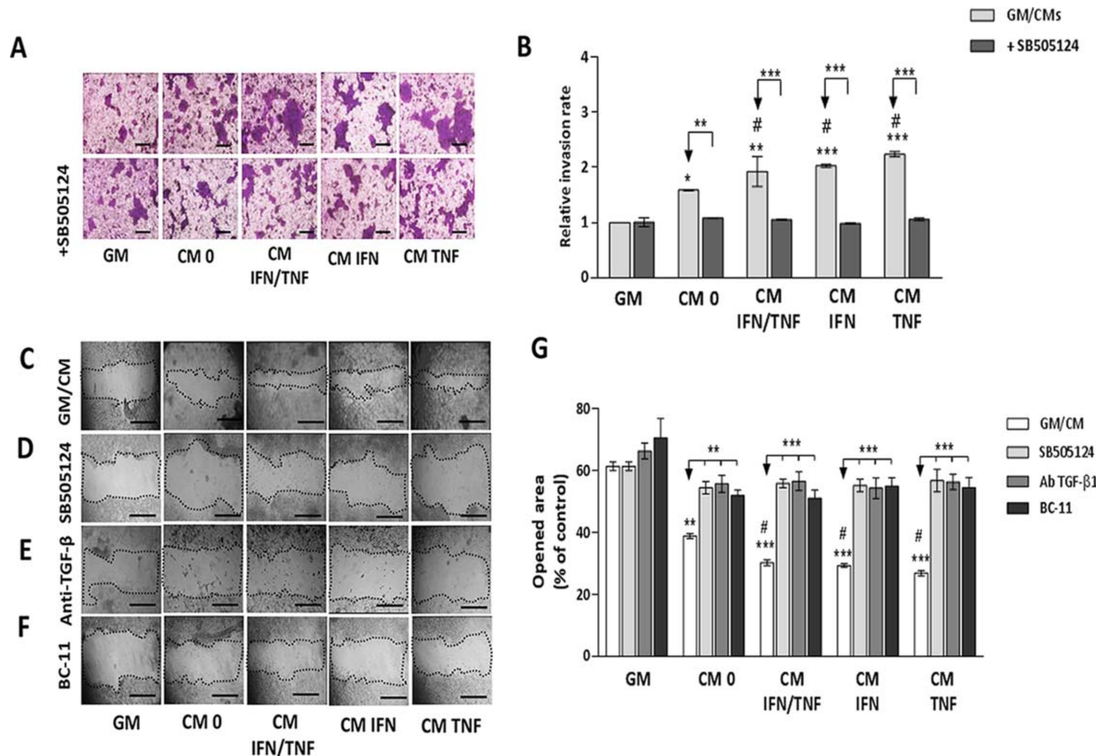

FIG 5 *CM derived from hASCs enhanced MCF-7 cells invasion and migration. Invasion and migration level were determined by transwell system and wound healing assay, respectively, after cultivating MCF-7 cells for 6 days. (A and B) Invasion rate of MCF-7 cells cultivated in GM or CM w/o SB505124. Scale bars 50 μm. Migration level of MCF-7 cells cultivated in (C) GM or CM; (D) GM or CM in the presence of SB505124 inhibitor (2 μM) (E) GM/CM supplemented with neutralizing anti-TGFβ1 antibody (1 μg/mL) 40 min prior to treatment of MCF-7 cells; (F) GM or CM in the presence of BC-11 (25 μM, Tocris Bioscience). Scale bars 100 μm. Representative photos are shown. Graphs show means±SEM for three independent experiments performed in triplicate. Statistically significant changes in invasion/migration rate relative to control (GM w/o SB505124 for invasion and GM at zero time T_0 for migration): *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, while relative to CM 0: #$P < 0.05$.*

As the expression of ECM proteinases, such as uPA, is essential for migration of tumor cells (37,38), we next determined whether changes in uPA expression occur in MCF-7 cells after treatment with CM. Furthermore, as TGF-β1 has been demonstrated as a potent inductor of uPA expression in cancer cells (33), we determined whether TGF-β1 is implicated in the enhancement of uPA expression by CM. We showed that increased uPA levels correlate with enhanced cell migration (Figs. 5 and 6). When compared with cells cultured in GM only, MCF-7 cells treated with all CM, showed higher expression of cell-associated uPA, while inhibition of TGF-β1 receptor on MCF-7 cells nullified the stimulatory effects of CM on cell-associated uPA activity (Fig. 6A). As shown for invasion and migration of MCF-7, CM 0 stimulates the activity of cell-associated uPA in MCF-7 cells, however, in lesser extent than CM IFN/TNF, CM IFN and CM TNF. Similarly, CM derived from cytokines-primed hASCs stimulated uPA protein expression, while inhibition of TGF- β1 receptor in MCF-7 abolished these effects (Fig. 6B). Obtained results also showed that all CM increased mRNA expression of uPA in MCF-7 cells and that inhibition of TGF- β1 receptor on MCF-7 cells abolished these effects (Fig. 6C). Taken together, these results confirmed the role of TGF- β1 and uPA in the effects of inflammatory cytokines-primed hASCs CM in enhanced invasion and migration capacity of MCF-7 cells.

Discussion

Adipose tissue is currently recognized as rich source of MSCs which, owing to their stem cell properties, self-renewal and differentiation ability, participate in tissue regeneration and repair. MSCs within mammary adipose tissue represent one of the major factors in mammary tissue development, but also in tumorigenesis and progression of diverse breast cancer types (39,40). Contrary to acute inflammation which has protective role in tissue homeostasis, chronic inflammation could be related to neoplastic processes and to all cancer development stages, thus contributing to the complexity of inflammation and cancer crosstalk (41–43). Inflammatory factors present in tumor microenvironment render MSCs to adopt an immunosuppressive phenotype, thus recruiting more tolerogenic types of lymphocytes, macrophages and myeloid-derived suppressor cells to the tumor site. However, how inflammatory conditions

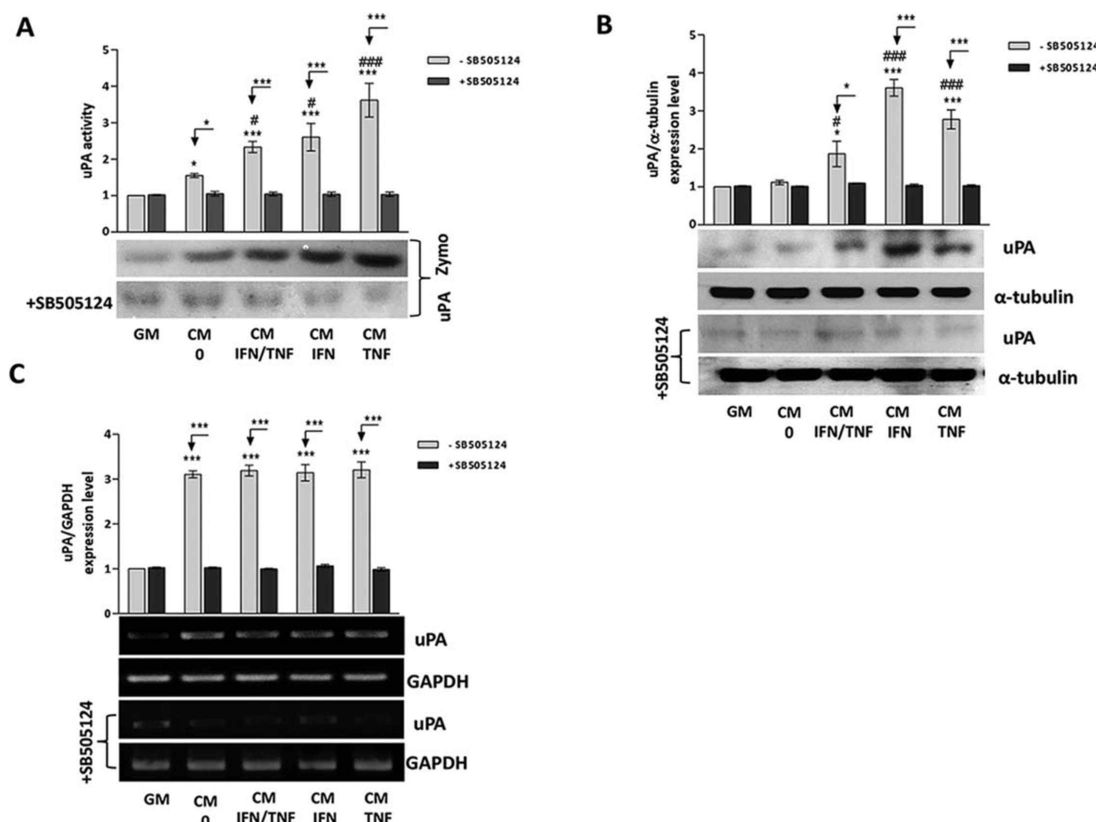

FIG 6 *Inhibition of TGF-β1 receptor nullified the effects of hASCs-derived CM on increased uPA expression in MCF-7 cells. MCF-7 cells were cultivated for 6 days in GM or CM in the presence or absence of SB505124. After treatment, zymography, Western blot and RT-PCR were performed to detect the level of (A) cell-associated enzyme activity, (B) protein expression and (C) mRNA expression, respectively, of uPA in MCF-7 cells. α-tubulin and GAPDH were used as gel loading controls for WB and RT-PCR, respectively. Representative blots and gels are shown. Graphs represent the activity or expression level of uPA (means ± SEM for at least three experiments). Statistically significant changes in expression relative to control (GM w/o SB505124): *P < 0.05, ***P < 0.001, while relative to CM 0 w/o SB505124: #P < 0.05, ###P < 0.001.*

affect the hASCs and cancer cells interplay is still not fully elucidated. Here, we provide results which demonstrate that proinflammatory cytokines (*i.e.* IFN-γ and/or TNF-α) act on hASCs in a way to promote expression and secretion of TGF-β1 into conditioned media, which further increases the malignancy of MCF-7 cells.

Pretreatment of MSCs with IFN-γ and TNF-α has previously been shown to upregulate TGF-β1 expression in bone marrow MSCs (44), while its association with promotion of tumor growth has been reported for IL-1α-treated mouse MSCs (45). In addition, previously reported expression of membrane-bound form of TGF-β1 in bone marrow MSCs induced by present colorectal tumor cells (46) provided evidence that TGF-β1 expression in MSCs could be regulated by present tumor cells. Our results are in accordance with mentioned results, adding to the diversity of MSCs which are affected by inflammatory cytokines in a similar way.

TGF-β in complex with its type I and type II receptors, leads to activation of Smad proteins, with an important role in the mammary development and tumorigenesis (33,47,48). We showed that CM from cytokines-primed hASCs induced phosphorylation and nuclear translocation of Smad3 in MCF-7 cells, thus supporting previous findings which showed that Smad3 phosphorylation can be induced by direct cell contacts/interactions between bone marrow MSCs and colorectal cancer cells (46). Since CM derived from IFN-γ and/or TNF-α-primed ASCs were capable to stimulate Smad3 activation in MCF-7 cells, it is plausible that, similar to its essential role in TGF-β1-induced effects in epithelial cells (34), Smad3 activation may reflect the capacity of TGF-β1, present in the CM, to stimulate EMT, cell migration and uPA expression in cancer cells. As dual role of TGF-β in breast cancer development depends on Smad3 mutations (47), further research would be necessary to elucidate the role of Smad3 activation in EMT and migration of MCF-7 cells.

TGF-β1 induces EMT-related phenotypic changes in epithelial cells, as characterized by reduced expression of epithelial markers, such as E-Cadherin, and increased expression of mesenchymal markers, such as Vimentin (34,46,49,50). Results of our study demonstrated that CM from IFN-γ and/or TNF-α-primed hASCs had the capacity to promote EMT of MCF-7 cells via TGF β1, along with lower E-cadherin and

higher Vimentin expression in these cells. These findings are in accordance with previous data which demonstrated the supporting role of human bone marrow MSCs in the EMT of hepatocellular carcinoma cells (31).

Our results demonstrated that CM derived from hASCs stimulated the migration and invasion capability of MCF-7 cells via TGF β1. CM derived from cytokines-primed hASCs possessed higher stimulatory effect on migration of MCF-7 cells than CM derived from non-primed hASCs, emphasizing the role of inflammatory conditions in the effects of MSCs on tumor development, which must be considered as crucial factors in the investigation of MSCs and tumor cells interaction. We found that CM derived from primed hASCs increased the production of uPA in MCF-7 cells via TGF β1. Although it was previously reported that CM from MSCs affect EMT of tumor cells via uPA-TGF β1 system (51), here we for the first time demonstrated that inflammatory cytokines enhanced capacity of hASCs-derived CM to increase motility and uPA expression in MCF-7 cells via TGF β1.

Interestingly, CM derived from non-primed hASCs (CM 0) modified the expression of E-cadherin, Vimentin and uPA at mRNA level in MCF-7 cells, while it failed to modify their expression at protein level, thus implying that inflammatory cytokines are responsible for modulation of these markers at protein level. All treatments applied, *i.e.* either IFN-γ or TNF-α alone, or their combination, induced comparable increase of EMT, migration and uPA expression and no additive or synergistic effects were observed with the combined treatments with these cytokines.

In summary, priming of hASCs with inflammatory cytokines enhances their ability to promote EMT, migration, invasion and uPA expression in MCF-7 tumor cells. The results indicate that all these features, which highly participate in breast cancer cell malignancy and metastasis, are mediated, at least in part, by TGF-β1, and point to the importance of the inflammatory tumor microenvironment in educating the hASCs to adopt the breast tumor-promoting characteristics.

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