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Macrophage-secreted TNFα and TGFβ1 Influence Migration Speed and Persistence of Cancer Cells in 3D Tissue Culture via Independent Pathways

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Abstract

The ability of a cancer cell to migrate through the dense extracellular matrix (ECM) within and surrounding the solid tumor is a critical determinant of metastasis. Macrophages enhance invasion and metastasis in the tumor microenvironment but the basis for their effects are not fully understood. Using a microfluidic 3D cell migration assay, we found that the presence of macrophages enhanced the speed and persistence of cancer cell migration through a 3D extracellular matrix in a matrix metalloproteinases (MMP)-dependent fashion. Mechanistic investigations revealed that macrophage-released TNFα and TGFβ1 mediated the observed behaviors by two distinct pathways. These factors synergistically enhanced migration persistence through a synergistic induction of NF-κB-dependent MMP1 expression in cancer cells. In contrast, macrophage-released TGFβ1 enhanced migration speed primarily by inducing MT1-MMP expression. Taken together, our results reveal new insights into how macrophages enhance cancer cell metastasis, and they identify TNFα and TGFβ1 dual blockade as an anti-metastatic strategy in solid tumors.

Introduction

Cancer cells are surrounded by a complex tumor microenvironment consisting of extracellular matrix (ECM), tumor-associated stromal cells, and a myriad of signaling molecules (1), which can significantly influence tumor growth and metastasis (2). ECM in the tumor microenvironment acts as a barrier to metastasis, and cancer cells have enhanced capabilities to navigate through the dense 3D collagen ECM surrounding the tumor (3). To migrate through the ECM, cancer cells employ proteases such as matrix metalloproteinases

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(MMPs) to degrade ECM, kinases to assist in forming protrusions, and integrins to adhere to the matrix to enable movement (4). Indeed, the activities and/or expressions of these molecules have been shown to be elevated in cancer cells (5–7).

Macrophages, one of the most abundant stromal cell types in the tumor microenvironment, are key promoters of tumor metastasis (8). Various clinical data have revealed that the infiltration of macrophages in tumor tissues correlates with poor prognosis in cases of breast cancer, prostate cancer, and melanoma (9,10). Moreover, *in vivo* and *in vitro* studies have shown that macrophages enhance cancer cell intravasation (11,12) and invasion through various signaling pathways (13,14). However, many of these *in vitro* migration studies were performed on 2D tissue culture substrates, which fail to capture the 3D microenvironment present *in vivo*. In addition, the majority of these studies were carried out using transwell assays, which only yield an end-point readout of cell behaviors (15) and thereby provide little information on how macrophages affect different aspects of cancer cell migration, such as how fast or how persistently the cancer cell moves. These distinct features of migration (speed vs. persistence) describe cell migration dynamics, and they can be quantified using metrics such as total speed and directedness. Total speed (the total distance that cell migrated divided by the migration time) defines how fast a cancer cell migrates. In contrast, directedness (the ratio of cell displacement to the total distance that the cell travelled) measures the persistence of the cell movement (15,16).

Recently, it has become increasingly clear that both migration speed and persistence determine the metastatic potential of a cancer cell (17), and stimuli that increase both of these factors can greatly enhance metastasis. More importantly, speed and persistence can be modulated independently of one another by a single stimulus. For example, inhibiting integrin has been shown to decrease cancer cell migration speed but has no effect on persistence (17). On the other hand, interstitial flow can increase cancer cell migration persistence without altering the speed of migration (18). Lending further complexity, a stimulus can affect speed and persistence of migration differently when cells are cultured on 2D substrates compared to in 3D matrix. Specifically, EGF has been shown to increase cancer cell migration speed and decrease persistence when cells are migrating on 2D surfaces. However, this same growth factor enhances both cancer cell migration speed and persistence when cells are cultured in 3D ECM (19). Collectively, these results highlight the importance of characterizing how a stimulus affects different aspects of cancer cell migration (i.e. speed and persistence) in 3D ECM to gain a detailed and quantitative understanding of metastasis. However, to our knowledge, the effects of macrophages on the **dynamics (speed and persistence)** of cancer cell migration in 3D ECM have not been explored.

In the present study, we employed a microfluidic 3D migration assay to examine how macrophages affect different aspects (speed and persistence) of cancer cell migration. This microfluidic assay allows us to perform real-time high-resolution imaging of cancer cells migrating in 3D collagen I ECM in the presence of macrophages, which recapitulates key aspects of their interactions in the primary tumor sites *in vivo*. By tracking the movement of cancer cells within the 3D ECM, we can evaluate the effects of macrophages on the dynamics of cancer cell migration in a more physiologically relevant environment than 2D

in vitro assays. In addition, this microfluidic assay is better suited for the detailed mechanistic study of macrophage-assisted cancer cell migration than *in vivo* assays (such as intravital imaging), as it is easier to operate and offers a tightly controlled experimental environment. Using this microfluidic assay, we show that macrophages release TNFα and TGFβ1 that increase both migration speed (total speed) and persistence (directedness) of cancer cells in 3D ECM. Interestingly, macrophage-released TNFα and TGFβ1 were found to promote cancer cell migration speed and persistence through two different mechanisms. Specifically, macrophages enhance cancer cell migration speed mainly through TGFβ1-induced MT1-MMP expression in cancer cells. In comparison, macrophage-released TNFα and TGFβ1 synergistically enhance cancer cell migration persistence via NF-κB-dependent MMP1 expression. These results demonstrate, for the first time, that speed and persistence of cancer cell migration in 3D can be modulated by macrophages via different pathways, which strongly suggests that both of these pathways need to be targeted to effectively mitigate macrophage-induced metastasis.

Methods

Cell culture and reagents

MDA-MB-231 human breast carcinoma cells expressing GFP (MDA231) were kindly provided by Dr. Frank Gertler, MIT. PC3 human prostate carcinoma cells (PC3), MDA-MB-435S human melanoma cells (MDA435), and Raw 264.7 mouse macrophages (Raw) were obtained from American Type Culture Collection. MDA231, MDA435, and Raw cells were cultured in DMEM. PC3 were cultured in RPMI. All media were supplemented with 10% fetal bovine serum (FBS), and 100 U/mL penicillin/streptomycin. Cell lines were authenticated using Short Tandem Repeat profiling (Promega).

To generate primary bone marrow-derived macrophages (BMDM), bone marrow cells were first isolated from the femurs of C57BL/6 mice. These cells were then differentiated in RPMI supplemented with 10% FBS, 1% HEPES, 40 ng/mL MCSF (Peprotech) and 50 μM β-Mercaptoethanol for 7 days to produce BMDM. Primary human monocyte-derived macrophages (MDMΦ) were generated from monocytes isolated from whole blood (Research Blood Component) using a Ficoll-Paque gradient and the EasySep™ Monocyte Enrichment Kit (StemCell Tech.). These cells were cultured with IMDM supplemented with 2% L-glutamine and AB serum for 7 days to generate MDMΦ. All cells were cultured in a humidified incubator at 5% CO_2 and 37 °C.

Microfluidic 3D cell migration assay

To quantify macrophage-assisted cancer cell migration in 3D ECM, a microfluidic cell migration assay was used (Fig. 1A and Fig. S1A in supplementary information, SI). This assay consists of a polydimethylsiloxane (PDMS) microfluidic device (20) with a collagen gel flanked by two micro-channels containing media. 2.3×10^6 cells/mL of cancer cells and/or 0.92×10^6 cells/mL of macrophages treated with Cell Tracker Red CMTPX were suspended in 2.5 mg/mL rat-tail collagen type I ECM (BD Bioscience) introduced to the central chamber of the device. For a detailed description of seeding protocols, see SI.

After overnight incubation, the microfluidic device was transferred to a fluorescent microscope (Zeiss) fitted with an environmental chamber operating at 37 °C and 5% CO_2. Time-lapse microscopy was employed to record cancer cell movement in the 3D collagen I ECM. Images were taken every 15 mins for 18 hrs.

ImageJ (NIH) was used to track cancer cell movement to produce cell migration trajectories. The migration trajectories were analyzed with Chemotaxis and Migration software (Ibidi) to quantify the dynamics of cell migration such as total speed and directedness. Total speed was calculated as total distance the cell travelled divided by migration time, while directedness was calculated as the displacement of the cell divided by total distance (Fig. S1B).

As appropriate, various concentrations of neutralizing antibodies, inhibitors, or recombinant growth factors (listed in SI) were added to the cell culture media in the device.

Real-time PCR and western blot analysis

Total RNA was extracted and purified using the RNeasy Mini Kit and RNase-free DNase Set (QIAGEN). Expression levels of mRNA were analyzed by real-time RT-PCR using High Capacity RNA-to-cDNA Kit and SYBR Green Master Mixture (Applied Biosystems). The sequences of the primers used can be found in SI. Data were analyzed according to the comparative Ct method and were normalized to GAPDH expression in each sample.

Cell lysate was extracted with RIPA buffer containing protease inhibitor and PMSF. Equal amount of total protein (30 μg) was resolved on 4–12% NuPAGE electrophoresis gels (Invitrogen) and transferred onto nitrocellulose membranes. The membranes were probed with various primary antibodies listed in SI, followed by secondary antibodies conjugated to horseradish peroxidase. The immunoreactive bands were detected with ECL Chemiluminescent substrates. Densitometry analysis was performed using Alpha Innotech software to quantify western blot images. The densitometry quantification for each protein was normalized to the appropriate loading control (β-actin, GAPDH, Lamin B1) before further normalized to the control group of each experiment.

Statistical analysis

All statistical analyses were performed using GraphPad Prism with a P-value of <0.05 considered statistically significant. The difference between groups was evaluated by two-tailed student t-test or one-way ANOVA. In all figures, ns represents not significant, * represents $p<0.05$, ** represents $p<0.01$, and *** represents $p<0.001$. For cell migration quantification, bars represent mean ± standard error of mean (SEM) of data from 40–100 cells from 3 independent experiments. For western blot and qRT-PCR quantification, bars represent mean ± SEM of data (fold increase relative to no-treatment or mono-culture control) from 3 independent experiments.

Results

Macrophages enhance cancer cell migration total speed and directedness in 3D ECM

To determine the effects of macrophages on the dynamics of cancer cell migration in 3D matrix, we tracked the movement of cancer cells inside the collagen I ECM in the microfluidic devices. We chose to use collagen I ECM to mimic the tumor matrix since collagen I has been shown to be the major component of tumor-associated stromal tissue (3,21) and it has also been implicated in metastasis (22). From the cell tracking, we quantified cancer cell migration total speed and directedness (defined in Fig. S1B), and we compared the migration dynamics of cancer cells cultured alone to that of cancer cells co-cultured with Raw macrophages (Fig.1B and Fig. S1C). We found that Raw macrophages significantly enhanced cancer cell migration total speed and directedness (Fig. 1C–D) in 3D ECM for MDA-MB-231, PC3, and MDA-MB-435S cells. Similar to Raw macrophages, primary macrophages such as human MDMΦ and murine BMDM were also observed to increase cancer cell migration total speed and directedness (Fig. 1E–F) in 3D ECM. These results indicate that macrophages allow cancer cells to move faster and more persistently, contributing to increases in cancer cell invasion rate (ratio between the displacement of cell and migration time), which is an end-point measurement of cell invasiveness (Fig. S2). These results are in stark contrast to results obtained from a 2D migration assay, where we found that macrophages only slightly enhanced cancer cell migration total speed but markedly reduced cancer cell migration directedness (Fig. S2F). Moreover, the abilities of macrophages to enhance cancer cell migration dynamics in 3D ECM were not affected by the seeding ratio of the cells or the addition of Matrigel into the collagen I ECM (Fig. S2G–H). Hence, these results suggest that there are fundamental differences in how macrophages affect cancer cell migration on 2D substrates versus in 3D ECM.

Macrophage-induced cancer cell migration in 3D ECM is mediated via cancer cell MMP expression

Next, we investigated the molecular mechanisms that control how fast and how persistently the cancer cell migrates in 3D ECM. We hypothesized that MMPs produced by cancer cells are involved, since the migration of cells in the dense 3D matrix critically depends on their ability to degrade ECM (4,19). To test this hypothesis, we treated MDA231 cancer cells with a pan-MMP inhibitor GM6001. We found that inhibiting MMP activities in cancer cells significantly reduced cancer cell migration total speed and directedness (Fig. 2B–C). Further evidence for the role of MMPs was obtained using confocal reflectance microscopy, which revealed that the migration of MDA231 cells in ECM produced micro-tracks of empty space (Fig. 2A). However, when these cells were treated with GM6001, the formation of cell protrusions, as well as the ability of cells to degrade ECM, was reduced compared to control samples (Fig. S3A–B). These results illustrate that cancer cells migrate in our experimental system in an MMP-dependent fashion, and the production of MMPs is a critical determinant of cancer cell migration dynamics (total speed and directedness) in 3D ECM.

Based on these findings, we examined the role of macrophages in regulating MMP1 and MT1-MMP expression by cancer cells. We chose to study these two MMPs since these proteases are responsible for the breakdown of collagen I matrix. Moreover, MT1-MMP and

MMP1 have been shown to be present in the tumor microenvironment, and they have been implicated in tumor metastasis (23–27). To study how macrophages influence MMP expressions in cancer cells, we co-cultured cancer cells with macrophages in a transwell system, and assessed the cancer cell expression of MMP1 and MT1-MMP via western blotting. We found that co-culture of MDA231 cancer cells with Raw macrophages, as well as BMDM, significantly enhanced cancer cell expression of MMP1 and MT1-MMP (Fig. 2D–E and S3C). This result was reproduced in PC3 prostate cancer cells co-cultured with macrophages (Fig. S3D).

Next, we tested whether it is necessary for macrophages to be in direct physical contact with cancer cells to promote migration. Instead of culturing Raw macrophages together with MDA231 cancer cells in the ECM, we cultured macrophages in the micro-channels flanking the ECM (Fig. S4A). The macrophages seeded in the micro-channels were not in physical contact with the ECM or the cancer cells, but they were able to communicate with the cancer cells via the secretion of paracrine factors. Interestingly, we found that macrophages cultured in the micro-channel increased cancer cell migration total speed and directedness to the same degree as macrophages cultured in the collagen ECM (Fig. S4B), suggesting that direct contact between macrophages and cancer cells is not necessary to enhance cancer cell migration. We also found that the conditioned media from Raw macrophages and BMDM significantly up-regulated the expression of MMP1 and MT1-MMP (Fig. S4C–G) in cancer cells. These results indicate that the effects of macrophages on cancer cell migration dynamics and MMP expressions are mediated primarily through paracrine factors secreted by macrophages.

Macrophage-released TNFα and TGFβ1 are responsible for the increases in cancer cell migration total speed and directedness

We next performed experiments to identify the paracrine factors released by macrophages that were responsible for the increases in cancer cell migration dynamics. We hypothesized that TNFα and TGFβ1 secreted by macrophages are involved in promoting cancer cell migration, since these two factors are major secretory products of macrophages in the tumor microenvironment (28–31), and they have been implicated in tumor metastasis (32,33). Indeed, primary macrophages such as MDMΦ and BMDM have been shown to secrete TNFα and TGFβ1 (34–37). We first verified, using ELISA, that Raw macrophages used in our study also secreted TNFα and TGFβ1 (Fig. S4H). To test our hypothesis further, we treated cancer cell-macrophage co-culture with neutralizing antibodies against TNFα and/or TGFβ1, and measured cancer cell migration total speed and directedness as before. The antibodies used in this study were designed to act against mouse TNFα and TGFβ1. This allowed us to specifically inhibit TNFα and TGFβ1 secreted by Raw 264.7 mouse macrophages. Antibody blocking results showed that neutralizing TNFα in co-culture slightly decreased macrophage-enhanced cancer cell migration total speed, while blocking TGFβ1 almost completely abrogated the effects of macrophages on total speed (Fig. 3A–B). Co-blocking both TNFα **and** TGFβ1 did not further reduce cancer cell migration total speed when compared to the blocking of only TGFβ1 (Fig. 3C). These results suggest that TGFβ1 is primarily responsible for the ability of macrophages to enhance cancer cell migration total speed.

Surprisingly, when we assessed the effects of antibody blocking on cancer cell migration directedness, we found that inhibiting either TNFα **or** TGFβ1 in co-culture did not lead to significant decreases in cancer cell migration directedness (Fig. 3D–E). In contrast, blocking TNFα **and** TGFβ1 simultaneously almost completely abolished the ability of macrophages to promote migration directedness (Fig. 3F), suggesting that both TNFα **and** TGFβ1 are important to macrophage-enhanced migration directedness. Interestingly, this result is in contrast to the antibody blocking results for total speed, which seems to suggest that macrophage-enhanced cancer cell migration total speed and directedness are controlled by two different pathways. Finally, to verify if blocking antibody treatments were specific to macrophage-secreted TNFα and TGFβ1, we treated cancer cell monocultures with anti-mouse neutralizing antibodies that we used in co-culture experiments. We found this to have no significant effect on MDA231 cell migration total speed and directedness (Fig. S5), indicating that the antibody inhibition was macrophage-specific.

We next demonstrated that co-blocking of both TNFα and TGFβ1 in co-culture resulted in almost complete inhibition of macrophage-induced MMP1 and MT1-MMP protein expression in cancer cells (Fig. S6A–B). These results further support our previous conclusion that macrophage-enhanced cancer cell migration total speed and directedness in 3D ECM are controlled by MMPs. Finally, as expected, since both migration total speed and directedness contribute to cancer cell invasion rate (Fig. S2), blocking of TNFα **or** TGFβ1 cannot completely abrogate macrophage-enhanced cancer cell invasion rate. In contrast, when both macrophage TNFα and TGFβ1 were inhibited, cancer cell invasion rate in co-culture was reduced to the level of the cancer cell monoculture control (Fig. S6C).

Macrophage-released TGFβ1 enhances cancer cell migration total speed via MT1-MMP, while macrophage-released TNFα and TGFβ1 synergistically increase cancer cell migration directedness via MMP1

We then proceeded to examine the detailed mechanisms by which macrophage-released TNFα and TGFβ1 affect cancer cell migration dynamics. We also sought to elucidate the seemingly distinct pathways that are involved in promoting migration total speed and directedness. Since it is difficult to perform a detailed and well-controlled study on molecular mechanism with blocking antibodies alone, we elected to treat cancer cell monocultures with TNFα and/or TGFβ1 and assess the resulting cell migration dynamics and MMP expressions. We found that the treatment of MDA231 cancer cell with TNFα slightly increased cancer cell migration total speed, while TGFβ1 treatment significantly enhanced total speed. No additional increase in migration total speed was observed for TNFα and TGFβ1 co-treatment over the TGFβ1 mono-treatment condition (Fig. 4A). These results parallel the blocking antibody experiments and further support our prior conclusion that macrophage-released TGFβ1 is the main contributor to the increase in cancer cell migration total speed.

In contrast to its effects on total speed, TNFα or TGFβ1 mono-treatment did not significantly enhance cancer cell migration directedness. When the cancer cells were treated with both TNFα and TGFβ1, however, there was a synergistic increase in cancer cell migration directedness that cannot be explained by the additive effects of TNFα and TGFβ1

mono-treatment (Fig. 4D). Combined, these results provide further evidence that macrophage-induced cancer cell 3D migration total speed and directedness are controlled by two distinct mechanisms. Specifically, cancer cell migration total speed is controlled primarily by macrophage-released TGFβ1, while the directedness is controlled by the combined effects of macrophage-released TNFα and TGFβ1. Finally, we found that co-treatment of cancer cell monoculture with TNFα and TGFβ1 led to levels of migration total speed and directedness (Fig. S7) comparable to those in co-culture, indicating that TNFα and TGFβ1 from macrophages are, indeed, the main factors responsible for the enhancement in cancer cell migration.

For further verification that cancer cell migration total speed and directedness are controlled through two distinct pathways, we varied the concentration of TNFα and TGFβ1 in the co-treatment condition. Specifically, we treated MDA231 cancer cells with 5 ng/mL TNFα + 0.5 ng/mL TGFβ1, or 0.5 ng/mL TNFα + 5 ng/mL TGFβ1, or 5 ng/mL TNFα + 5 ng/mL TGFβ1. Interestingly, treating cancer cells with a low concentration of TGFβ1 (0.5 ng/mL), even in the co-treatment conditions, resulted in slight or no increase in the migration total speed (Fig. S8A). These results further illustrate that cancer cell migration total speed is mainly controlled by TGFβ1. In comparison, treating cancer cells with various concentrations of TNFα or TGFβ1 in the co-treatment regimen resulted in similar levels of increase in cancer cell migration directedness over the no-treatment control. Moreover, addition of even a minute amount (0.5 ng/mL) of TGFβ1 to TNFα mono-treatment resulted in sharp increases in cancer cell migration directedness. A similar response was observed when a minute amount of TNFα (0.5 ng/mL) was added to TGFβ1 mono-treatment (Fig. S8B). These results further verify that TNFα and TGFβ1 synergistically enhance cancer cell migration directedness.

Since cancer cell migration in 3D ECM depends on the cell's ability to express MMPs, it seemed that the effects of TNFα and TGFβ1 on cancer cell migration dynamics might also be mediated through MMPs. To test for this hypothesis, we treated MDA231 monoculture with TNFα and/or TGFβ1 and evaluated the resulting MMP1 and MT1-MMP mRNA and protein expression. We found that the treatment of cancer cells with TNFα resulted in a slight increase in MT1-MMP mRNA and protein expression. In comparison, the treatment of cells with TGFβ1 alone markedly enhanced MT1-MMP mRNA and protein expression, while co-treatment of both TNFα and TGFβ1 led to no further increase in MT1-MMP expressions (Fig. 4B–C). We noted that these trends in the increases in MT1-MMP mRNA and protein expressions match the trend in the increases in cancer cell migration total speed (Fig. 4A). This observation points to the possibility that TGFβ1-induced increase in cell migration total speed is mediated mainly via MT1-MMP. Furthermore, we observed that TNFα and TGFβ1 synergistically enhanced cancer cell expression of MMP1 mRNA and protein (Fig. 4E–F). These findings are similar to the observation that TNFα and TGFβ1 synergistically promote cancer cell migration directedness (Fig. 4D), suggesting that TNFα/TGFβ1-induced cancer cell migration directedness is mediated mainly by MMP1 expression. Indeed, Pearson correlation analysis revealed that MT1-MMP expression levels resulting from TNFα and/or TGFβ1 treatments strongly correlate with cancer cell migration total speed, but not directedness Similarly, MMP1 expression levels in cancer cells strongly correlate with migration directedness, but not total speed (Fig. S9). These results led us to

hypothesize that macrophage-induced cancer cell migration total speed is controlled by MT1-MMP expression in cancer cells, while the directedness is controlled by MMP1 expression.

To test whether or not macrophage-induced cancer cell migration total speed and directedness are controlled by two different MMPs, we treated cancer cell-macrophage co-culture with blocking antibodies against MT1-MMP and MMP1. We found that treating the co-culture with anti-MT1-MMP antibody resulted in a significant decrease in cancer cell migration total speed with little effect on directedness (Fig. 5A–B). In contrast, we observed that blocking MMP1 in co-culture with anti-MMP1 antibody had almost no effect on macrophage-induced increase in cancer cell migration total speed, while the increase in cancer cell migration directedness was significantly reduced (Fig. 5C–D). Furthermore, we treated cancer cell monoculture with exogenously supplied recombinant MMP1 and observed an enhancement in cancer cell migration directedness but no significant change in migration total speed (Fig. 5E–F). These findings, coupled with previous observations that macrophage-released TNFα and TGFβ1 up-regulated cancer cell expression of MMPs (Fig S6), strongly support the conclusion that macrophage-induced MMP1 expression is responsible for the increase in cancer cell migration directedness, while the induction of MT1-MMP expression is responsible for the increase in total speed. Taken together, these results (Fig. 3–5) demonstrate that macrophages influence cancer cell migration in 3D ECM via two different mechanisms: 1) macrophage-released TGFβ1 increase cancer cell migration total speed (speed) via the up-regulation of MT1-MMP expression, and 2) macrophage-released TNFα and TGFβ1 synergistically enhance cancer cell migration directedness (persistence) through the induction of MMP1 expression. Hence, these results strongly suggest that both of these two pathways need to be inhibited in order to effectively reduce metastasis. Indeed, using a 4T1 orthotopic breast tumor model in BALB/c mice, we found that inhibiting both TNFα and TGFβ1 in these mice resulted in a more significant reduction in lung metastasis formation compared to inhibiting TNFα or TGFβ1 alone (Fig. S10).

Finally, we found a similar synergistic response in MMP1 secretion due to TNFα and TGFβ1 co-treatment (Fig. S11A), which mirrors the results of cancer cell migration directedness (Fig. 4D). The synergistic induction in MMP1 protein production was also observed in MDA435 and PC3 cells (Fig. S11B–C).

TNFα and TGFβ1 synergistically increase nuclear localization of NF-κB

To further understand the synergistic effects of TNFα and TGFβ1 on the expression of MMP1 in cancer cells, we tested whether TNFα and/or TGFβ1 could alter the expression or nuclear localization of NF-κB, a transcription factor for MMP1 (38). We first treated MDA231 cancer cells with TNFα and/or TGFβ1 for 48 hrs, and found that these two factors did not change the protein production of NF-κB by cancer cells (Fig. 6A). We then tested whether the treatment of these two factors could alter the nuclear localization of NF-κB. Indeed, co-treatment of TNFα and TGFβ1 synergistically enhanced NF-κB expression inside the nucleus of the cancer cells (Fig. 6B–C). These results support the conclusion that TNFα and TGFβ1 act together to enhance the expression of MMP1 via the synergistic

induction of NF-κB nuclear translocation. Similar results were also observed in MDA435 cells (Fig. S12).

Discussion

Macrophages in the tumor microenvironment are key promoters of cancer cell metastasis (8), suggesting that the control of these cells and their released factors can be a viable strategy in treating metastasis. Yet, it is still unclear how macrophages affect different aspects of cancer cell migration, such as speed and persistence, especially in 3D ECM that closely mimics the *in vivo* tumor microenvironment. To address this gap in knowledge, we utilized a microfluidic 3D cell migration assay that allows us to study, in high resolution, the effects of macrophages on cancer cell migration speed (total speed) and persistence (directedness) in 3D ECM.

From our study, we found that macrophages increase cancer cell migration speed and persistence in 3D collagen I ECM, suggesting that macrophages may help cancer cells invade and gain access to intravasation sites more efficiently. In contrast to the 3D results, we discovered that on 2D tissue culture plastic, macrophages tend to increase cancer cell migration speed but decrease persistence, so that cancer cells move faster, but more randomly. This disparity, similar to results obtained from previous works, illustrates a fundamental difference in how cancer cells migrate in 2D compared to 3D (19). We also note that the cancer cells in our 3D microfluidic system migrated at a total speed of 5–11 μm/hr, a value which closely matches the speed values obtained from *in vivo* intravital imaging experiments (39). In sum, these results demonstrate the advantages of our microfluidic assay, which allows us to perform physiologically relevant studies with precise control of experimental conditions.

In 3D, cell migration critically depends on the ability of cancer cells to degrade ECM. Indeed, MMP expression is dispensable for 2D cell migration, but not for 3D (19). In the present study, we showed that macrophages enhanced cancer cell migration in 3D ECM via the up-regulation of MMP expression in cancer cells. We further identified that macrophage-released TGFβ1 increased cancer cell migration speed, while macrophage-released TNFα and TGFβ1 synergistically enhanced cancer cell migration persistence. Previous studies have shown that EGF released by macrophages can promote cancer cell migration (14). Here, we report that macrophage-released TNFα and TGFβ1 can also promote cancer cell migration. The clinical relevance of this finding is demonstrated by the fact that the expression levels of TNFα and TGFβ1 in tumor-associated macrophages correlates with metastasis for human tumors (28,40). Moreover, this study, to our knowledge, is the first to report that macrophage-released TNFα and TGFβ1 control different aspects of cancer cell migration (speed vs. persistence) differently. Thus, although prior studies have implicated TNFα and TGFβ1 in cancer cell invasion and metastasis (32,33), our results now demonstrate subtle but important differences in their effects on cancer cell migration.

We also found that TGFβ1 released by macrophages promotes cancer cell migration speed through up-regulation of MT1-MMP. We suspect this is due to the fact that MT1-MMP can influence cell intrinsic migration behaviors as well as cell extrinsic matrix properties, both of

which are known determinants of cell migration speed in 3D matrix (19,41). Two examples of cell intrinsic behaviors that control cell migration speed are the activities of kinases and the expression of integrin. It has been shown that intermediate levels of integrin α2β1 contribute to an optimum cell migration speed (42,43); and inhibiting integrin could lead to a decrease in cell migration speed, but not persistence (17). Similarly, inhibiting Akt/PI3K activities in cells has been reported to result in a decrease in migration speed (44). In addition to cell intrinsic properties, cell extrinsic matrix properties, such as the pore size of the matrix that can be modified by MMPs, also affect cell migration speed in 3D (41). Unlike MMP1, which primarily degrades collagen I matrix to alter the cell extrinsic properties, MT1-MMP modifies both cell intrinsic and cell extrinsic properties. Besides degrading collagen I matrix, MT1-MMP can process integrin (45), mediate Akt phosphorylation (46), and promote syndecan shedding (47), all of which are parts of cell intrinsic pathways of migration. Hence, since both intrinsic and extrinsic properties control cell migration speed, it stands to reason that MT1-MMP should be the major determinant of migration speed over MMP1.

We further demonstrated that macrophage-enhanced cancer cell migration persistence, in contrast to total speed, was mediated primarily by the expression of MMP1, but not MT1-MMP. This result may be explained by the fact that MMP1 is more efficient in degrading collagen I matrix and altering extrinsic matrix properties (pore size) than MT1-MMP (48). Although cell intrinsic properties (such as Rac activities (49)) control migration persistence in 2D, it has been reported that cell extrinsic matrix properties seem to dominate over intrinsic property as the primary determinant of 3D migration persistence (19,50). Since MMP1 is more efficient in degrading collagen I ECM and altering extrinsic matrix properties than MT1-MMP, MMP1 should therefore be a major contributor to migration persistence.

Based on our findings, we propose a novel mechanism whereby macrophages promote cancer cell migration speed (total speed) and persistence (directedness) via two distinct mechanisms (Fig. 7). First, macrophage-released TNFα and TGFβ1 synergistically induce nuclear translocation of NF-κB in cancer cells, leading to synergistic increases in the expressions of MMP1, which result in a synergistic enhancement in cancer cell migration persistence. In contrast to the mechanism for the persistence, macrophages increase cancer cell migration speed, mainly through TGFβ1, by the up-regulation of cancer cell MT1-MMP expression. These findings establish that TNFα and TGFβ1 released by macrophages influence speed and persistence of cancer cell migration differently, and both of these factors need to be targeted to effectively inhibit macrophage-assisted cancer cell 3D migration and metastasis. Moreover, these findings also broaden our current view on the molecular determinants of 3D migration, suggesting that MT1-MMP primarily controls cell migration speed, whereas MMP1 mainly controls migration persistence. In conclusion, our findings provide new insights into macrophage-assisted cancer cell migration in 3D tumor microenvironment, and these could ultimately lead to novel therapeutic strategies to effectively inhibit tumor invasion and metastasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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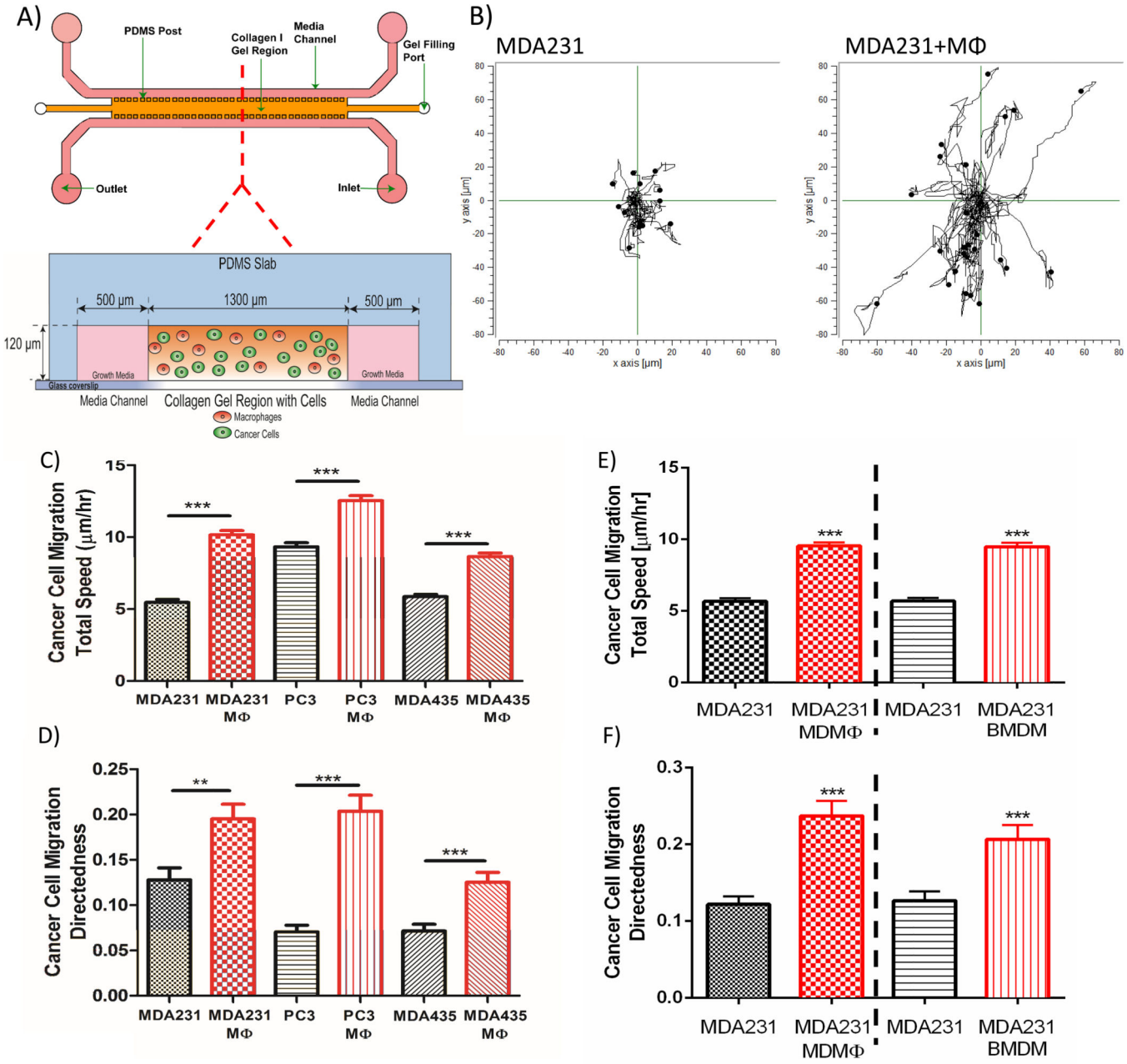

Figure 1. Macrophages enhance cancer cell migration total speed and directedness in 3D ECM

(A) Schematics of the microfluidic device. Cancer cells and macrophages were suspended in 3D collagen I ECM (orange) encased in the device. **(B)** Representative MDA-MB-231 cancer cell (MDA231) migration trajectories for cancer cell monoculture (left) and cancer cell-Raw 264.7 macrophages (MΦ) co-culture (right). **(C and D)** Co-culture of Raw macrophages (MΦ) with cancer cells significantly enhanced cancer cell migration total speed (C) and directedness (D) for MDA231 cells, PC3 prostate cancer cells, and MDA-MB-435S melanoma cells (MDA435). **(E and F)** Co-culture of primary human monocyte-derived macrophages (MDMΦ), as well as murine bone marrow-derived macrophages

(BMDM), with MDA231 cells enhanced migration total speed (E) and directedness (F) of MDA231 cells.

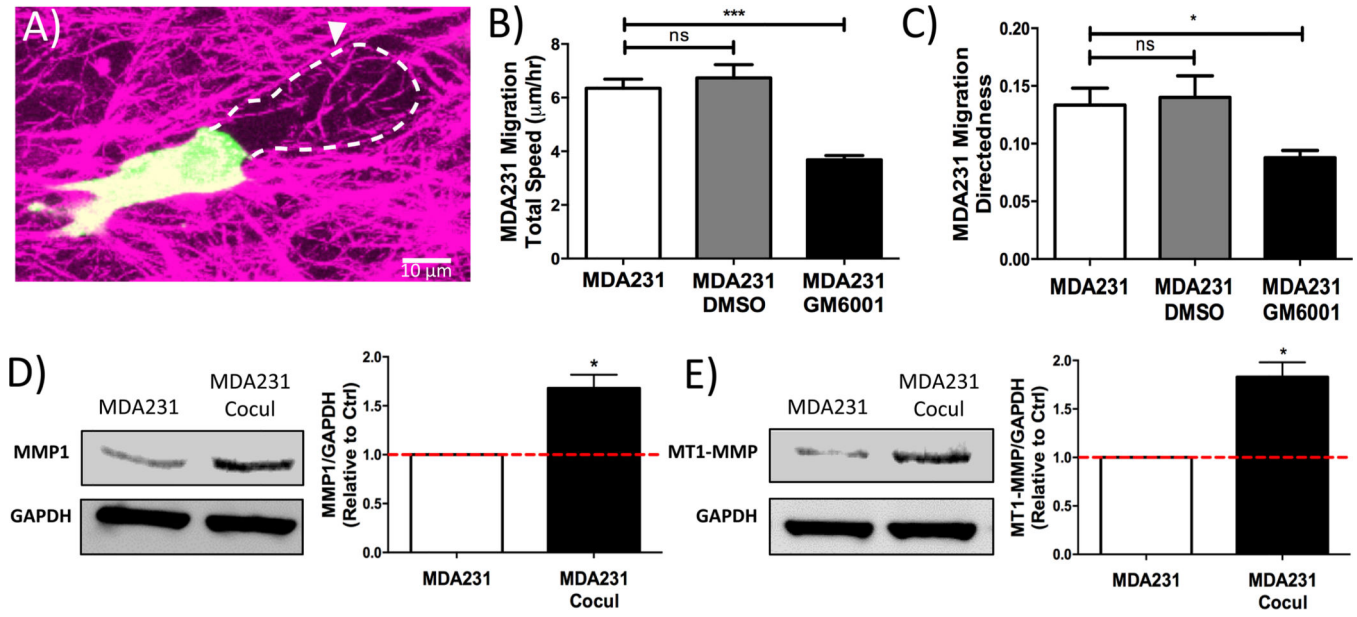

Figure 2. Cancer cell migration speed and directedness are MMP-dependent, and macrophages enhance cancer cell MMP expression

(A) Representative confocal image showing MDA231 cells (green) migrating through dense collagen I ECM (magenta) by degrading the matrix, leaving behind a micro-track (arrow). (**B and C**) Compared to the untreated and DMSO controls, inhibition of MMP activity by GM6001 significantly reduced MDA231 migration total speed (B) and directedness (C). **(D and E)** Representative western blot images (left) and quantification (right) showing that co-culture of Raw macrophages with MDA231 cells (MDA231 Cocul) in 3D collagen I gels significantly enhanced the expression of MMP1 (D) and MT1-MMP protein (E) in MDA231 relative to monoculture control (MDA231, Ctrl=Control).

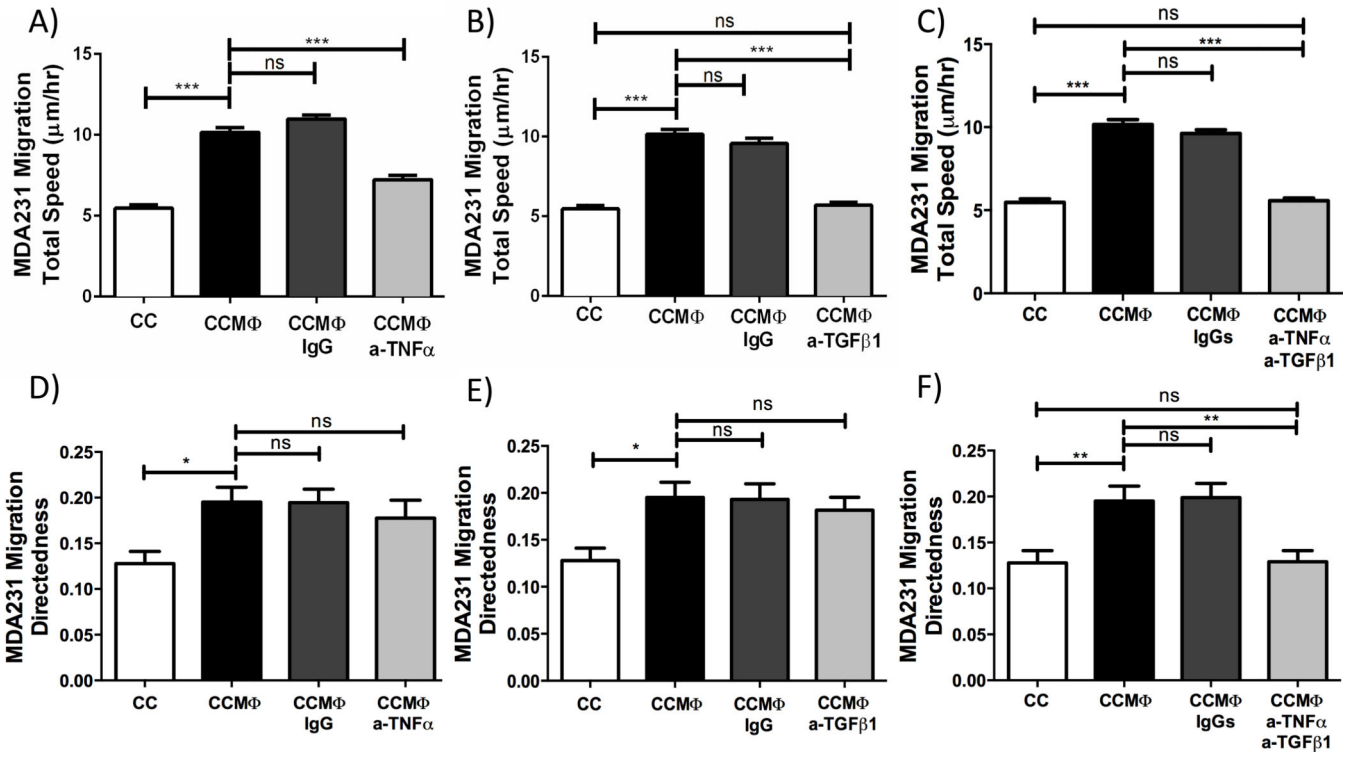

Figure 3. Macrophage-released TNFα and TGFβ1 are responsible for the increase in cancer cell migration total speed and directedness

MDA231 cancer cells (CC) co-cultured with Raw cells (MΦ) were treated with neutralizing antibodies against TNFα (a-TNFα) and/or TGFβ1 (a-TGFβ1). **(A, B, and C)** Neutralizing TNFα released by macrophages (CCMΦ a-TNFα) led to a decrease in MDA231 migration total speed compared to no-treatment control (CCMΦ) (A). However, inhibiting macrophage-released TGFβ1 (CCMΦ a-TGFβ1) led to an almost complete inhibition of macrophage's effect on MDA231 migration total speed (B), similar to the simultaneous inhibition of both TNFα and TGFβ1 (C). **(D, E, and F)** Neutralizing macrophage-released TNFα (CCMΦ a-TNFα) or TGFβ1 (CCMΦ a-TGFβ1) alone did not significantly reduce MDA231 migration directedness (D and E). However, simultaneous inhibition of both TNFα and TGFβ1 led to an almost complete abolishment of macrophage-enhanced MDA231 migration directedness (F).

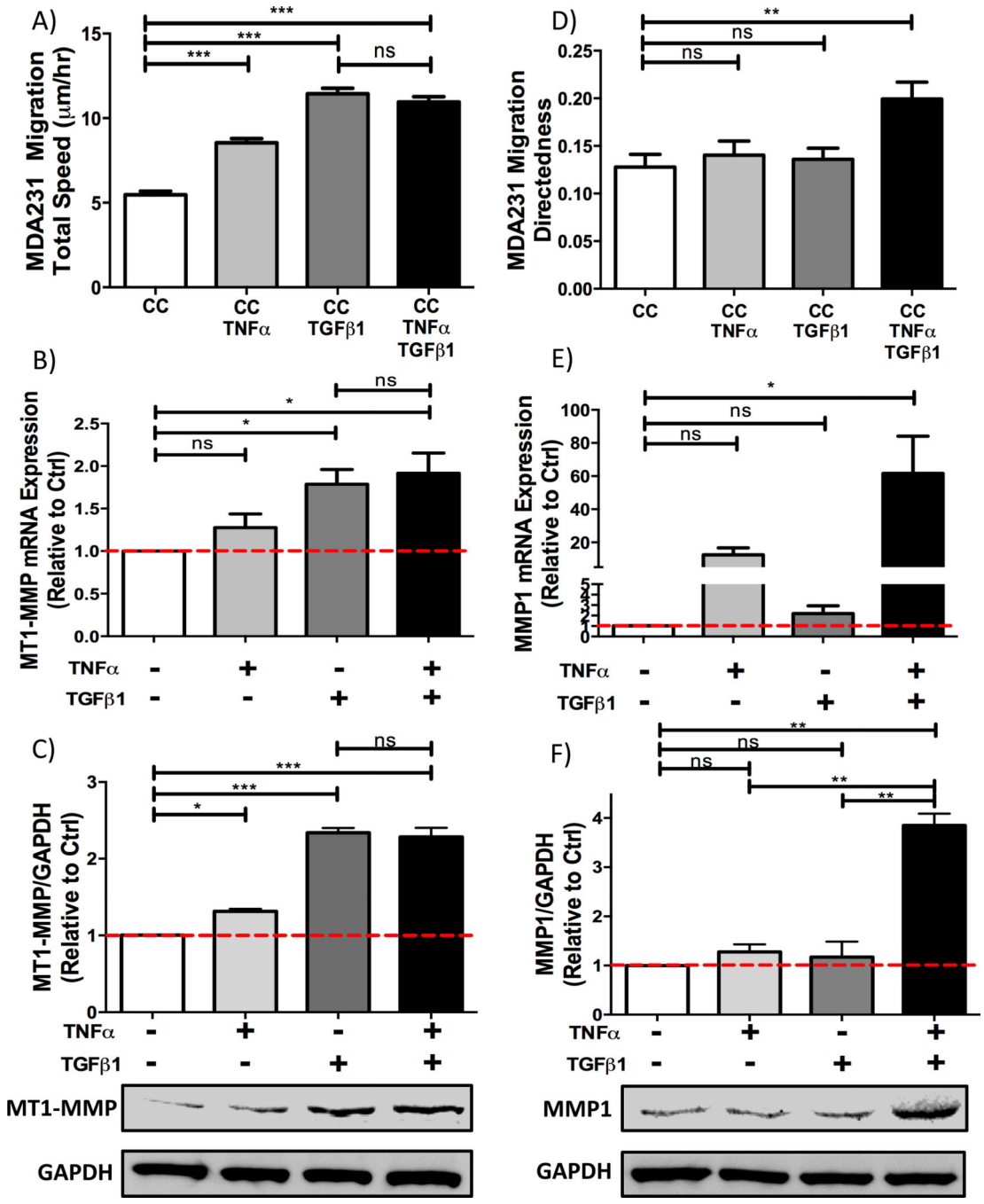

Figure 4. TGFβ1 increases cancer cell migration total speed via the induction of MT1-MMP expression, while TNFα and TGFβ1 synergistically increase cancer cell migration directedness via the induction of MMP1 expression

MDA231 monoculture (CC) was treated with TNFα and/or TGFβ1, and the resulting cell migration dynamics and MMP expressions were analyzed. **(A–C)** Treatment of MDA231 with TGFβ1 (CC TGFβ1) led to larger increases in MDA231 migration total speed (A), MT1-MMP mRNA (B) and protein (C) expressions than TNFα mono-treatment (CC TNFα). However, co-treatment of both TNFα and TGFβ1 led to no further increase in migration total speed, MT1-MMP mRNA and protein expressions compared to TGFβ1

mono-treatment. Data in (A), (B), and (C) follow a similar trend. **(D–F)** TNFα and TGFβ1 synergistically increased MDA231 migration directedness (D), MMP1 mRNA expression (E), and MMP1 protein production (F) when compared to mono-treatment conditions. Data in (D), (E), and (F) follow a similar trend. Data in (C) and (F) were obtained from cells cultured in 3D collagen I ECM.

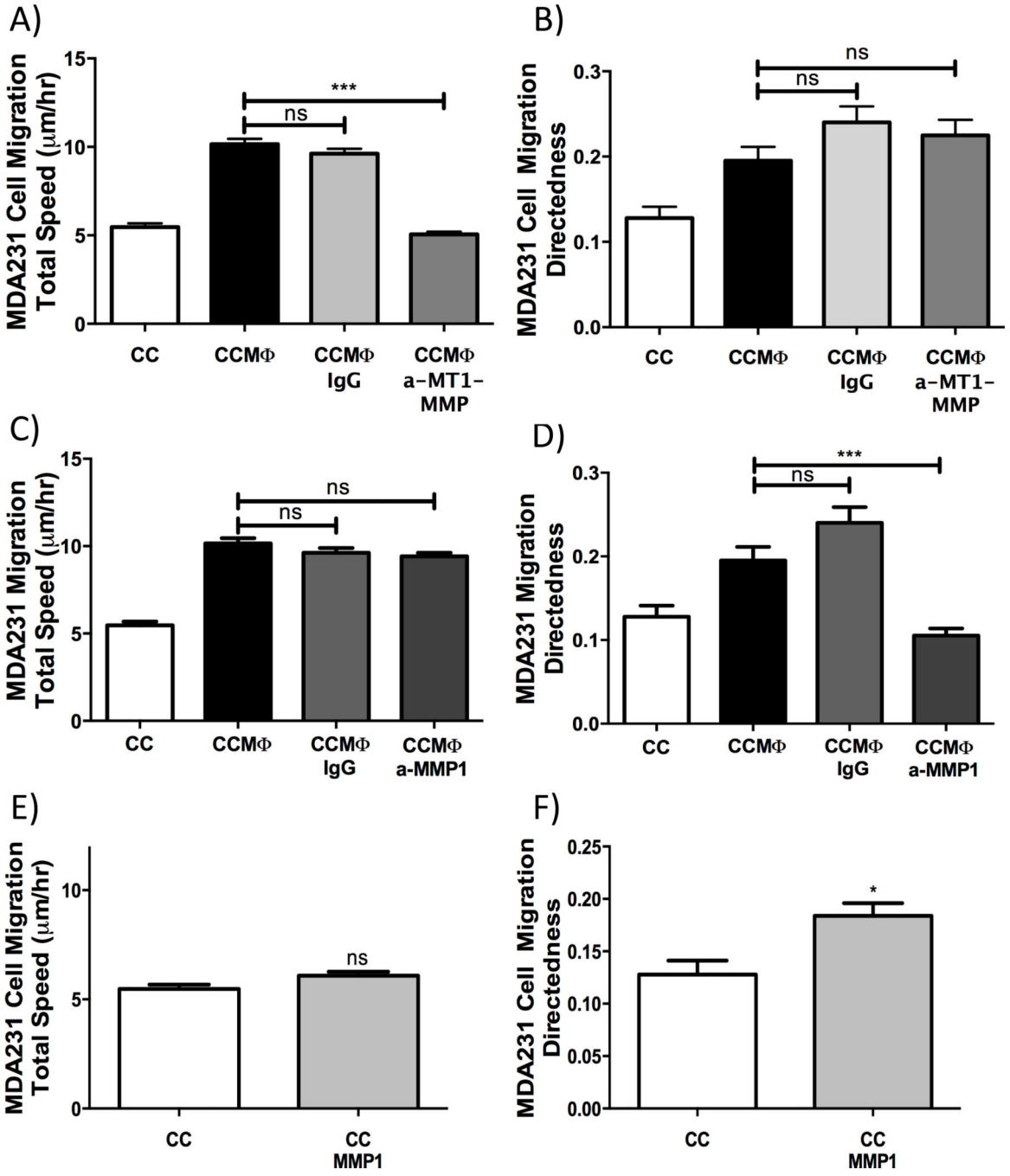

Figure 5. Macrophage-induced cancer cell migration total speed is mediated via MT1-MMP, while directedness is mediated by MMP1

MDA231 cancer cells (CC)-Raw macrophages (MΦ) co-culture was treated with blocking antibodies against MT1-MMP or MMP1. **(A and B)** Treatment of co-culture with anti-MT1-MMP antibody (CCMΦ a-MT1-MMP) decreased MDA231 migration total speed (A) but not directedness (B). **(C and D)** Treatment of co-culture with anti-MMP1 antibody (CCMΦ a-MMP1) reduced MDA231 migration directedness (D) while having a minimal effect on total speed (C). **(E and F)** Treatment of MDA231 cancer cell monoculture with recombinant

MMP1 (CC MMP1) enhanced MDA231 migration directedness (F), while having a minimal effect on total speed (E).

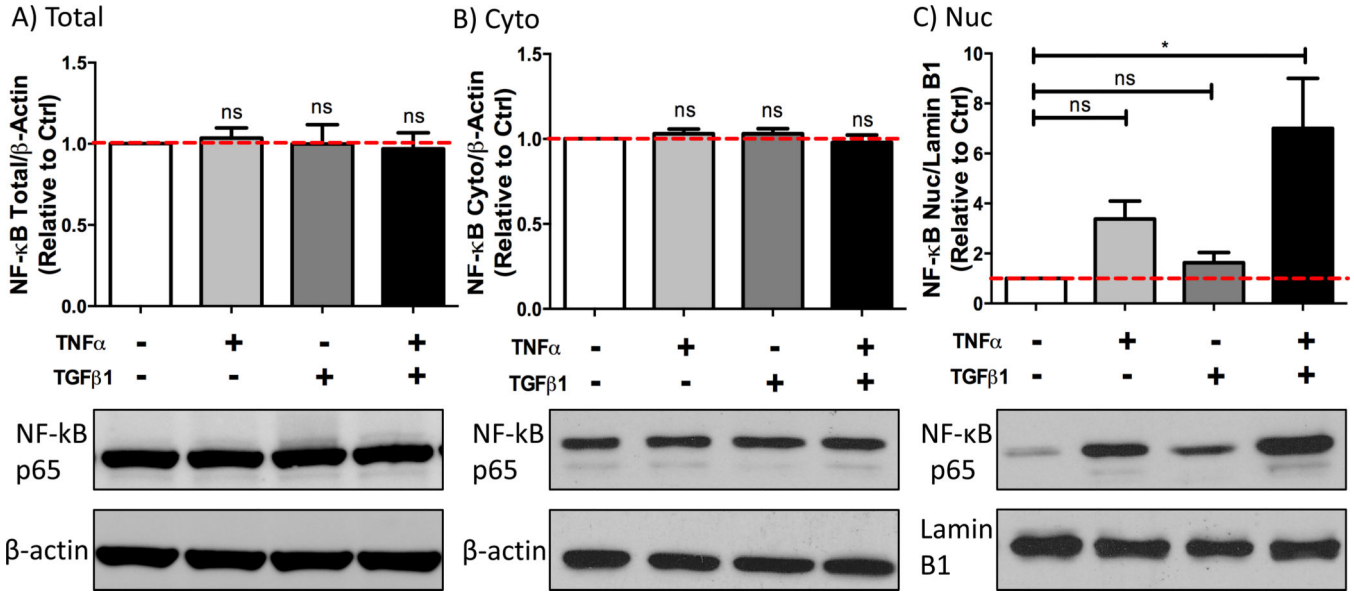

Figure 6. TNFα and TGFβ1 synergistically increase NF-κB nuclear localization

(A) Western blot quantification (top) and representative images (bottom) showing 48-hr TNFα and/or TGFβ1 treatments of MDA231 cells did not alter the production of NF-κB (NF-κB total). **(B and C)** Western blot quantifications (top) and representative images (bottom) showing 2-hr TNFα and TGFβ1 co-treatment of MDA231 synergistically increased the level of NF-κB in the nuclear fraction of MDA231 (NF-κB Nuc, C), but not in the cytoplasmic fraction of the cells (NF-κB Cyto, B).

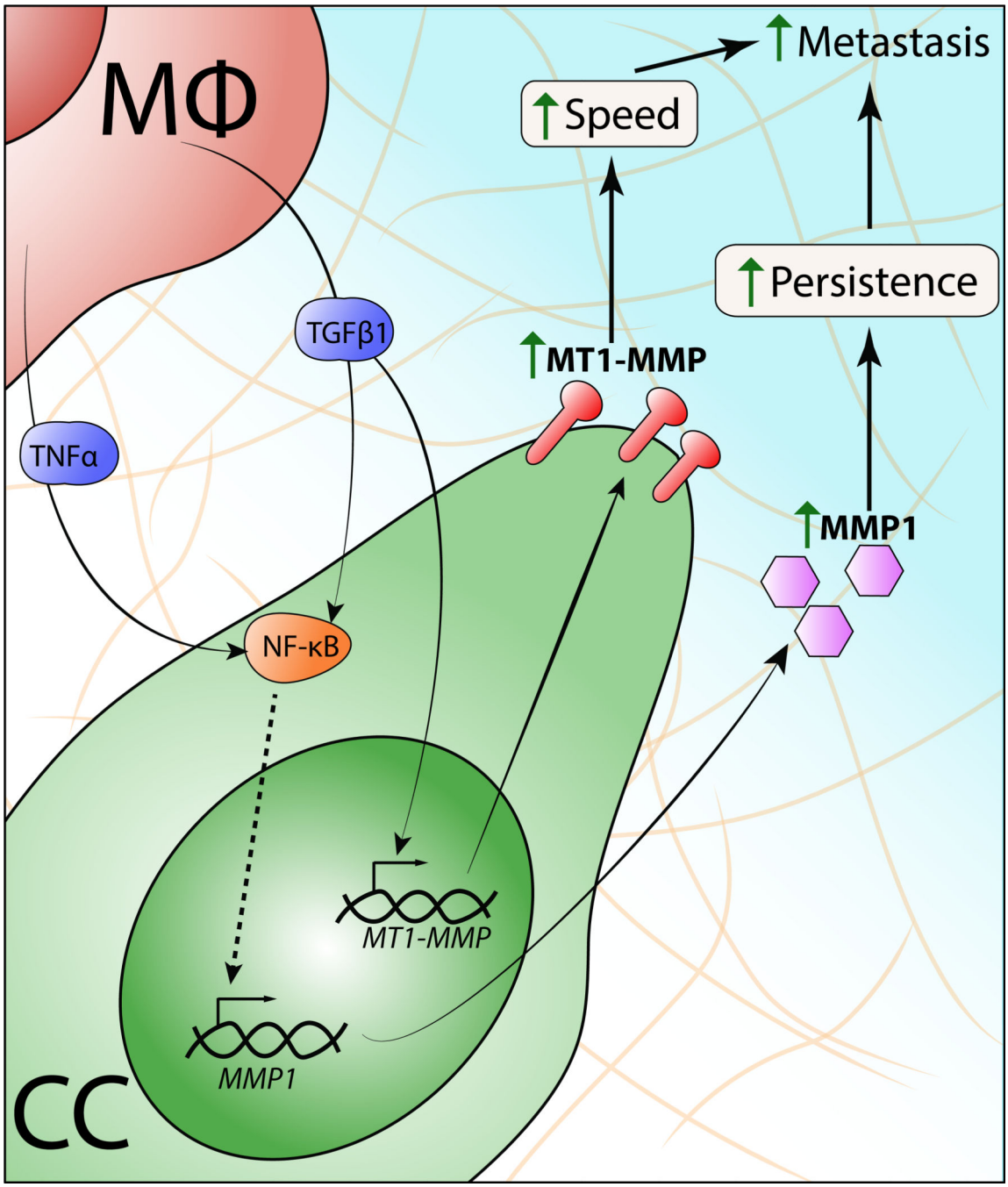

Figure 7. Proposed mechanism explaining the effects of macrophages (MΦ) on cancer cell (CC) migration speed and persistence

Macrophage-released TNFα and TGFβ1 synergistically enhance NF-κB nuclear localization in cancer cells, leading to synergistic increases in cancer cell MMP1 mRNA expression, protein production, and protein secretion. This increase in MMP1 secretion by cancer cells leads to an increase in cancer cell migration persistence (directedness). Meanwhile, macrophages increase cancer cell migration speed (total speed), mainly through TGFβ1-induced cancer cell expression of MT1-MMP.