PRECLINICAL STUDY

Daintain/AIF-1 promotes breast cancer cell migration by up-regulated TNF-α via activate p38 MAPK signaling pathway

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Abstract Tumor-associated macrophages can release a vast diversity of growth factors, proteolytic enzymes, cytokines, and inflammatory mediators. Many of these factors are key agents in cancer metastasis. Daintain/AIF-1 is a macrophage-derived inflammatory cytokine which defined a distinct subset of tumor-associated activated macrophages/microglial cells. Previous study demonstrated that daintain/AIF-1 could promote breast cancer proliferation through activating NF-κB/cyclin D1 pathway and facilitate tumor growth. However, the effect of Daintain/AIF-1 on cell migration and cancer metastasis has never been reported. Herein, we used a mimic tumor microenvironment by incubating breast cancer cell lines, MDA-MB-231 and MCF-7 cells, with macrophage-conditioned medium with or without purified daintain/AIF-1 polypeptide to evaluate cell migration. Results indicated that daintain/AIF-1 enhanced the migration of MDA-MB-231 and MCF-7 cells in the manner of TNF-α up-regulation. Further study found that daintain/AIF-1 activates p38 MAPK signaling pathway contributing to up-regulation of TNF-α in MDA-MB-231 and MCF-7 cells. Therefore, this novel daintain/AIF-1-p38-TNF-α pathway and insight into daintain/AIF-1 might have potential benefits in the control of tumor metastasis during cancer therapy.

Abbreviations

NF-κB	Nuclear factor-κB
MAPK	Mitogen-activated protein kinase
TNF-α	Tumor necrosis α
CM	Conditioned medium
AIF-1 siRNA	siRNA knockdown of daintain/AIF-1 in U937 cell
EMT	Epithelial–mesenchymal transition

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Introduction

Inflammation is highly correlated with the cancer. Many studies demonstrated that inflammatory mediators are detected in human cancers and experimental cancer models [1–4]. Cancer-associated inflammation promotes the proliferation and survival of malignant cells, stimulates angiogenesis and metastasis, subverts adaptive immunity, and induces the resistant response to hormones and chemotherapy [5]. Macrophages can execute the functions of infiltrate inflammatory cells in solid tumors, which make a significant contribution to tumor microenvironment by secreting a wide range of inflammatory cytokines [5]. Tumor necrosis factor-α (TNF-α) is one of major mediators generated from cancer-related inflammation. Many malignant cells can constitutively produce a small amount of TNF-α [6]. Animal models also provide the evidence that malignant cell-derived TNF-α enhances the growth and

metastasis of syngeneic, xenogeneic, and carcinogen-induced tumors in skin, ovary, pancreas, pleural cavity, and bowel [7–11].

Daintain/allograft inflammatory factor-1 (AIF-1) is a macrophage-derived polypeptide. It is an evolutionarily conserved calcium-binding protein with 143 amino acids in cytoplasm [12]. The daintain/AIF-1 gene, located in the major histocompatibility complex (MHC) class III region on chromosome 6p21.3, is densely clustered with genes involved in the inflammatory response, including surface glycoproteins, complement cascade proteins, TNF-α, TNF-β, and nuclear factor-κB (NF-κB) [13]. Daintain/AIF-1 expression is a marker for astrocyte activation in experimental autoimmune neuritis [14] and correlates with inflammation severity in liver allograft rejection [15] and endometriosis [16]. Daintain/AIF-1 expression also modulates vasculopathy by affecting VSMC migration [17].

Our previous study described that high-level expression of daintain/AIF-1 is observed in ductal breast tumor epithelial cells. However, weak or negative expression is detected in the adjacent histologically normal ductal epithelial cells. Daintain/AIF-1 can promote the development of breast cancer by activating NF-κB/cyclin D1 pathway and facilitate tumor growth in nude mice [18].

Inflammatory cells and mediators are key components of tumor microenvironment, and cancer-related inflammation has been proposed to represent the seventh hallmark of cancers [3]. Daintain/AIF-1 defined a distinct subset of tumor-associated activated macrophages/microglial cells [19], and it is crucial for the survival and pro-inflammatory activity of macrophages [20], but the characterization of daintain/AIF-1 in breast tumor microenvironment has not been described. In order to explore the potential properties of daintain/AIF-1, we incubated MDA-MB-231 and MCF-7 cells with macrophage-conditioned media or purified polypeptide daintain/AIF-1, then assessed cell migration. Results indicated that daintain/AIF-1 significantly enhanced the migration of MCF-7 and MDA-MB-231 cells. Meanwhile, the up-regulation of TNF-α was involved in the daintain/AIF-1-induced cell migration, which was confirmed by gene expression profiling analysis. Further study demonstrated that daintain/AIF-1-activated p38 MAPK pathway contributed to TNF-α up-regulation to modulate the migration of breast cancer cells.

Materials and methods

Cell culture

The human monocytic U937 (myelomonocytic) cell line and human breast cancer cell lines, MDA-MB-231 and MCF-7, were purchased from the American Type Tissue Collection (ATCC) and maintained in RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Gaithersburg, MD). Monocytic U937 cells were induced to differentiate by treating with 20 nM PMA (Amresco, OH, USA) for 48 h. Purified daintain/AIF-1 was prepared as described previously [21].

Construction of recombinant plasmids

The recombinant human daintain/AIF-1-pRNAT-H1.1/Shuttle (pRNAT-DT) and siRNA expression vector pRNAT-H1.1/Shuttle (pRNAT) were constructed as described previously in our laboratory [18].

Transfection

The cultured human monocytic U937 cells were washed with phosphate-buffered saline (PBS), and resuspended in complete growth medium at a density of 5×10^7 cells/ml. A total of 25 μg DNA was added to 500 μl of cell suspension in a 0.4-cm electroporation cuvette (BioRad Laboratories, CA, USA). Electroporation was carried out in a BioRad Gene-Pulser electroporation apparatus (BioRad Laboratories, CA, USA) at the condition of 300 V and 950 mF. In order to obtain stable transfected cell line, 600 μg/ml G418 (Amresco, OH, USA) was used to eliminate untransfected cells. Transfectants were pooled and expanded in the complete growth medium supplemented with 300 μg/ml G418.

Western blot

Proteins were extracted using the complete RIPA (Millipore Corporation, MA, USA) buffer. Totally 25 μg protein was loaded and separated on 15% SDS-PAGE gel, and then transferred onto PVDF membrane (Millipore Corporation, MA, USA). The target proteins were probed for daintain/AIF-1 (PTGLAB, Chicago, IL, USA), TNF-α, p38 MAPK, phosphor-p38, and β-actin (Santa Cruz Biotechnology, CA, USA). Antibody reactions were visualized with enhanced chemiluminescence reagents according to the manufacturer's instructions (Amersham International, Bucks, UK). Optical densities of the bands were measured using the software of Image J.

Conditioned medium

Human monocytic U937 cells were plated at 5×10^5 per well (6-well plate format, Corning Costar, Cambridge, MA, USA) and were then treated with 20 nM PMA overnight. On the next day, the medium was aspirated, and 1 ml of

fresh serum-free medium was replaced. Conditioned medium was harvested at 24 h later.

Migration assays

The conditioned medium pre-treated cells at the density of 5×10^5 cells/well were seeded into the upper chambers of an 8-μm pore Costar Transwell (12-well plate format, Corning Costar, Cambridge, MA, USA), with bottom wells containing complete growth medium. After 12 h incubation in the incubator with 5% CO_2 at 37°C, the cells migrated to the bottom surface of the porous membrane from the upper chambers were wiped with a cotton swab, fixed with 100% ice-cold methanol and then stained with crystal violet in 0.1 mM borate buffer (pH 9). Migrated cells were quantified by counting in 12 fields under a microscope with a 400× magnification. All experiments were in triplicate.

RT-PCR

Total RNA was prepared using the TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. RT-PCR was performed using a Superscript One-Step RT-PCR kit (Invitrogen). The PCR products were analyzed by electrophoresis on a 2% agarose gel containing ethidium bromide, visualized, and photographed under UV light. The primer sequences and reaction conditions of RT-PCR for TNF-α, TGF-β, EGF, VEGF-A, FGF-2, and β-actin were shown in Table 1.
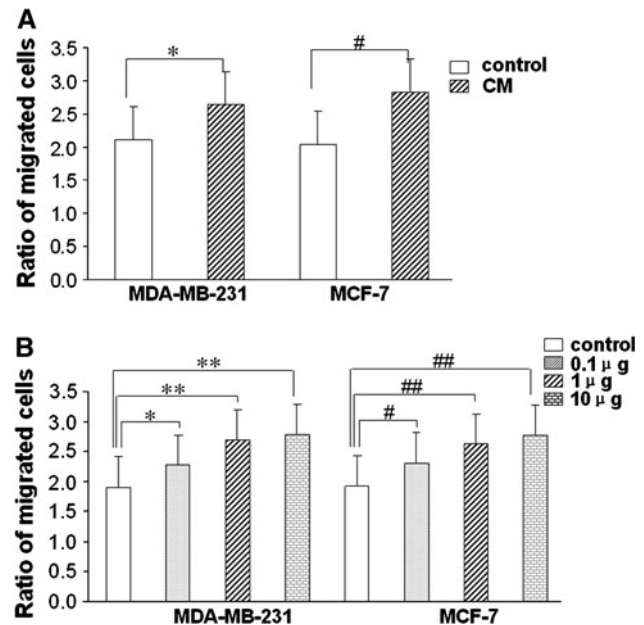

Fig. 1 Assessment of the migration of MDA-MB-231 and MCF-7 cells. **a** Compared with the control cells cultured with serum-free medium (*empty bar*), the cells with treatment (*slash line bar*) of U937 cell-conditioned medium for 12 h exhibited an enhanced migration of MDA-MB-231 and MCF-7 cells. **b** Compared with the control cells cultured with serum-free medium (*empty bar*), the cells treated with daintain/AIF-1 at the concentrations of 0.1 (*think slash line bar*), 1 (*thick slash line bar*), and 10 μg/ml (*grid bar*) significantly promoted the migration of MDA-MB-231 and MCF-7 cells. The experiment was performed three times. *Bars* SE. * $P < 0.05$; ** $P < 0.01$; # $P < 0.05$; ## $P < 0.01$

Table 1 Primer sequences and reaction conditions used in RT-PCR

Genes	Sequences	Annealing temperature (°C)	Cycle number
TNF-α			
Forward	GTGGAGCTGAGAGATAACCA	55	30
Reverse	AAAGTAGACCTGCCCAGACT		
TGF-β			
Forward	CAGCAGGGATAACACACTG	55	30
Reverse	GTCCAGGCTCCAAATGTAG		
EGF			
Forward	GGCTGTACTCTTGGGTGTAA	55	30
Reverse	ACAGGGTCATGATCTAGGG		
FGF-2			
Forward	CCAGTGAAGCTTACCTAGAGC	56	30
Reverse	CAATGACCTGGCGAAGAC		
VEGF-A			
Forward	CCTCCGAAACCATGAACT	56	
Reverse	GCCTTGGTGAGGTTTGAT		
β-actin			
Forward	CTC CAT CCT GGC CTC GCT GT	56	30
Reverse	GCT GTC ACC TTC ACC GTT CC		

Statistical analysis

Data were analyzed using the Student t test. The significant difference was considered at $P < 0.05$.

Results

Daintain/AIF-1 regulate MDA-MB-231 and MCF-7 cells mobility

In order to assess the effect of secreted factors generated by macrophages in breast cancer cells on tumor metastasis, conditioned medium from U937 cells was used for cell migration assay in vitro. Transwell chambers were used to evaluate the migration potential of MDA-MB-231 and MCF-7 cells. The cells in the upper wells of the chamber were seeded in starvation medium, and the bottom wells of the chamber were filled with complete growth medium. In the presence of pre-incubated breast cancer cells with conditioned medium, the average number of the migrated cells in each field was 21 for MDA-MB-231 cells and 20 for MCF-7 cells. In contrast, the average number of the

migrated cells in each field was 10 for MDA-MB-231 cells and 10 for MCF-7 cells in the control group without treatment of conditioned medium (Fig. 1a). In both cell lines, the conditioned medium-treated breast cancer cells exhibited a higher migration rate than the control groups ($P < 0.05$). In addition, compared with the control group, the migration of the cells incubated with purified daintain/AIF-1 polypeptide for 12 h was significantly enhanced (Fig. 1b). Therefore, daintain/AIF-1 may play an important role in cell migration.

Daintain/AIF-1 siRNA/CM decrease cell migration

In order to further characterize the enhancement of daintain/AIF-1-induced migration, the inhibition of daintain/AIF-1 in U937 cells was conducted using stable, vector-based siRNA. Several siRNA constructs based on human daintain/AIF-1 cDNA were cloned into the pRNAT vector. Macrophages were then transfected, stable transfectants were isolated by antibiotic selection, and the resistant cells were pooled. Consequently, conditioned medium of U937 cells with siRNA knockdown of daintain/AIF-1 was prepared. Daintain/AIF-1 expression was verified using
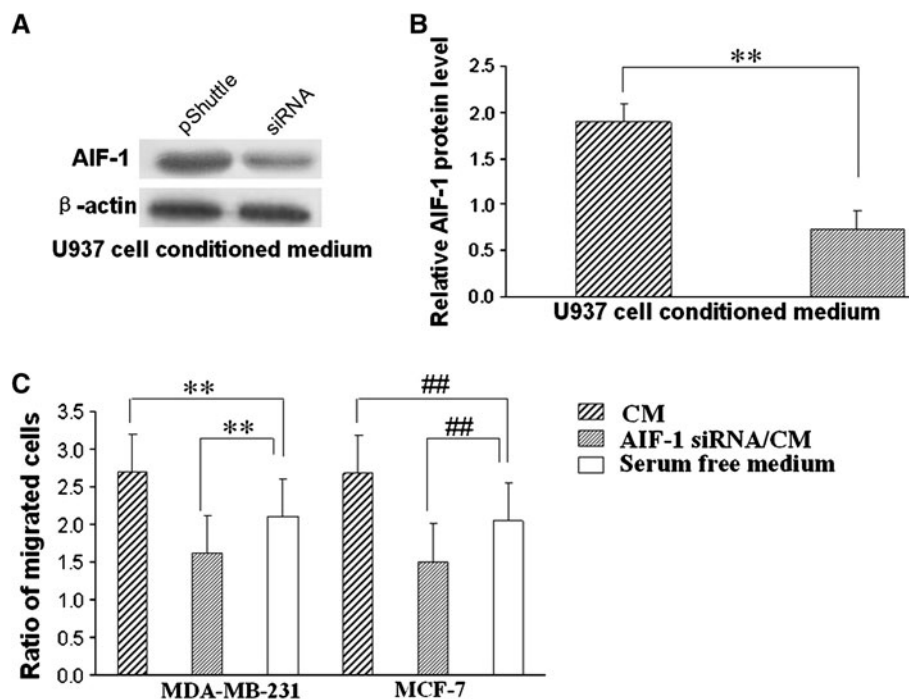

Fig. 2 Daintain/AIF-1 with low expression levels attenuated cell migration. **a** The knockdown efficiency of daintain/AIF-1 in conditioned medium of U937 cells was evaluated by western blot, and the daintain/AIF-1 was partially silenced in U937 cells. **b** Protein expression levels of daintain/AIF-1 in conditioned medium of U937 cells without (*thick slash line bar*) and with (*slash line bar*) of siRNA knockdown of daintain/AIF-1 were semi-quantitatively analyzed by Imaging J on the basis of optical density of bands in western blot. The daintain/AIF-1 expression in conditioned medium of U937 cells

exhibited a decrease of 62% due to siRNA knockdown. **c** MDA-MB-231 and MCF-7 cells were treated with U937 cell-conditioned medium (*thick slash line bar*) and conditioned medium of U937 cells with siRNA knockdown of daintain/AIF-1 (*slash line bar*) and serum-free medium (*empty bar*). The reduced daintain/AIF-1 expression in U937 cell-conditioned medium significantly decreases the migration of MDA-MB-231 and MCF-7 cells. The experiment was performed three times. *Bars* SE. **$P < 0.01$; ##$P < 0.01$

western blotting (Fig. 2a). The pRNAT-DT reduced daintain/AIF-1 expression in conditioned medium by an average of 62% (Fig. 2b). MDA-MB-231 and MCF-7 cells pre-incubated with low-expressing daintain/AIF-1-conditioned medium exhibited weak migration (Fig. 2c).

Daintain/AIF-1 up-regulate TNF-α expression

In order to understand the mechanism of daintain/AIF-1-activated migration of breast cancer cells, the expression levels of TNF-α, TGF-β, EGF, FGF-2, and VEGF-A which are involved in cancer metastasis were evaluated by RT-PCR. Compared with the controls, a significant increase of TNF-α expression in MDA-MB-231 cells pre-treated by U937 cell-conditioned medium was observed, whereas no obvious difference in the expression of TGF-β, EGF, FGF-2, and VEGF-A was observed (Fig. 3a). Western blotting analysis showed a significant decrease in TNF-α expression in MDA-MB-231 and MCF-7 cells stimulated by conditioned medium from daintain/AIF-1-siRNA knockdown U937 cells (Fig. 3b, d). In order to further validate the direct regulation of daintain/AIF-1 on TNF-α expression, MDA-MB-231 cells were treated with

purified daintain/AIF-1 polypeptide for 12 h. A significantly increased TNF-α expression level in MDA-MB-231 cells was validated by western blot (Fig. 3c, e). Thus, the daintain/AIF-1-regulated TNF-α expression played a critical role in the migration of breast cancer cells.

Daintain/AIF-1 up-regulate TNF-α expression via p38 MAPK activation

To elucidate the signaling pathways that are triggered by daintain/AIF-1 stimulation and that contribute to the cell migration, we initially focused on the MAPK pathway. The p38 MAPK activation in response to daintain/AIF-1 was measured in MDA-MB-231 and MCF-7 cells, respectively. The p38 MAPK was significantly activated by daintain/AIF-1, as shown in Fig. 4a. In contrast, daintain/AIF-1-induced phosphorylation of p38 MAPK was inhibited by SB203580, a specific kinase inhibitor (Fig. 4b). Moreover, the p38 MAPK activity exhibited an obvious contribution to the daintain/AIF-1-induced TNF-α expression in MDA-MB-231 and MCF-7 cells, as shown in Fig. 4b. The cell migration mediated by daintain/AIF-1-p38-TNF-α pathway was further characterized by adding TNF-α neutralizing
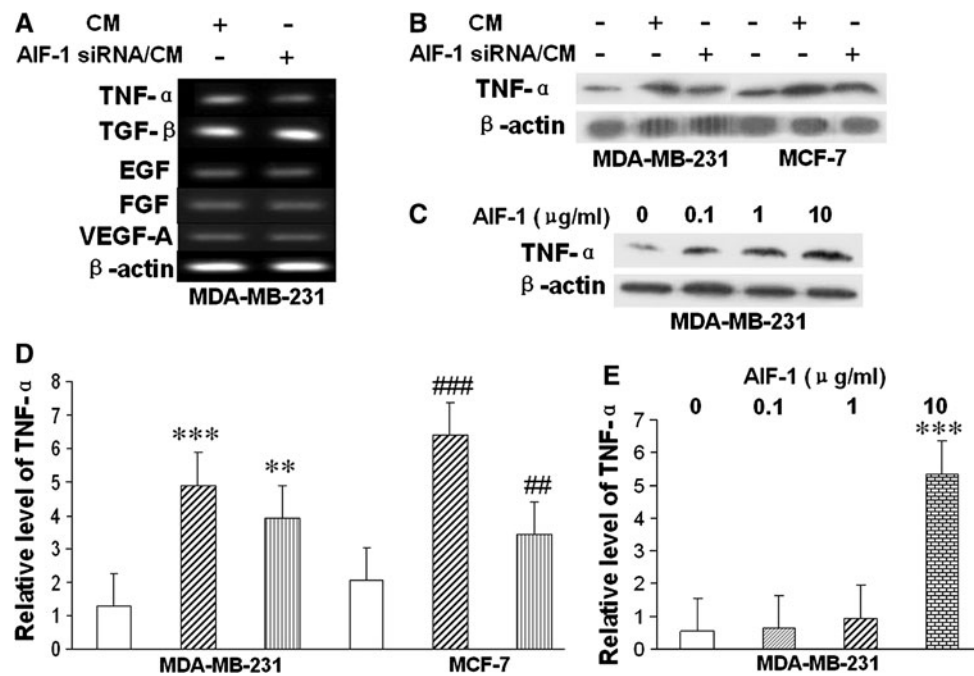

Fig. 3 Daintain/AIF-1 upregulated TNF-α expression. a The mRNA level of TNF-α, TGF-β, EGF, FGF-2, and VEGF-A in MDA-MB-231 cells was detected by RT-PCR. b Protein levels of TNF-α in MDA-MB-231 and MCF-7 cells at the condition of conditioned medium (CM) treatment and siRNA knockdown of daintain/AIF-1 were detected by Western blot. c Protein level of TNF-α in MDA-MB-231 cells treated with purified daintain/AIF-1 for 12 h was detected by western blot. d Relative protein levels of TNF-α in MDA-MB-231 and MCF-7 cells at the condition of without treatment as the control (empty bar), conditioned medium treatment (slash line bar), and siRNA knockdown of daintain/AIF-1 (vertical line bar) were semi-quantitatively determined on the basis of optical density of bands in western blot. e Protein expression levels of TNF-α induced by purified daintain/AIF-1 at the concentration of 0 (empty bar), 0.1 (slash line bar), 1 (thick slash line bar), and 10 μg/ml (grid bar) in breast cancer cell were semi-quantitatively determined. The experiment was performed three times. Bars SE. **$P < 0.01$; ***$P < 0.001$; ## $P < 0.01$; ### $P < 0.001$
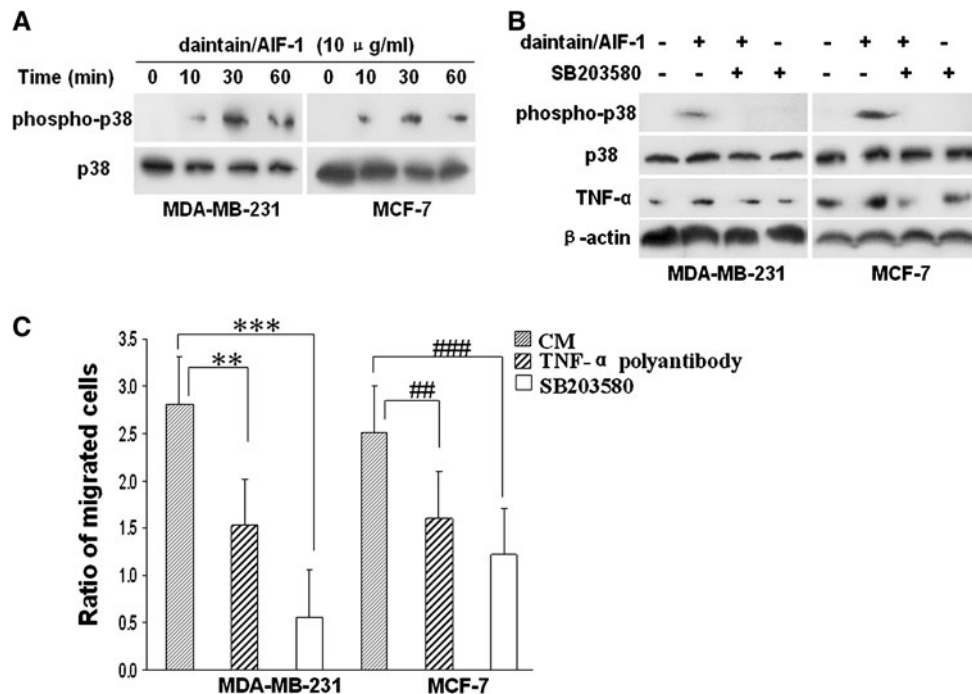
Fig. 4 Daintain/AIF-1 upregulated TNF-α expression via p38 MAPK activation. **a** The expression levels of p38 MAPK and phosphorylated p38 MAPK in MDA-MB-231 and MCF-7 cells treated by 10 μg/ml daintain/AIF-1 for 0, 10, 30, and 60 min was evaluated by western blot. **b** The expression levels of p38 MAPK, phosphorylated p38 MAPK, and TNF-α in MDA-MB-231 and MCF-7 cells treated by 10 μg/ml daintain/AIF-1 and 20 μM SB203580 for 90 min were evaluated by western blot. **c** Blocked p38MAPK pathway or TNF-α

neutralization inhibited cell migration. MDA-MB-231 and MCF-7 cells with neutralization by 20 μg/ml TNF-α polyantibody (*thick slash line bar*) and p38 MAPK inhibition by 20 μM SB203580 (*empty bar*) under the conditioned medium treatment exhibited a reduced cell migration, when compared with the cells only treated with conditioned medium (*thin slash line bar*). The experiment was performed three times. *Bars* SE. **$P < 0.01$; ***$P < 0.001$; $^{\#\#} P < 0.01$; $^{\#\#\#} P < 0.001$

antibody to the chambers. Compared with the controls, both the blockage of polyantibody and the inhibition of p38 MAPK resulted in less cell migration, as shown in Fig. 4c.

Discussion

Immune cells supply soluble growth and survival factors, matrix remodeling enzymes, reactive oxygen species, and other bioactive molecules that affect the proliferation, angiogenesis, invasion, and metastasis of cancer cells [22–24]. Daintain/AIF-1 is a macrophage-derived polypeptide. Previous studies described that daintain/AIF-1 promotes breast cancer proliferation by activating the NF-κB/cyclin D1 pathway and facilitates breast tumor growth in nude mice [18]. Recent studies have demonstrated that daintain/AIF-1 stimulates the release of cytokines including FGF-2, PDGF, TGF-β, G-CSF, and MCP-1 [14, 20, 25]. However, its roles in tumor microenvironment and its interactions with other cytokines have not been elucidated. In the present study, we demonstrate that daintain/AIF-1 enhances the migration of MDA-MB-231 and MCF-7 cells by augmenting TNF-α expression.

TNF-α is an important inflammatory factor as a master switch in establishing an intricate link between inflammation and cancer. A wide variety of evidences have described the critical role of TNF-α in tumor proliferation, migration, invasion, and angiogenesis [26]. It is well recognized as a regulator of tumor microenvironment. In an ovarian cancer model, TNF-α is observed to be an important component in malignant cell-autonomous network of inflammatory cytokines including stromal cell-derived factors, monocyte chemoattractant protein 1, interleukin-6, macrophage inhibitory factors, and vascular endothelial growth factors [27]. TNF-α in tumor microenvironment may induce epithelial–mesenchymal transition (EMT) of malignant cells in colorectal cancer [9], which provides some reasonable explanations for its capability to increase the metastatic activity of tumor cells [7, 28, 29]. In addition, it is also crucial for the right place, the right time, and the appropriate context for TNF-α production. Thus, restricted production of TNF-α in specific cell types may be a strategy to control its functions [30].

Our previous works show that daintain/AIF-1 can activate NF-κB pathway [18]. Several data in previous reports also exhibit that AIF-1 binds to and participates in F-actin

rearrangement, suggesting that AIF-1 may be involved in the cytoskeletal signaling network and contribute to the progression of EMT [17, 31, 32].

Tumor macrophage infiltrating can promote metastasis, which is a normal immune response, but during tumor infiltrating process, macrophages can secrete many kinds of inflammatory factors, lead to malignant tumor and prognosis negative [33–35]. In this study, macrophage-derived inflammatory cytokine daintain/AIF-1 activates p38 MAPK pathway, contributes to the up-regulation of TNF-α, and consequently enhances breast cancer cell migration. Since daintain/AIF-1 could promote breast cancer cell proliferation and enhance cell migration, it is suggested that it play an important role in breast cancer progression. Although the regulatory mechanisms of TNF-α in the migration of breast cancer cells remain elusive, the new role of the daintain/AIF-1-p38-TNF-α pathway and insight into daintain/AIF-1 will benefit to the control of tumor metastasis during cancer therapy.

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