Biochemical and Biophysical Research Communications 443 (2014) 1218–1225

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Tumor-conditioned Gr-1$^+$CD11b$^+$ myeloid cells induce angiogenesis through the synergistic action of CCL2 and CXCL16 in vitro

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ARTICLE INFO

Article history:
Received 16 December 2013
Available online 2 January 2014

Keywords:
Gr-1$^+$CD11b$^+$ myeloid cells
Angiogenesis
CCL2
CXCL16

ABSTRACT

Gr-1$^+$CD11b$^+$ cells can suppress innate and adaptive immunity, and the functional immunosuppressive characteristics of these cells can be modulated by the tumor microenvironment. Since Gr-1$^+$CD11$^+$ cells are also involved in tumor-associated angiogenesis, we hypothesized that the angiogenic nature of Gr-1$^+$CD11b$^+$ cells could be regulated by the tumor milieu. To address this hypothesis, we imitated a tumor microenvironment by exposing Gr-1$^+$CD11b$^+$ cells isolated from spleen of 4T1 mammary carcinoma-bearing mice to tumor-conditioned medium. Supernatants from tumor-conditioned Gr-1$^+$CD11b$^+$ cells significantly induced capillary-like tube formation and migration of human umbilical vein endothelial cells (HUVECs) compared to naive Gr-1$^+$CD11b$^+$ cells. Incubation of Gr-1$^+$CD11b$^+$ cells with tumor-conditioned medium induced production of pro-angiogenic chemokines CCL2 and CXCL16. Pretreatment with an anti-CCL2 antibody, but not an anti-CXCL16 antibody, suppressed the angiogenic effects of tumor-conditioned Gr-1$^+$CD11b$^+$ cells on HUVECs. Simultaneous neutralization of CCL2 and CXCL16 significantly inhibited tube formation and migration of HUVECs compared to the sole neutralization against CCL2. Supernatants from tumor-conditioned Gr-1$^+$CD11b$^+$ cells induced phosphorylation of ERK1/2 in HUVECs, and inhibition of the ERK pathway blocked angiogenic effects. ERK pathway activity was partially abrogated by neutralization of CCL2 and more suppressed by simultaneous neutralization of CCL2 and CXCL16. These results collectively indicate that CCL2 and CXCL16 chemokines produced by tumor-conditioned Gr-1$^+$CD11b$^+$ myeloid cells synergistically induce angiogenesis in vitro by stimulating the ERK1/2 signaling pathway. Thus, regulation of Gr-1$^+$CD11b$^+$ cells in the tumor microenvironment may contribute to angiogenesis through the secretion of pro-angiogenic chemokines.

1. Introduction

Angiogenesis is essential for tumor growth and metastasis, and therefore, its regulation could be a valuable target for cancer therapy [1]. The process of angiogenesis involves several cellular events including proliferation, migration, formation of capillary-like structures, and extracellular matrix (ECM) degradation [2]. Although tumor cells have been considered the main cellular component driving tumor angiogenesis, emerging evidence suggests that tumor-infiltrating cells, such as a subset of myeloid cells [3,4], and stromal cells including fibroblasts [5] or mesenchymal stem cells [6], may also be involved in tumor angiogenesis.

Gr-1$^+$CD11b$^+$ myeloid cells, also known as myeloid-derived suppressor cells (MDSCs), are a heterogeneous population of myeloid cells consisting of immature myeloid cells including macrophages, granulocytes, and dendritic cells [7]. These cell subsets significantly accumulate in spleens of tumor-bearing mice and have been found in peripheral blood of various patients with cancer [8–10]. Gr-1$^+$CD11b$^+$ cells are immunosuppressive through suppression of T cell and expansion of regulatory T cells [7]. In addition to immunosuppressive function, Gr-1$^+$CD11b$^+$ cells have been reported to promote angiogenesis by producing matrix metalloproteinase 9 (MMP9), which releases vascular endothelial growth factor (VEGF) from the matrix of tumor tissue [11]. Gr-1$^+$CD11b$^+$ cells have also been determined to mediate tumor resistance to anti-VEGF treatment [12].

Immunosuppressive functions of Gr-1$^+$CD11b$^+$ cells differ in peripheral lymphoid organs and within tumor tissues [7]. In peripheral lymphoid organs, Gr-1$^+$CD11b$^+$ cells inhibit CD8$^+$ T cells in an antigen-dependent manner by altering a T cell receptor required for antigen recognition, while tumor-derived factors, such as IL-1β, and prostaglandin E2 (PGE2), or hypoxia, induce arginase I and inducible nitric oxide synthase, which are essential mediators

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0006-291X/$ - see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.12.117

for the suppression of $CD8^+$ T cells in an antigen-independent manner in tumor tissues [7,13].

Although the tumor-associated regulation of immunosuppressive functions of Gr-1^+CD11b^+ cells have been well studied in recent years, the effect of the tumor microenvironment on alternative functions of these cells remains unclear. We hypothesized that the tumor microenvironment may modulate angiogenic properties of Gr-1^+CD11b^+ cells derived from peripheral lymphoid organs. In the present study, we used a murine orthotropic 4T1 murine mammary carcinoma model to examine our hypothesis. To mimic a tumor microenvironment, Gr-1^+CD11b^+ cells isolated from spleen of tumor-bearing mice were exposed to tumor-conditioned medium (TCM).

In this study, we found that these angiogenic processes are synergistically mediated by chemokines CCL2 and CXCL16, and that the ERK pathway is involved in tumor-conditioned Gr-1^+CD11b^+ cells-mediated angiogenesis.

2. Materials and methods

2.1. Cell culture and TCM preparation

The 4T1 murine mammary cancer cell line was maintained in RPMI-1640 medium with 10% fetal bovine serum (FBS), 25 mM HEPES, 2 mM L-glutamine, and 100 U/ml penicillin G/streptomycin in a CO_2 incubator at 37 °C. Human umbilical vein endothelial cells (HUVECs) were grown in EGM-2 Bulletkit medium (Lonza Biologics, Hopkinton, MA) [14]. HUVECs from passages three to six were used for all experiments.

2.2. Reagents

Normal rat IgG, anti-CCL2, and anti-CXCL16 mouse monoclonal antibodies were purchased from R&D Systems (Minneapolis, MN). FITC-conjugated anti-mouse Ly-6G, PE-labeled anti-mouse Ly-6C, and Gr-1 antibodies were obtained from eBioscience (San Diego, CA). PerCP-Cy5.5-conjugated anti-mouse CD45.2, APC-labeled anti-mouse CD11b and purified anti-mouse CD16/CD32 antibodies were purchased from BD Biosciences (Bedford, MA). Anti-mouse-Gr-1-biotin and streptavidin microbeads were obtained from Milteny Biotec (Bergish Gladbach, Germany). Antibodies for phospho-AKT (Ser473), AKT, phospho-ERK (Thy202/204), ERK, and GAPDH were obtained from Cell Signaling Technology (Beverly, MA). U0126 and U0124 were purchased from Calbiochem (La Jolla, CA).

2.3. Mice and tumor models

Six- to eight-week-old female Balb/c mice were obtained from Orient Bio (Seongnam, Republic of Korea). To make the mouse tumor model, 4T1 cells (3×10^5) were injected into the fourth mammary fat pad of mice. Tumor size ranged from 1.0 to 1.5 cm in diameter 3 weeks after tumor inoculation. At this time, mice were euthanized to harvest spleen and tumor mass. All procedures were approved by the Institutional Animal Care and Use Committee of the Korea Advanced Institute of Science and Technology (KAIST).

2.4. Isolation of cells and Flow cytometry

Gr-1^+CD11b^+ cells were isolated using anti-mouse-Gr-1-biotin and streptavidin microbeads. $Ly6G^+Ly6C^{low}$ granulocytic and $Ly6G^-Ly6C^{high}$ monocytic cells were purified using a FACSAria cell sorter (BD Biosciences). Spleens and tumors were removed from mice under sterile conditions. Single cell suspensions were prepared and stained with the relevant fluorescence-conjugated antibodies. Stained cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences). Data analysis was performed using FlowJo software (Tree Star, Ashland, OR).

2.5. Cell proliferation

Cell proliferation was determined using a WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium) assay as described [14]. HUVECs (5×10^3/well) were seeded into 96-well plates (Nunc, Roskilde, Denmark). Cells were starved in endothelial growth medium-2 (EBM-2) with 0.5% FBS for 15 h and treated with 4T1 TCM or Gr-1^+CD11b^+ cells supernatant. Cells were then incubated at 37 °C for an additional 72 h.

2.6. Cell migration

Cell migration assays were performed using 8.0-μm pore size Transwell permeable supports (Corning Costar, Lowell, MA). The lower chamber was filled with 500 μl 4T1 TCM or Gr-1^+CD11b^+ cells supernatant. HUVECs (2×10^4 cells/well) were suspended in 100 μl serum-free EBM and loaded into each upper chamber. After 4 h at 37 °C, cells on the upper surface of the filter were removed with a cotton swab. Filters were then fixed and stained with 1% Crystal Violet. Cells adhering to the lower surface of the filter were counted.

2.7. Tube formation

Tube formation assays were performed as previously described with some modifications [15]. Briefly, 200 μl growth factor-reduced Matrigel (BD Biosciences) was used to coat 4-well cell culture slides (SPL Life Sciences, Pocheon, Republic of Korea) and allowed to polymerize at 37 °C for 30 min. HUVECs (3×10^4 cells/well) were suspended in 500 μl 4T1 TCM or Gr-1^+CD11b^+ cells supernatant. After incubation for 4 h at 37 °C, photographs of five representative fields per well were taken using phase contrast microscopy. Endothelial tubes were quantified by counting the number of branches from each endothelial cell, and junctions were defined as the origin of two or more branch protrusions.

2.8. Western blot analysis

HUVECs were seeded into 6-well plates and incubated in EBM-2 with 0.5% FBS for 15 h before stimulation with the supernatant from 4T1 tumor-conditioned Gr-1^+CD11^+ cells. In some cases, the supernatant was pretreated with anti-CCL2 or anti-CXCL16 antibodies, or both anti-CCL2 and anti-CXCL16 antibodies. AKT, phospho-AKT, ERK, and phospho-ERK expression was determined as previously described [14].

2.9. Mouse angiogenesis antibody array and enzyme-linked immunosorbent assay (ELISA)

Angiogenesis-related protein profiles from cell-free supernatants were determined using a mouse angiogenesis antibody array kit (R&D Systems) according to the manufacturer's instructions. CCL2 and CXCL16 production from cell-free supernatants were determined using a mouse CCL2 and CXCL16 ELISA kit (RayBiotech, Redwood City, CA) according to the manufacturer's instructions.

2.10. RNA extraction and qRT-PCR analysis

Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany) and converted to cDNA using a high-capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA) according

to the manufacturers' protocols. Real-time PCR analysis was performed using a StepOne Real-Time PCR system (Applied Biosystems) according to the manufacturer's protocol. Expression levels were normalized to GAPDH gene levels. Primers for mouse CCL2, CXCL16, and GAPDH genes were designed using Primer Express 3.0 (Applied Biosystems). Primer sequences are as follows: CCL2, forward: 5′-GTCTGTGCTGACCCCAAGAAG-3′; reverse: 5′-TGGTTC CGATCCAGGTTTTTA-3′; CXCL16, forward: 5′-CACTCAGCACTCCA CTCTTCCA-3′; reverse: 5′-CAGGGTTGGGTGTGCTCTTT-3′; and GA PDH, forward: 5′-TGGCCTCCAAGGAGTAAGAAAC-3′; reverse: 5′-GG GATAGGGCCTCTCTTGCT-3′.

2.11. Statistical analysis

All data are expressed as the mean ± standard error of the mean (SEM). Levels of significance for comparisons between two independent samples were determined using Student's *t*-test. *p* values <0.05 were considered statistically significant.

3. Results

3.1. Supernatants from tumor-conditioned Gr-1$^+$CD11b$^+$ cells induce endothelial cell tube formation and migration

Significant increases in the number and proportion of Gr-1$^+$CD11b$^+$ cells in spleens were observed after 3 weeks of 4T1 cell inoculation. The total number of splenocytes increased eightfold. In 4T1 tumors, Gr-1$^+$CD11b$^+$ cells comprised an average of 18.2% of CD45.2$^+$ hematopoietic cells (Fig. S1). To mimic a tumor microenvironment, Gr-1$^+$CD11$^+$ cells isolated from the spleen of 4T1 tumor-bearing mice were exposed to varying concentrations (0%, 10%, or 50%) of 4T1 TCM. To determine whether tumor-conditioned Gr-1$^+$CD11b$^+$ cells promote angiogenesis, we assessed the effect of supernatants from Gr-1$^+$CD11b$^+$ cells exposed to the TCM on endothelial cell migration and tube formation. Supernatants from 10% or 50% TCM-treated Gr-1$^+$CD11b$^+$ cells induced capillary-like structures and migration to a higher degree compared to supernatants obtained from Gr-1$^+$CD11b$^+$ cells cultured in RPMI medium (control) (Fig. 1A and B). However, supernatants from Gr-1$^+$CD11b$^+$ cells exposed to the TCM did not affect HUVECs proliferation (Fig. 1C). Collectively, these results suggest that tumor-conditioned Gr-1$^+$CD11b$^+$ cells enhance endothelial cell tube formation and migration, but not proliferation.

3.2. TCM stimulates CCL2 and CXCL16 production by Gr-1$^+$CD11b$^+$ cells

To determine angiogenic factors produced by tumor-conditioned Gr-1$^+$CD11b$^+$ cells, an antibody array kit of angiogenesis-related proteins was used. Since the supernatant from Gr-1$^+$CD11b$^+$ cells exposed to 10% TCM induced endothelial tube formation and migration efficiently, we examined protein levels in 10% TCM and supernatants from Gr-1$^+$CD11b$^+$ cells cultured in control medium and exposed to 10% TCM, respectively. Elevation of CCL2 and CXCL16 was observed in supernatants obtained from Gr-1$^+$CD11b$^+$ cells exposed to 10% TCM (Fig. 2A). Incubation of Gr-1$^+$CD11b$^+$ cells with the TCM significantly increased the secretion of CCL2 and CXCL16 (Fig. 2B). Quantitative real-time PCR analysis confirmed the increased expression of CCL2 and CXCL16 at the transcription level in tumor-conditioned Gr-1$^+$CD11b$^+$ cells (Fig. 2C). Collectively, these results demonstrate that tumor-derived factors upregulate transcription and the subsequent release of CCL2 and CXCL16 by Gr-1$^+$CD11b$^+$ cells.

Gr-1$^+$CD11b$^+$ cells could be further subdivided into Ly6G$^+$Ly6C^{low} granulocytic and Ly6G$^-$Ly6C^{high} monocytic cells. Ly6G$^+$Ly6C^{low} cells were 13 times more abundant in the spleen of 4T1 tumor-bearing mice (Fig. 2D). To evaluate the ability of CCL2 and CXCL16 production in each cell subset, these two populations were sorted from the spleen of tumor-bearing mice and then exposed to the TCM for 24 h. Ly6G$^+$Ly6C^{low} cells produced significantly higher levels of CCL2 than Ly6G$^-$Ly6C^{high} cells. The majority of CXCL16 was produced by Ly6G$^+$Ly6C^{low} cells (Fig. 2E).

3.3. CCL2 and CXCL16 synergistically mediate the pro-angiogenic effects of tumor-conditioned Gr-1$^+$CD11b$^+$ cells

To determine the contribution of CCL2 and CXCL16 in endothelial tube formation and migration, we utilized CCL2 and CXCL16 blocking antibodies. Treatment with a CCL2 neutralizing antibody inhibited tube formation of HUVECs induced by the supernatants taken from TCM-treated Gr-1$^+$CD11b$^+$ cells. Simultaneous neutralization of both CCL2 and CXCL16 significantly inhibited tube formation and migration of HUVECs compared to CCL2 neutralization alone; however, blocking of CXCL16 alone had little effect (Fig. 3A and B). These results indicate that CCL2 and CXCL16 mediate the pro-angiogenic effects of tumor-conditioned Gr-1$^+$CD11b$^+$ cells in a synergistic manner.

3.4. ERK activation is involved in tumor-conditioned Gr-1$^+$CD11b$^+$ cells-mediated angiogenesis

Several studies have shown that ERK activation is involved in endothelial cell proliferation, migration, and morphogenesis [16–18]. We examined whether supernatants from tumor-conditioned Gr-1$^+$CD11b$^+$ cells stimulate the ERK pathway in HUVECs. Supernatants from tumor-conditioned Gr-1$^+$CD11b$^+$ cells induced ERK1/2 phosphorylation in a time-dependent manner. Phosphorylation of ERK1/2 peaked at 10 and 30 min after stimulation and declined thereafter, whereas no significant change in Akt phosphorylation was induced by the same treatment (Fig. 4A). Consistent with previous results, we found that CCL2 neutralization inhibited ERK phosphorylation in HUVECs induced by the supernatants taken from TCM-treated Gr-1$^+$CD11b$^+$ cells (Fig. 4B). Simultaneous neutralization of both CCL2 and CXCL16 significantly inhibited ERK phosphorylation of HUVECs compared to CCL2 neutralization alone (Fig. 4B). These data indicate that CCL2 and CXCL16 secreted by tumor-conditioned Gr-1$^+$CD11b$^+$ cells are involved in ERK1/2 phosphorylation.

Activation of the ERK pathway by tumor-conditioned Gr-1$^+$CD11b$^+$ cells prompted us to examine whether this event is essential for angiogenic activity in HUVECs. HUVECs were incubated in the absence or presence of a MEK1/2 inhibitor (U0126; 10 μmole/l) or an inactive structural analog (U0124; 10 μmole/l) for 30 min before supernatants of tumor-conditioned Gr-1$^+$CD11b$^+$ cells were added for an additional 4 h. Pretreatment of cells with U0126 inhibited tube formation and migration of HUVECs (Fig. 4C and D). These data indicate that ERK activation is involved in tumor-conditioned Gr-1$^+$CD11b$^+$ cells-induced angiogenesis in vitro.

4. Discussion

In this study, we demonstrated that tumor-derived factors potentiate the angiogenic function of Gr-1$^+$CD11b$^+$ myeloid cells. Among pro-angiogenic factors produced by tumor-conditioned Gr-1$^+$CD11b$^+$ cells, we focused on CCL2 and CXCL16 due to a novel combination of CC and CXC chemokines. These chemokines are involved in angiogenesis synergistically via the ERK signaling pathway. Although some reports have been published on Gr-1$^+$CD11b$^+$ cells-associated angiogenesis [3,11,19], the effect of the tumor

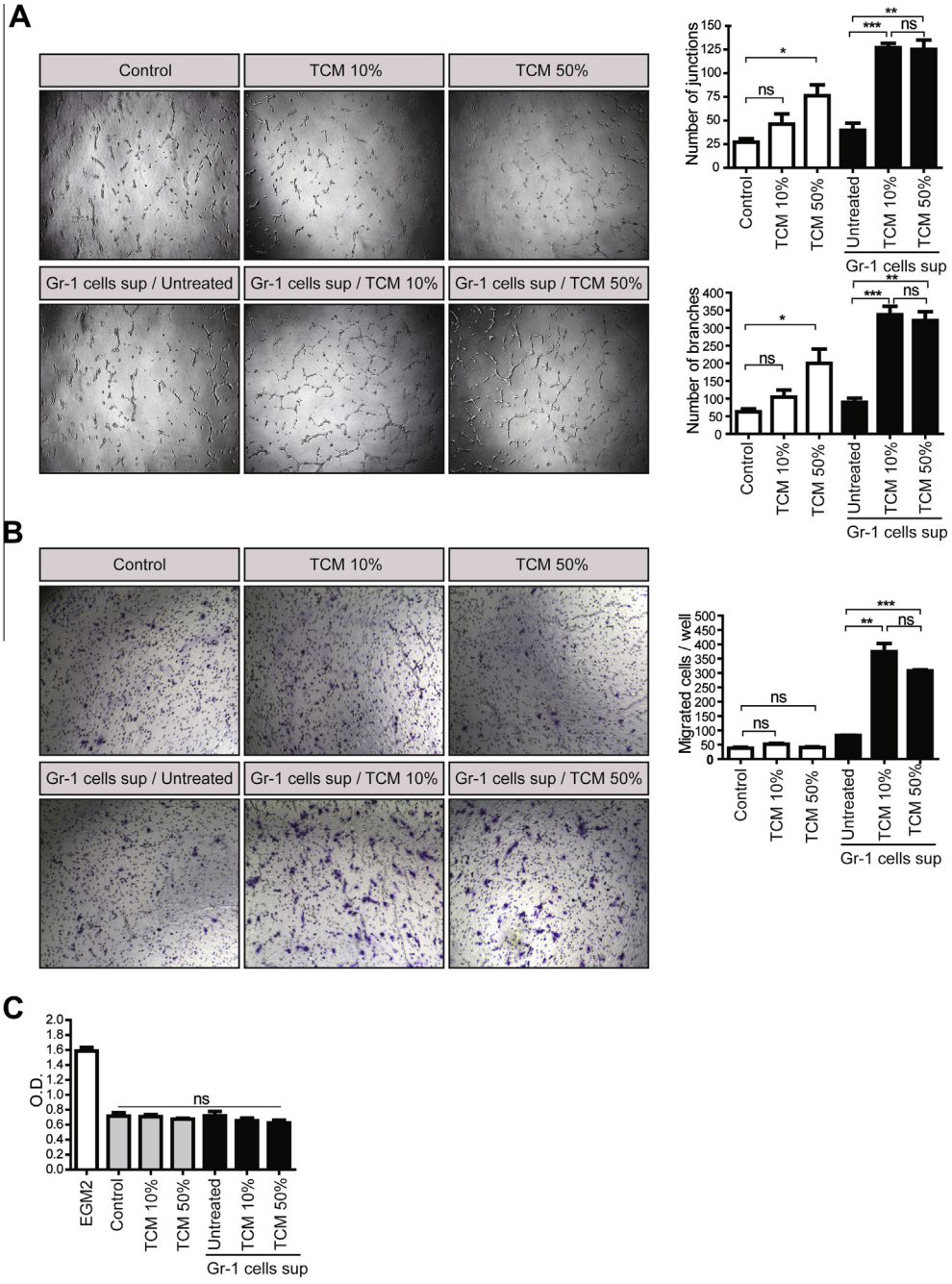

Fig. 1. Supernatants from tumor-conditioned Gr-1$^+$CD11b$^+$ cells induce endothelial cell tube formation and migration. (A) HUVECs were plated on growth factor-reduced Matrigel in tumor-conditioned medium (TCM) and supernatant (sup) from Gr-1$^+$CD11b$^+$ (Gr-1) cells. The number of branches and junctions by HUVECs was counted as described in Section 2. (B) HUVECs were placed in the upper chamber while the lower chamber was filled with TCM and Gr-1$^+$CD11b$^+$ cells supernatants. The number of cells that migrated and adhered to the undersurface of the filter was counted in the whole field. (C) HUVECs were cultured with TCM and Gr-1$^+$CD11b$^+$ cells supernatants for 72 h. HUVECs cultured in EGM2 served as a positive control. Cell proliferation was evaluated by a WST-1 assay. Control: serum-free RPMI medium; Error bars. SEM: ns: nonsignificant, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

microenvironment on angiogenic regulation of these cells has not been adequately studied. The present study suggests a link between the chemokine network induced by Gr-1$^+$CD11b$^+$ cells and the tumor microenvironment in mediating angiogenesis in vitro.

Previous studies have demonstrated that tumor cells or soluble tumor-derived factors regulate the property of tumor-infiltrating cells such as supportive stromal cells and immune cells in the tumor microenvironment [20,21,22,23]. Tumor-derived factors inhibit myeloid precursor differentiation into dendritic cells and render these immature myeloid cells immunosuppressive [22,23]. Furthermore, the interactions of cancer cells with tumor-infiltrating cells are essential components of cancer progression including angiogenesis, neoplastic cell growth and metastasis [20,21]. We have shown that one of the major components of the tumor microenvironment, tumor-derived factors, can modulate the angiogenic activity of Gr-1$^+$CD11b$^+$ cells. Many cytokines and growth factors secreted by tumor cells can stimulate numerous cell types to secrete angiogenic factors. Kujawski et al. showed that tumor-conditioned Gr-1$^+$CD11b$^+$ cells secreted both VEGF and bFGF, which induced endothelial cell tube formation in a B16 murine melanoma model [19]. However, we did not detect VEGF and

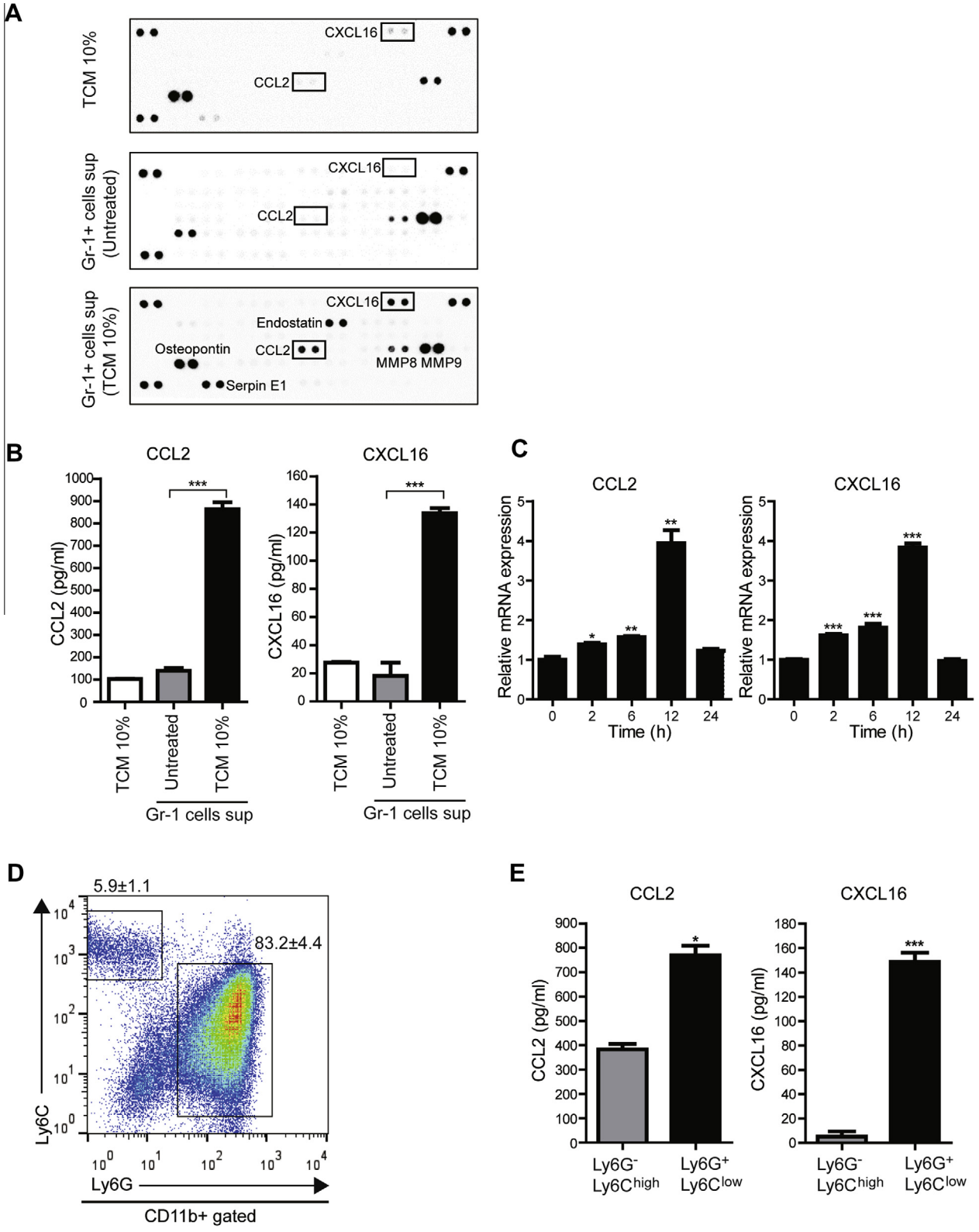

Fig. 2. TCM promotes CCL2 and CXCL16 release by Gr-1$^+$CD11b$^+$ cells. (A) Mouse angiogenesis antibody array analysis was used to determine differences in the release of angiogenic factors from 10% TCM, or supernatants of Gr-1$^+$CD11b$^+$ cells untreated or treated with 10% TCM for 24 h. Expression changes in CCL2 and CXCL16 are presented in a block square. (B) CCL2 and CXCL16 protein levels in cell supernatant were determined by ELISA. (C) mRNA expression of CCL2 and CXCL16 was analyzed by quantitative real-time PCR in 10% TCM treated Gr-1$^+$CD11b$^+$ cells at different time. (D) Proportion of Ly6G$^+$Ly6C^{low} and Ly6G$^-$Ly6C^{high} cells in spleens of 4T1 tumor-bearing mice. (E) CCL2 and CXCL16 protein levels in supernatant of Ly6G$^+$Ly6C^{low} and Ly6G$^-$Ly6C^{high} cells exposed to 10% TCM for 24 h measured by ELISA. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

bFGF in tumor-conditioned Gr-1$^+$CD11b$^+$ cells using an angiogenic array; however, the two main chemokines (i.e., CCL2 and CXCL16) were detected as major mediators of angiogenesis in vitro. This variation could be attributable by the use of a different tumor

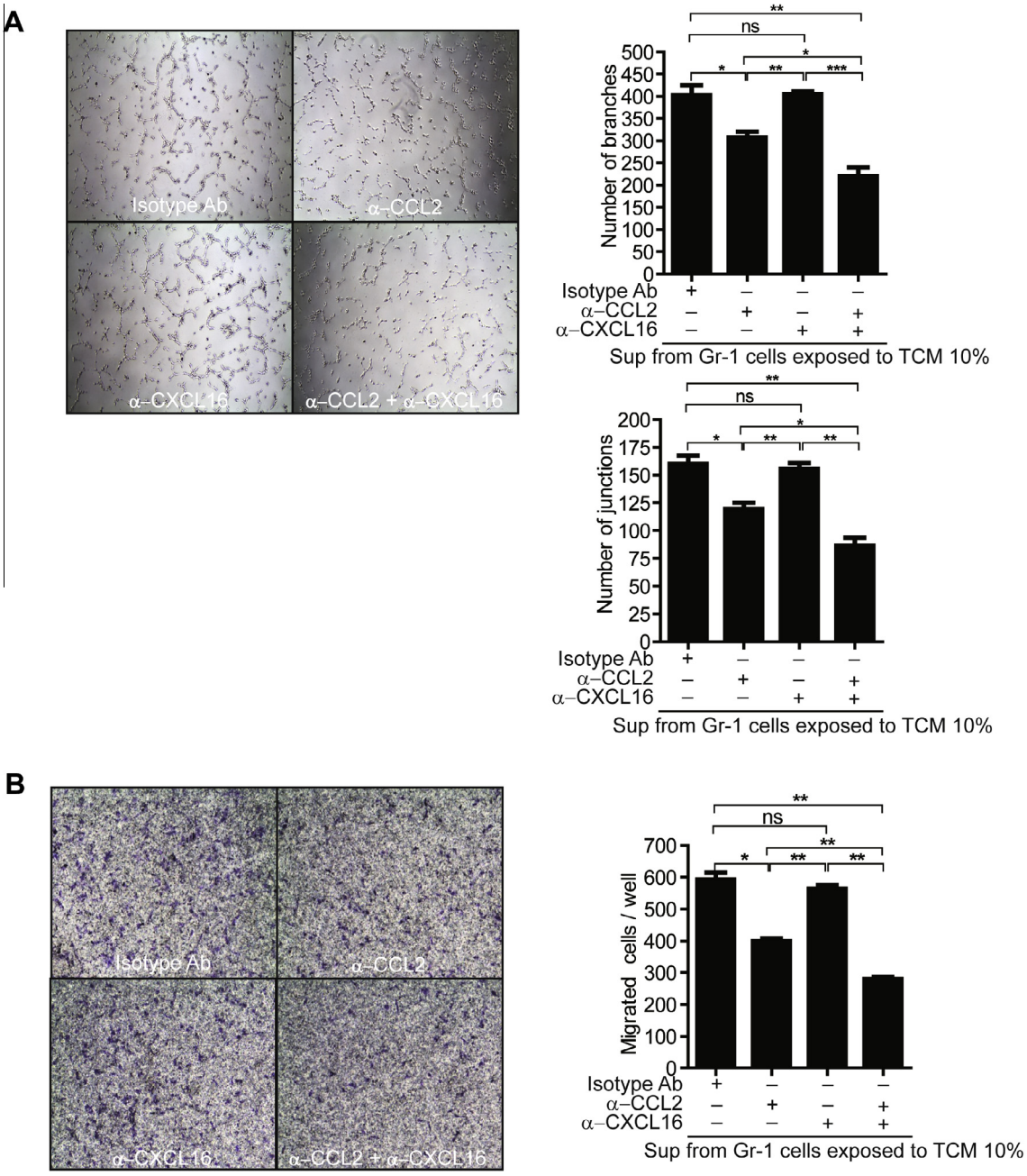

Fig. 3. CCL2 and CXCL16 synergistically mediate the pro-angiogenic effects of tumor-conditioned Gr-1$^+$CD11b$^+$ cells. (A) Effect of CCL2 and CXCL16 neutralizing antibodies on endothelial cell tube formation. Supernatants (sup) from Gr-1$^+$CD11b$^+$ (Gr-1) cells exposed to 10% TCM for 24 h were pretreated with normal IgG (isotype), anti-CCL2 mouse antibody, anti-CXCL16 mouse antibody, or both anti-CCL2 and anti-CXCL16 antibodies for 1 h before performing the tube formation assay (concentration: 10 μg/ml). (B) Effect of CCL2 and CXCL16 neutralizing antibodies on endothelial cell migration. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

model in these studies. Our study also implies that the angiogenic function of Gr-1$^+$CD11b$^+$ cells is context-dependent.

According to our angiogenic protein array data, tumor-derived factors activate Gr-1$^+$CD11b$^+$ cells to produce pro-angiogenic factors, chemokines CCL2 and CXCL16, serpin E1, and MMP-9. As previously reported, we also observed MMP-9 production in Gr-1$^+$CD11b$^+$ cells, but this production did not increase by tumor-derived factors [11]. Serpin E1 has been demonstrated to promote angiogenesis by inhibiting excessive proteolysis to maintain an optimal degree of ECM integrity required for endothelial cell migration [24,25]. In our study, tumor-conditioned Gr-1$^+$CD11b$^+$ cells secreted both proteolytic enzyme MMP and serpin E1, which inhibit uncontrolled proteolysis of the ECM. Thus, these cells might play a role in the maintenance of optimal balance in the ECM for angiogenesis.

Although chemokines play a crucial role in the regulation of leukocyte trafficking to sites of inflammation, recent studies indicate that the chemokine network has an important role in tumor progression, including tumor cell proliferation, survival, invasion, metastasis, and angiogenesis [26]. Several members of the CXC and CC chemokine families are critical inducers of angiogenesis [27]. CCL2 has been found to exert a direct angiogenic effect independent of its action on leukocyte recruitment [28,29]. Zhuge et al. reported that CXCL16 can trigger proliferation and migration of HUVECs in vitro [30]. In our studies, tumor-derived factors induced an increase in CCL2 and CXCL16 at protein and mRNA expression

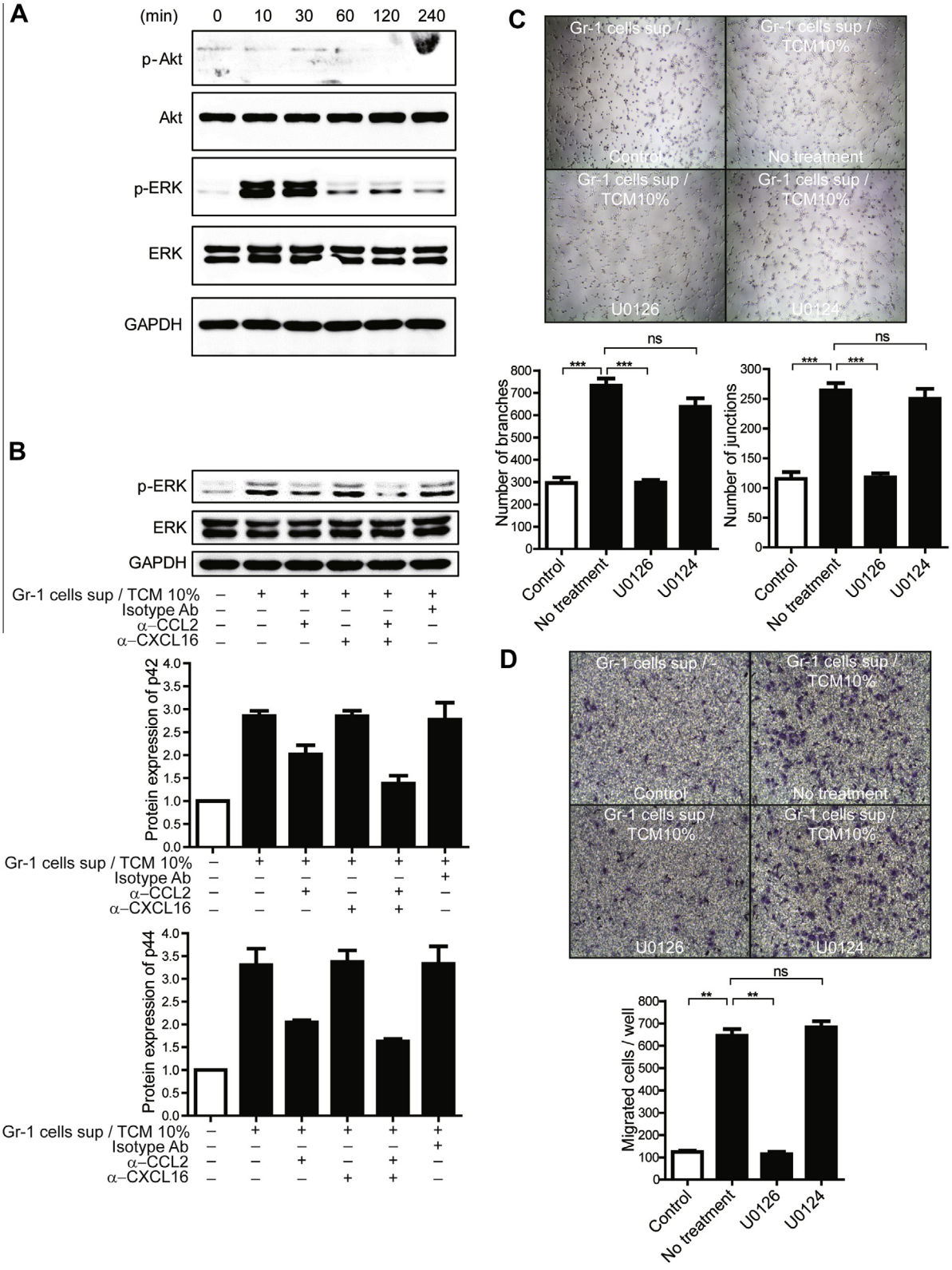

Fig. 4. ERK activation is involved in tumor-conditioned Gr-1$^+$CD11b$^+$ cells-mediated angiogenesis. (A) HUVECs were treated with the supernatant from Gr-1$^+$CD11b$^+$ cells exposed to 10% TCM for various times (0–240 min). Cell lysates were analyzed by Western blotting for Akt, phospho-Akt (p-Akt), ERK, and phosphor-ERK (p-ERK). (B) Effect of CCL2 and CXCL16 neutralizing antibodies on ERK1/2 phosphorylation measured using Western blotting. Preparation of Gr-1$^+$CD11b$^+$ cell supernatants and neutralizing antibody pretreatment before treatment in HUVECs were the same as described in Fig. 3. Band intensities were calculated by ImageJ software. (C) Effect of the MEK1/2 inhibitor U0126 on endothelial cell tube formation. HUVECs were preincubated with U0126 (10 μmole/l) or an inactive structural analog (U0124; 10 μmole/l), respectively, for 30 min before supernatants of tumor-conditioned Gr-1$^+$CD11b$^+$ cells were added for 4 h. (D) Effect of U0126 on endothelial cell migration. Control: supernatants from Gr-1$^+$CD11b$^+$ cells cultured in RPMI for 24 h. ns: nonsignificant, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.01$.

levels by Gr-1$^+$CD11b$^+$ cells. CCL2 neutralization inhibited endothelial cell tube formation and migration mediated by tumor-conditioned Gr-1$^+$CD11b$^+$ cells. However, CXCL16 neutralization alone did not suppress tube formation and migration of HUVECs. The concentration of CXCL16 produced by tumor-conditioned Gr-1$^+$CD11b$^+$ cells was 130 pg/ml, which was tenfold lower than the concentration required for inducing HUVECs migration suggested by others [30].

The synergistic action of chemokines has been well described in leukocyte recruitment during inflammation [31,32]. In our study, simultaneous neutralization of both CCL2 and CXCL16 significantly inhibited tube formation of HUVECs compared to CCL2 neutralization alone. These results suggest a synergism between coproduced CCL2 and CCL16 in angiogenic activity on endothelial cells. Although the individual effects of several chemokines on angiogenic regulation have been studied, no report has described chemokine synergism on endothelial cells.

ERK has been reported as being required for endothelial cell migration and tube formation by CCL2 and CXCL16 [29,30]. Consistent with published data, the present study demonstrated that supernatants from tumor-conditioned Gr-1$^+$CD11b$^+$ cells induced ERK1/2 phosphorylation in HUVECs. The angiogenic effect of tumor-conditioned Gr-1$^+$CD11b$^+$ cells was abolished by inhibition of this pathway, and simultaneous neutralization of both CCL2 and CXCL16 significantly inhibited ERK1/2 phosphorylation in endothelial cells compared with CCL2 neutralization alone. These findings suggest that synergy between CCL2 and CXCL16 can be at least in part explained by intracellular cooperation in ERK signaling. Although this study indicates a role for CCL2 and CXCL16 as important pro-angiogenic factors, other angiogenic chemokines are likely to be involved as well.

Our study may not only help to understand the regulation of myeloid cell-related angiogenesis, but also suggests that the effect of the microenvironment should be considered in formulating therapeutic strategies against tumor-associated angiogenesis. Further studies are required to elucidate the intracellular mechanisms and external signals essential for regulating the angiogenic properties of Gr-1$^+$CD11b$^+$ cells.

Acknowledgment

This work was supported by National Research Foundation grant funded by the Korea government (MEST) (No. 2012M3A9C5048707).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.12.117.

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